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ORIGINAL ARTICLE

Effects Study of Some Common Food Additives on Gene Expression of TPO Gene in Young Male Rats

SARMAD A. ABBAS[1], HUSSEIN A. AL-HAMADAWI[1]
[1]Department Biology, College of Education, University of Al-Qadisiyah, Iraq.
Corresponding author: Sarmad A. Abbas

ABSTRACT

This study was conducted in an animal house / Department of biology / Al-Qadisiyah University from November 2021 to February 2022, To study the effect of food additives on gene expression Thyroid Peroxidase (TPO) for the thyroid gland This study included 32 young male rats, It was randomly divided into four equal groups, The control group, which dosed 1 ml of normal drinking water for the duration of the experiment and for a period of 30 days, The first treatment group (T1) dosed tetrazine dye at a concentration of 25 mg / kg of body weight for 30 days, The second treatment group (T2) was dosed with chocolate brown dye HT at a concentration of 200 mg / kg of body weight during the 30-day trial period, The third treatment group (T3), which was dosed with the preservative sodium benzoate at a concentration of 25 mg / kg of body weight during the 30-day trial period.

The results of the statistical analysis of the molecular study of the gene expression of the enzyme thyroid peroxidase(TPO) No significant change ($p > 0.05$) In the first group (T1) , second group (T2) And the third group (T3) when compared to the control group.

And there was a significant increase ($p < 0.05$) in the third group (T3) compared to the first group (T1) and the second (T2). On the other hand, the results of the immunohistochemistry study of the immune reaction showed the presence of TPO enzyme in the cytoplasm and tops of the follicular cells of the thyroid gland in the control group, and the intensity of (TPO) immunostaining was high in the mentioned sites. s for the first treatment group (T1) and the second treatment group (T2), the immune reaction was weak, meaning that the (TPO) enzyme was absent its gene expression disappeared or decreased in the cytoplasm and the tops of the membranes of the vesicular cells of the thyroid gland. The results of the third treatment group (T3) also indicated that it retained the dye because the strength of the immunostaining of the enzyme (TPO) was clear, but with a lower intensity than the control group. (TPO) gene expression was present in the cytoplasm and tops of the follicular cells of this group, but less than that of the control group. We conclude that the additives have negative effects on the expression of the enzyme (TPO) enzyme, as they lead to a defect in the function of the thyroid gland and should be minimized, especially in children's food containing it.

Keyword: thyroid gland, immunnohistochemical ,tartrazine , benzoatsodium, chocolatebrown HT E 155, expression of TPO

INTRODUCTION

Because of the fast pace of life, people prefer to eat preserved foods and ready-made foods at the expense of eating fresh foods. In recent years, many restaurants and food and beverage manufacturers have become hotbeds of diseases due to the addition of certain chemicals to foods for the purpose of improving their appearance, composition, taste and preservation (Seetaramaiah et al., 2011). Food additives are substances that have no nutritional value added to foods with the aim of preserving them from spoilage or improving their physical characteristics such as taste, colour, texture and smell, making foods easy to prepare and cooking and increasing consumer attraction Food additives are many and varied, some of which add color to food or improve its appearance and a composition called food coloring. Some of them prevent biological and chemical spoilage and spoilage of food called preservatives, or they are added with the aim of giving certain flavors to food and they are called flavors. (Pundir & Rawal 2013). The excessive and continuous use of food additives is likely to expose the consumer to danger, especially in foods, drinks and juices that are eaten randomly. (Al-Harthy et al.,2016). The European Food Safety Authority(EFSA) agreed In 2010 and the World Health Organization (WHO), To develop a system for evaluating food additives and using special numbers preceded by the letter E, which refers to the word Europe, It showed the permissible limits for the use of these additives per dayAcceptable Daily Intake (ADI) (Carocho et al., 2014) .

