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THE AMELIORATION OF PSEUDOMONAS AERUGINOSA INDUCED THYROID GLAND TOXICITY IN FEMALE SWISS ALBINO MICE BY MITOCHONDRIA-TARGETED GALLIC ACID

Endocrinology

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ABSTRACT

The aim of the present investigation is to evaluate the protective effect of mitochondria-targeted gallic acid against Pseudomonas aeruginosa induced thyroid gland toxicity in female Swiss albino mice. Female Swiss albino mice weighing 28-32gm randomly divided into three groups and each group consist of five animals. The first group considers as control treated with only DMSO, the second group is DMSO + Pseudomonas aeruginosa treated and the third group is DMSO + Pseudomonas aeruginosa + mt-G treated, a whole experiment performed for fourteen days. The presence of the microflora population in the gut in balanced form showed the normal function of the thyroid gland whereas the gut microflora population are imbalanced affects thyroid function. Pseudomonas aeruginosa cause increased body weight, level of T3, T4, decreased, level of TSH did not show significantly changes and increased the activity of catalase, SOD,LPO, LDH, MDH and decreased the activity of GPx were determined. The mt-G is influential sufficient to ameliorate the thyroid toxicity caused by bacteria Pseudomonas aeruginosa.

KEYWORDS

Pseudomonas aeruginosa, mt-G, thyroid toxicity, female Swiss albino mice DMSO.

INTRODUCTION

Bacteria are found in all type of natural habitat from extreme cold to very hot place also inside as well as outside of the body of the organisms. Most of the bacteria are pathogenic and some are beneficial to humans. The beneficial bacteria are called commensal bacteria. The bacteria present in environment are contaminating the air, water and soil, the bacteria enters in human body via contaminated food, water and air. More than 120 species of *Pseudomonas* are pathogenic to humans and other animals. The bacteria through contaminated food and water reach to the site of commensal bacteria and start to decrease the count of commensal bacteria. The decreased commensal bacteria counts results in decreased uptake of minerals like iodine. This result in deficiency of iodine simultaneously affects the thyroid gland and generates the free radicals in thyroid [1]. The thyroid problem is considered to be most generic problem of the world. However, some studies have been signifying that a bacterial cell and their metabolic product present in the extraneous environment of the host compound that affects the thyroid function [2]. *Escherichia coli* secrete antithyroid compound in the cell free and broth culture medium [3, 4]. *E.coil* and Cholera produced an enterotoxin that stimulates cAMP in the thyroid gland and produced antithyroid compound [5]. Gallic acid is a secondary metabolite product of plant. Its bioactive natural compound constitutes the phenolic compounds combined with alkaloids and terpenoids [6]. The structure of gallic acid has three hydroxyl group binds to an aromatic ring. Its biological activity is due to its structural diversity [7, 8]. Mitochondria is a essential organiles to play role in metabolic activities like amino acid biosynthesis, lipid metabolism, homeostasis of steroid hormones, apoptosis and intermediate metabolic pathways [9]. The alterations of mitochondrial functions generate more reactive oxygen species (ROS) [10]. High level of ROS increase lipid peroxidation (LPO), damage nucleic acid, oxidized proteins and effect function of other cellular organelles. One of the most efficient ways to decrease oxidative effect to use compounds, which has antioxidant property. Although several conventional non-enzymatic antioxidants used such as vitamin-E and vitamin-C have less protective value [11].

One of the potential explanations for this response, antioxidant therapies may be specific in nature[12]. After that it is reported that the antioxidants have limited potential because they do not accumulate sufficiently inside the mitochondria to reduce oxidative damage. Thus, efficient mitochondria targeted antioxidants need to develop which have higher ability to reach the inner mitochondrial membrane [13]. The mitochondrial respiratory chain participates in the transfer of an electron to O_2, thus this electron transport generates a proton gradient which is used to drive the production of ATP by ATP synthase. Current drug strategies that selectively target to mitochondria take advantage of the electrochemical gradient from the outer plasma membrane m = -30 to -60mV to the inner mitochondrial matrixm = -150mV to -180mV. This gradient provides a large driving force for the selective targeting and concentration of large lipophilic cations to the mitochondria [14,15] and reported that treatment of mt-c with hypothyroidism increased the antioxidant defence of the cells and attenuates infection of reactive oxygen metabolism [16].

