Biochemical, Histopathological and Immunohistochemical study of the repeated environmental toxicant Ultra Violet B radiations induced Hyperthyroidism alleviates oxidative stress and prevented by Naringin in male Swiss albino mice

SHASHANK SHAKYAWAL[1], GAYATRI RAI[1], and PAYAL MAHOBIYA[1]

[1]Dr Hari Singh Gour University

June 6, 2023

Abstract

Global warming rise has been observed as a result of ozone layer destruction, which makes it possible for UVB radiation to pass through atmosphere and kill people. It is believed that daily exposure to UVB radiation has biological effects on both humans and other living things. Concerning effect of UVB radiations, we examine the instinctive mechanism by which a specific amount of radiation induces oxidative stress and hyperthyroidism, which is further controlled by the antioxidant activity of Naringin (NG), due to the detrimental effects of UVB radiation on the biological system. For these examinations twenty-four adult male albino mice were divided into four equal groups; control group, UVB exposed group (2 hrs/day), UVB+NG treated group (80 mg/kg b.w), and NG-treated group. Doses were given orally, daily for 15 consecutive days. Specimens of the thyroid gland were processed for hormonal, biochemical, histological, immunohistochemical and immunofluorescence examinations. A morphometric study and statistical analysis of the findings were performed for different groups. Compared to the control group, the specimens of the UVB exposed mice showed alteration in mean height of the thyroid follicular epithelial cells and caspase-3, NOS-2 and THRβ-1 expression. In contrast, naringin prevent the changes caused due to repeated UVB radiations.

1. Introduction

Radiation being an environmental toxicant from several sources is always present around all living things on earth. Irradiation of the entire body damages cellular, physiological, and biochemical reactions within living cells. Radiation effects are influenced by various factors, including age, gender, exposure time, and affected tissue. These radiations are emitted and transmitted by various sources and are absorbed by the animal body. UV rays are non-ionizing rays ranging from 200nm to 400nm. UV radiations are found in sunlight and have enough energy to easily penetrate body cells, causing changes in chemical and biological activities. UVB radiation has a wide range of effects on all living things on Earth, including the infliction of cancer.[1] The amount of ozone in the stratosphere determines the amount of UVB radiation that reaches the Earth's surface. Animals and humans may be harmed by biological reactions to changes in UVB radiation. The United Nations has conducted regular assessments of the effects of UVB radiation on people and the environment.[2]

Furthermore, radiations have been discovered to be possible endocrine disruptors. This has been proven to have a deleterious influence on human and animal reproductive, immunological, and hormone systems.[3] Environmental factors may be playing a role in the rise in thyroid cancer cases. Chemicals having endocrine-disrupting effects, in particular, have sparked interest as possible danger factors.

Thyroid gland and its hormones have essential biological functions in children as well as adults. They possess essential role in growth and development especially for brain. In adults, the gland hormones are involved in the regulation of protein, carbohydrates and lipid metabolic processes. Moreover, they are essential in regulating heart rate, reproductive functions, emotional steadiness and gastrointestinal functions.[4,5] Thyroid hormones regulate metabolic and energy homeostasis, thermogenesis, and the transcription of genes that

control cell proliferation and basal metabolic rate to control an organism's growth throughout.[6]

Flavonoids are diphenyl propanoids that occur naturally in plant foods such as fruits, grains, and nuts.[7] Naringin, the main flavonoid of grapefruit and nearly only found in citrus fruits, is one of the most common flavonoids in the diet. The average human dietary intake of these three flavonoids is unknown, but polyphenol intake is reported to be nearly 1 g/day, with two-thirds flavonoids such as naringin. According to epidemiological studies, flavonoids have biological activities such as antiallergy, antiviral, anti-inflammatory, antioxidative, and vasodilating properties. An extensive range of pharmacological and therapeutic properties, including lipid-lowering, anti-inflammatory, free radical-scavenging/antioxidative, antihyperlipidemic, anti-apoptotic, and anti-atherogenic properties, have been reported for naringin (NG), the compound that gives fruits their sour flavour and bitter taste.[8,9]

Despite these reported beneficial effects of flavonoids, in vivo studies are negligible.[10] The other two reported investigations are based on in vitro studies, which very often do not comply with the in vivo results.[11] There is not a single report on the effect of Naringin on male *Swiss albino* mice exposed to UVB irradiation. Furthermore, radiations were used for the first time to demonstrate the direct free radical scavenging potential of this flavonoid in thyroid tissue. The present study is the novel approach to elucidate UVB radiations induced hyperthyroidism by the treatment of Naringin in male mice.

2. Methods and Materials

2.1. Chemicals

Analytical-grade chemicals and reagents were utilized. In Mumbai, India, Himedia Laboratories Pvt. Ltd. sold naringin (C27H32O14). NBT, NADPH, Methionine, Reduced Glutathione, and the remaining chemicals were acquired from Central Drug House (P) Ltd, New Delhi, India. Thiobarbituric Acid, Hematoxylin, Eosin, and the other chemicals were acquired from Himedia, India, thyroid hormone receptor beta-1 (THRβ-1) and Caspase-3 antibodies were purchased from science emporium and NOS-2 antibody purchased from Santa Cruz Biotechnology. Avidin/Biotin blocking kit Cat No.

2.2. UVB irradiations

The UVB light, produced in Germany with a wavelength of 280 nm, was used to expose the *Swiss albino* mice. For 15 days, the irradiance was set at two hours each day .[12]

2.3. Study animals

We bought male *Swiss albino* mice from Mhow, India's College of Veterinary Sciences and Animal Husbandry, measuring 22-28 grammes. Animals were acclimated to the housing at a temperature of 20 to 25 °C, relative humidity of 50 to 55 %, and a cycle of 12 hours of light and 12 hours of darkness.

2.4. Ethical statement

Institutional animal ethics committee**,** Dr Harisingh Gour Vishwavidyalaya, Sagar (M.P.) (A Central University) (Approval No.:379/CPCSEA/IAEC-2021/004), at Department of Pharmaceutical Sciences, provided its ethical endorsement. International standards were followed in the handling and usage of laboratory animals. The study was conducted in accordance with the Basic & Clinical Pharmacology & Toxicology policy for experimental and clinical studies.[13]

2.5. Study test design

The animals were randomly divided into four groups with six mice in each group. A timeline of dosage and experimental test design was portrait in Fig. 1.

