Immunohistochemistry Assay of Sodium Nitrate in White Mice Protected with Alpha Lipoic Acid

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Abstract
Colorants, preservatives, stabilizers, antioxidants, thickeners, emulsifiers, acidity regulator agents, flavor enhancers, and anti-caking agents are all examples of food additives. Sodium nitrate is a white crystalline powder that is slightly hygroscopic. It used as a preservative, color fixative, and antimicrobial in the food industry, primarily in cheese, meats, and fish. The aim of this research was to study the immunotoxic effects of NaNO3 in addition to the antioxidant effects of α-lipoic acid in activating immunity and decreasing the toxic effect of sodium nitrate. Eighty adult male and female White mice, aged eight weeks, were separated into 4 groups: The first group received 1/10 LD50 (302.2 mg per kg b.w.) sodium nitrate orally by gavage daily for 8 weeks. The second group received 30 mg per kg B.W of α-lipoic acid orally by gavage daily for 8 weeks. The third group received 1/10 LD50 (302.2 mg per kg b.w.) sodium nitrate and 30 mg/ kg b.w. α -lipoic acid orally by gavage daily for 8 weeks. The fourth group was the negative control group. The result exhibited that revealed significant increasing ($p<0.05$) level of CD4+T cell in animals treated with ALA (G2) compared to control(G4), (G1) and (G3) group.

1. Introduction

Since the beginning of time, people have used food additives. They are the most important for adding artificial flavors and improving the quality of food and beverages. Colorants, preservatives, stabilizers, antioxidants, thickeners, emulsifiers, acidity regulator agents, flavor enhancers, and anti-caking agents are all important (shafaq et al., 2020). A preservative is defined as any substance that can suppress, slow, or stop the growth of microorganisms. Chemical preservatives bind to the cell membranes of microorganisms, as well as their enzymes and genetic mechanisms. Preservatives prevent dangerous poisons such as botulism a (food poisoning illness) from developing in foods such as cured meats (Sunitha and Preethi, 2000). Nitrites and nitrates improve and maintain the color of meats and dried fruits (Pandey and Upadhyay, 2012). People are exposed to nitrites and nitrates via the consumption of vegetables and processed meat products, as well as, to a lesser extent, through water and other foods (Marija and Mirna et al., 2017). NaNO3 is a white crystalline powder that is slightly hygroscopic (EFSA ANS Panel, 2017b).It used in the food as a preservative, colour fixative, and antimicrobial agent, primarily in cheese, meats, and fish (Santamaria, 2006). Nitrate is not poisonous to humans. Only when it is converted to nitrite in the human body does it become a problem (Majumdar, 2003).
Nitrate, when consumed during contaminated drinking water or food, mainly affects the gastrointestinal tract and small intestine (Lundberg et al., 2004). The acidic pH less than (2) environment of the stomach promotes the conversion of nitrate into a nitrosating agent, which may result in the formation of nitrosamines (Ohshima et al., 1998). Both nitrite and N-nitroso compounds are toxic and can cause severe diseases in humans when nitrite links to other substances before or after ingestion (Speijers and van, 2003). Nitrate was thought to be dangerous due to its ability to create nitrosamines that are cancer-causing beneath certain conditions, like acidic stomach. Nitrosamines have been linked to cancer of esophageal, gastric, and colon (Bedale et al., 2016, Park et al., 2015).
Th1 cells secrete IL-2, which promotes T- cell proliferation (including CD4+ cell proliferation in an autocrine response). IL-2 promotes CD8+T cell proliferation and cytotoxicity by lowering activation thresholds (Newport et al., 1996). CD4 T cells are essential for immune protection. They accomplish this by directing neutrophils, eosinophils, and basophils to areas of infection and inflammation, helping B-cells produce antibodies that encourage macrophages to develop enhanced microbicide activity, and producing cytokines and chemokines that coordinate the full spectrum of immune responses. Beginning with the seminal work of (Mossman and Coffman, 1986), which demonstrated that long-term CD4 T-cell lines could be divided into 2 groups, those that produced interferon as their signature cytokine and those that produced IL-4, it was secreted that CD4 T cells comprise a variety of diverse cell types with various roles rather than being a single entity. While several of these CD4 T-cell populations, like "natural regulatory T" (n-Treg) cells (Sakaguchi, 2004 ; Shevach, 2006) and natural killer T cells (NKT cells), are distinct lineages of cells when they elicit from the thymus (Bendelac et al.,2007).
ALA is a potent mitochondrial antioxidant agent that improves anti-inflammatory and anti-thrombotic pathways while also positively influencing nitric oxide-mediated vasodilation (Heitzer et al., 2001). Plants, animals, and humans all produce small