These food additives are divided into:

Food Colors are substances that are added to foodstuffs, drinks, juices, cosmetics and medicines with the aim of giving a special color or reaching the natural color of food. These dyes have a visual and sensory effect on the consumer's choice of the manufactured product(Arnold et al., 2012; Bawazir, 2016). The most common formulations are the dye Carmoisine (E122) and the dye Tartrazine (E102) is a common synthetic azo dye that imparts a lemon yellow color and is widely used in the manufacture of many food products such as sweets, ice cream, jams and soft drinks, as well as In pharmaceuticals and cosmetics (Floriano, et al.,2018). Chocolate brown dye HT E 155 bis -azo,it odorless, brown in color, and widely used for coloring various types of foods and beverages (Neshe , et al 2016) . The researcher noted (De campos & maria,2013) Azo-dye industrial dyes are stable and do not decompose, especially in the aquatic environment because they contain aromatic and toxic rings and may cause genetic mutations, some of which are carcinogenic. There is a lot of research that has shown that metabolic disorders and toxicity that occur due to these industrial dyes by noting their effect on rats and some other types of milk, in addition, many azo compounds have a toxic or carcinogenic effect on laboratory animals. (Reyes et al., 1996 ; El Golli,2016; Bawazir, 2016).

Preservatives They are substances that are added to food to maintain in order to extend its shelf life and the use of these materials has become more important in order to provide a wide variety of foods for long periods of time and for a large number of people. and yeasts) and its main purpose is to increase the preservation period and prevent chemical and biological spoilage of foods As chemical corruption is the oxidation and coloration of food in brown, while biological corruption works on the decomposition of food by microorganisms and producing undesirable changes in the appearance and taste of food as well as its nutritional value and the production of toxins that pose a great danger to human health.(Stanojevic et al., 2009; Sharma, 2015) classified into natural preservatives such as sugar and salt, organic acids such as acetic acid, lactic acid, honey, spices and their oil, and artificial preservatives such as Sodium benzoate(SB), Sodium acetate (SA), Potassium Sorbate (PS) and Butyl Parabens (BP) Watch widely used in pharmaceutical and food industries (Gupta,2021).

Thyroid Gland: The thyroid and parathyroid glands are endocrine glands located in the neck and are characterized by the absence of ducts. The thyroid gland is one of the largest endocrine glands and produces thyroid hormonestriiodothyronine (T3) thyroxine (T4) , These hormones are directly related to the body's metabolism and

play a particularly important role in the maturation of the brain during fetal development and stimulating physical and psychological growth, in addition to their vital role in calcium and phosphate metabolism,It is distinguished from the rest of the glands by the ability of its cells to absorb iodine and to produce and store its hormones in the gland itself and secrete them when needed where the thyroid gland secretes its hormones directly into a stream where these hormones are transferred to the target cells (Ravichand etal.,2005; Mohebati and Shaha, 2012). The thyroid gland consists of two lobes and resembles the shape of a butterfly. The two lobes are connected by Isthmus It is called the third lobe (Akudu et al.,2018). The thyroid gland is made up of three main types of cells: C Cell, thyroid cells and endothelial cells C cells These are epithelial cells associated with follicles that produce calcitonin Thyroid cells are polarized epithelial cells organized into monolayers in follicles containing iodothyroglobulin locatedlocated in Colloid It is responsible for the production of hormones derived from thyroglobulin Whoever T3 & T4 A dense network of endothelial cells surrounds each thyroid follicle (Degosserie, 2018). The production and secretion of hormones into the blood is regulated by Negative fee back mechanism Hypothalamic-pituitary-thyroid axis by hormone Thyroid Stimulating Hormone (TSH) It plays a major role in stimulating the activity of the thyroid gland and stimulates secretion (TSH) hormone (TRH) Thyrotropin releasing hormone which secretes from Hypothalamus It stimulates the pituitary gland to release a hormone (TSH) which in turn stimulates The thyroid gland secretes its hormones (T3,T4) When there is a deficiency in the bloodstream (Qatato et al., 2018).

MATERIALS AND METHODS

Experimental animals: This study was conducted in an animal house Located in the Department of biology/ Faculty of Education / Al-Qadisiyah University Used 32 young white male rats Which was brought from the animal house in the Faculty of Science/ Al-Qadisiyah University The weights of the rats were calculated (90-110)g It was placed in plastic cages with dimensions 50*15*35 cm And in each cage, eight rats, and in a room of space 4*3m² All rats were exposed to the same temperature conditions and were 20-25 c̆ Where organized by air conditioner. With an average of 12 hours of light and 12 hours of darkness, the rats were given a concentrated diet for free feeding and water. It was randomly distributed and left for two weeks to acclimatize Their weight was calculated to determine the appropriate dose, and they were divided into four groups, each group of eight animals.