Group 1: The animals received standard food and water ad libitum for 15 days (control group).

Group 2: The animals was exposed to UVB irradiation at 280 nm for two hours every day for 15 days.

Groups 3: The animals exposed to UVB irradiation along with Naringin (at a concentration of 80 mg/kg body weight) for 15 days orally.[14]

Group 4: The animals only received NG (at a concentration of 80 mg/kg body weight) for 15 days orally.

Throughout the therapy, body weight and daily food and drink intake were noted. Animals were starved overnight and then killed by cervical decapitation after the study period. After the connective tissues around the thyroid gland were removed, the thyroid gland was removed through a transverse abdominal incision and weighed.

2.6. Sample collection

After being extracted, whole blood samples were put in evacuated tubes containing an EDTA anticoagulant solution. The plasma obtained after centrifuging the second tube at 2200 rpm for 15 minutes was stored at -20°C until the levels of the thyroid hormones were determined.

2.7. Measurement of body weight and thyroid weight

Body weight was recorded on the beginning, sixth, eleventh, and fifteenth days of the trial. Thyroid weight was assessed following exsanguinations. An electronic weighing balance (Sartorius BP 210S) was used to measure thyroid weight.

2.8. Tissue homogenization

The thyroid gland was removed for biochemical testing, cleaned with PBS solution, and kept at -20°C until needed. The thyroid gland extract was made with homogenization in 0.02 M Tris-Cl (pH 7.4). After centrifuging homogenate tissue at 12500 rpm for 30 min at 4°C, the supernatant was kept at -20°C for the biochemical tests.

2.9. Hormonal Analysis

Using an enzyme-linked immunosorbent test kit given by The Calbiotech Inc. (California, USA), serum samples were examined for T3, T4, TSH and fT3 and fT4 levels.[15]

2.10. Analysis of Enzymatic and Non-Enzymatic assays

The remaining part of testis was used for biochemical analysis to measure Lipid peroxidation[16], Catalase[17], Superoxide dismutase[18], GSH[19], Hydrogen peroxide[20], Glutathione Reductase[21] and Nitric oxide[22] levels in all the above groups.

2.11. Reactive oxygen species (ROS) Level

The oxidizing agents known as reactive oxygen species (ROS) are heavily linked to tissue damage. The respiratory redox chain in the mitochondria is thought to be the main source of ROS and free radicals in the cell. ROS can come from both external and endogenous sources. This implies that ROS production is inevitably linked to ATP synthesis in most cells. ROS and other related radicals serve diverse physiological functions in the cell. Dichlorofluorescein diacetate (H2DCF-DA) is a widely used cell-permeable fluorescence-based sensor for detecting ROS both in vitro and in vivo. Endogenous esterases deacetylate DCFDA to dichlorofluorescein (H2DCF), which reacts with different ROS to produce the fluorophore DCF, measured fluorometrically at excitation wavelengths of 488 nm and emission wavelengths of 525 nm.[23]

2.12. Activity staining of antioxidants (SOD and CAT)

The active levels of all antioxidant enzymes were determined using non-denaturing PAGE of thyroid extracts using the method described above.[24] In each lane of 10% and 8% non-denaturing PAGE, a thyroid extract containing 100 µg protein for SOD and catalase was loaded. Following electrophoresis at 4±2°C, the gels were stained with substrate specific antioxidant enzymes. The SOD staining solution contained 2.5 mM NBT, 28 mM riboflavin, and 28 mM TEMED. Gels were incubated in the dark for 20 minutes before being exposed to fluorescent light until achromatic SOD bands developed against a dark blue background. In the case of

catalase, gels were first soaked in 0.003% H2O2 for 10 minutes before being incubated in a staining mixture containing 2% potassium ferricyanide and 2% ferric chloride. Catalase bands appeared achromatic against a blue-green background.

Gel densitometry was used to calculate the intensities of all bands using alpha imager gel documentation software. PAGE was performed 3-4 times in each case, and the mean SD of densitometry values of the bands as a percentage of the control lane are presented in the result along with one representative gel photograph.

2.13. Histopathological Analysis

Through the heart, 0.02M phosphate-buffered saline (PBS) and 4% PFA were used to perform whole-body perfusion.[25] After being removed, the thyroid gland was placed in the fixative for two days. For haematoxylin and eosin staining, fixed tissues were treated, dried, and cut into 5 μm slices from paraffin-embedded thyroid. 10X and 40X magnification microscope analyses of images.

2.14. Immunohistochemistry

Paraffin sections of thyroid samples were deparaffinized and rehydrated. Antigens were retrieved by incubating the sections in a microwave oven in sodium citrate buffer (10 mM; pH 6.0) for 15 min. Sections were brought to room temperature and rinsed with PBS. Then the sections were blocked using 5% bovine serum albumin at room temperature for 20 min. Subsequently, the tissues were incubated with primary antibodies overnight at 4@C. Antibodies against THRβ-1 (cat. no. PA1213A), caspase-3 (cat. no. BS-0081R) and NOS-2 (cat no. sc-7271) were diluted at 1:20, 1:50 and 1:50 in PBS, respectively. After 12 h, the sections were washed with PBS and incubated for 60 min with biotin labeled goat anti-rabbit IgG secondary antibodies (cat. no. HPO3 provided by science emporium) at room temperature. Sections were stained using diaminobenzidine (DAB) chromogenic agent at room temperature for 5 min. Sections were counterstained with Mayer's hematoxylin at room temperature for 5 min.[26] The photographs were taken using a compound microscope. Finally, the mean optical density values were analyzed with ImageJ software.