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amounts of alpha lipoic acid. It was detected to be a cofactor for numerous mitochondrial enzyme complexes participated in energy production (Mohammad and Baraa, 2019). Antioxidant, cognitive, removing toxicity, anti-inflammatory, anti-aging, cardiovascular, anti-cancer, and neuroprotective pharmacological properties have been demonstrated for ALA and DHLA (Goraca et al., 2011).ALA antioxidant activity includes scavenging hydroxyl radicals, hypochlorous acid, and singlet oxygen, as well as transition metal chelation, While DHLA can repair oxidative damage and regenerate endogenous antioxidants including vitamin E, vitamin C, and glutathione. The majority of ALA's therapeutic effects are due to its antioxidant properties (Medical research Institute, 2005). Previous research has shown that ALA supplementation improves antioxidant enzyme activities in the liver, muscles, and plasma, as well as in chicken meat (Zhang et al., 2009; Chen et al., 2011; Guo et al., 2014).

Materials and Methods

Albino mice (n=80), both males and females, aged 7-8 weeks and weighing 20-25gm, were obtained from the animal's house of the College of Vet.Medicine/University of Baghdad. Mice were housed and cared for in a standard animal facility under temperature control (20 ±5°C). Mice were given unlimited access to food and water. Every group of mice was housed in cage with hard-wood chip bedding throughout the experiments. To maintain a clean environment.

Experimental design

80 adult's Swiss albino mice males and females, aged 7-8 weeks old, and divided into four groups (n=20), and treated as following:

1. For 8 weeks, 1/10 LD50 (302.2 mg/kg b.w) sodium nitrate were administered orally by gavage to the first group of mice (n=20).
2. For 8 weeks, mice in the second group (n=20) were administered alpha lipoic acid (30 mg/kg b.w) orally via gavage.
3. The third group (n=20) of mice were treated orally by gavage with 1/10 LD50 (302.2 mg/kg b.w) sodium nitrate and 30 mg alpha lipoic acid/kg B.W daily for 8 weeks.
4. The fourth group was designated as the negative control group and was given distilled water.

At the ending of the experiment (8 weeks), all animals were sacrificed, and specimens were taken from liver. The tissues were fixed in a 10% formaldehyde solution and then tissue processing was done and immunohistochemical analysis to detect the level of CD4 in the liver.

Preparation of sodium nitrate

The LD50 of sodium nitrate was calculated based on the outcome of the toxicity study "UP& down method" Dixon, 1980). (Reyam, 2019) determined the LD50 (302.2 mg/kg b.w). 302.2 mg per kg b.w. was dissolved in 10 ml of D.W. to make the dose. As a result, the sodium nitrate dosage volume was 0.1 ml per 10 gm b.w of mice.

Preparation of dosage rate 30 mg /kg B.W of α-Lipoic acid

In mice, the dosage rate is 0.3 mg per 10 gm body weight. To make a chief, we dissolved 300 mg of lipoic acid (Melhem et al., 2001) in 100 ml of distilled water. As a result, the lipoic acid dosage volume was 0.1 ml per 10 gm b.w of mice.

