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Histopathological and Immunohistochemical Study in Liver of Mice *Muse Musculus* Treated with B-Carotene

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Abstract

β-Carotene is one of the natural bioactive products that has received attention as additives to some cancer-suppressing drugs, so the current study aims to detect the relationship of this substance in stimulating the pathways of apoptosis. The present study evaluated the role of β-Carotene *in vivo* mice, it included detection of histopathological changes in the liver of mice *Mus musculus* treated with β-Carotene, and immunological detection of apoptosis proteins (caspase 8, caspase 9 and p53) in mentioned organ. There are variety of reversible and irreversible histopathological changes were observed in liver of mice treated with 300 µg/ml of β-Carotene while the samples that treated with 150 µg/ml did not suffered irreversible changes. The immunohistochemistry assay of apoptotic proteins (caspase 8, caspase 9 and P53) showed staining caspase8 protein in Large areas of liver tissue, of mice treated with high concentrations of β-Carotene, with brown of DAB stain, while the caspase9 and p53 proteins were stained in small separate area of tissue. In conclusion, the β-Carotene has a toxic effect with high concentrations on liver tissue and have no toxic effect in low concentration. The injury of tissue as a result the potential of the β-Carotene to induce apoptosis.

Key word: Histopathology, immunohistochemistry, liver, mice, β-Carotene

Introduction

The β-Carotene is one type of the carotenoids, It has an orange color, it is available in fruits and vegetables, and in human body at low concentrations. Many studies have indicated that β-Carotene is an antioxidant factor (Teodoro *et al*., 2012). Gumpricht *et al*.(2004) observe that a β-carotene have potent to decreased generation of reactive oxygen species(ROS) by >50% in rat hepatocyte that were exposed to the toxic hydrophobic bile acidglycochenodeoxycholic acid (100 or 500 µM) and β-Carotene (100 µM). Lyama *et al*.(1996) showed that oral administration of β-Carotene for 10 days led to its accumulation in mouse various tissues and excrete a protective role

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against oxidative stress. Park *et al*. (2020) observe that Low-β-carotene concentration have an active role in reducing the level of ROS. Also, hepato protective effect of β-carotene against cadmium toxicity in rats has been reported, It reduced the concentration of H_2O_2 in rat blood (Bashandy and Alhazza, 2008). Other researchers showed to the pro-oxidant properties of β-carotene and trigger ROS-mediated apoptosis (Shin *et al*. 2020), Which gives it a clear role to inhibiting growth of some cancers by inducing $p27^{kip1}$ expression and decreasing SKp2 expression (Freedman *et al*., 2008). β-carotene-induced increases in ROS may lead to caspase-3 activation causing apoptosis (Park *et al*., 2015). Treatment with β-carotene lead to increased intracellular ROS level, which then triggere caspase activation (Prasad *et al*., 2006). The β-carotene can be picked up from the bloodstream by body tissues, to be stored or metabolized directly to vitamin A. In mammalian , liver is the main organ for storage a large quantities of β-carotene (Sy *et al*., 2012). An accumulation of β-carotene in liver may cause injury in liver tissue.
The current study aimed to detection the β-carotene histopathological effects in mice liver, and its relationship in inducing apoptosis proteins (caspase8,9 and p53).

Material and methods

Male mice were divided into three groups A, B and C. Group A was injected (inter peritoneal) with 150µg/ml of β-Carotene, B was injected with 300µg/ml of β-Carotene and C injected with normal saline 0.9 .

The mice were dissected at two periods (15, 30 D), then extracted their livers and fixed with formalin 10% for 48h to study histopathological changes and immunhistochemistry of apoptosis proteins (caspase8, caspase9 and P53).

Histopathological study

Humason (1972) assay was adopted to preparation of tissue sections for study histopathological changes, which is briefly:

After fixation, the tissues are washed with tap water for 24h. Then dehydrated by passing through series of diluted ethanol (70% for 4h, 85% for 2h, 100% for 2h). Then the tissues were cleared by xylene, and infiltrated with paraffin wax at 50 C° for 2h, then embedded with paraffin wax. The tissues were sectioned at 5 µm , then the section were floated and mounted on the slides. After the sections dried up, it was stained with Haematoxylin and Eosin after it passed with a series of solutions(xylene for 20 minute, ethanol (100%, 90% and 70% respectively) for 5minute for each one, D.W. for 2 minute, Haematoxylin stain for 5 minute, T.W. for 2 minute, Eosin stain for 5

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minute, D.W. for 2 minute, ethanol (70%, 90% and 100% respectively) for 5minute for each one and xylene for 5 minute). Then the sections were coverd with cover slid by Canada balsam and examined by light microscope and visualization by digital camera.