The aim of this investigation is to find the effect of *Pseudomonas aeruginosa* to induced thyroid toxicity and disturb thyroid hormones level and enzymatic and non-enzymatic reactions. We have to investigate mitochondria targeted gallic acid ameliorative effect on thyroid gland.

MATERIAL AND METHODS

Chemicals

All the chemicals of analytical grade are Nitroblue tetrazolium (NBT), triphenylphosphonium oxide (TPP) HCl, 48% Hydrobromic acid (HBr), thiobarburetic acid (TBA), tricholoroacetic acid (TCA), ethyl acetate, ethanol, dimethosulpho-oxide (DMSO), GSH (Glutathione reduced), riboflavin, phenozaine methosulphate (PMS), lithium lactate, H2O2 (30%), NADH, NADPH procured from Merck, Himedia and sigma chemical co. USA respectively. Gallic acid procured from Himedia.

Bacteria Culture

The bacteria *Pseudomonas aeruginosa* (MTCC 1748) were purchased from IMTECH Chandigarh and then cultured at the Endocrinology lab, Department of Zoology, Dr Harisingh Gour University Sagar, M.P. The bacteria were cultured in antifungal agar medium after incubating them at 37º C for 18-24 hours. Then the broth culture streaked on nutrient agar and after 18-24 hours incubation the microbial colonies developed. The *Pseudomonas aeruginosa* produces smooth, large, translucent colonies with the greenish blue pigment over the nutrient agar. The bacteria culture and gram negative culture plate of bacteria *Pseudomonas aeruginosa* is shown in fig. 1.

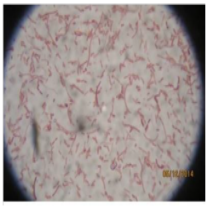


Fig.1- Gram Negative Bacteria Pseudomonas Aeuriginosa (MTCC-1748)

Gram's Staining

A thin smear of the bacterial culture was prepared over a slide and fixed with heat. The smear was stained with crystal violet for 60 sec and followed by washing slowly with running tap water. Then Gram's Iodine added for 30 sec and then washed with tap water. After washing, slides were dipped in 90% ethanol for 10-15 sec and in the end; Saffranin was added for 30 sec, followed by washing with running tap

water. After drying the slides were observed under the light microscope. Pink color indicated the presence of gram negative bacteria Pseudo monas aeruginosa.

Experimental Animals

Female adult Swiss albino mice weighing 28-32 gm were purchased from the college of Veterinary science and Animal husbandry Mhow, India. The ethical approval was taken from ADINA Institute, Sagar (M.P.) India (Ragistration no. AIPS/2015/2459/IAEC-03). Animal housing and caring followed by international guideline of ethical. All animals were housed in plastic cages on laboratory condition and were fed on standard laboratory diet daily food and water ad libitum.

Experimental Design

The experimental animal were divided into three groups and all groups administrated different doses as follows

Group I: control group treated with DMSO (0.02%) for 14 days daily.

Group II: Treated with DMSO + Pseudomonas aeruginosa (5X106 /kg bw/ day ip) for 14 days daily.

Group III: Treated with DMSO + Pseudomonas aeruginosa (5X106 /kg bw/ day ip) + mt- G (0.12mg/kg bw/ day, orally) for 14 days daily.

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 to synthesize the targeted derivative of curcumin, a solution of lipophilic cation refluxed with curcumin and evaporated to obtained mitochondrial targeted curcumin (mt-c) [17].

In Vivo Study

Reverse osmosis (RO) water 200 µl in (5×106) 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

Animals were sacrificed by the cervical dislocation after the experiment; thyroid was dissected out, washed in ice cold phosphate buffer saline and stored at -80ºC for biochemical analysis. 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

Estimatimation of T3, T4, and TSH

At the end of the experiment blood was collect by cardiac puncture, leave the room temperature for 15-30 minute and centrifuge at the 1500 rpm for 15 minute. Take the serum from micropipettes and stored at -20ºC for hormonal test. Detection of T3, T4, and Thyroid stimulating hormone (TSH) measured using ELISA provided by The Calbiotech Inc. (California, USA) [18].

