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Protective effect of benzethonium chloride on diethylnitrosamine-induced hepatocellular carcinoma

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A B S T R A C T

This study aims to evaluate the protective effect of Benzethonium Chloride (BZC) on-diethylnitrosamine (DEN)-induced hepatocellular carcinoma. Thirty-two male albino rats were used (eight animal per group). The hepatocarcinogenesis was induced in male Wistar albino rats by DEN (20 mg/kg b.w, orally (five times per week for 6 weeks). The treatment group rats received a similar dosage as above then injected with benzethonium chloride (5 mg/ kg b.w i.p.) for 4 weeks. Blood samples for serum parameters were collected or determination of biochemical markers such as alanine amino transaminase (ALT), aspartate amino transaminase (AST), alkaline phosphatase (ALP), and total bilirubin, albumin and total protein. Also, liver tissues were taken for histopathological investigation. The obtained result revealed that treatment with benzethonium chloride significantly reduced DEN-induced elevation of ALT, AST and ALP activities and total bilirubin concentration. However, significant increase in the serum albumin and total protein concentration were observed in benzethonium chloride-treated rats as compared with DEN-treated group. The obtained results suggest that benzethonium chloride possesses chemopreventive activity against DEN-induced liver cancer.

Keywords: Diethylnitrosamine, Benzethonium Chloride, Hepatocellular carcinoma, Liver marker enzymes

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1. INTRODUCTION

Hepatocellular carcinoma (HCC) is an aggressive cancer, it is one of the most frequent primary cancer of the liver at which its incidence rate has been increased to become the fifth most common malignancy worldwide (Zhong *et al.*, 2014). The HCC incidence rate is one million cases every year (Liovet *et al.*, 2008). The pathogenesis of HCC is multifactorial, highly associated with many risk factors but it is mainly develops after exposition of the cellular machinery to a mutation that results in replication of the cell in higher rate and/or avoiding of apoptosis (Youns *et al.*, 2013). The main way for HCC treatment is surgical resection and liver transplantation (Zekri *et al.*, 2013; Zhang *et al.*, 2013; Zhu *et al.*, 2013). For unresectable HCC other methods were developed such as chemotherapies by interferon or 5 Flouro-uracil (Parker *et al.*, 2004). Hepatocellular carcinoma (HCC), originating from epithelium of hepatocytes and accounting

for 80% of primary liver cancers, ranks as 4th place in causing tumor-related deaths globally. Abnormal activation of molecular signaling pathways contributes to initiation and progression of liver cancer (Chen and Wang, 2015).

Benzethonium chloride [($C_{27}H_{42}ClNO_2$)] is benzyldimethyl (2-{2- [4-(2, 4, 4-trimethylpentan-2-yl) phenoxy] ethoxy} ethyl) azanium chloride (Rodríguez *et al.,* 2012). It is used as a topical antimicrobial agent in aid antiseptics (Shintre *et al.,* 2006). BZC is found in cosmetics and toiletries such as mouthwashes, anti-itch ointments, antibacterial moist towelettes (Elder, 1984), and as a hard surface disinfectant (Bearden *et al.,* 2008). It is also found in several grapefruit seed extract preparations, and can be used as a preservative (Takeoka *et al.,* 2001), such as in the anesthetic Ketamines (Coates and Flood, 2001). It was identified as a novel specific anti-cancer agent by using a cell-based small molecule screen (Yip *et al.,* 2006). BZC is used as an anti-microbial agent in anthrax vaccine (Puziss *et al.,* 1963). It is also a common component in other injectable and nasal medications, such as thrombin, ketamine, orphenadrine, and butorphanol (Montvale, 2000).

Accordingly, this study aimed to evaluate the antitumor activity of BZC against hepatocellular carcinoma induced in rats.

2. MATERIALS AND METHODS

2.1. Chemicals

Diethylnitrosamine (DEN) and benzethonium chloride (BZC) were obtained from Sigma Aldrich Chemical Co., St. Louis, Mo. USA. All other chemicals and reagents were used of analytical grade.

2.2. Animals

Thirty-two Wistar rats (weighing 110–120 g) were obtained from the Nile Pharmaceutical Co., Cairo, Egypt. They were housed at the animal facility at the National Center for Radiation Research and Technology. The animals were left to acclimatize for one week before starting the experiment. The animals were kept under standard laboratory conditions of light/dark cycle (12/12 h), a temperature of 25 ± 2 °C and humidity of 60 ± 5%. The rats were housed in cages with free access to food and drinking water *ad libitum*. They were provided with a nutritionally adequate standard laboratory (pellet) diet. All animals procedures were carried out in accordance with the Ethics committee of National Research Center conformed to the Guide for the care and use of laboratory animals" published by the US National Institute of Health (NIH publication, No.85-23, 1996).

