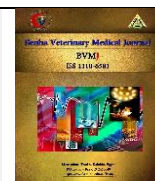




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The anti-proliferative and immunosuppressor effect of Newcastle and Doxorubicin against DEN induced HCC experimentally in rats

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ABSTRACT

Doxorubicin is a broad-spectrum antitumor antibiotic. Newcastle disease virus (NDV) is one of the viruses with an inherent oncolytic property. This study was done to investigate the antitumor effects of Doxorubicin and NDV on Diethylnitrosamine (DEN) induced Hepatocellular carcinoma in rats. Seventy-five male albino rats were divided into five groups. Group (1): rats administered distilled water only. Group (2): rats received diethylnitrosamine (200 mg/kg b. wt./i. p.), three times at an interval of 15 days at experimental weeks 2, 4 and 6. Group (3): rats received DEN then treated with Doxorubicin at experimental week 10 at a dose level (2 mg/kg b. wt./i. p.). Group (4): rats received DEN then treated with Hitchiner B1 at experimental week 10 at a dose level (10⁷ PFU/mouse/i. p.). Group (5): rats received DEN then treated with Doxorubicin at experimental week 10 at a dose level (2 mg/kg b. wt./i. p.) and with Hitchiner B1 at experimental week 10 at a dose level (10⁷ PFU/mouse/i. p.). All animals were sacrificed after the end of experiment. DEN induced HCC showed elevation in serum ALT, AST, ALP, GGT, bilirubin. Also, resulted in upregulation in serum IL-6 and TNF- α . On the other hand, resulted in a significant decrease in serum Albumin, total protein compared with control group. Treatment with doxorubicin or/ and NDV showed that, all the parameters changed towards normal control group. The obtained results confirmed that doxorubicin and NDV can inhibit the proliferation of HCC cells through improving hepatocytes

1. INTRODUCTION

Hepatocellular carcinoma (HCC) is a leading cause of cancer mortality. It is the fifth most common cancer in men and the seventh in women. Hepatocellular carcinoma often occurs in the background of a cirrhotic liver. Major risk factors for HCC include infection with HBV or HCV, alcoholic liver disease, and most probably nonalcoholic fatty liver disease. The distribution of these risk factors among patients with HCC is highly variable, depending on geographic region and race or ethnic group (El-Serag, 2011; Raza and Sood, 2014).

Diethylnitrosamine is a well-known potent hepatocarcinogenic agent present in tobacco smoke, water, cured and fried meals, cheddar cheese, agricultural chemicals, cosmetics, and pharmaceutical products. It is a hepatocarcinogen that is known to cause perturbations in the nuclear enzymes involved in DNA repair/replication. It is normally used as a carcinogen to induce liver cancer in animal models. It has been reported that DEN after its metabolic biotransformation produces the promutagenic adducts, O6-ethyl deoxy guanosine and O4- and O6-ethyl deoxy thymidine that may initiate liver carcinogenesis (Ramakrishnan *et al.*, 2006).

Doxorubicin acts on cancer cells through intercalation into DNA resulting in the inhibition of DNA synthesis and function. It inhibits transcription through inhibition of

DNA-dependent RNA polymerase (Panno, 2005). Doxorubicin is a DNA topoisomerase II inhibitor, DNA intercalator leading to DNA strand breaks and formation of Reactive Oxygen Species (ROS) in cells (Yurtcu *et al.*, 2015).

Oncolytic viruses (OVs) provide a new promising way to treat cancer. Such biological agents replicate selectively in tumor cells and induce tumor-selective cell death (oncolysis). Oncolytic viral therapy has an initial phase in which the virus mediates direct oncolysis of tumor cells, followed by a second phase of post-oncolytic immune response. This post-oncolytic immune response is directed towards tumor-associated antigens (TAs) and is considered as a key factor for an efficient therapeutic activity (Fournier and Schirmacher, 2014). Among the oncolytic viruses, Newcastle disease virus (NDV) is one such virus with an inherent oncolytic property (Omar *et al.*, 2003).

The aim of this study was to demonstrate the possibility of Newcastle virus (NDV) and doxorubicin to treatment HCC induced by DEN through evaluation of some biochemical parameters.

