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Research Article

# IMMUNE RESPONSE AFTER TARTRAZINE SUBCHRONIC INGESTION IN SWISS ALBINO MICE

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**ABSTRACT**

Objective: Tartrazine is one of the most widely used dyes in food products, drugs and cosmetics. The present study was conducted to evaluate the immune response after subchronic ingestion of Tartrazine in Swiss mice.

Methods: Tartrazine was administered to adult mice at doses of 0.45 and 1% for 13 weeks. Subsequently, 6 mice in each group were immunized with an emulsion of bovine serum albumin (BSA) and complete Freund's adjuvant (CFA).

Results: Our results show that Tartrazine induced a significant decrease in body weight in male and female treated with 0.45 and 1% (**p<0.01 and *p<0.05 respectively). However, spleen and thymus relative weight and titles of IgG anti-BSA were decreased significantly only in male treated groups (*p <0.05 and **p <0.01). The histological and cytological examinations revealed an expansion of medullar sinuses in the spleen of mice treated with 0.45% and 1% of Tartrazine with hemosiderin deposits and an inflammation with partial villous atrophy in the intestine, as well as cell density and morphology modification in thymus, spleen and intestine tissues of mice treated with 1%.

Conclusion: This study demonstrates that subchronic ingestion of Tartrazine at dose of 1% alters the humoral immune response in male mice and cellular lymphoid organs, thus it affects the immune system function.

**Keywords:** Food dye, Immunoglobulin G, lymphoid organs.

## INTRODUCTION

Synthetic colorants are very important class of food additives. They are widely used to compensate for loss of natural colors in food, which can be destroyed during processing and storage, and to provide the desired colored appearance.

Tartrazine is a salt of 3-carboxy-5-hydroxy-1 (p-sulfophenyl) -4-(sulfophenyl azo) pyrazolone, widely used in dye sweets, jellies, juices, jams, mustard, sodas, drugs and cosmetics [1, 2]. Also, this food colorant is many used in cooking in developing countries as a substitute for saffron [3]. The ADI for human is 0-7.5 mg/kg body weight [4].

Numerous studies are incriminated Tartrazine to be responsible for exacerbations of asthma, allergic rhinitis and urticaria in atopic patients [5, 6, 7, 8]. *In vitro,* Tartrazine induces a cytotoxic activity and an immunosuppressive effect on the human peripheral blood lymphocytes [9]. In addition, prolonged use of this dye increases the number of gastric mucosa eosinophils and lymphocytes of Wistar rats [10].

Other effects are attributed to Tartrazine, such as genotoxicity [11, 12], hyperactivity in children [13, 14] and reduction of fertility in swiss mice male [3].

Considerable knowledge about the immunotoxic insult by Tartrazine was not documented so far. Hence, this study is carried out in order to evaluate the humoral immune response to bovine serum albumin (BSA) after Tartrazine subchronic ingestion in swiss albino mice and to observe its toxic effects on the lymphoid organs structure.

## MATERIALS AND METHODS

### Chemicals

The chemical name of the colorant is Tartrazine also known as (FD & C Yellow No. 5, C.I. No. 19140, and Food Yellow No. 4). The purity of this azo dye is 86.7%, it was provided from Courtex International, France. The BSA, sulphuric acid, di sodium hydrogeno- phosphate, calcium chlorure, hematoxylin-eosin stains were purchased from Merck (Germany). The diamine orthophenylene, anti-mouse IgG biotinylated, extravidin peroxydase, tween 20, complete and incomplete freund's adjuvant, di potassium hydrogeno-phosphate, $H_2O_2$ were purchased from Sigma (USA). Potassium chloride, sodium chloride, potassium chlorure were purchased from Prolabo (France), ethanol was purchased from Biochem (UK), toluidine blue and fushin were purchased from Panreac (Spain).

### Experimental animals and treatments

Seventy two swiss albino mice aged of 4 weeks old and weighing (19.50±0.25) g obtained from Pasteur institute, Algiers, Algeria, were kept under proper conditions of ambient temperature and adequate humidity. Tartrazine was diluted in water. Mice were divided into three groups, each one including 12 males and 12 females. The first group was given drinking water as a control group, the second group was given drinking water containing 0.45% of Tartrazine and the third one was given drinking water containing 1% of Tartrazine, for a period of 13 weeks. Standard food pellets diet and water were given *ad libitum* for the duration of the experiment. Water was measured daily, whereas body weight was measured weekly. After 13 weeks of administration, 6 mice in each group were fasted for 18 hours. The animals were sacrificed on the day of necropsy by cervical dislocation and then spleen and thymus were removed and weighed. The relative weights of these organs compared with the body weight were calculated according to Chavalittumrong et al. [15] using the following formula: Relative organ weight = [organ weight (g) /body weight (g)] × 100. All the animals were used following the instructions of Oran University guidelines for animal use.

### Immunization protocol

After 13 weeks of experiment, 6 mice of every group received a first subcutaneous injection with 0.3 ml of a mixture. The solutions injected were prepared with 0.2 ml of an emulsified Freund's complete adjuvant and a volume of normal saline solution (NaCl 0.9%) containing 15 µg of BSA. The injection was distributed on 3 detached points along the backbone. A recall was made on the 14$^{th}$ day with an emulsion of BSA and the incomplete adjuvant of Freund. Blood was collected from orbital venous plexus on days (0, 7, 14, 21 and 28) to measure the content of the IgG anti-BSA by ELISA method using sera frozen after centrifuging the whole blood (3500 rpm for 15 min) (Sigma 4 K10 bioblock scientific, Germany).