2.15. Immunofluorescence

THRβ-1, caspase-3 and NOS-2 immunoreactivity was detected in the thyroid by immunofluorescence by using method of Niranjan & Srivastava[27], with slight modifications. Paraffin sections of thyroid samples were deparaffinized and rehydrated. Antigens were retrieved by incubating the sections in a microwave oven in sodium citrate buffer (10 mM; pH 6.0) for 15 min. Sections were brought to room temperature and rinsed with PBS. Sections were incubated with primary antibodies overnight at 4@C. Antibodies against THRβ-1 (cat. no. PA1213A), caspase-3 (cat. no. BS-0081R) and NOS-2 (cat no. sc-7271) were diluted at 1:20, 1:50 and 1:50 in PBS, respectively. Slides were incubated with the secondary antibody FITC at a dilution of 1:20 for 60 min at 4@C. The sections were counterstained using DAPI for 4 minutes at RT, then slides were mounted by glycerine-based media. The immunofluorescence images were taken using EVOS 5000 Invitrogen fluorescent microscope. For semiquantitative analysis of THRβ-1, caspase-3 and NOS-2, the immunoreactive cells were analysed with Image J software.

2.16. Statistical analysis

All statistical data analyses employed one-way ANOVA (analysis of variance), and the results are presented as mean SE (standard error). The irradiated and control groups were compared using Dunnett's test. The threshold for significance was established at, correspondingly, *$p<0.05$, **$p<0.01$, and ***$p<0.001$.

3. Result

3.1. Body and Thyroid weight

The body weight measured over the study period was analysed using the body weight mean. Body weight differences between the UVB-irradiated group and the control group were found to be statistically significant ($p<0.01$). The body weight of the UVB+NG treated group was significantly greater than that of the UVB

irradiated group ($p<0.05$), but there were no discernible changes between the administrative group of NG and the control group (Fig.2.A).

In comparison to the control group, the thyroid weight in the UVB-irradiated groups reduced considerably (p 0.01), according to the findings. However, as compared to the group that had received UVB radiation, the thyroid weight considerably increased ($p<0.05$). Additionally, in compared to the control, the Naringin administrative group (NG) showed significant modifications ($p<0.05$). (Fig.2.B).

3.2. Effects of UVB irradiation on thyroid hormones

Thyroid hormone levels are noticeably higher in the UVB-irradiated group compared to the control group. T3, T4, fT3, and fT4 levels significantly increased in the UVB-irradiated group compared to the control group, while TSH levels significantly decreased. As shown in figure 3, the UVB+NG treated group's T3, T4, fT3, and fT4 levels were significantly lower than those of the UVB irradiated group, and the administrative NG group exhibited substantial differences from the control group.

3.3. Effect of UVB irradiation on enzymatic and non-enzymatic assays

LPO estimation- Thyroid MDA levels in the UVB-irradiated group were considerably higher than those in the control group. When compared to the UVB-irradiated group, the distribution of Naringin to the UVB+NG treated group resulted in a considerable drop in MDA levels (Fig.4.A).

SOD estimation- In comparison to the control group, the UVB-irradiated group's SOD activity was significantly higher. When Naringin was administered to the UVB+NG treated group, the activity of SOD was markedly reduced in comparison to the UVB irradiated group (Fig.4.B).

Catalase activity- The UVB-irradiated group had significantly more catalase activity than the control group. The endogenous accumulation of hazardous H2O2 brought on by the lack of catalase activity is predicted to cause a multitude of genetic modifications, including single-strand and double-strand breaks, which may eventually result in the pathological illness. The catalase level was found to be lower in the UVB+NG treated group than it was in the UVB irradiated group after naringin administration (Fig.4.C).

H2O2 activity- When compared to the control group, the H2O2 activity seen in the UVB-irradiated group was significantly higher. When naringin was administered to the UVB+NG treated group, the activity of H2O2 was markedly reduced in comparison to the UVB irradiated group (Fig.4.D).

NO activity- NO activity was substantially higher in the UVB-irradiated group than it was in the control group. When Naringin was administered to the UVB+NG treated group, it was observed that the NO level was lower than in the UVB alone group (Fig.4.E).

GR activity- In comparison to the control group, the GR activity was significantly higher in the UVB-irradiated group. When Naringin was administered to the UVB+NG treated group, the activity of GR was markedly reduced in comparison to the UVB irradiated group (Fig.4.F).

GSH activity- When compared to the control group, the GSH activity seen in the UVB-irradiated group was significantly lower. When naringin was administered to the UVB+NG treated group, the activity of GSH was markedly increased in comparison to the UVB irradiated group (Fig.4.G).

Changes in the ROS level- Reactive oxygen species is commonly employed as a marker to detect tissue injury in toxicological and clinical chemistry. Variations in ROS level are a sign of antioxidant enzymes alterations occurring in the afflicted tissue. In comparison to the control group, the ROS activity was significantly higher in the UVB-irradiated group. When Naringin was administered to the UVB+NG treated group, the activity of ROS was markedly reduced in comparison to the UVB irradiated group (Fig.4.H).

3.4. Profiles of Antioxidant Enzymes

The quantitative estimation of the enzyme activity was done to support the results of PAGE profile. SOD is the committed enzyme of central antioxidant pathway and according to Fig. 4. B, only Cu-Zn SOD could

be detected in the thyroid tissue of untreated as well as UVB exposed sets. Moreover, as compared to the untreated set active level of SOD protein (Fig. 5.1. A, B) were observed to be significantly increased ($P < 0.05/0.01$) in the thyroid tissue exposed to UVB radiations.

Catalase is an enzyme, which are responsible for the detoxification of H2O2. In the present investigation, there was a significant increase ($P < 0.05$) in the level of catalase in UVB exposed group, which was restored toward normal in UVB exposed mice treated with naringin ($P < 0.05$). An increase in the level of catalase is expected to result in the accumulation of harmful H2O2, which can cause DNA damage and initiate follicular damage. Treatment of UVB exposed mice with naringin restored catalase level in UVB exposed mice toward normal and thus, could exert antiproliferative effect by detoxifying H2O2, thereby, minimizing its adverse DNA damaging effects (Figure 5.2. A, B).