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 [19] 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-

The activity of superoxide dismutase (EC: 1.15.1.1) was determined method [20]. 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 30º 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.

Catalase Activity-

The Catalase activity (EC: 1.11.1.6) was measured as described [21] with some modification. Briefly, the reaction mixture 1 ml consisted of 0.067 M phosphate buffer (pH 7.0) and 0.003% H2O2. 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 H2O2 consumed/min/mg protein.

Glutathione Reductase Assay

The activity of glutathione reductase (EC: 1.6.4.2) was measured following the method of [22]. 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.

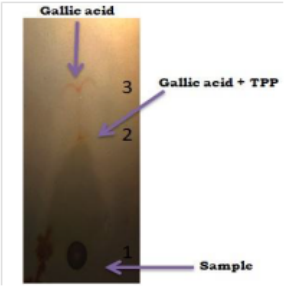
Statistical Analysis

Results expressed as Mean±SD and student t-test was applied for determining the level of significance between control and treated groups. Significance value expressed *p<0.05, **p<0.01, ***p<0.001 [23].

RESULTS AND DISCUSSION

1.10.1 Mitochondria targeted Gallic acid-

The TLC (thin layer chromatography) and HPTLC (High profile liquid chromatography) of mt-G are shown in fig. 2 and 3 respectively. The photograph depicted TLC and three spots are seen on the TLC plate, the first spot indicates the sample and second spot indicates the gallic acid that binds to the TPP (Thiamine Pyrophosphate) - mt-G and third spot indicates the gallic acid. The molecular weight of second spot is higher than the third spot because gallic acid binds with TPP and its molecular weight increases, so its spot appeared in middle region. TLC and HPTLC results are positive in HPTLC 2200-1400 peak shows gallic acid bind with TPP.



Net Body Weight Gain

After 14 days, female mice treated with *Pseudomonas aeruginosa* ($5x10^6$ /kg bw/day, ip) the body weight was increased. Co-treatment of mice with bacteria ($5x10^6$/kg bw/day, ip) and mt-G (1.63mg/kg bw/day, orally) the adverse effect of bacterial toxicity reduced and decreased the net body weight. The net body weights of mice are summarized in table 1 and fig. 4.

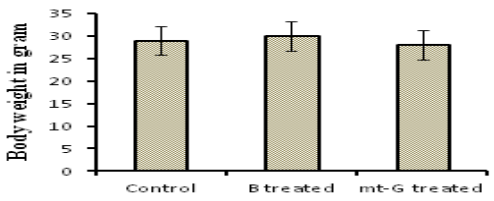


Fig. 4- Effect of *P. aeruginosa,* and antioxidant mt- G treatment on mice, weight increased and decreased respectively. The values represent mean + SD. Significance value are presented *p<0.05, **p<0.01, ***p<0.001

Serum Hormone Levels

A significant decreased levels of serum T3, T4 were observed in *Pseudomonas aeruginosa* treated female mice while the treatment with mt-G prevent the decrease level of T3 and T4 in serum significantly, However level of thyroid stimulating hormone does not change in treated group while treatment with mt-G increase the level of TSH in serum (***p<0.001) significantly elucidated in fig. 5.

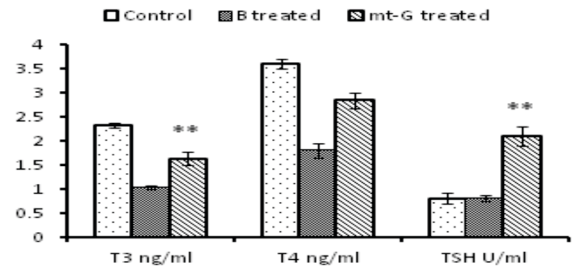


Fig. 5- Effect of *P. aeruginosa* and mt-G on mice serum, hormone levels in the group studied. Treatment of mt-G, Level of T3, T4 and TSH (***p<0.001). Thyroid stimulating hormone (TSH) significantly increases.