2. MATERIAL AND METHODS

2.1. Experimental animals:

Seventy-five albino rats, 5-6 weeks old and average body weight 80-120 g were used in the experimental

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investigation of this study. Rats were obtained from the production center for natural toxins and raw plasma at the Helwan farm of the Egyptian Company for Vaccines, Vaccines and Medicines. Animals were housed in separate metal cages. Pure drinking water was supplied ad-libitum through specific nipple. Rats were kept at constant environmental and nutritional conditions throughout the period of experiment. The animals were left 7 days before the beginning of the experiment for acclimatization.

Ration and additives:

The animals were fed on constant ration through the course of the experiment in the form of concentrated diet composed of concentrated mixture 10%, yellow corn 60.91 %, soya bean meal 22.3 %, fat 4.74 %, di calcium phosphate 0.83 %, ground limestone 0.67 %, methionine supplement 0.05 %, mineral and vitamin mixture 0.5%. Concentrate mixture composed of corn gluten meal 60 %, sunflower meal 44%, fish meal 45%, meat and bone meal 50%, di calcium phosphate, common salt limestone, vitamin and mineral, premix, L. lysine and methionine according to (NRC, 1995).

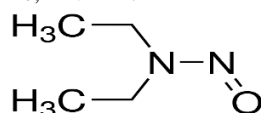
2.2. Chemicals:

The chemicals and natural agents used in the present study were:

2.2.1. Diethylnitrosoamine (DEN):

Common name: N-Nitrosodiethylamine ISOPAC®

Synonym: DEN; DENA; Diethylnitrosamine, N-Nitroso-N,N diethyleamine; NDEA.



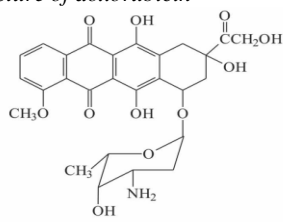
Induction of Hepatocarcinogenesis:

HCC was induced in rats by I.P injection of DEN in normal saline (200 mg/kg bw), three times at an interval of 15 days (Khan *et al.*, 2011).

2.2.2. Doxorubicin

Chemical anti-cancer drug, it is used at a dose level (2 mg/kg b. wt./i. p.) (Wakharde *et al.*, 2018).

Chemical structure of doxorubicin



2.2.3. Hitchner B1:

Purchased from Egypt Masters Co. for veterinary products. Live Newcastle vaccine (Hitchner B1), It is used at a dose level (10⁷ PFU/mouse/i. p.) (Sharma *et al.*, 2017).

2.3. Experimental design:

Rats were divided into five main equal groups each one contains 15 rats as follow:

Group 1: Control Normal group:

Consisted of 15 male rats, rats fed with ordinary diet only without any treatment during the entire experimental period (13 weeks).

Group 2: DEN- induced hepatocarcinogenesis group: Rats considered as the carcinogen control injected with DEN at a dose of (200 mg/kg body weight i.p) three times at an interval of 15 day at experimental weeks 2, 4 and 6.

Group 3: DEN- induced hepatocarcinogenesis + Doxorubicin treated group. Rats considered as the carcinogen control injected with DEN at a dose of (200 mg/kg b. wt./ i. p) three times at an interval of 15 day at experimental weeks 2, 4 and 6 then treated with Doxorubicin at experimental week 10 at a dose level (2 mg/kg b. wt./ i. p.) until the end of experiment.

Group 4: DEN- induced hepatocarcinogenesis + Hitchner B1 treated group. Rats considered as the carcinogen control injected with DEN at a dose of (200 mg/kg body weight i. p) three times at an interval of 15 day at experimental weeks 2, 4 and 6 then treated with Hitchner B1 at experimental week 10 at a dose levels (10⁷ PFU/rat/ip) until the end of experiment.

Group 5: DEN- induced hepatocarcinogenesis + Doxorubicin + Hitchner B1 treated group. Rats considered as the carcinogen control injected with DEN at a dose of (200 mg/kg b. wt. i. p) three times at an interval of 15 day at experimental weeks 2, 4 and 6 then treated with Doxorubicin at experimental week 10 at a dose level (2 mg/kg b. wt./i. p) and with Hitchner B1 at experimental week 10 at a dose levels (10⁷ PFU/rat/i. p.) until the end of experiment.