2.3. Experimental Design

Rats were randomly divided into four main equal groups (eight animals per group) as follows: Group I (Control): Rats were received no drug, served as control for all groups. Group II (BZC): Rats injected daily with benzethonium chloride (5 mg/ kg b.wt.) interaperitionealy for 4 weeks (Yip *et al.,* 2006). Group III (DEN): Rats received 20 mg/kg b.w. of DEN orally (five times per week for 6 weeks) and left a live for 4 weeks.

Group IV (DEN+ BZC): Rats received DEN as in-group III, after the dose of DEN, rats injected with benzethonium chloride as in-group II.

At the end of the experiment, rats were fasted overnight. Blood samples were withdrawn from the heart of each animal, under light anesthesia by diethyl ether. Blood was allowed to coagulate and then was centrifuged at 3000 rpm for 15 min for

serum separation. Immediately after blood sampling, animals were sacrificed by cervical dislocation; Liver tissue specimens from all animals groups were dissected and was kept in 10% formalin for histopathological examination.

2.4. Biochemical analysis

Serum aspartate aminotransaminase (AST), alanine aminotransaminase (ALT) and alkaline phosphatase (ALP) activities were determined by the methods described by Reitman and Frankel (1957) and King (1965) respectively. Also, total bilirubin, albumin and total protein concentrations were determined by method of Walter and Gerade (1970), Lowry et al. (1951) and Gornal et al. (1949) respectively.

2.5. Histopathological examination

Liver tissue specimen from of all animal groups were washed, dehydrated in ascending grades of ethyl alcohol, cleared in xylene and embedded in paraffin wax. Sections of 5–6 μm in thickness were cut out, deparaffinized and stained with Hematoxylin and Eosin (H & E) for examination under the light microscope (Banchroft *et al.,* 1996).

2.6. Statistical Analyses.

The SPSS (version 20) was used in data analyses. Data were analyzed with one-way analysis of variance (ANOVA) followed by a post hoc test (LSD) for multiple comparisons. The data were expressed as mean ± standard error (SE). P values < 0.05 were considered statistically significant.

3. RESULTS

3.1. Biochemical results

3.1.1. Effect of benzethonium chloride on liver function tests in serum of normal and HCC induced- rats is presented in figures 1 and 2

Rats administered with DEN showed significant increase in serum ALT, AST, ALP, and total bilirubin levels when compared with normal group. The treatment group (benzethonium chloride) significantly overcame the deleterious effects when compared with DEN group.

3.1.2. Effect of benzethonium chloride on serum albumin and total protein concentrations in normal and HCC induced rats is presented in Fig. (3)

Rats administered with DEN showed significant decrease in albumin and total protein when compared with normal group. The treatment group (benzethonium chloride) significantly overcame the deleterious effects when compared with DEN group.

3.2. Histopathological findings

Microscopical examination of liver in benzethonium chloride treated in DEN-induced HCC in rats is given in Fig. (4). Group A: rats revealed normal liver parenchyma cells with granulated cytoplasm, small uniform nuclei, and central vein surrounded by cords of hepatocytes. Group B: rats exhibited normal architecture, indicating the non-toxic nature of benzethonium chloride. Group C: DEN-treated rats showed dysplastic changes and the anisonucleosis, irregular chromatin pattern and prominent nucleoli. Groups D: rats co-treated with benzethonium chloride showed hepatocellular carcinoma with degenerative changes, congested portal blood vessels and inflammatory infiltrate.