Procedure of immunohistochemistry:

By using primary Ab (Mouse anti CD4) / (USA).

Immunohistochemistry procedure:

Liver specimens measuring 1x1x1cm were collected.Immediately after removal, the tissues fixed in a 10% formaldehyde solution. After 72 hours, the specimens washed with tap water and then tissue processing routinely this done with a set of upgrading alcoholic concentrations from 70% - 100% for 2 hours for every concentration to eliminate water from the tissues, then clearance was done by utilizing xylol, then the specimens were infiltrated with semi-liquid paraffin wax at 58 °C on two stages, then blocks of specimens were made with paraffin wax and sectioned by microtome (Luna, 1968).

Each specimen's paraffin-embedded sections were cut into 4 µm, thick sections, mounted on glass, and dried overnight at 37 °C. Additionally, for each immunohistochemistry run, negative and positive control slides were included to determine the signal specificity.

deparaffinized in xylene (2×10min) and with graded alcohols for rehydration as following

Twice alters of xylene for 15 minutes, and twice alters of absolute ethanol for 5 minutes. 95% ethanol for 5 minutes, then 70% ethanol for 5 minutes. Dip in distill water for 5 minutes. Using H2O2 (1.5 %) (1.5ml) to block the endogenous peroxidase activity for 5-10 minutes. Distill water for 5 minutes, then for 5 minutes in PBS. Dip in retrival solution for 10 minutes at 100 ˚C. Dip in D.W. for 5 minutes. In PBS dip for 5 minutes. Section had additional buffer taped and cleaned around it. Block the serum (0.8 ml from PBS + 10 µl from blocking serum) at 37 °C for 1 hour. Dip in D.W and PBS every 5 minutes. Use blocking serum (0.8 ml from PBS+12 µl from blocking serum) + 16 µl primary Ab (primary Ab 1 ul 50 µl from blocking serum) use for overnight at 37 °C. In distill water and PBS for 5 minutes. Next, wiping. Secondary Ab (biotinylated) (0.8 ml from PBS +10 µl from blocking serum +10 µl biotinylated Ab) for 1.5 -2 hours at 37 °C. For 5 minutes in distilled water and PBS, next, wiping. Usage of Ab enzyme (0.75 ml from PBS+15 µl from solution A +15 µl from solution B) at 37 °C for 30 minutes. For 5 min in distilled water and PBS, next, wiped.Usage of chromogen solution (DAB) for 30 minutes. For 5 minutes in D.W. For 2 minutes in tap water for washing. Use hematoxylin stain for 15 seconds. Then wash in tap water.

After that done dehydration process.Dip in ethanol 70% for 2 minutes. Dip in ethanol 95% for2minutes. Dip in ethanol 100% for 2 minutes (twice).Dip in Xylene 100% for 2 minutes (twice).Finally we apply cover slipping and mounting.

Scoring

At least 10 high power fields were chosen at random on every section to count the number of positive cells in the staining tissues samples. The same two observers examined all immune stained sections under the light microscope with a 400x objective, numerating 100 cells from every section. The expression of CD4 was measured quantitatively like 0 (cells not stained), score1 (1-25 positive cells), score2 (26-50 positive cells), score3 (51-75 positive cells) and score4 (75 and over). The level of intensity was calculated as 0 (nil), (low), (moderate), or (high). The pattern and intensity of staining in the various cell kinds of liver samples were evaluated by two independent observers, using a light microscope at a magnification of 200X(objective 20x and ocular 10x). The grade of staining in each kind of liver cell was graded as characterized by (Zenclussen et al., 2003).