LDH Activity

For spectrophotometric based analysis of LDH isozymes, the LDH activity obtained from extracts tissue of normal mice. *Pseudomonas aeruginosa* causes the significant increase the activity of LDH isozymes. However, the LDH activity significantly declines towards normal tissues of bacteria affected mice co-treated with mt-G. Treatment of normal mice with bacteria (5x106/kg bw/day, ip) for 14 days caused a significant increase LDH activity in thyroid gland as compared to control indicates tissue injury. The level of LDH declined significantly in bacteria mice treated with mt-G(1.63mg/kg bw/day, orally) for 14 days elucidated in fig. 6.

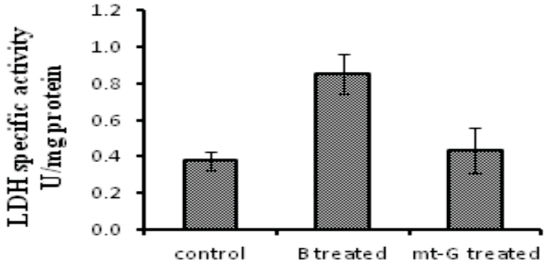


Fig. 6- Effect of *P. aeruginosa* and mt-G on the specific activity of lactate dehydrogenase in the t hyroid g land. Data in panel represents mean± SD.

Lip Id Peroxidation

Mice treated with bacteria caused a significant increase the level of MDA (p<0.001) in thyroid gland compared to control group. Co-treatment with mt-G bacteria treated mice significantly decreased MDA level (***p<0.001) in the thyroid gland as described in fig. 7.

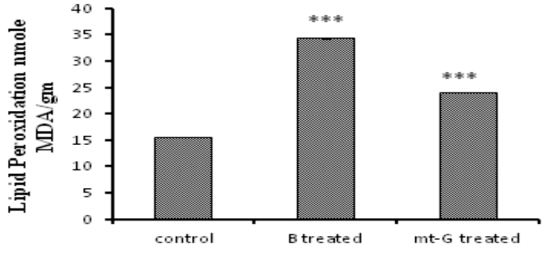


Fig. 7- Effects of *P. aeruginosa* and mt-G, LPO in the thyroid gland. Data in panel represents mean± SD. (***p<0.001)

GSH-

Treated mice with bacteria caused a significant decrease the level of GSH in the thyroid gland whereas co-treatment of bacteria treated mice with mt-G prevent this decreased level of antioxidant GSH and maintained it towards normal level and elucidated in fig. 8.

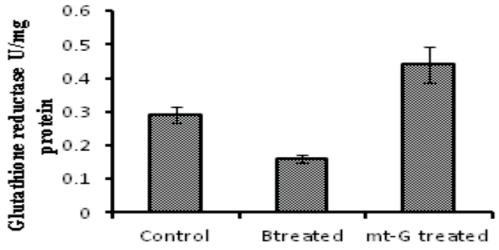


Fig. 8- Effect of *P. aeruginosa* and mt-G on the specific activity of glutathione reductase in the thyroid gland of mice. Data panel represents mean± SD.

Catalase Activity

Treated mice with bacteria cause a significant increaseH_2O_2 in thyroid gland compared to control group. Co-treatment of mt-G with bacteria treated mice significantly prevented the increase H_2O_2in the thyroid gland and the measured activity of catalase is summarized in fig. 9.

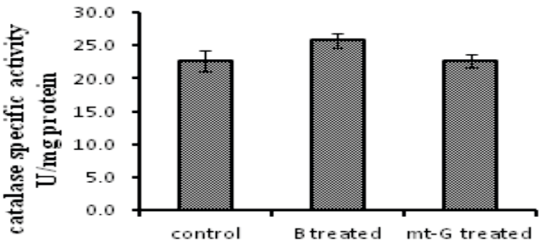


Fig. 9- Effect of *P. aeruginosa* and mt-G on the specific activity of catalase in the thyroid gland. Data in panel represents mean± SD.

Superoxide Dismutase

The level of active SOD significantly increases in the thyroid gland. However, co-treatment of bacteria treated mice with mt-G prevents this increased level of total SOD in the thyroid gland significantly summarized in fig. 10.