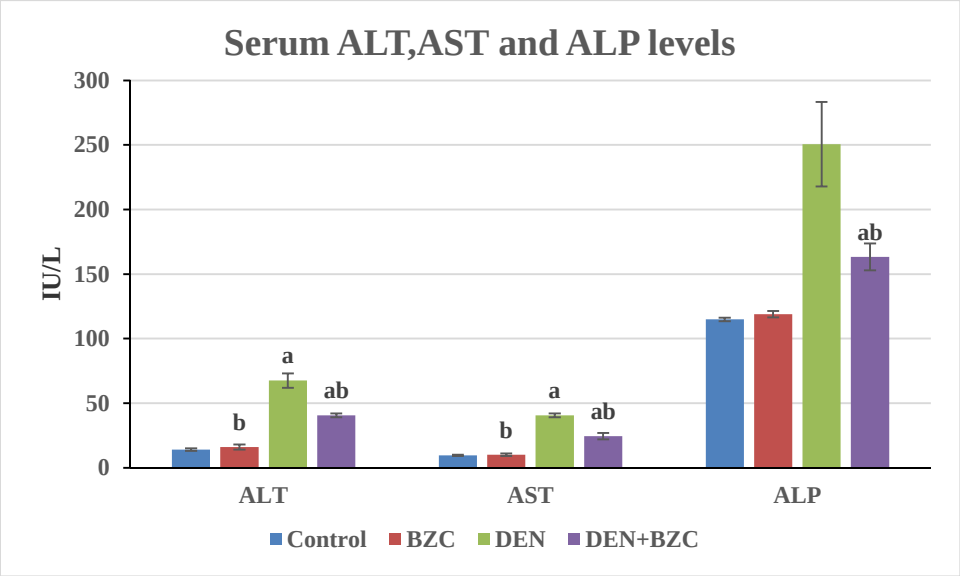

Figure 1: Effect of benzethonium chloride on serum ALT, AST and ALP activities of normal and experimental animals. Values are expressed as mean ± standard error of the mean (n = 6). Comparisons are made between: (a) normal rats (Group I) and (b) diethylnitrosamine-treated rats (Group III).

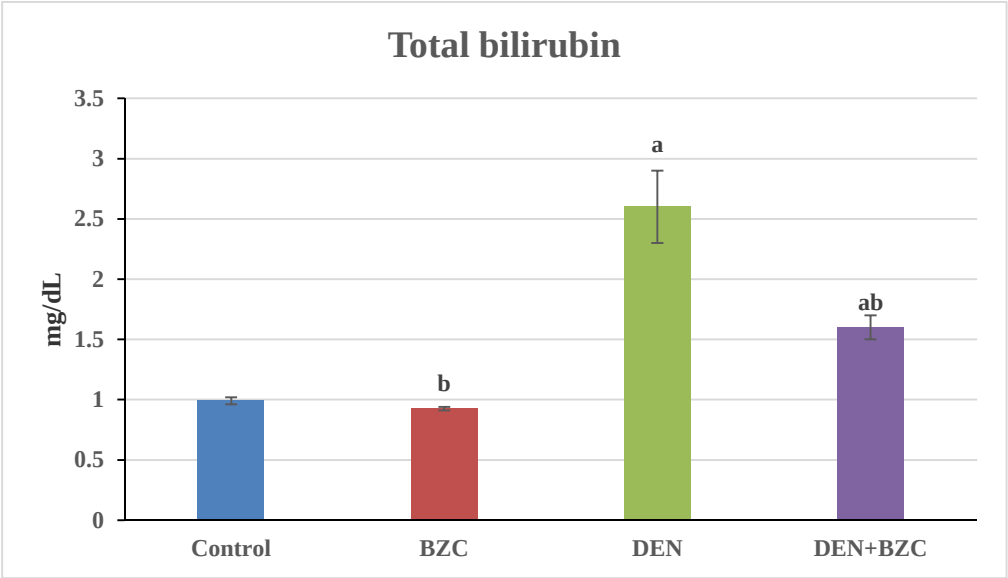

Figure 2: Effect of benzethonium chloride on serum total bilirubin level of normal and experimental animals. Values are expressed as mean ± standard error of the mean (n = 6). Comparisons are made between: (a) normal rats (Group I) and (b) diethylnitrosamine-treated rats (Group III).