### Cytological examination

After the sacrifice of mice and the withdrawal of their organs, the inner sides of the spleen and the thymus were immediately put in contact with the blade in order to take their print. The intestinal

mucous membranes were scraped with a blade and spread on another one. After eliminating excess cells, the blades were fixed for few seconds in pure acetone then stained by a polychrome stain (Toluidine blue 73%, Basic fushin 27% and ethanol 30% (V/V)) according to Hould [16].

**Histological examination**

Histological examination of thymus, spleen and intestine were performed. The organs were fixed in 10% buffered formalin solution for routine processing. Six microns thick paraffin sections were stained with hematoxylin and eosin and examined by light microscopy (Optica Axiom 5000, China).

**Length of villi**

The measurements of the length of villi were taken using a micrometer eyepiece. Length of villi was expressed in µm.

**Dosage of the IgG anti-BSA by ELISA**

A multi-well microtiter plate (Maxisorp; Nunc, Roskilde, Denmark) was coated with 100 µl of BSA (10 µg/ml) and incubated overnight at 4°C. The plates were after that washed with phosphate buffered saline containing 0.05% Tween 20, pH 7.4 (ELx50 Auto Strip washer, BioTEK instruments, USA). Residual free binding sites were blocked with 200 µl/ well with 2% of gelatine of fish PBS at 37°C for 1hour. After washing, 100 µl of serially diluted sera ($1/10^2$– $1/10^7$) was added in duplicate to each well and incubated at 37°C for 2 hours.

The plates were again washed and 100 µl of anti-mouse IgG biotinylated (1:2000) was added to the wells, the plates were left at 37°C of temperature for 1 hour and 30 min.

The plates were again washed and 100 µl of extravidin peroxydase (1:5000) was added to the wells, the plates were left for 30 min at 37°C of temperature. After further washing, peroxidase activity has been assayed by addition of 50 µl $H_2O_2$ (30%, 0.25 ml/l) associated with diamine-orthophenylene (0.5 mg/ml) in 0.05 M citrate buffer, pH 5.1. The stained wells were kept in the dark at room temperature for 30 min. The reaction has been stopped with 6N $H_2SO_4$ (50 µl/ well) then absorbance was measured at 492 nm with an automated ELISA reader (ELx 800 universal microplate reader, BioTEK instruments, USA). Positive titers were given as the last dilution with an optical density above the background.

**Statistical analysis**

Statistical analyses were performed using analysis of variance (ANOVA) followed by student *t*-test. The results were presented as means with standard errors and $p< 0.05$ was considered statistically significant.

**RESULTS**

**Body weight**

The mean values of body weight in treated groups and controls were similar during the first week. From the 4th week till the end of the administration period, body weights of males in the group treated with 0.45% and 1% of Tartrazine were lower than the body weight of males in the control group ($p<0.01$) (Figure 1). However, from the 1st to the 5th week and at the end of the experiment, female body weight decreased significantly in the groups treated with 0.45% and 1% Tartrazine compared with the body weight of the controls ($p<0.05$) (Figure 1).

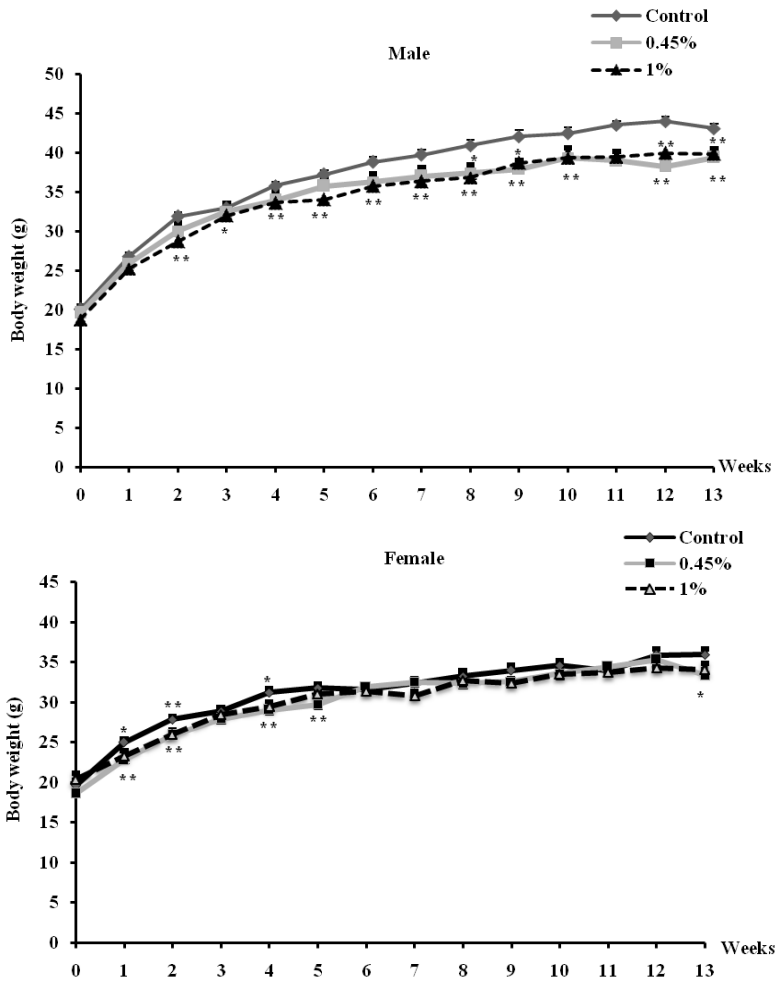



**Fig. 1: Body weight changes in male and female mice consuming a liquid containing Tartrazine for 13 weeks.**

Values are means ± SE for 6 animals in each group. Control, 0.45% Tartrazine, 1% Tartrazine. *p<0.05,**p<0.01 significantly different from the control group (student *t*-test).