3.5. Effect of UVB irradiation on histology of thyroid gland

The thyroid follicular structure was shown in the control group (A) to have a normal histological appearance, but the UVB-irradiated group (B) showed disruption of the follicular structure. The thyroid follicles were practically normal in the UVB+NG treated group (C) (Fig.6.A).

3.6. Immunohistochemistry of Thyroid gland showing expression of THRβ-1, Caspase-3 and NOS-2

There was a marked reduction in the expression of THRβ-1 receptor in the thyroid of UVB exposed mice as compared to control. The thyroid of naringin treated showed similar TRHβ-1 receptor expression like that in the thyroid of control rats (Fig. 7). Moreover, the level of caspase-3 protein expression was significantly increased in UVB exposed mice compared to the expression in control. The intensity of activated caspase-3 immunostaining (deep brown) is pre-dominant on thyroid follicular disruption (Fig. 8). The expression of NOS-2 was seen to be increased in UVB exposed group as compared to control. The group of naringin treated showed the intensity of NOS-2 antibody somewhat similar to control (Fig. 9).

3.7. Immunofluorescence expression of THRβ-1 in thyroid gland

The fluorescence expression of THRβ-1 was seen to be decreased in case on UVB irradiated group as compared to control. The administration of naringin shows somewhat enhanced fluorescence as compared to UVB irradiated group (Fig. 10).

3.8. Immunofluorescence expression of Caspase-3 in thyroid gland

The fluorescence expression of Caspase-3 was seen to be increased in case on UVB irradiated group as compared to control. The administration of naringin shows somewhat lower fluorescence as compared to UVB irradiated group (Fig. 11).

3.9. Immunofluorescence expression of NOS-2 in thyroid gland

The fluorescence expression of NOS-2 was seen to be increased in case on UVB irradiated group as compared to control. The administration of naringin shows somewhat lower fluorescence as compared to UVB irradiated group (Fig. 12).

4. Discussion

In order to provide hopeful evidence for a link between exposure to EMF and an increased prevalence of health risks, possible impacts of EMF on biological systems were widely examined.[28] Numerous in-depth studies have been conducted over the last few decades to examine the potential biological effects of electromagnetic fields (EMF) on various human systems. The endocrine system, namely the pineal gland, has received the most attention out of all the researched systems.[29] Effects from low radiation doses are more challenging to forecast and analyse. There is no limit to how efficient adaptive mechanisms promote cellular protection. As a result, it is impossible to draw a firm judgement concerning exposure to low radiation levels.[30,31] Due to the sensitivity of the thyroid gland to EMFs, this exposure led to morphological modifications and a decrease in blood T4 and T3 levels. These alterations remained over the whole trial, proving that normal thyroid function takes more time to rebound after exposure to EMFs.

Loss of weight is typically linked to hyperthyroidism.[32] Our findings were consistent with previous studies in that UVB irradiation resulted in considerable thyroid and body weight loss. [33,34] The thyroid gland is impacted by UV radiation, and long-term radiation exposure predominantly targets many genes, proteins, and lipids and leads to cancer.[35] We discovered that UVB radiation-induced oxidative stress produced free radicals and induced hyperthyroidism. T3, T4, FT3, and FT4 were all considerably higher in hyperthyroidism, whereas TSH was much lower. By causing damage to crucial components, including body protein and thyroid tissue, UVB irradiation was shown to dramatically diminish the body weight and thyroid weight of the experimental mice. Naringin was utilised as an antioxidant in our analysis. Additionally, compared to the UVB-irradiated group, the administration of naringin resulted in a substantial drop in T3, T4, FT3, and FT4 levels and a large increase in TSH levels. In vivo cellular oxidative stress is thought to be modulated by any fluctuation in circulatory thyroid hormones. Reactive oxygen species are produced due to increased mitochondrial respiration, which is thought to be the primary mechanism of this physiological/pathological alteration.[36]

We observed that *Swiss albino* male mouse thyroid glands exposed to UVB radiations experienced a heightened rise in ROS. Other investigations have revealed elevated levels of oxidative stress in several organs, including the testis, skeletal muscle, heart, pancreas, and brain, which is mediated by the hyperthyroid condition.[37,38] Physiological levels of ROS are created during typical cell metabolism. However, excessive ROS production can result in cell apoptosis, necrosis, or autophagy in several pathological circumstances, including inflammatory bowel disease, diabetes, cancer, or obesity.[39] Organisms have complete, integrated endogenous enzymatic repair mechanisms to deal with ROS damage. Important non-enzymatic antioxidants include glutathione (GSH), vitamin E, vitamin C, h-carotene, and uric acid, which are either ingested with food or produced endogenously. The endogenous enzymatic antioxidants are represented by Cu^{2+}, Zn^{2+} and MnSODs, catalase, and GPx.[40] The decrease in antioxidant capacity in hyperthyroid patients is most likely due to increased free radical production. Increased enzyme activity in hyperthyroidism patients is likely due to increased ROS production.[41] In the mouse thyroid, exposure to UVB significantly increased the level of lipid peroxidation. Earlier research on lipid peroxidation showed a similar impact.[42,43] Lipid peroxidation is inversely related to oxidative stress, reducing some defensive systems effectiveness. Exogenous chemicals may be added to boost defence systems. Treatment with NG dramatically decreased the production of TBARS in the mouse thyroid. According to reports, NG prevents lipid peroxidation by H_2O_2.[44]