Emmunohistochemistry study

For immunohistochemical detection of apoptosis proteins (caspase8, caspase9 and P53) in mice liver tissue we have used Ultra Cruz® kit (Eckle *et al*., 2004), according to the following steps:-

1- Deparaffinization tissue sections by placing it in xylene for 30 minutes.
2- Rehydration the tissue sections by passing it with a series of ethanol dilutions (100%, 90%, 80% and 70%) for 15 minutes each one and then washed with distilled water (W.D.) for 1 minute.
3- Soaking the sections with H_2O_2 1% W.D. for 5 minutes, then wash with PBS for 5 minutes.
4- Soaking the sections with Ultra Cruz® Blocking Reagent for 1h.
5- Incubation the sections in 4µg/ml of diluted primary antibody with blocking reagent at 4°C for 24h, then wash with phosphate buffer saline (PBS) for 5 minutes.
6- Expose sections to secondary antibody diluted by blocking reagent (25-1) for 1 hour, then wash with PBS for 5 minutes.
7- Expose sections to DAB-H_2O_2 stain (2% DAB with 0.015 H_2O_2) for 10 minutes, then wash with W.D.
8- Counterstaining for 5second.
9- Dehydrating by passing with sires ethanol (70% 90% and 100%,) for 5minute each one.
10-Clearing with xylene for 10 minutes.
11- Stabilizing with mounting medium.
12- Examining by light microscope, then visualization by digital camera.

Results

The microscopic examination of mice livers tissue treated with 150µg/ml of β-Carotene for 15 days (Figure1(B)) showed no histopathological changes compared to the control group (Figure1(A)), while minor histopathological changes were observed in mice treated at the same concentration for 30 days, it represented by a slight expansion of sinusoids and a vacuolar degeneration of a few hepatic cells (Figure 1 (C)).

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Histopathological changes have become more clear in mice's liver treated with 300µg/ml of β-Carotene, the vacuolar degeneration become surrounding the hepatic cell nuclei (Figure 1 (D)), many hepatic cells were suffered necrosis(Figure 1 (D, E)), and lyses their nucleus , and inflammation adjacent the central vein were observed (Figure 1(E).