**Organ relative weight**

The thymus relative weight of the male treated with 0.45% of Tartrazine decreased significantly ($p<0.05$) (Figure 2), however, the spleen relative weight increased significantly in the group treated with 0.45% of Tartrazine ($p<0.05$) and decreased in the group treated with 1% of Tartrazine ($p<0.05$) compared to the control group (Figure 2). In females, the relative weights of spleen and thymus were not affected in the group of mice treated with 0.45% and 1% of Tartrazine compared to the control group.

**Histological and cytological examination**

The cytological and histological studies were conducted in spleen, thymus and jejunum. We observed an expansion of medullar sinuses in the spleen of mice treated with 0.45% and 1% of Tartrazine with hemosiderin deposits (Figure 3). Cytological observations showed an increased macrophages cell population in both groups treated with Tartrazine and cell atrophy and malformation in groups treated with 1% Tartrazine compared to controls (Figure 4). These observations were similar in both sexes.

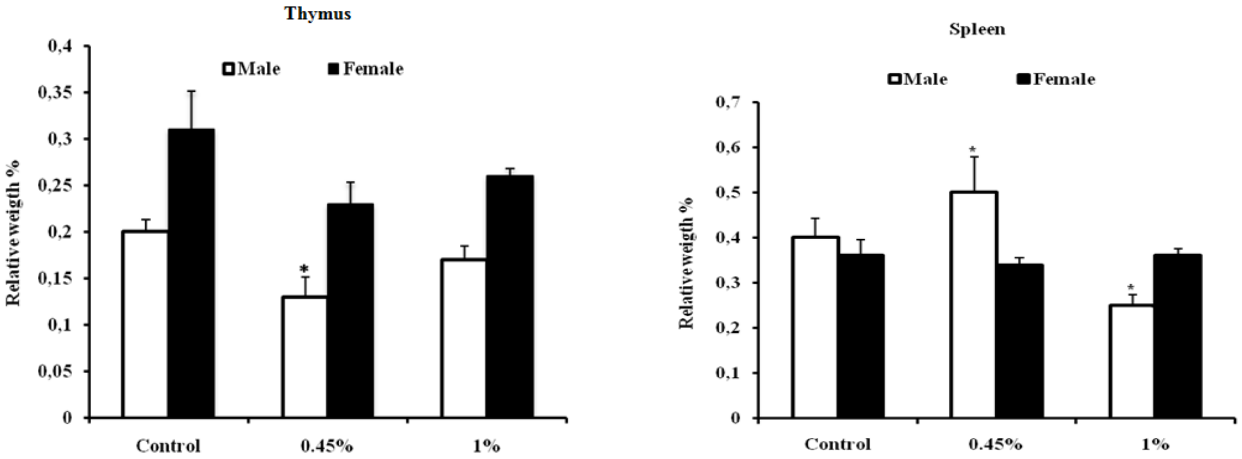



**Fig. 2: Effect of Tartrazine on the relative weights of the thymus and the spleen.**

Values are means ± SE for 6 animals in each group. Control, 0.45% Tartrazine, 1% Tartrazine. *$p<0.05$ significantly different from the control group (student *t*-test).