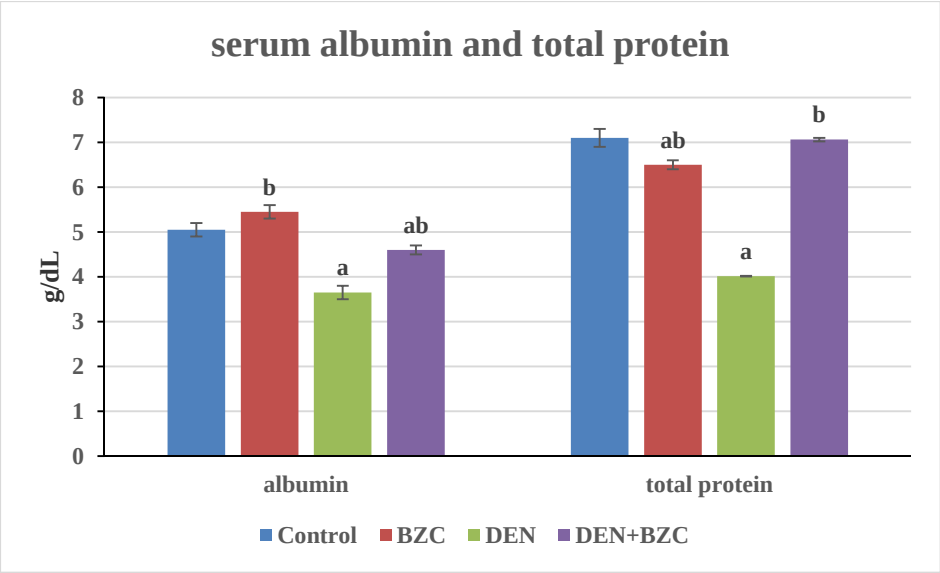

Figure 3: Effect of benzethonium chloride on serum albumin and total protein levels of normal and experimental animals. Values are expressed as mean ± standard error of the mean (n = 6). Comparisons are made between: (a) normal rats (Group I) and (b) diethylnitrosamine-treated rats (Group III).

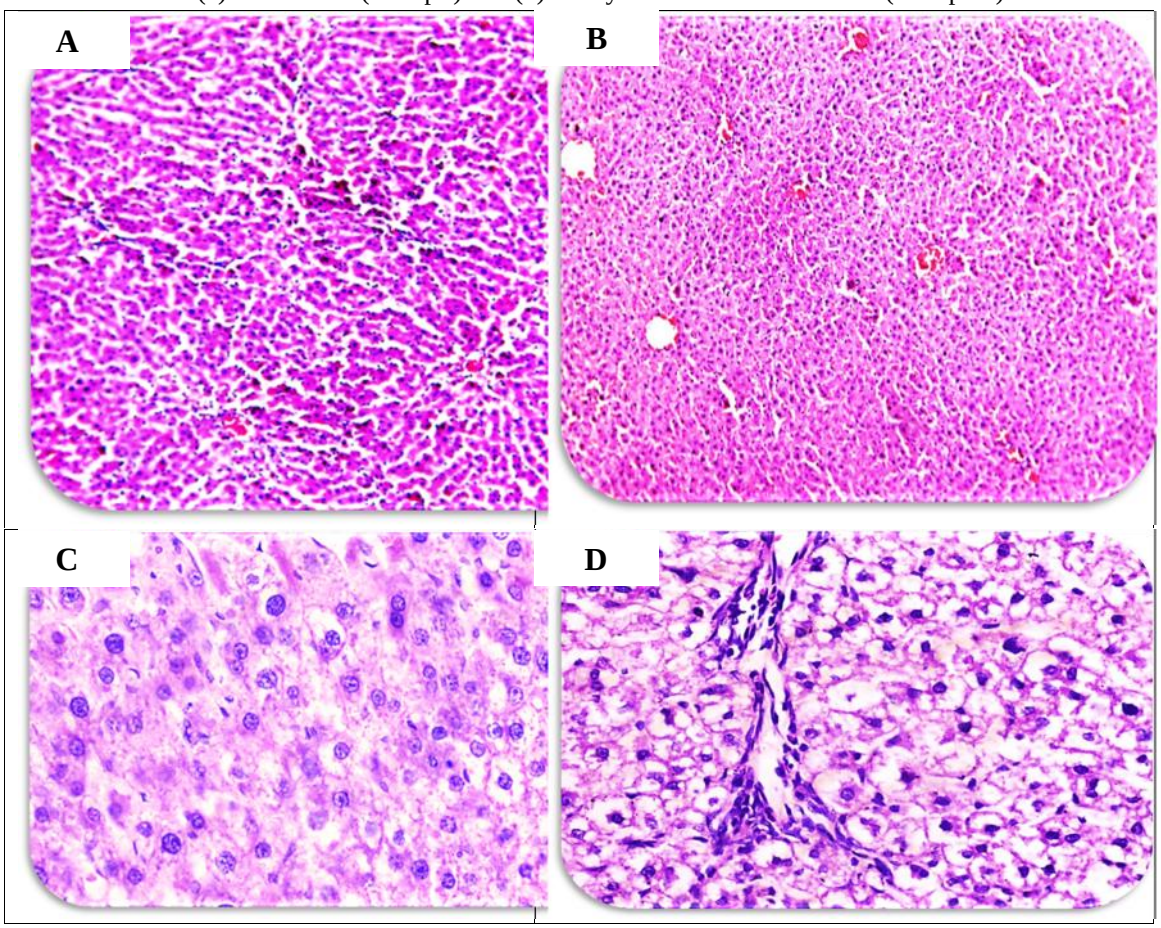

Fig. 4: Light microscopy of liver sections showing: (A) Control group; A normal rat liver tissue (H&E x40), (B) benzethonium chloride group; A liver of rat receiving benzethonium chloride showing dilated congested central vein and sinusoids (H&E x100), (C) DEN-treated group; A liver of rat receiving DEN showing dysplastic changes (note the anisonucleosis, irregular chromatin pattern and prominent nucleoli) (H&E x400), (D) DEN+ benzethonium chloride group; A liver of rat treated with benzethonium chloride after receiving DEN showing hepatocellular carcinoma with degenerative changes, congested portal blood vessels and inflammatory infiltrate (H&E x400).