Additionally, it has been demonstrated that melatonin, ascorbic acid, and Ocimum flavonoids guard against lipid peroxidation in mice.[45] There are several potential strategies for reducing the damage that oxidative stress causes to macromolecular structures. The preventive effectiveness of NG against radiation-induced lipid peroxidation was further evaluated by detecting some antioxidants in the thyroid of mice exposed to UVB radiation. Whole-body UVB exposure reduces an organism's potential for general antioxidant defence and depletes known antioxidants like GSH.[39] Mice exposed to UVB radiations exhibited significantly reduced thyroid GSH activity. It has been observed that GSH depletion enhances lipid peroxidation since it is known to impair glutathione peroxidase activity both in vitro and in vivo. The current study shows a similar relationship between the decrease in GSH and the rise in lipid peroxidation. Treatment with NG considerably slowed the reduction in thyroid gland GSH content in mice. According to reports, NG's antioxidant activity is comparable to GSH.[43] According to our study, thyroid Glutathione Reductase (GR) activity significantly increased due to UVB exposure. According to some authors, the thyroid hormone impact directly contributes to the enhanced activity of glutathione reductase, and administration of peroxidative products to rats has been shown to dramatically promote the activity of several detoxification enzymes.[46] Catalase, hydrogen peroxide, and superoxide dismutase activity were significantly increased throughout our research. As per the previous study, hyperthyroid rats' hearts and erythrocytes had greater SOD, CAT, and H2O2 activity levels.[47] The expression of antioxidant enzymes may be impacted by hyperthyroidism depending on the cell type, mitochondrial activity, and ROS concentration inside the cell.[48] The lipid-soluble antioxidant probucol has been shown in both in vivo and in vitro studies to halt oxidative damage caused by gamma radiation.[49] Supplemental NG and probucol have been demonstrated to boost antioxidant reserve in rabbits fed a high cholesterol diet.[50]

The alterations in thyroid gland function in the exposed group were further supported by the histological examination of the thyroid follicles in the current investigation, which exhibited glaring light microscopic and ultrastructural abnormalities. In many instances, these follicles were disorganised and lost. While some follicles seemed to have involuted walls, others had fragmented follicular walls. While other cells had vacuolated cytoplasm, the follicular cells lost their epithelial covering in the lumina. Additionally, some follicular cells possessed darkly coloured nuclei, and interfollicular septa had cellular infiltration. The efficiency of TSH's gland-stimulating actions and hyperactivity might be employed to explain the thyroid follicles' histological alterations. Morphometrical data supported the changes in epithelial height. Our histological findings agree with those of other investigations. Because low thyroid hormone levels weaken the feedback inhibition of TSH and induce the thyrotrophs to secrete more TSH, chronic stimulation of the pituitary can give rise to thyroid gland malignancy, which manifests as follicular cell proliferation, colloid region shrinkage, and thyroid gland enlargement.[51] Yu highlighted that the reactive oxygen species (ROS) and lipid peroxidation produced by chlorpyrifos may have contributed to the death and deterioration of follicular cells.[52] Furthermore, nuclear alterations are a marker of Necrosis and apoptosis of cells, according to previous study.[53] The thyroid follicles in the group treated with naringin (80 mg/kg) were healthy and free of any pathological alterations. The study suggests that under typical circumstances, naringin has no negative consequences. The results suggest that the test flavonoids may be useful in managing hyperthyroidism.

Immunohistochemistry and immunofluorescence revealed increased amounts of cells positive for THRβ-1, Caspase and NOS-2 after UVB exposure. In the present study, the expression of THRβ-1 was lowered down in UVB exposed group while enhanced Caspase-3 and NOS-2 expression was detected in thyroid follicular cells in the UVB exposed group. In UVB irradiated group thyroid showed reduced expression and fluorescence of TRHβ-1 in thyroid follicular cell. The administration of naringin in UVB irradiated group showed enhanced expression and fluorescence in thyroid follicles. Thyroid hormones regulate mammals' growth, development and differentiation. This is because as the oxidative stress generated by UVB radiations breaks the thyroid hormones receptors, as such the breakdown of receptors did not allow the antibodies to bind over the receptors, thus the expression got decreased.[54]Thus, the study showed the degenerative effect of UVB radiations which lowered down the expression of TRHβ-1 in UVB exposed group as compared to control group. To date, little information exists on the effect of UVB radiation on caspase-3 expression as well as the effect of naringin in correction of the UVB radiation's alteration. In the present study, UVB radiation induced significant increase in caspase-3 expression indicating UVB provokes apoptosis in the mice thyroid cells. Interestingly, the addition of naringin improved the condition by lowering the caspase-3 expressions. Apoptosis is a physiological process of selected cell deletion. As an antagonist of cell proliferation, apoptosis contributes to keeping the cell number in thyroid tissue and helps to remove superfluous and damaged cells, but excessive apoptosis could cause destruction of thyroid tissue.[55] In a similar study in China to explore the effects of expressions of caspase-3 in mice testes at different concentrations and time of lead acetate, it increased the expressions of caspase-3, which induces apoptosis of germ cells.[56] The occurrence of thyroid cell apoptosis and the expression of caspase-3 in the adult male mice following UVB radiation administration were investigated. Compared with the control group, the protein levels of caspase-3 were significantly higher in UVB exposed group. Excessive NO production due to elevated NOS-2 causes cytotoxicity and induces germ cell apoptosis.[57]Furthermore, NO is a signalling molecule that has an important role in the pathogenesis of inflammation.[58] In UVB irradiated group, NOS-2 expression was detected. These results suggested that thyroid follicular cells are also affected by UVB radiation. Furthermore, UVB exposure led to inflammation in the thyroid follicular cell apoptosis, as demonstrated in the DAB and DAPI-FITC assay.

Nevertheless, it is necessary to establish therapeutic dosages for human treatment. At dosages of 1 g/day or greater, naringin is widely considered safe, and no side effects have been noted. Because the concentration of naringin utilised in this investigation equates to 88.09 mg/kg in a 70-kg human, based on a comparison of body surface area, the flavonoid dosages employed in this study are thus more or less realistic for people.[59]