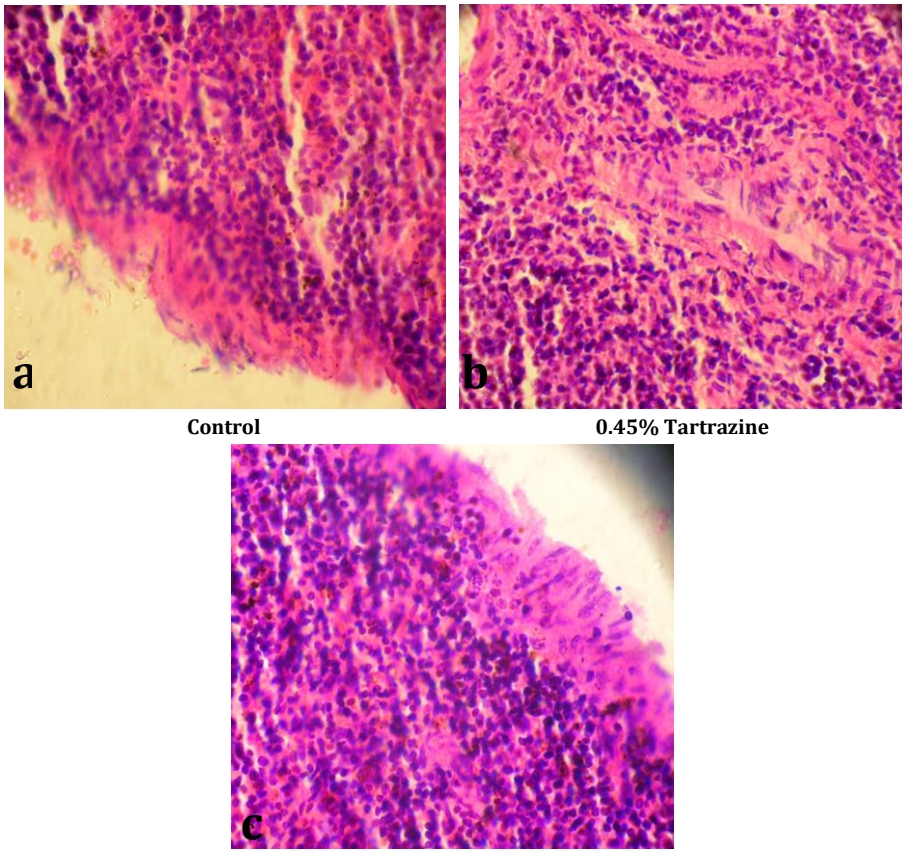
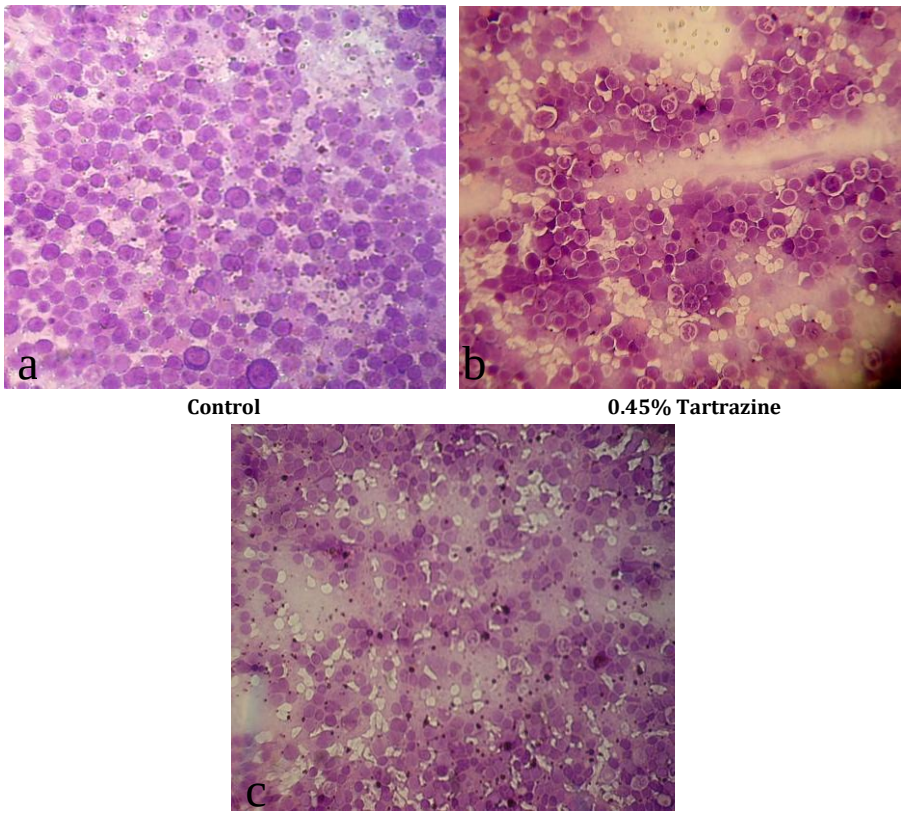



**Fig. 3: Histological sections of spleen stained with haematoxyline–eosine stain [magnification ×400].**

[a] Control mouse spleen. [b] Mouse treated with 0.45% of Tartrazine, a widening of the medullary sinus. [c] Mouse treated with 1% of Tartrazine, marked hemosiderin deposits with increased cell density.

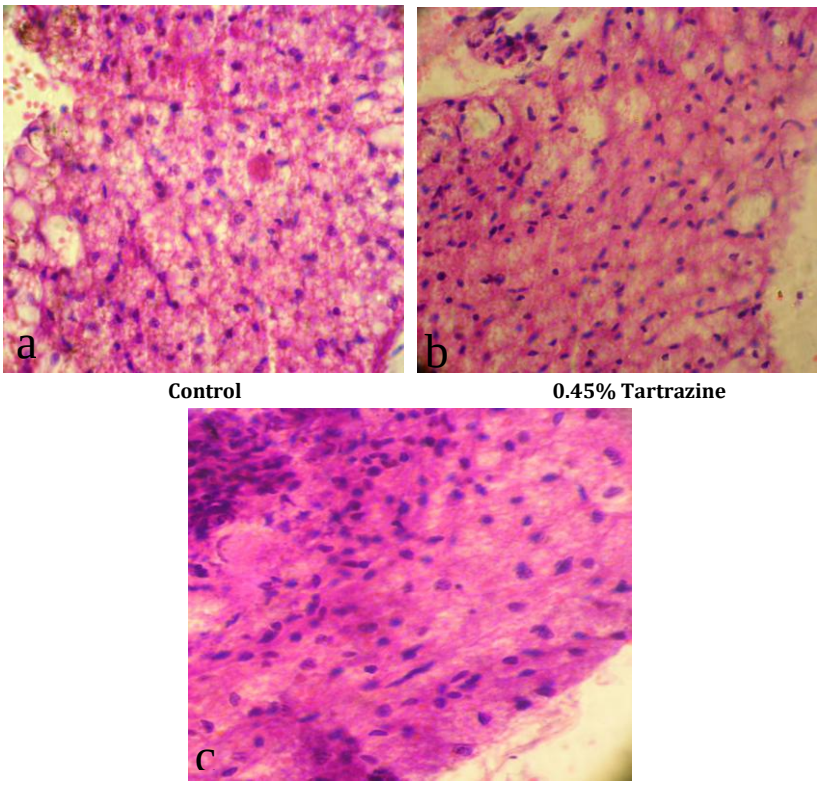



**Control** **0.45% Tartrazine**

**1% Tartrazine**

**Fig. 4: Cytological sections of spleen stained with polychrome stain [magnification ×400].**

[a] Control mouse spleen. [b] Mouse treated with 0.45% of Tartrazine, macrophages hyperplasia. [c] Mouse treated with 1% of Tartrazine, macrophages hyperplasia and cell atrophy and/or abnormal.