2.4. Sampling:

2.4.1. Blood samples:

Blood samples were collected by ocular vein puncture from all animal groups after overnight fasting in dry, clean tubes and allowed to clot for 30 minutes and serum was separated by centrifugation at 3000 rpm for 15 min. The serum was taken by automatic pipette and received in dry sterile tubes, then kept in deep freeze at -20 °C until use for subsequent biochemical analysis. All sera were analyzed for determination of the following parameters: (ALT, AST (Schumann *et al.*, 2002), ALP (Tietz *et al.*, 1983), GGT (Szasz, 1969), Total protein (Burtis *et al.*, 2012), Albumin (Doumas *et al.*, 1971), Total bilirubin (Young, 1997), (IL-6 (Chan and Perlstein, 1987) and TNF- α (Beyaert and Fiers, 1998)

2.5. Statistical analysis:

The results were expressed as mean \pm SE using SPSS software program version 16 (SPSS© Inc., USA). The data were analyzed using one-way ANOVA to determine the statistical significance of differences among groups. Duncan's test was used for making a multiple comparison among the groups for testing the inter-grouping homogeneity. Values were considered statistically significant when $p < 0.05$.

3. RESULTS

The obtained results presented in Figures (1-4) revealed that, DEN induced HCC (group2) showed elevation in serum ALT, AST, ALP, GGT, bilirubin. On the other hand, there was a significant decreased in serum Albumin, total protein compared with control group. Treatment with doxorubicin or/ and NDV (group 3,4 and 5) showed that a significant reduction in serum ALT, AST, ALP, GGT, bilirubin. resulted in a significant elevation in serum Albumin, total protein when compared with DEN group.

The obtained results presented in Figure (5) revealed that, DEN induced HCC (group2) showed upregulation in serum IL-6 and TNF- α compared with control group. Treatment with doxorubicin or/ and NDV (group 3,4 and 5) showed that a significant down regulation in serum IL-6 and TNF- α when compared with DEN group.

4. DISCUSSION

Hepatocellular carcinoma is now the second leading cause of cancer death worldwide. It is a unique cancer that typically arises in the setting of chronic liver disease at a rate dependent upon the complex interplay between the host, disease and environmental factors (Wallace *et al.*, 2015). *N*-Nitrosodiethylamine (DEN) is a potent hepatic carcinogen agent (Mahmoud and Abdul-Hamid, 2012).

The obtained results revealed a significant elevation in serum ALT, AST, ALP and GGT activities and total bilirubin concentration resulted in a significant decreased in serum Albumin and total protein with DEN injection to rats.

Similar results were obtained in DEN-induced HCC in albino rats in many studies like (Mohammed *et al.*, 2014; Sunwoo *et al.*, 2015) who reported that, DEN injection resulted in a significant increase in these liver enzyme activities in rats. Additionally, (Wills *et al.*, 2006) reported that, rats intoxicated with DEN had elevated activities of serum AST and ALT. Moreover, (Ranju *et al.*, 2013) reported that, administration of DEN to rats lead to a marked increase in the levels of serum AST, ALT and total bilirubin which is indicative of hepatocellular damage. Also, (Kumar *et al.* 2014) stated that, the animals treated with DEN showed a significant increase in levels of diagnostic liver marker enzymes AST, ALT, and ALP activities and bilirubin concentration in serum. Furthermore, (Gnanaraja and Prakash, 2014) displayed that, tumor bearing mice (DEN treated) showed significant increase in bilirubin concentration and ALT, AST, ALP activities. In case of DEN-induced HCC, the decreased albumin and total protein serum levels often indicates the presence of impaired liver function and inability to fight infections (Mohammed *et al.*, 2014). Similar decreased levels of serum albumin and total protein were obtained in DEN-induced HCC in many studies like (Metwally *et al.*, 2011) who reported that, DEN injection revealed a significant decrease tissue total proteins and serum albumin albino rats. Also, (Salama *et al.*, 2017) discussed that, groups treated with DENA led to significant elevation in plasma liver functions tests AST, ALT, GGT activities and total bilirubin concentration. Elevations of activities of ALT and AST may indicate the presence of hepatocellular predominant disorders while elevations of ALP and GGT activities may implicate cholestatic predominant diseases (Hann *et al.*, 2012).