Experiment design: The animals were divided into four groups, as follows :

1 control group They were dosed with 1 ml of normal drinking water and diet during the 30-day experimental period.

2 The first treatment group (T1): They were dosed 1 ml of Tartrazine dye at a concentration of 25 mg/kg of body weight and the diet during the 30-day trial period.

3 The second treatment group (T2): They were dosed 1 ml of Chocolate Brown HT E155 dye at a concentration of 200 mg / kg of body weight and the diet during the 30-day trial period.

4 The third treatment group (T3): dosed 1 ml of the preservative sodium benzoate at a concentration of 50 mg / kg of body weight and the diet throughout the 30-day trial period.

Animal Sacrifice: After 24 hours the last dosing, young male white rats were anesthetized with (ketamin and xylaine) Partially, and then blood was drawn using a 5 ml medical syringe from the heart directly and during anesthesia Heart Puncture The blood was placed in plastic tubes and placed in a centrifuge for 15 minutes at a speed of (3000 rpm). To obtain blood serum, it was drawn using micropipette after withdrawing the blood serum and keeping it in the marked Alapndrove tubes, it was placed at a temperature of (-20) C for examinations. The animals were dissected and the thyroid gland was removed, placed in petri dishes, a container of physiological solution, cleaned of residues, and part of it was placed with formalin at a concentration of 10% until the tissue sections were prepared.

Molecalar Study :

Quantitative Reverse Transcription Real – time PCR (RT – QPCR): assay Real-time quantitative polymerase chain reaction For the purpose of measuring the quantitative level of (mRNA transcript levels) o indicate the amount of Gene expression gene(TPO) and use gene (GAPDH) As a standard regulator gene for quantification of gene expression according to the manufacturer's method for kit As in the following steps.

1 Total RNA Extraction RNA has been extracted By using Trizol Kit And use it according to the company's instructions.

2 Assessing RNA Yield and quality has been detected RNA Extracted by using a special device Nanodrop Spectrophotometer using RNA ng/ml concentration DNA purity measurement RNA By reading the absorbance degree(260/280 nm) .

3 cDNA synthesis DNA synthesis method was used cDNA complement of DNA samples Extract using KIT Universal RT-PCR Kit (M-MLV) The manufacturing method was carried out according to the method of work KIT Enzyme is used M-MLV For the reverse transcription reaction, a long sized transcription product can be obtained.

4 Quantitative Real-Time PCR (qPCR) Check done (qPCR) for samples cDNA Experimental rat groups using EnTurbo™ SYBRGreen PCR SuperMix (2x Green star qPCR kit) Primers were used in this study to detect gene expression (Hatem,2012).

Table 1: primers that were used in the qRT-PCR reaction to detect gene expression

primer	Sequence		Amplicon
Tpo	AAGGAGCACTCTTTGGCAAC	F	119 bp
	TTGCGCAACTGCTTCTCAAC	R	124 bp
GAPDH	ATGCCCCCATGTTTGTGATG	F	124 bp
	TCCACGATGCCAAAGTTGTC	R	136 bp

5 Real-Time PCR data analysis: The data from the polymeric chain reaction is analyzed in real time quantitatively by using the . method (Livak and schmittgen, 2001) Which depends on extracting the relative quantity Relative Quantitive and absolute Quantitive Through the process of correcting and neutralizing the gene Target with control samples so that the results are biologically meaningful. Each target sample is corrected with a control sample to produce a defined level of relative expression.

Study Immunohistochemistry (IHC): The term immunohistochemistry is defined as the process of detecting specific antigens in biological tissues, such as proteins, and staining them with marked antibodies, depending on the principle of binding between antigens and Target antigens and Antibodies. In this way, the required protein is detected, and according to the manufacturer's instructions, the immunostaining was done For antibodies that recognize the target protein (thyroid peroxidase) in Thyroid tissue.

1 Preparation of solutions and reagents used in the technology Immunohistochemical

A Prepare (Phosphate buffered saline) PBS (0.01ml from PBS) Dissolve ten PBS tablets in 1000 ml of distilled water.