There have been reports of NG's ability to scavenge free radicals. In vitro studies have shown that it can scavenge the free radicals OH, O_2^-, DPPH, and ABTS.[60] When O_2^- is inhibited by NG, less hydrogen peroxide and hence less OH is produced. While oxygen receives one electron and generates superoxide at

a rate constant of $1.9X10^{10}M^{-1}s^{-1}$, NG rapidly accepts electrons at a rate of $(1.0\pm0.1)X10^{10}M^{-1}s^{-1}$.[61] As a result, oxygen and NG continually conflict with one another for available electrons. By removing the radiation-induced electrons that oxygen molecules compete with, NG molecules may limit potential harm. One of NG's key characteristics in its defence against oxidative stress is its capacity to scavenge free radicals. Earlier investigations, found that NG provided radioprotection by scavenging free radicals and improving the antioxidant state of radio-exposed mice.[60] Numerous medications, including felodipine, cyclosporine, simvastatin, and lovastatin, are affected by NG and other grapefruit flavonoids. According to reports, NG contains antiestrogenic, antiatherogenic, anti-apoptosis[62] and cholesterol-lowering properties[9]. According to reports, up to 2 g in humans, it is non-toxic. NG may function as a radioprotector in individuals undergoing radiation because its protective dosage is 2 mg/kg, and the human tolerance dose is larger than 2 g.[63] Human radioprotection of normal tissue is a promising way to advance radiotherapy. The key characteristic of a radioprotector that only protects normal tissue is the exclusion of tumour tissue. It might not be impossible for NG to protect tumours in some cases. As a result, further study is required in this area before NG may be used in radiation for cancer. More research is planned to determine if NG is specifically rejected from neoplastic cells and whether it could offer distinct radiation protection.

5. Conclusion

Our findings conclusively demonstrate that giving flavonoids to mice with UVB irradiation-induced hyperthyroidism caused a drop in thyroid hormone levels. Finally, one of the test flavonoids, naringin, may be able to reverse the changes induced by UVB exposure. UVB exposure has significant negative impacts on thyroid function and weight. When the thyroid and body weight are out of balance, naringin can control them while simultaneously acting as a scavenger. The study also indicates how naringin has antioxidant capabilities that can reduce ROS, enhance antioxidant status via free radical scavenging pathways, and quench ROS to protect against UVB irradiation-induced hyperthyroidism. Our study's results are novel to demonstrate how naringin can protect male mice against UVB-induced hyperthyroidism and prevent the deformation of thyroid follicles and the alterations in the expression of THRβ-1, caspase-3 and NOS-2 antibodies. As a result, naringin has a potential antioxidant and anti-apoptosis and anti-inflammation activity and is a strong medication for the treatment of hyperthyroidism. Clarifying the chemical mechanisms that result in naringin's antioxidant, anti-apoptosis and anti-inflammation effect will require more study. The findings suggest that environmental toxicants like radiations effect the endocrine systems and disturbed the metabolism thus when evaluating foods for potential health benefits, it may be important to characterise the naringin concentration of such foods.

6. Conflict of interest statement

The authors declare that there is no conflict of interest.

7. Authors' contributions

Payal Mahobiya designed the experiment plan. Shashank Shakyawal managed the experimental animals, performed the treatment and completed data analysis and wrote the manuscript. Gayatri Rai and Shashank contributed to the editing and completion of the manuscript. All the authors read and approved the final manuscript.

8. Acknowledgment

This research work was financially assisted by UGC NON-NET FELLOWSHIP for carrying out this research work successfully. The authors wish to thank the Department of Zoology, School of Biological Sciences, Dr. Harisingh Gour Vishwavidyalaya, Sagar, M.P., India, for providing infrastructure facilities.

9. References

1. S. Neugart, S. Monika, UVB and UVA as eustressors in horticultural and agricultural crops, Scientia Horticulturae. 234 (2018) 370-381.

2. A. Andrady, P. Aucamp, A. Bais, C. Ballare, L. Bjorn, J.R. Bornman, M. Caldwell, T. Callaghan, A.P. Cullen, D.J. Erickson, F.R. de Gruijl, Environmental effects of ozone depletion and its interactions with climate change, Progress report. 2005.

3. E. Ausó Monreal, R. Lavado Autric, E. Cuevas, F. Escobar del Rey, G. Morreale de Escobar, P. Berbel, A moderate and transient deficiency of maternal thyroid function at the beginning of fetal neocorticogenesis alters neuronal migration, Endo. Society. 145 (2004) 4037-4047.

4. M.E. Gilbert, J. Rovet, Z. Chen, N. Koibuchi, Developmental thyroid hormone disruption: prevalence, environmental contaminants and neurodevelopmental consequences, Neurotoxicology. 33 (2012) 842-852.

5. M.G. Akande, M. Shittu, C. Uchendu, L.S. Yaqub, Taurine ameliorated thyroid function in rats co-administered with chlorpyrifos and lead, Vet Res Commun. 40 (2016) 123-129.

6. M. Schriks, J.M. Roessig, A.J. Murk, J.D. Furlow, Thyroid hormone receptor isoform selectivity of thyroid hormone disrupting compounds quantified with an in vitro reporter gene assay, Environmental Toxicology and Pharmacology. 23 (2007) 302-307.

7. S.J. Tsai, C.S. Huang, M.C. Mong, W.Y. Kam, H.Y. Huang, M.C. Yin, Anti-inflammatory and antifibrotic effects of naringenin in diabetic mice, Journal of agricultural and food chemistry. 60 (2012) 514-521.

8. R.P. Constantin, R.P. Constantin, A. Bracht, N.S. Yamamoto, E.L. Ishii-Iwamoto, J. Constantin, Molecular mechanisms of citrus flavanones on hepatic gluconeogenesis, Fitoterapia. 92 (2014) 148-162.

9. S. Shakyawal, G. Rai, P. Mahobiya, Retaliating Properties of Naringin: A Mini-Review, American Journal of Biomedical Science & Research. (2022).

10. A.K. Chandra, N. De, Catechin induced modulation in the activities of thyroid hormone synthesizing enzymes leading to hypothyroidism, Molecular and cellular biochemistry. 374 (2013) 37-48.

11. A.C. Ferreira, J.C. Neto, A.C. da Silva, R.M. Kuster, D.P. Carvalho, Inhibition of thyroid peroxidase by Myrcia uniflora flavonoids, Chemical research in toxicology. 19 (2006) 351-355.

12. A.J. Akindele, A.A. Adeneye, O.S. Salau, M.O. Sofidiya, A.S. Benebo, Dose and time-dependent sub-chronic toxicity study of hydroethanolic leaf extract of Flabellaria paniculata Cav.(Malpighiaceae) in rodents, Frontiers in pharmacology. 5 (2014) 78.