Statistical analysis

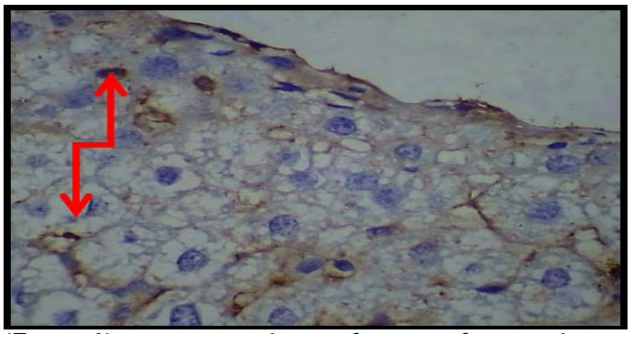
Two ways ANOVA was used for statistical analysis, and the mean variance was significant at the (P≤0.05) level by the statistical package for social sciences (SPSS). (SPSS, 2008).

2. Results

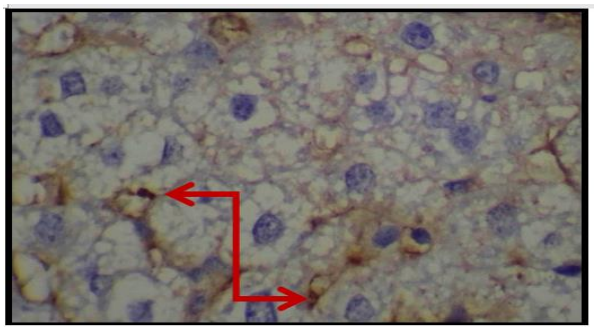
Immunohistochemistry examinations:
Comparative between treated animals and control negative group.
Table (1): Show in (G2) appear high level of CD4+T cell in liver (76.5± 2.5A), compared with G1 (19.0 ± 1.0 D), G3 (45.5 ± 2.5 B), and control negative group (25.0 ± 0.0 C) respectively.

Table (1): CD4+T cell density in G1, G2, and G3 compared to control negative group.

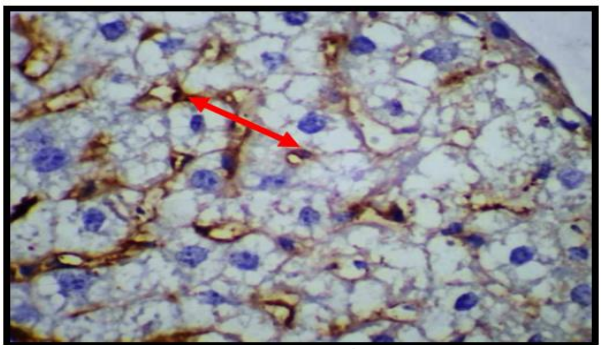
Intensity	Score	Mean (Std. Error)	Groups
Low	1	19.0 ± 1.0 D	G1
High	4	76.5 ± 2.5 A	G2
Moderate	2	45.5 ± 2.5 B	G3
Low	1	25.0 ± 0.0 C	G4
Data represented as mean ± SE N=10 per group			



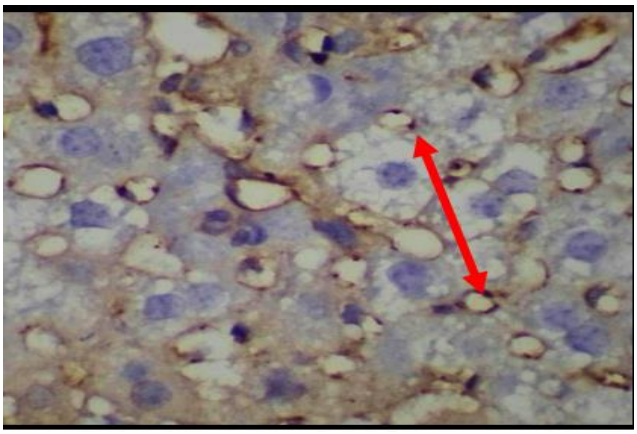
(Figure1): section in liver of mice of control negative group treated with distilled water showed CD4 score1,low intensity in the liver parenchyma(red arrow) by using (DAB stain (brown color), 40 x).