4. DISCUSSION

Hepatocellular carcinoma is one of the most common malignancies worldwide and the main form of liver cancer due to its bad prognosis (Bruix, J. and Sherman, 2011). It is considered to be a multi-gene and muti-step disease process and likely involves various pathogenic factors, such as hepatitis B or hepatitis C infection, aflatoxin, and chronic, heavy ethanol consumption (Altekruse *et al.,* 2009). DEN-induced HCC model, similar to human HCC, is one of the most accepted and widely used experimental models to study hepatocarcinogenesis and screen potential anti-HCC agents (Lee *et al.,* 1991; Balamurugan and Karthkeyan, 2012; Khan *et al.,* 2011).

The obtained results showed that the values of serum ALT, AST/aminotransferase, ALP, and total bilirubin were significantly increased in DEN-induced HCC model than in normal group. Many pathological abnormalities were observed in liver tissues of DEN-induced HCC model group by histological analysis. Previous studies have claimed similar rise in the activity of serum liver markers upon DEN administration (Bansal *et al.,* 2005). These markers enzymes are mainly engaged in the diagnosis of hepatic injury with HCC lesions and parenchymal necrosis where these marker enzymes are released from the damaged hepatocytes into the bloodstream since these are primarily localized in the liver (Ramakrishnan *et al.,* 2007; Sallie *et al.,* 1991). Moreover, Ramakrishnan *et al*. (2007) attributed the increases in serum aminotransferase enzyme activities to their intracellular location in the cytosol, so toxicity affecting the liver with subsequent breakdown in membrane architecture of the cells leads to their spillage into serum where their concentration rises. ALP, another key hepatic marker enzyme, its elevated activity in serum indicates pathological alterations in bile flow. Studies have shown that dividing cells shed more of ALP as they are located in the bile canalicular plasma membrane (Frederiks *et al.,* 1990). Moreover, Thapa and Walia, (2007) stated that , the structural integrity of the cells has been damaged in cancer induced animals, and this results in cytoplasmic leakage of the enzyme into the blood stream, leads to the elevated levels of these enzymes in blood with a subsequent fall in the tissues. Kim *et al.* (1999) showed that the increase in the activities of serum ALT, ALP and AST occurred when hepatocellular damage caused abnormalities of liver function, and the activity of these enzymes increased remarkably in hepatoma.

Treatment with benzethonium chloride significantly reduced the level of the serum liver marker enzyme activity as compared to DEN group. This indicates the membrane stabilizing activity or the ability to repair the liver injury by maintaining the integrity of the plasma membrane, hence suppressing the leakage of enzymes through membranes, providing hepatoprotective action and inhibition of carcinogenesis.

Serum proteins have many functions, including the transport of substances, immune defense, blood clotting, and inflammation defense. Serum protein levels are useful for evaluating nutritional status, infection, and various other disorders. Within the human body, albumin is an important component of life (Aiad *et al.,* 2004; Goyal and Soni, 2011, Honarmand *et al.,* 2011). Albumin is synthesized in liver. In the human body albumin transports essential fatty acids

from adipose tissue, to muscle tissue. Consequently, decreased albumin levels may be associated with liver diseases (Honarmand *et al.*, 2011).

The total protein levels including albumin and globulin levels that have been reported to decrease in hepatotoxic conditions due to defective protein biosynthesis in liver (Clawson, 1989). The DEN intoxication causes disruption and disassociation of polyribosomes on endoplasmic reticulum and thereby reduces the biosynthesis of protein. Reduction in serum total protein and albumin in DEN treated group could be ascribed to the development of hepatic lesions observed in the present study that affected protein synthesis, which concurred with earlier findings (Ha *et al.*, 2001).

5. CONCLUSION

It could be concluded that, benzethonium chloride has potential hepatoprotective property as a food additive in HCC by restoring the hepatic marker enzymes and reversing cancer-indicating parameters during DEN-induced stress in rats. However, further studies are required to elucidate the molecular mechanism of benzethonium chloride.

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