13. Tveden-Nyborg, P., Bergmann, T. K., Jessen, N., Simonsen, U., & Lykkesfeldt, J. (2021). BCPT policy for experimental and clinical studies. Basic Clin Pharmacol Toxicol, 128(1), 4-8.

14. A. Kumar, A. Prakash, S. Dogra, Naringin alleviates cognitive impairment, mitochondrial dysfunction and oxidative stress induced by D-galactose in mice, Food and Chemical Toxicology. 48 (2010) 626-632.

15. M. Sachidhanandam, S.N. Singh, A.K. Salhan, U.S. Ray, Evaluation of plasma hormone concentrations using Enzyme-Immunoassay/Enzyme-linked Immunosorbent assay in healthy Indian men: Effect of ethnicity, Indian Journal of Clinical Biochemistry. 25 (2010) 153-157.

16. Z.A. Placer, L.L. Cushman, B.C. Johnson, Estimation of lipid peroxidation, malindialdehyde in biochemical system, Anal. Biochem. 16 (1996) 359-367.

17. H.U. Bergmeyer. Methods of enzymatic analysis, Weinheim, Germany: Verlag Chemie. 3 (1983) 273-286.

18. C. Beauchamp, I. Fridovich, Superoxide dismutase: improved assays and an assay applicable to acrylamide gels, Analytical biochemistry. 44 (1971) 276-87.

19. J. Sedlak, R.H. Lindsay, Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent, Analytical biochemistry. 25 (1968) 192-205.

20. A.K. Sinha, Colorimetric assay of catalase, Analytical biochemistry. 47 (1972) 389-394.

21. I.N. Carlberg, B.E. Mannervik, Purification and characterization of the flavoenzyme glutathione reductase from rat liver, Journal of biological chemistry. 250 (1975) 5475-5480.

22. I. Guevara, J. Iwanejko, A. Dembińska-Kieć, J. Pankiewicz, A. Wanat, P. Anna, I. Gołabek, S. Bartuś, M. Malczewska-Malec, A. Szczudlik, Determination of nitrite/nitrate in human biological material by the simple Griess reaction, J. Clinica chimica acta. 274 (1998) 177-188.

23. O. Myhre, J.M. Andersen, H. Aarnes, F. Fonnum, Evaluation of the probes 2', 7'-dichlorofluorescin diacetate, luminol, and lucigenin as indicators of reactive species formation, Biochemical pharmacology. 65 (2003) 1575-1582.

24. S. Singh, S.K. Trigun, Activation of neuronal nitric oxide synthase in cerebellum of chronic hepatic encephalopathy rats is associated with up-regulation of NADPH-producing pathway, The Cerebellum. 9 (2010) 384-397.

25. M. Stefanini, C.D. Martino, L. Zamboni, Fixation of ejaculated spermatozoa for electron microscopy, Nature. 216 (1967) 173-174.

26. R. Srivastava, C.M. Chaturvedi, Age, photoperiod and estrogen dependent variations in the shell gland and the expression of AVT in the ovary of Japanese quail, Steroids. 77 (2012) 578-588.

27. M.K. Niranjan, R. Srivastava, Expression of estrogen receptor alpha in developing brain, ovary and shell gland of Gallus gallus domesticus: Impact of stress and estrogen, Steroids. 146 (2019) 21-33.

28. F.I. Wolf, A. Torsello, B. Tedesco, S. Fasanella, A. Boninsegna, M. D'Ascenzo, C. Grassi, G.B. Azzena, A. Cittadini, 50-Hz extremely low frequency electromagnetic fields enhance cell proliferation and DNA damage: possible involvement of a redox mechanism, Biochimica et Biophysica Acta (BBA)-Molecular Cell Research. 1743 (2005) 120-129.

29. C. Graham, M.R. Cook, A. Sastre, M.M. Gerkovich, R. Kavet, Cardiac autonomic control mechanisms in power-frequency magnetic fields: a multistudy analysis, Environmental health perspectives. 108 (2000) 737-42.

30. K. Daino, M. Nishimura, T. Imaoka, M. Takabatake, T. Morioka, Y. Nishimura, Y. Shimada, S. Kakinuma, Epigenetic dysregulation of key developmental genes in radiation-induced rat mammary carcinomas, International journal of cancer. 143 (2018) 343-354.

31. S. Sharma, N. Singla, V.D. Chadha, D.K. Dhawan, A concept of radiation hormesis: stimulation of antioxidant machinery in rats by low dose ionizing radiation, Hellenic journal of nuclear medicine. 22 (2019) 43-48.

32. W.F. Ganong, Review of medical physiology, Dynamics of blood and lymph flow. 30 (1995) 525-541.

33. P. Venditti, S.D. Meo, Thyroid hormone-induced oxidative stress, Cellular and Molecular Life Sciences. 63 (2006) 414-434.

34. M. Petrulea, A. Muresan, I. Duncea, Oxidative stress and antioxidant status in hypo-and hyperthyroidism, Antioxidant enzyme. 8 (2012) 197-236.

35. B. Sinnott, E. Ron, A.B. Schneider, Exposing the thyroid to radiation: a review of its current extent, risks, and implications, Endocrine reviews. 31 (2010) 756-773.

36. A. Guerrero, R. Pamplona, M. Portero-Otín, G. Barja, M. López-Torres, Effect of thyroid status on lipid composition and peroxidation in the mouse liver, Free Radical Biology and Medicine. 26 (1999) 73-80.

37. M.T. Elnakish, A.A. Ahmed, P.J. Mohler, P.M. Janssen, Role of oxidative stress in thyroid hormone-induced cardiomyocyte hypertrophy and associated cardiac dysfunction: an undisclosed story, Oxidative Medicine and Cellular Longevity. 2015.

38. G. Rao, R. Verma, A. Mukherjee, C. Haldar, N.K. Agrawal, Melatonin alleviates hyperthyroidism induced oxidative stress and neuronal cell death in hippocampus of aged female golden hamster, Mesocricetus auratus, Experimental Gerontology. 82 (2016) 125-130.

39. S.K. Niture, R. Khatri, A.K. Jaiswal, Regulation of Nrf2—an update, Free Radical Biology and Medicine. 66 (2014) 36-44.