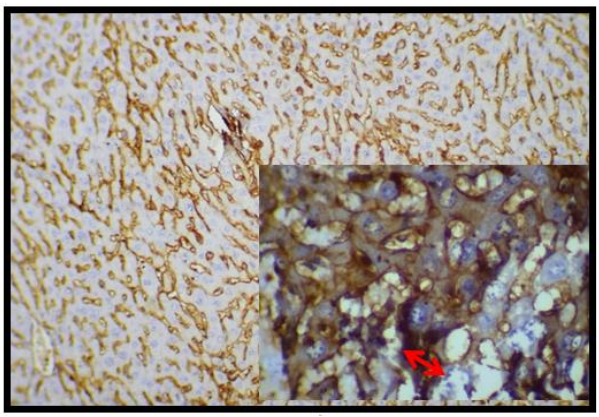
(Figure 2): section in liver of mice control negative group showed CD4 score1, low intensity in the liver parenchyma (red arrow) by using (DAB stain (brown color), 40 x).



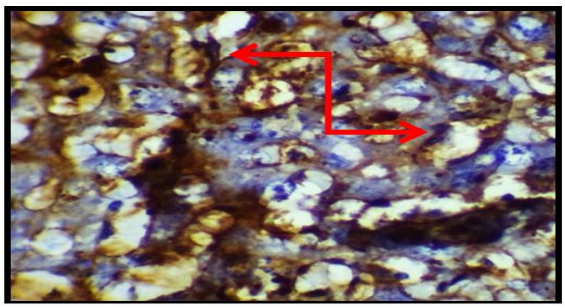
(Figure3): section in liver of mice treated with 302.2mg/kg B.W. of sodium nitrate showed CD4 score1, low intensity in the liver parenchyma(red arrow) by using (DAB stain (brown color), 40 x).



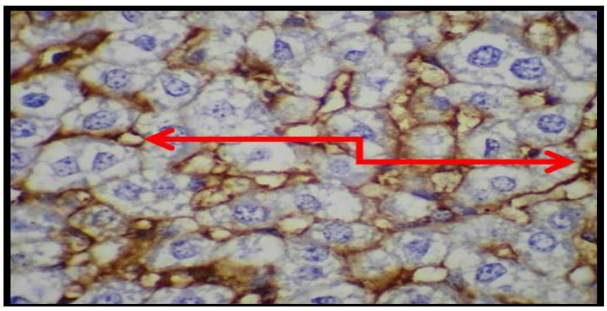
(Figure 4): section in liver of mice treated with 302.2mg/kg B.W. of sodium nitrate showed CD4 score1, low intensity in the liver parenchyma (red arrow) by using (DAB stain (brown color), 40 x).



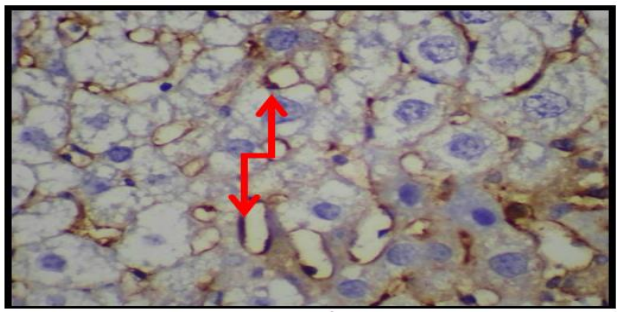
(Figure 5): section in liver of mice treated with 30 mg/ kg b.w. ALA showed CD4 score4,high intensity in the liver parenchyma(red arrow) stained by(DAB stain (brown color), 10 X & 40 X).



(Figure 6): section in liver of mice treated with 30 mg/ kg b.w. ALA showed CD4 score4, high intensity in the liver parenchyma(red arrow) by using (DAB stain (brown color), 40 x).



(Figure 7): section in liver of mice treated with 302.2 mg/ kg b.w. sodium nitrate and 30 mg/ kg b.w ALA showed CD4 score3, moderate intensity in the liver parenchyma(red arrow) by using (DAB stain (brown color), 40 x).