B Preparation Antigen Retrival solution Prepare 0.01ml of sodium citrate solution PH (6.0) (Stock Solution). Solution. A (0.1 ml citric acid) It is prepared by dissolving 21 gm of citric acid monohydrate (C 6 H 8 O 7 .H 2 O) in 1000 distilled water to obtain a solution with (6.0) pH.

C Solution.B(0.1 ml of sodium citrate solution) It is prepared by dissolving 29.4 g of tri-sodium citrate (C 6 H 5 Na 3 O 7 . 2 H 2O) in 1000 distilled water.

D working solution: Add 9 ml of solution (Stock solution A) 41 ml from (Stock solution B) to 450 ml distilled water.

E Preparation of the diluted primary antibody: Dilution of primary antibody :Monoclonal rat antithyroid peroxidase used at adilution range of 1:100 in formalin-fixed ,embedded sections.

Method Work: Using a microtome, sections of the paraffin-embedded tissues are cut to a thickness (5- 8Mm) They are placed

in a water bath and then the tissue sections are loaded onto special tissue strips(posive charge) To confirm the results, two tissue slices were made, one of them is a positive control (It is a glass slide containing a piece of tissue from another tissue stained with an enzyme immunoassay dye (TPO) And the second is negative control, which is a glass slide containing a tissue section of the thyroid tissue, but it is not added to it (Anti body –TPO).then Paraffin is removed from the sections by passing them twice with xylene for five minutes each time and then passing Slides with alcohol in concentration (100%) Twice for 5 minutes each time and then pass it in a series of concentrations (95% ,85 % ,75 %) of alcohol for 5 minutes for each concentration, respectively then The slides are incubated in an electric oven after being placed in a sodium citrate solution at a temperature 125C For 15 minutes, when they come out of the electric oven, they are left to cool at room temperature for 5-10 minutes, then the slides are washed with PBS solution for five minutes, and the process is repeated three times. Slides are incubated in solution (H2O2) concentrated 3% for 10 minutes To prevent peroxidase intrinsic activity, at room temperature, the sections were then washed with PBS solution three times for 5 minutes each time.then Incubate the slides with the solution Bovine Serum Albumin (BSA) At a concentration of 5% for 30 minutes, then washed three times with PBS solution for three minutes, Primary incubation of antibodies We add the primary antibody to each slide, which is the antibody Enzyme (TPO) incubated at 4˚C for a day, then wash slides three times with (PBS) solution for three minutes, Second incubation of the antibodies utilizing the surplus PBS solution Add 50 ml of secondary antibody conjugation solution and incubate for 20-30 minutes at room temperature, then wash the slides three times with PBS solution for three minutes.The Staining Incubate the slides with a solution Diaminobenzidine (DAB) The first DAB solution and the second DAB solution are mixed in a 1:1 ratio, and then the slides are washed with tap water when the required density is obtained. Then Put the stain-resistant slides in hematoxylin stain for three minutes, then examine them with a light microscope to control the intensity of staining after adding 1% of the acid differentiation solution, then wash them with tap water for 10 minutes, The slides are immersed in ethanol alcohol according to the following concentrations for five minutes for each concentration (75%, 85%, 95%, 100%), and the slides are again immersed in ethanol alcohol at a concentration of 100% for five minutes.Then The slides are immersed in xylene twice for three minutes in the end The slides were examined by a light microscope to observe the staining of the antibodies and the identification of the antigen required for the enzyme (TPO) in the histological section.

Statistical Analysis: The results are subject to statistical analysis to identify the significant differences between the rates of the studied criteria in the groups, and the significant differences were determined at the level of probability (P<0.05) by Using the statistical program (SPSS2010) The statistical analysis included obtaining the average Mean and Standard error ignificant differences were determined using one-way analysis of variance (ANOVA) and extract (LSD) (Schefler,1980).