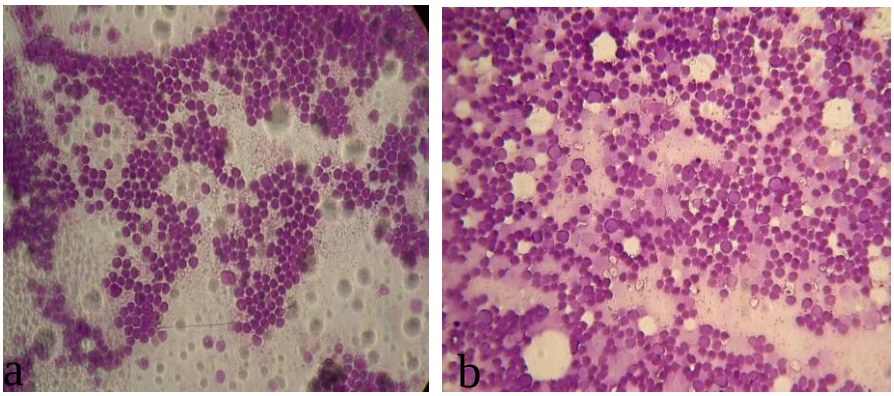



**Control** **0.45% Tartrazine**

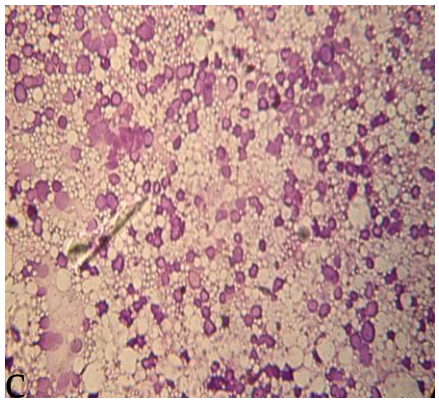
**1% Tartrazine**

**Fig. 5: Histological sections of thymus stained with haematoxyline–eosine stain [magnification×400].**

[a] Control mouse thymus. [b] Mouse treated with 0.45 % of Tartrazine showed normal appearance. [c] Mouse treated with 1% of Tartrazine showed cell hyperplasia.



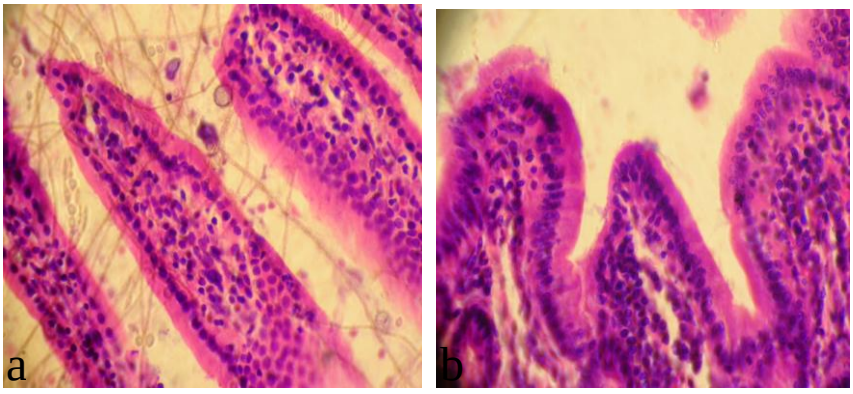

**Control** **0.45% Tartrazine**



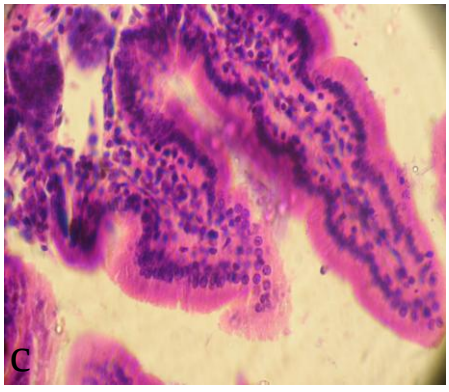

**1% Tartrazine**

**Fig. 6: Cytological sections of thymus stained with polychrome stain [magnification ×400].**

[a] Control mouse thymus. [b] Mouse treated with 0.45% of Tartrazine, no abnormalities detected. [c] Mouse treated with 1% of Tartrazine, cell atrophy and malformation.




**Control** **0.45% Tartrazine**




**1% Tartrazine**

**Fig. 7: Histological sections of jejunum stained with haematoxyline–eosine stain [magnification × 400].**

[a] Control mouse jejunum. [b] Mouse treated with 0.45 % of Tartrazine showed a partial atrophy. [c] Mouse treated with 1% of Tartrazine showed immune cell hyperplasia.