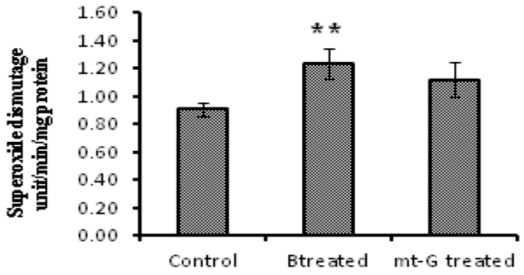


Fig. 10- Effect of *P. aeruginosa* and mt-G on the specific activity of superoxide dismutase on the thyroid gland of mice. Data panel represents mean± SD. (**p<0.01)

CONCLUSION AND DISCUSSION

It has predicted that the mitochondrial targeted antioxidants like mt-Gallic acid can be easily reached the mitochondrial matrix and was more effective than non-targeted antioxidants. The *Pseudomonas* affected the T3 & T4 hormone synthesis [24]. The 14 days exposure to *Pseudomonas aeruginosa* leads to increase in net body weight which in turn means inhibition of DNA replication by bacteria treatment which altered the concentration of enzymes, structural proteins and receptors. This is all due to reduced food intake, decreased proteins levels and altered metabolic activities. The results of present investigation showed that the gain in body weight is prevented by mt-G after treatment with bacteria [25, 26].The concentration of serum hormone level of T3, T4 and TSH is an important parameter for measurement of growth, development and metabolism of the individual. The suggested results showed that the level of T3 and T4 reduced and no change in TSH level after treatment with bacteria. It is

reported that the intake of contaminated food and water can inhibit growth, changes structural proteins and alter the metabolic activities [27, 28]. After treatment with mt-G the serum hormone level of T3, T4 and TSH increases. The thyroid follicles are rich in poly unsaturated acid and has high oxygen supply so the changes of lipid peroxidation is more and the reduced in T3 and T4 is indicator of generation of oxidative stress in follicles but no changes hypothalamo-pituitary-thyroid axis because *Pseudomonas* secretes enterotoxin which binds to kinase receptor has no effect on cAMP hence the hypothalamus-thyroid-pituitary axis is not affected and the level of TSH is constant. During the cell proliferation and pathological condition the glucose uptake and lactate formation increase many time and lactate dehydration helps in it which convert pyruvic acid into lactate acid and vice–versa [29].

The result of present study shows that a linear view between LDH and MDA concentration in tissue extracts exposed to bacteria *Pseudomonas* indicates that the tissue toxicity by bacteria cause the increase the lipid peroxidation [30].. The results of present study showed that the lipid peroxidation plays an important role in modifying LDH activity. The increased lactate production shows that the bacterial toxin induces cell stress. In this study shows that the mt-G inhibit the LDH production activity and prevent the mice from *Pseudomonas* induced toxicity.

The oxidative stress is generally associated with the excessive production of ROS and reduced action of antioxidant defence system and the oxidant and anti-oxidant regulates the thyroid hormone and protects the cell [31].

In this investigation LPO is evaluated as an oxidative stress biomarker in thyroid gland of bacteria treated mice have significant elevated MDA level and the results show that mt-G inhibit the production of MDA level and protect the mice from bacterial induced toxicity [32, 33]. The results of present study shows that the bacterial toxin decreased the level of GSH in thyroid tissues this is generally due to the oxidative stress produced by bacteria that reduces glutathione loss electrons. The results have shown that the mt-G treatment induces the production of GSH and protect the mice from bacterial associated toxicity.

The results showed that level of CAT and SOD increases in case of hypothyroidism. These results showed that the mt-G inhibits the production of CAT and SOD the mt-G neutralizes the toxicity. The increased level of H_2O_2 is due to thyrotropin, this H_2O_2 is acts as a substrate for the thyroperoxidase enzyme which catalyses and synthesizes T3 and T4 hormone in hypothyroidism due to bacterial toxicity decrease the thyroid hormone production and H_2O_2 accumulates and deforms the thyroid gland [16].

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