RESULTS AND DISCUSSION

The results of the current study showed, as in Table No. (2), that there was no change at the level of significant(P>0.05) In the gene expression of genes in the first group treated with tetrazine dye (T1) , the second group treated with chocolate brown dye (T2) and the third group treated with the preservative sodium benzoate (T3) when compared to the control group. There was a significant increase(P<0.05) in the third group (T3) compared to the first group (T1) and the second group (T2). These changes in height, which reached a significant level, may be due to the activity of neurons and their secretion of stimulating hormones and an increase in the activity of the anterior lobe of the pituitary gland, which secretes different types of hormones, including an increase in thyroid stimulating hormone (TSH), which leads to an increase in thyroid activity, which is manifested by an increase in thyroid hormone (T3,T4). In the third group, low body weight compared to other groups (Qatato et al., 2018). The reason for the difference in gene expression in the first, second and third groups, compared to the control group, may be due to a change in the structure of proteins and cell differentiation, an increase or decrease in the expression of proteins responsible for the manufacture of thyroid hormones, or a disorder in the enzyme (TPO) and hormone imbalance (TSH), which is the main regulator of (TPO) gene expression in thyroid cells (Zarrilli etal., 1990).

Table 2: Effect of food additives on total RNA concentration and purity in thyroid tissue of young male albino rats.

Descriptives	
qRtPCR	(ΔCT Values)
C	1.43±15.01 AB
T1	0.40±16.30 B
T2	0.76±17.36 B
T3	0.39±14.22 A
LSD	2.656

The numbers represent averages ± standard errors

The results of the current study of the immunological reaction showed the presence of (TPO) enzyme in the cytoplasm and close to the plasma membrane of thyroid follicular cells in young male rats in the control group, and the intensity of (TPO) immunostaining was high in the mentioned sites as in Figure (1). The results of the third treatment group (T3) indicated that it preserved the dye because the strength of immunostaining of the enzyme (TPO) was clear, but in a lower intensity than the control group, meaning that the gene expression of (TPO) was present in the cytoplasm and tops of the follicular cells of this group, but less than the control group. As in Figure (1). The reason for the decrease in TPO gene expression in the thyroid tissues of the treatment groups (T1, T2) may be attributed to the free radicals and oxidative stress resulting from isotrazine and chocolate brown dyes, which cause disturbances in thyroid function and metabolic processes, including hormone formation (T3 & T4) (Kurebayashi et al.,1988; Shakoor et al.,2022) Some studies also indicated that low (TPO) gene expression is an early event for the emergence of malignant tumors in the follicular cells of the thyroid gland and may be used as an important predictive indicator for the occurrence of cancerous diseases and for the diagnosis of malignant tumors (Garcia et al.,1998; Caballero et al.,2015) In this study, we observed a decrease in TPO enzyme immunostaining in the first and second treatment group (T1, T2) that dosed the food dyes titrazine and chocolate brown, respectively It causes many cancerous diseases in different parts of the body (Muller et a.l,2017; Merinas-Amo et al.,2019).

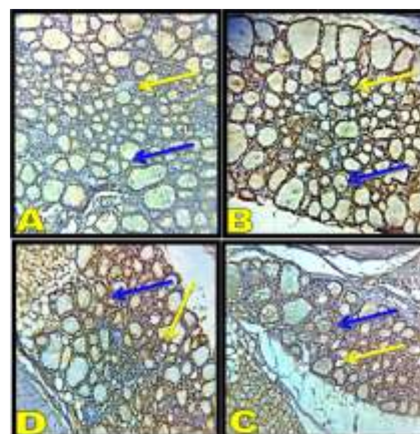

Figure (1) A cross section of thyroid tissue in young male albino rats showing the immune reaction to an enzyme(TPO) Picture (A) shows the control group, where we notice the intensity of TPO(++) enzyme immunostaining in Cytoplasm and tops of thyroid follicle cells (yellow arrow) and most of the follicles were circular and colloid-filled (blue arrow) as for the picture (C). (B) represents the immunoreactivity of TPO enzyme for the first treatment group and the second treatment group, respectively, where we notice the lack and disappearance of immunostaining for (TPO) enzyme (-) in the cytoplasm and tops of thyroid cells (blue arrow) and most of the vesicles were small (yellow arrow). As for the picture (D) representing the third treatment group, we note the presence of the immune reaction in the cytoplasm and tops of the follicular cells of the thyroid gland to TPO enzyme (+), but less compared to the control group, as we note hyperplasia.

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