(Figure8): section in liver of mice treated with 302.2 mg/kg B.W. sodium nitrate and 30 mg/ kg b.w ALA showed CD4 score3, moderate intensity in the liver parenchyma (red arrow) by using (DAB chromogen immunostaining (Brown color), 40 X).

3. Discussion

It was reported that the mean CD4 values of animals treated with lipoic acid showed higher than animals treated with NaNO3, implying that α-lipoic acid may possess immunomodulatory effects. With the advancement of basic and clinical immunology research, the function of oxidative stress in the pathogenesis of several autoimmune diseases has become widely accepted, and the interaction of ROS with the immune system has been well established. Furthermore, ROS may play a physiological function in the signaling of all types of immune cells. For example, macrophages produced ROS to damage bacteria, and regulatory T-cells (T-reg) produced ROS to prevent other T-cells from activating (Nathan and Cunningham-Bussel, 2013). Furthermore, in pathological states, immune cells produce too much ROS, exacerbating inflammation and disrupting immune system balance.like, oxidative stress was one of the agents that contributed to immune system dysregulation and dysfunction (Perl, 2013).

More research has revealed that ALA can influence T cell function in a variety of ways. ALA has been shown to improve the impaired mitochondrial function of CD4+T cells in AIDS patients (Milazzo *et al*,2010), downregulate the expression of CD4 molecules in human peripheral blood T cells (Marracci *et al*,2006), and suppress factor of nuclear kappa B (NF-B) activation in Jurkat T-cells (Suzuki *et al.*,1992). Inhibit interferon- (IFN) and interleukin -4 (IL-4) production by CD4+T cells to reduce the severity of atopic dermatitis lesions in a mouse model (Kim *et al.*,2011), and stimulate lymphocyte progression from G0/G1 to S phase, which may be related to restoring immune system function in advanced cancer patients (Mantovani *et al.*,2003). The reduced mean CD4 levels in animals treated with sodium nitrite may be due to nitrite toxicity. Nitrite is converted to nitrosonium ions in the body, which then react with amines and amides to form nitros amines and, respectively. Diethylnitrosamine has been shown to stimulate the production of free radicals in liver tissue of rat in vivo (Yamada *et al.*, 2006).

Another line of research has shown that nitric oxide (NO), a free radical produced by the action of induced nitric oxide synthase (iNOS) via the hydrolysis of L-arginine, can downregulate the immune response mediated by CD4+ T lymphocytes. Nitric oxide participates in physiological events such as vascular relaxation, neurotransmission, pathogen defense, and the regulation of in vivo adaptive immune response (Sellers *et al.*, 2013). Over production of NO by activated phagocytes, on the other hand, has been linked to the destruction of healthy tissue in the inflammatory area, Possibility of influencing the pathophysiology of some diseases (Lintomen *et al.*, 2009). Furthermore, NO release in the supernatant reduced CD4+T lymphocyte proliferation and induced T-cell apoptosis (Hoffman *et al.*, 2002). Furthermore, the lower mean CD4 values of animals treated with NaNO3 may be attributed to the hematopoietic tissues' inability to produce new leucocytes (Sperandio *et al.*, 2009). As a result, the defense mechanism for dealing with foreign antigens and initiating a primary immune response was weakened. After 21 days of NaNO3 exposure, a dose-dependent decrease in lymphocyte percentages and an increase in neutrophil count were observed in Balb/c mice (Abuharfeil *et al.*, 2001). Stated natural killer (NK) cell activity, IgM titer, and IgG titer were all significantly reduced after 100 mg per kg of sodium nitrite treatment. It was observed that the immunosuppressive effect of NaNO3 can be changeful after exposure.

4. Conclusion

Sodium nitrate induces immunotoxic effect. We noted high levels of CD4 appear in the liver of mice

treated with α-lipoic acid compared with animals treated with sodium nitrate.

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