40. M. Karbownik, R.J. Reiter, Antioxidative effects of melatonin in protection against cellular damage caused by ionizing radiation, Proceedings of the Society for Experimental Biology and Medicine. 225 (2000) 9-22.

41. S. Naazeri, M. Rostamian, M. Hedayati, Impact of thyroid dysfunction on antioxidant capacity, superoxide dismutase and catalase activity. (2014) 51-54.

42. G.C. Jagetia, V.A. Venkatesha, T.K. Reddy, Naringin, a citrus flavonone, protects against radiation-induced chromosome damage in mouse bone marrow, Mutagenesis. 18 (2003) 337-343.

43. M. Koc, S. Taysi, M. Emin Buyukokuroglu, N. Bakan, The effect of melatonin against oxidative damage during total-body irradiation in rats, Radiation research. 160 (2003) 251-255.

44. S.I. Kanno, A. Shouji, K. Asou, M. Ishikawa, Effects of naringin on hydrogen peroxide-induced cytotoxicity and apoptosis in P388 cells, Journal of pharmacological sciences. 92 (2003) 166-170.

45. P. Uma Devi, A. Ganasoundari, B. Vrinda, K.K. Srinivasan, M.K. Unnikrishnan, Radiation protection by the ocimum flavonoids orientin and vicenin: mechanisms of action, Radiation research. 154 (2000) 455-60.

46. P. Morini, E. Casalino, C. Sblano, C. Landriscina. The response of rat liver lipid peroxidation, antioxidant enzyme activities and lathione concentration to the thyroid hormone, Int. J. Biochem. 23(1991) 1025-1030.

47. M. Messarah, M. Saoudi, A. Boumendjel, M.S. Boulakoud, A. El Feki, Oxidative stress induced by thyroid dysfunction in rat erythrocytes and heart, Environmental toxicology and pharmacology. 31 (2011) 33-41.

48. M. Costilla, R. Macri Delbono, A. Klecha, G.A. Cremaschi, M.L. Barreiro Arcos, Oxidative stress produced by hyperthyroidism status induces the antioxidant enzyme transcription through the activation of the Nrf-2 factor in lymphoid tissues of Balb/c mice, Oxidative medicine and cellular longevity. 2019.

49. M.E. Bonsack, I. Felemovicius, M.L. Baptista, J.P. Delaney, Radioprotection of the intestinal mucosa of rats by probucol, Radiation research. 151 (1999) 69-73.

50. Jeon SM, Bok SH, Jang MK, Kim YH, Nam KT, Jeong TS, Park YB, Choi MS. Comparison of antioxidant effects of naringin and probucol in cholesterol-fed rabbits. Clinica Chimica Acta. 2002 Mar 1;317(1-2):181-90.

51. M.I. Chiamolera, F.E. Wondisford, Thyrotropin-releasing hormone and the thyroid hormone feedback mechanism, J.E. 150 (2009) 1091-1096.

52. F. Yu, Z. Wang, B. Ju, Y. Wang, J. Wang, D. Bai, T. Pathology, Apoptotic effect of organophosphorus insecticide chlorpyrifos on mouse retina in vivo via oxidative stress and protection of combination of vitamins C and E, J. Expe.Toxicol. Pathology. 59 (2008) 415-423.

53. M. Zhang, M. Jiang, Y. Bi, H. Zhu, Z. Zhou, J. Sha, Autophagy and apoptosis act as partners to induce germ cell death after heat stress in mice, J.P.o.7 (2012) e41412.

54. J.H. Oppenheimer, The molecular basis of thyroid hormone action: scattered pieces of jigsaw puzzle, Progress in clinical and biological research. 74 (1981) 45-55.

55. J.H. Richburg, The relevance of spontaneous-and chemically-induced alterations in testicular germ cell apoptosis to toxicology, Toxicology letters. 112 (2000) 79-86.

56. Z.H. Yan, W.A. Chunhong, Z.H. Duanlian, Effects of lead on expressions of TGFβ1 and Caspase-3 in mice testes, Chinese Journal of Public Health. 22 (2006) 869-870.

57. Y. Lue, A.P. Sinha Hikim, C. Wang, A. Leung, R.S. Swerdloff, Functional role of inducible nitric oxide synthase in the induction of male germ cell apoptosis, regulation of sperm number, and determination of testes size: evidence from null mutant mice, Endocrinology. 144 (2003) 3092-100.

58. J.N. Sharma, A. Al-Omran, S.S. Parvathy, Role of nitric oxide in inflammatory diseases, Inflammopharmacology.15 (2007) 252-259.

59. S. Reagan-Shaw, M. Nihal, N. Ahmad, Dose translation from animal to human studies revisited, The FASEB journal. 22 (2008) 659-661.

60. G.C. Jagetia, T.K. Reddy. The grapefruit flavanone naringin protects against the radiation-induced genomic instability in the mice bone marrow: a micronucleus study, Mutation Research/Genetic Toxicology and Environmental Mutagenesiss. 519 (2002) 37-48.

61. Z. Cai, X. Li, Y. Katsumura, Interaction of hydrated electron with dietary flavonoids and phenolic acids: Rate constants and transient spectra studied by pulse radiolysis, Free radical biology and medicine. 27 (1999) 822-829.

62. M. Ghanbari-Movahed, G. Jackson, M.H. Farzaei, A. Bishayee, A systematic review of the preventive and therapeutic effects of naringin against human malignancies, Frontiers in pharmacology. 12 (2021) 639840.

63. L.J. Wilcox, N.M. Borradaile, M.W. Huff, Antiatherogenic properties of naringenin, a citrus flavonoid, Cardiovascular drug reviews. 17 (1999) 160-178.

Hosted file

`Figures.docx` available at https://authorea.com/users/626234/articles/647750-biochemical-histopathological-and-immunohistochemical-study-of-the-repeated-environmental-toxicant-ultra-violet-b-radiations-induced-hyperthyroidism-alleviates-oxidative-stress-and-prevented-by-naringin-in-male-swiss-albino-mice