Treatment with doxorubicin to DEN induced HCC in rats caused a significant decrease in serum ALT, AST, ALP and GGT activities and total bilirubin concentration as well as increase in serum total protein and albumin concentration when compared with DEN non-treated group. These results accorded with (Zhao *et al.*, 2015) who concluded that, DOX/Cur-NPs and observed their enhanced anti-tumor activity in DNE-induced HCC in mice, which might be regulated through increased apoptosis, and inhibited proliferation and angiogenesis.

Cell analysis indicates that modulation on MDR- and hypoxia-related proteins may contribute together to the enhanced activity of DOX/Cur-NPs. Furthermore, In DEN-induced HCC in mice treated with DOX-NPs and DOX/Cur-NPs, (Zhao *et al.*, 2015) observed that, decrease in liver damage, which is in line with the reported increase in DOX sensitivity loaded in nanoparticles for HCC (Barraud *et al.*, 2005).

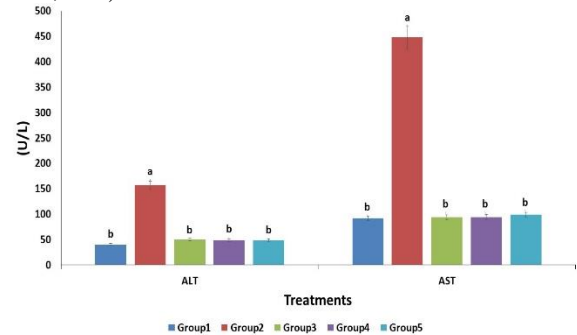


Figure 1 Effect of Newcastle disease virus on serum ALT and AST activities of DEN induced HCC in rats (U/L).

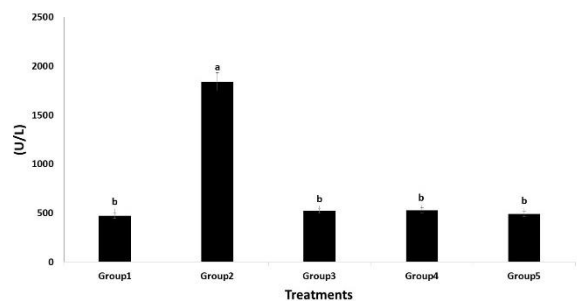


Figure 2 Effect of Newcastle disease virus on serum ALP activity of DEN induced HCC in rats (U/L).

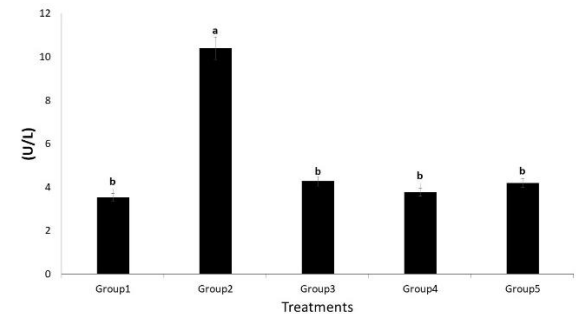


Figure 3 Effect of Newcastle disease virus on serum GGT activity of DEN induced HCC in rats (U/L).

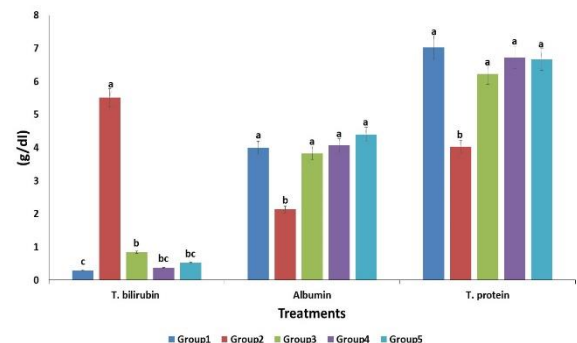


Figure 4 Effect of Newcastle disease virus on serum total bilirubin, Albumin and total protein concentration of DEN induced HCC in rats (g/dl).