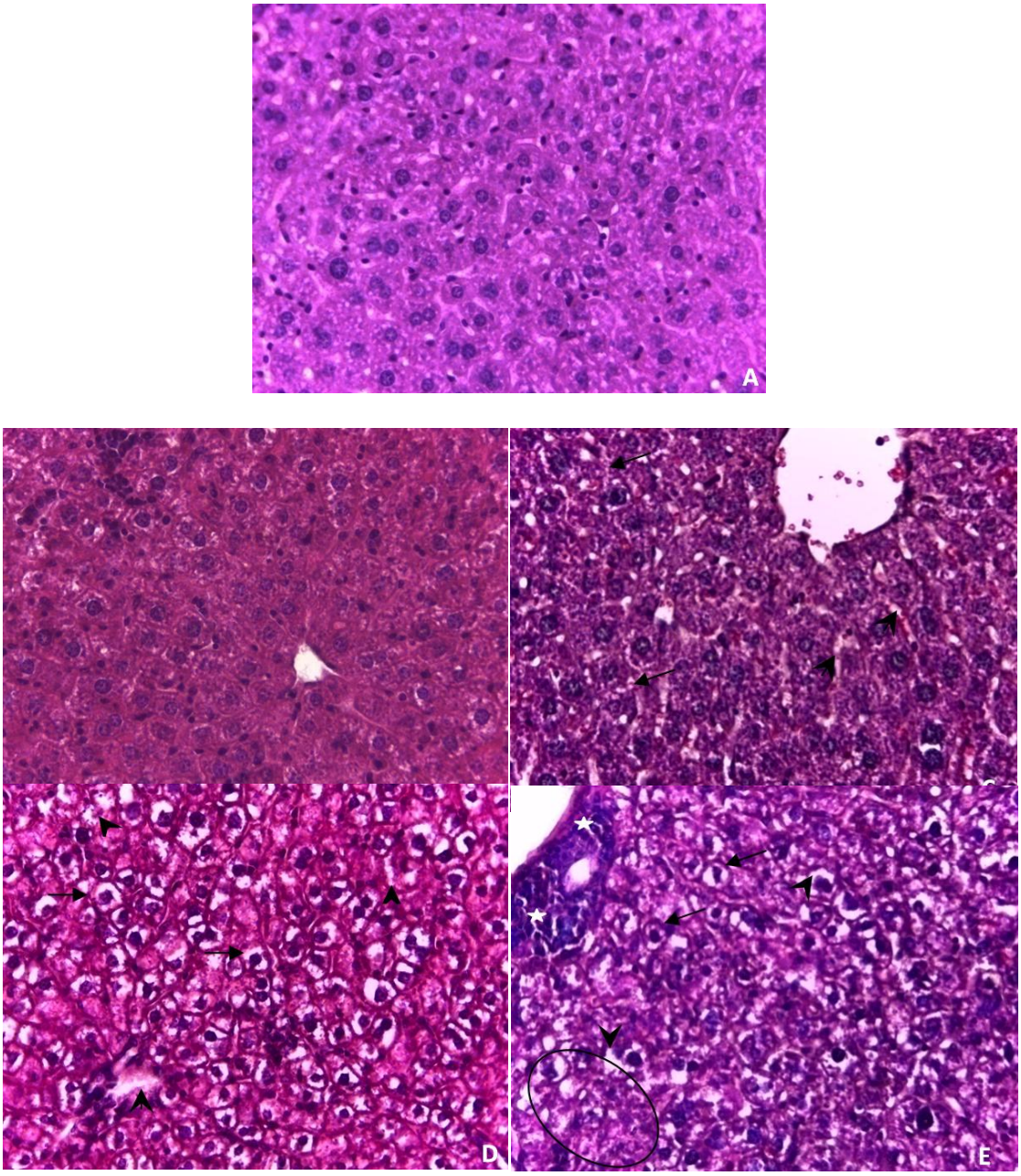

Figure 1: H&E staining sections of mice's liver treated with β-Carotene. (400x magnification) A- Mice treated with normal saline. B-Mice treated with 150 µg/ml for 15 days , showing hepatic cells are not affected. C- Mice treated with 150µg/ml for 30 days, showing degeneration (arrows), sinusoids expansion (arrow heads). D- Mice treated with 300µg/ml for 15 days, showing degeneration (arrows), necrosis (arrow heads). E- Mice treated with 300µg/ml for 30 days, showing degeneration (arrows), necrosis (arrow heads), lyses nucleus(ring) and inflammation(stares).

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Immunohistochemical detection showed that caspase8 proteins antibodis were stained with a brown DAB stain in large areas of mice liver tissue treated with 300µg/ml of β-Carotene for 30 days (Figure 2 (B)) compared to the control group (Figure 2 (A)).

While the immunohistochemical detection of caspase9 (Figure 2 (C)) and P53 protein antibodies (Figure D) 2) showed that its antibody was stained in small areas of liver's tissue.