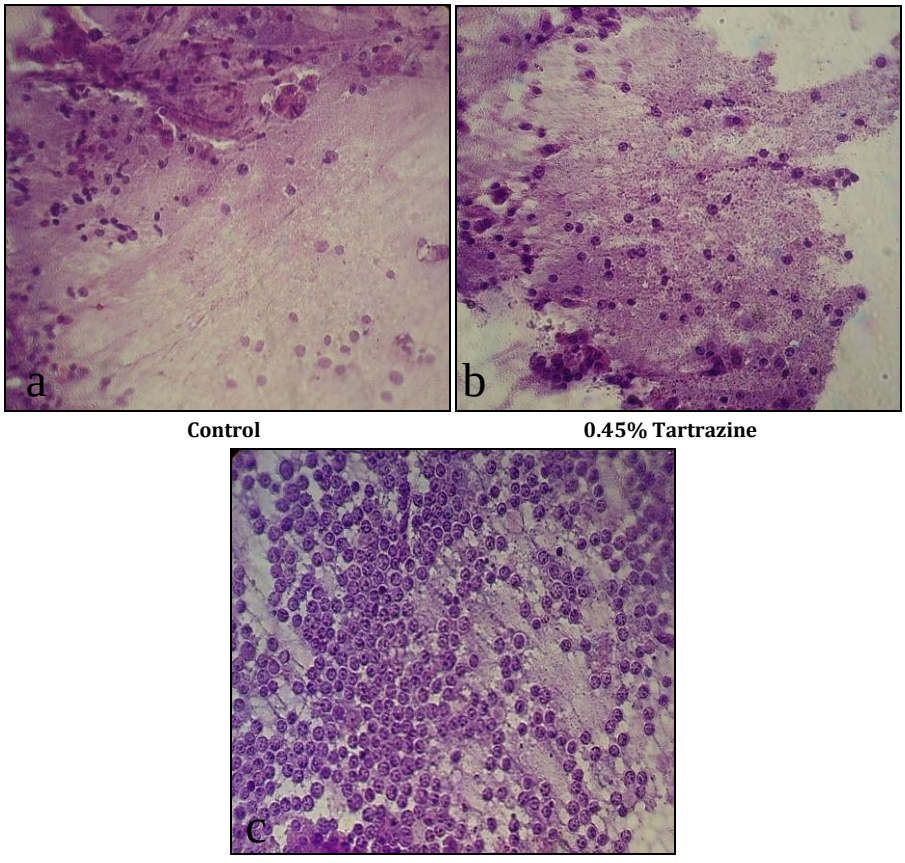



**Fig. 8: Cytological sections of jejunum stained with polychrome stain [magnification × 400].**

[a] Control mouse jejunum. [b] Mouse treated with 0.45% of Tartrazine, no abnormalities detected. [c] Mouse treated with 1% of Tartrazine, immune cell hyperplasia and hypertrophy.

Thymus histological observations revealed lymphocytes hyperplasia only in some male mice treated with 1% of Tartrazine but oral administration of Tartrazine in a dose of 0.45% did not affect the thymus architecture (Figure 5). On the other hand, cytological observations showed thymocytes/lymphocytes atrophy and malformation in all treated 1% male and female groups (Figure 6). However these anomalies were not detected in 0.45% Tartrazine treated groups.

The histological sections of the jejunum showed a significant lymphocyte infiltration presenting an inflammation in all treated groups; in addition a partial villous atrophy was observed in the most Tartrazine treated groups (Figure 7). Cytological observations showed lymphocyte cell hyperplasia and hypertrophy in mice treated with 1% as compared with the controls (Figure 8).

**Effect on Villus Length**

The mean values of villus length determined from sections of jejunum in the control mice and those treated with 0.45% and 1% of Tartrazine are shown in Figure 9. Length of villi measured was significantly reduced in the mice male and female treated with 0.45% and 1% of Tartrazine compared to controls mice ($p < 0.01$).

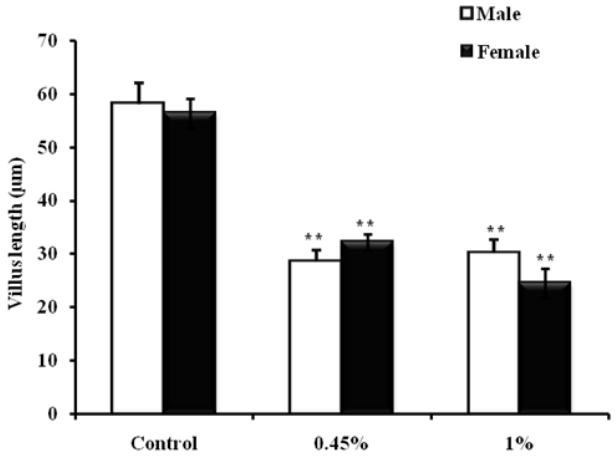



**Fig. 9: Effect of Tartrazine on the villus length in fragments of jejunum.**

Values are means ± SE for 6 animals in each group. Control, 0.45% Tartrazine, 1% Tartrazine. **p<0.01 significantly different from the control group (student *t*-test).