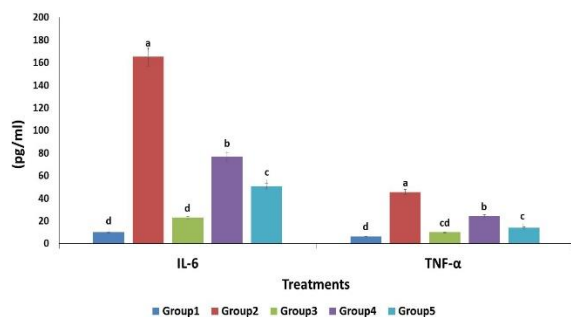


Figure 5 Effect of Newcastle disease virus on serum IL-6 and TNF- α concentration of DEN induced HCC in rats (pg/ml).

Treatment with oncolytic viruses, Newcastle disease virus (NDV) to DEN induced HCC in rats caused a significant decrease in serum ALT, AST, ALP and GGT activities and total bilirubin concentration as well as increase in serum total protein and albumin concentration when compared with DEN non-treated group. These results accorded with Shinozaki *et al.* (2004) who reported that, the effect oncolytic virus therapy on HCC and showed significant elevations of serum transaminases (aspartate aminotransferase (AST) and alanine aminotransferase (ALT)) were seen at day 1 after hepatic arterial administration of rVSV-h-gal, although the levels rapidly returned to baseline at day 3. To determine if the significant but transient rise in serum transaminases was caused by toxicity to the liver or oncolysis of the HCC lesions, they infused non-tumor-bearing animals with the same dose of rVSV-h-gal (n = 3) or PBS control (n = 3) in a follow-up experiment and measured serum transaminases 1 day later. The results indicated that rVSV-h gal infusion into the hepatic artery of non-tumor-bearing animals caused only mild elevations in serum transaminases compared to buffer-injected control animals. Therefore, the significant but transient elevation in serum transaminases on day 1 in tumor-bearing animals infused with rVSV-h-gal can be attributed to massive VSV-induced oncolysis of HCC cells and subsequent release of transaminases into the circulation. Serum transaminase levels in tumor-bearing animals also increased slightly at 14 days posttreatment, which might be indicative of tumor progression.

The obtained results revealed that, DEN induced HCC showed upregulation in serum IL-6 and TNF- α . Similar results were obtained by (Kumar *et al.*, 2016) who confirmed that, TNF- α level significantly upregulation in DEN treated animals. Moreover, (Song *et al.*, 2013) showed that, DEN-induced HCC increased TNF- α , such tumor necrosis factor alpha (TNF- α) is pro-inflammatory cytokines produced by macrophages and it plays an important role under tumor conditions (Lutsiak *et al.*, 2005). Similarity, (Tork *et al.*, 2015; Ahmed *et al.*, 2015) investigated that, there was significant increase in the serum levels of TNF α and IL-6 in the HCC group.

The obtained results revealed that, treatment with doxorubicin or/ and NDV showed that a significant down regulation in serum IL-6 and TNF- α when compared with DEN group. Similarity, Shinozaki *et al.*, (2004) compared the blood levels of proinflammatory cytokines (TNF- α , IFN-g, IL-6, and IL 1h) in the VSV-injected animals to those before injection of the virus at all time points and were well below concentrations associated with systemic toxicity in animals and in human clinical trials. The results indicated that hepatic arterial administration of recombinant VSV at the MTD did not induce a systemic proinflammatory cytokine response in the immune-competent rat model (Reid *et al.*, 2002).

5. CONCLUSION

The present findings exhibited that doxorubicin or Newcastle virus improve liver cells damage which led to improvement of liver cells and can inhibit the proliferation of HCC cells which decreased the serum ALT, AST, ALP, GGT, bilirubin concentration and down regulation in serum IL-6, TNF- α that increased by DEN induction. On the other hand, increase in serum albumin and total protein concentrations that decreased by DEN induction. It could be concluded that, the chemo preventive and anti-tumor effect of doxorubicin or Newcastle as powerful agents may be useful as a potent anti-tumor activity in hepatocellular carcinoma.

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