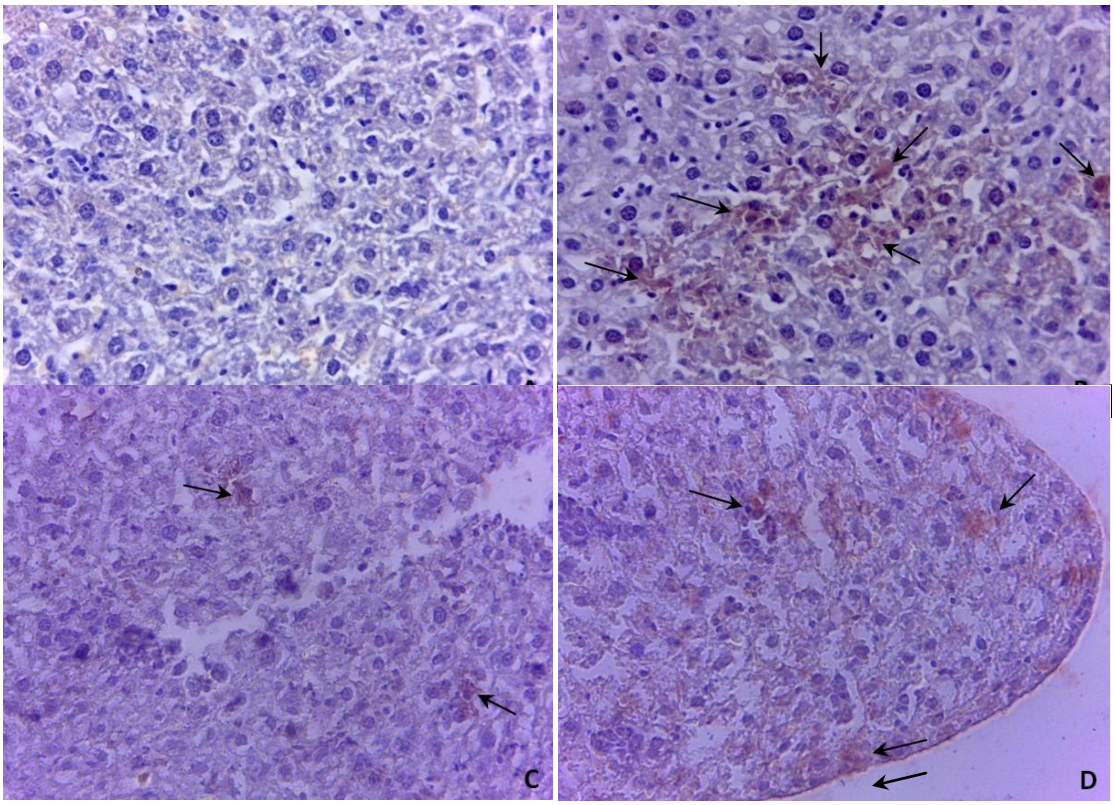

Figure 2: mice liver sections stained with Immunohistochemical stain (DAB-H_2O_2). (400x magnification). A- Control group. B- Mice treated with β-Carotene at 300 µg/ml for 30 days showing staining of caspase8 protein (arrows). C - Mice treated with β-Carotene at 300 µg/ml for 30 days showing staining of caspase9 protein (arrows). D - Mice treated with β-Carotene at 300 µg/ml for 30 days showing staining of caspase9 protein (arrows).

Discussion

Microscopic examination of liver sections of mice treated with β-Carotene showed minor changes that increased in a concentration-dependent manner. Mice liver treated with low concentration (150µg/ml) suffered very minor histopathological changes, while severe histopathological changes were observed in the liver of mice treated with high concentration, This may indicate the role of low concentration of β-Carotene, in reducing the level of oxidative potential(Das *et al*., 2014), while the high concentration of β-Carotene has transformed its role

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from an antioxidant to an oxidant agent, due to the toxicity of the high concentration of β-Carotene on tissues (Bjelakovic *et al*., 2013). Inflammation is one of the histopathological changes seen in mice liver treated with high concentration of β-Carotene, which may be due to the role of this material in cell damage, which caused attracting inflammatory cells to the damage area (Premanathan *et al*., 2011). Other histopathological changes such as degeneration and necrosis were observed, which may have occurred due to the effect of reactive oxygen species(ROS) which activated by materials(Monteiro *et al*., 2005). Excessive production of ROS will lead to disturbances in various bio reactions in the cell (Mekkawy *et al*.,2013) , May causing lipid peroxidation in plasma membranes, which led to permeability disruption, and causing degeneration(Stevens *et al*., 2009) . The continued effect of the material will lead to greater damage, such as cell death and necrosis (Levison *et al*., 2008), this explain the degeneration and necrosis which observed in large area of liver tissue (Thophon *et al*.,2003). The damage caused by the high concentration of β-Carotene may be due to the disorder of enzymatic activity of liver, and then the hepatic enzyme system of prevent toxicity will loss, causing histopathological changes in liver (Athikesavan *et al*.,2006).

Immuonohistochemistry assay was used to detect apoptosis proteins (caspase8, caspase9, p53) in mice liver tissue treated with β-Carotene. The results showed a variation in β-Carotene's ability to induce this proteins production , It has been observed that liver tissue is stained brown for DAB dye in varying areas. The caspase8 protein has been stained in large areas of tissue, while caspase9 and p53 proteins were stained in small areas of tissue, this suggests that β-Carotene has a role in stimulating the expression of caspase8 protein larger than its role in caspase9 and p53 proteins. This demonstrates the role of β-Carotene in inducing caspase-dependent apoptosis in tissue by extrinsic pathway (Palozza *et al*., 2009; Yu *et al*., 2012).

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