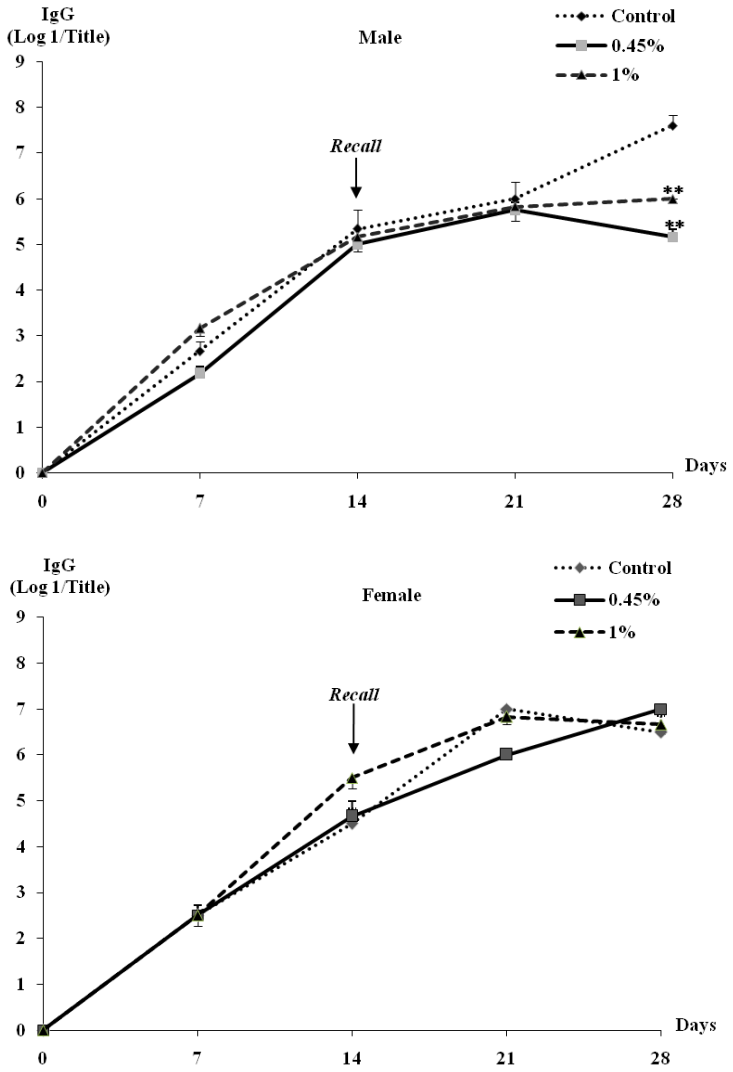



**Fig. 10: Cinetic of antibodies to BSA detected by ELISA in male and female mice after subchronic Tartrazine ingestion for 13 weeks.**

Values are means ± SE for 6 animals in each group. Control, 0.45% Tartrazine, 1% Tartrazine. **p<0.01 significantly different from the control group(student *t*-test).

**Effect on IgG antibodies to BSA**

The titles in IgG anti-BSA decreased significantly in male animals treated with 0.45% and 1% of Tartrazine compared to control group on the 28th days (p <0.01) (Figure 10). On the other hand, it did not affect the titles of IgG anti-BSA in female animals treated with 0.45% and 1% of Tartrazine compared to the control group (Figure 10).

**DISCUSSION**

In this work, we studied the effects of subchronic ingestion of Tartrazine on the humoral immune response and on lymphoid organs structure in swiss albino mice.

We observed during the period of treatment a significant decrease of body weight gain in several points throughout the experiment in male and female mice receiving 0.45% and 1% of Tartrazine compared to control groups. Weight loss or reduced weight gain is a sign of toxicity that may complicate the interpretation of possible direct effects on the immune system [17, 18]. Thus the body weight loss in the present study may represent the primary marker of dye bad effect.

These results are in accordance with the study of Amin et al.[19] which showed that oral administration of high (500 mg/kg bw) or low (15 mg/kg bw) doses of Tartrazine decreased significantly the body-weight gain in young male rats. These data are also in accordance with those of Aboel-Zahab et al., [20] who reported that Indigocarmine consumption in albino rats reduced the body-weight gain.

Oral administration of Tartrazine at doses of 0.45 and 1% for 13 weeks did not affect the relative weight of thymus and spleen of female mice compared to control group, but they have significantly decreased in male mice treated respectively with 0.45% and 1% of Tartrazine compared to control groups. It was shown that the decrease in the relative weight of the lymphoid organs is a sign of immunotoxicity [17, 21]. Reduced lymphoid organs weight suggests reduced immune cell numbers available to fight off infection, which could contribute to immunodeficiency.

This investigation is in accordance with the study conducted by Hashem [22], who showed that oral administration of Sunset Yellow an synthetic dye in daily dose of 315.0 mg/kg bw, for 4 weeks has significantly decreased the relative weight of thymus gland of the albino rats but did not affect the spleen relative weight.

The serum content of IgG was evolved in all the experimental groups from the day of the first injection, whereas, the titles in IgG anti-BSA were significantly decreased in male animals treated with 0.45% and 1% of Tartrazine compared to control group on the 28th day. At the same time, the relative weight of thymus was significantly decreased in these groups. Thomas et al. [21] indicated that alterations in both size and weight of the thymus reflected in a loss in cortical lymphocytes, often referred to as thymic atrophy. Also,

Wintrobe et al. [23], Petra et al. [24] and Weinreich and Hogquist [25] stated that the thymus supports the differentiation of multiple distinct T cell subsets so alteration in the development of this organ results in an altered immune function associated with B and T lymphocytes.

The amount of white blood cells (WBCs) was also decreased in male treated with 0.45% and 1% of Tartrazine but it was not affected in female treated groups (data not shown). This result is in accordance with that of Hashem et al. [22], they declared that Amaranth and Sunset Yellow in daily doses of 47.0 and 315 mg/kg bw, respectively for 4 weeks did not affect the total leucocytes count in female rats. Also, Himri et al. [26] showed that Tartrazine at doses of 5.0, 7.5 and 10 mg/kg bw administered in rats for 90 days did not affect the total leucocytes count but without specifying the animal sex. These differences may be due to differences of doses and animal sex.

The decrease in the amount of WBCs is known as leukopenia, it occurs when there is a toxic effect on the bone marrow [27]. It is well known that B- cells mature in the bone marrow [28], therefore their maturation may also be compromised. It is also well established that resistance to infection depends principally upon an interaction of phagocytes and humoral factors (antibody and complement) [29]. So it could suggest that the reduction of the titles in IgG anti-BSA reported in male mice treated with 0.45% and 1% of Tartrazine could affect the integrity of the humoral immune response. These differences may be due to those related to the animal sex; there are a number of documented differences concerning the disposition and toxicity of foreign compounds which are related to the sex of the animal [30, 31, 32]. Sex differences in metabolism are due to the influence of hormones and genetic factors. Many of the gender differences in both phase 1 and phase 2 enzymes in rodents are developed during puberty for the animal and are influenced by sex hormone concentrations or indirect effects of growth hormone [33].

Our results showed a widening of the medullary sinuses in the spleen of mice treated with 0.45% and 1% of Tartrazine with hemosiderin deposits. The presence of the macrophages cell population was very marked in both groups treated with Tartrazine and cell atrophy and malformation in groups treated with 1% Tartrazine compared to controls. The spleen is responsible for filtering/clearing blood borne particles. In the marginal zone, the blood leaves the terminals arterioles into open sinus, the blood flow is slowed down, and blood-borne particles are trapped with high efficiency [34, 35, 36]. Within the marginal zone, the macrophages form a ring at the outer border of the sinus. They are large with long cellular processes, are tightly bound the reticular meshwork, and appear to exhibit high phagocitic activity [37, 35].

Beside its crucial role in filtering the blood and recycling red blood cell components and iron, the spleen is also the largest secondary lymphoid organ of the body after the gut associated lymphoid tissue (GALT) [28]. The observations of Swirski et al. [38] uncover a role for the spleen as a site for storage and rapid deployment of monocytes and identify splenic monocytes as a resource that the body exploits to regulate inflammation. In the present study, the explanation of the medullary sinus was probably due to increase of blood filtration and increase recruitment of macrophages is essential for particle clearance of the blood and removal of effete red blood cells.

Thymus histological observations revealed lymphocytes hyperplasia only in some male animals treated with 1% of Tartrazine but oral administration of Tartrazine in a dose of 0.45% did not affect the thymus architecture. In addition, cytological observations showed cell atrophy and malformation (thymocytes or lymphocytes) in all male and female of 1% Tartrazine treated groups, however these anomalies were not detected in 0.45% treated groups.

These results are in accordance with the work of Maekawa et al. [39] who described degeneration and/or an atrophy of the hematopoietic organs (thymus gland, bone marrow, lymph nodes and the spleen) of albino rats receiving different doses of Tartrazine. Before thymocytes become fully mature and express the T-cell receptor (CD4 or CD8) with TCRαβ, they go through positive and negative selection. Homeostasis is maintained when there is control over the amount of cells being made and the amount of cells being destroyed [39]. If too many cells are killed, leukopenia and immunodeficiency occurs [40].

Jejunal architecture showed the presence of inflammation with partial villous atrophy at jejunal mucosa of animals treated with 0.45% and 1% of Tartrazine compared to the controls. These anomalies were more pronounced in female animals treated with Tartrazine. At the same time, length of villi measured was significantly reduced in the mice treated with 0.45% and 1% of Tartrazine compared to controls mice. Morphological aspect, there was an immune cell hyperplasia and hypertrophy in mice treated with 1% of Tartrazine. Inflammatory cell infiltration probably means an active chronic jejunitis. This lesion destroys the mucous membrane and can even stretch the mucosa layer [41]. Intestinal epithelial cells are always exposed to various stresses, including oxygen radicals, bacterial components, and environmental chemicals [42]. These stress factors induce stimulation of immune cells which is thought to be an adaptive response to risk factors and is called controlled inflammation [43]. However, excess response can induce uncontrollable inflammatory damage in the epithelial tissue [42]. These results are in accordance with data reported by Himri et al. [26] who revealed lymphoid infiltrates in jejunum of wistar rats fed with 7.5 and 10 mg/kg bw of Tartrazine. However, Raza et al. [44] showed that oral administration of Metanyl Yellow at the dose of 15mg/200g in rat decreased mucus secretion and McKee [45] showed an exfoliation of intestinal brush bordure in animal ingested Amaranth in their food.

The adverse immunotoxic effects of xenobiotics includes organ damage of the immune system, multiple histopathologic and cytopathologic effects in the thymus, the bone marrow, and the lymph nodes; chemical-induced cellular pathology including abnormal proliferation of stem cells in the bone marrow; altered maturation of immunocompetent cells and changes in B- and T-cell subpopulations; functional alterations of immunocompetent cells generally classified as altered humoral-mediated immunity (HMI), cell-mediated immunity (CMI), or nonspecific responses (NSR) [46, 47].

## CONCLUSION

In the present study subchronic ingestion of Tartrazine at dose of 1% causes structure alteration of the intestine and spleen, thymus cell damages as well as a depressing effect on the humoral immune response in male mice. It is advisable to limit the usage of synthetic food colorants especially those used by children and vulnerable people.

## ACKNOWLEDGEMENT

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