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Effect of Vascular Disrupting Agent (CA-4DP) and Ionizing Radiation on Tumor Growth in Rats

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Abstract

Hepatocellular carcinoma (HCC) is the most common primary liver cancer worldwide. This study was performed to explore the effect of low dose γ-irradiation (LDR) and Combretastatin A-4 disodium phosphate (CA-4DP) on HCC induced by N-nitrosodiethylamine (NDEA) in rats.

Male Wistar albino rats were orally administrated NDEA for 17 weeks. Rats bearing HCC were intravenously injected with 10 mg/kg of CA-4DP and/or exposed to 0.20 Gy of γ-irradiation. Histopathological examination of the liver tissues was performed following 6, 24, and 48 h post CA-4DP injection.

Treatment with LDR and CA-4DP resulted in extensive bleeding and necrosis in tumor tissue that was associated with HCC regression. The presented study demonstrated the promising therapeutic advantage of LDR along with CA-4DP in HCC.

Keywords: Hepatocellular carcinoma, Ionizing radiation, Combretastatin A-4 disodium phosphate.
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1. Introduction

Hepatocellular carcinoma represents a serious health problem in Egypt, contributing to 14.8% of all cancer mortality with a higher incidence in men 33.6 % than in women 13.5% [1]. Chronic infection with HCV is considered a main risk factor for HCC in Egypt as it was reported in 71% of the HCC cases [2].

N-nitrosodiethylamine is a potent hepatocarcinogen that induce the development of HCC through interaction with DNA bases and formation DNA strand-break and mutations. Hepatocarcinogenesis could be induced by the electrophilic moieties generated by NDEA that interfere with the cellular DNA repair mechanism, causing alterations in DNA, formation of adducts, deletions and activation of proto-oncogenes. This induce the transformation of normal hepatocyte to become an initiated cell that can develop focal lesions, which permit the development of HCC [3]. Moreover, reactive oxygen species (ROS) generated by NDEA was reported to elevate oxidative stress leading to membrane lipid peroxidation, DNA damage and mutagenesis associated with tumor formation [4].

Radiotherapy (RT) is a key component in the treatment of cancer. Over the past decades, high-dose radiation (HDR) has long been the focus of interest among researchers. However, it has a major limitation in the treatment of liver tumors, mainly due to the low tolerance of the liver to radiation. Many reports have reported the negative side effects of RT, including radiation-induced liver disease (RILD) [5, 6]. Dissimilar to high-dose radiation (HDR), LDR was reported to induce innate and adaptive immune responses, enhance anticancer immunity, and delay cancer progression [7]. Moreover, low dose radiation has been shown to induce protection against spontaneous genomic damage, spontaneous and induced mutations, spontaneous neoplastic transformation, and suppress cancer metastases [8].

Combretastatin A4 disodium phosphate (CA-4DP) is the prodrug of Combretastatin A-4 (CA-4), the natural vascular disrupting agent that was extracted from the South African tree *Combretum caffrum* by Pettit *et al.* [9]. Interestingly, dissimilar from other anti-vascular agents, CA-4 has been shown to target only blood vessels at the core of the tumor to avoid drug resistance resulting in hypoxia and subsequent tumor necrosis [10]. It is believed that the selectivity of CA-4 occurs due to the structural and the functional differences between the tumor vasculature and normal vessels [11]. This study was undertaken to investigate the synergistic effect of LDR along with CA-4DP on HCC induced by NDEA in rats.

2. Material and Methods:

Experimental animals:

Male Wistar albino rats, of about 140 - 160 g body weight, were supplied by The National Center for Radiation Research and Technology (NCRRT) (Nasr City, Egypt).

Rats were acclimatized in the animal house building of the (NCRRT) for one week with a 12:12-hour light, dark cycle and they were provided with a pellet concentrated diet containing the necessary nutritive elements and tap water *ad libitum*.

Chemicals:

N-nitrosodiethylamine (NDEA) (CAS no. 55-18-5) was purchased from Sigma-Aldrich Chemicals Co. (St Louis, MO, USA).

Combretastatin A4 disodium phosphate (CA-4DP) (CAS: 168555-66-6) was purchased from Chemleader Biomedical Co. (Shanghai, China).

Irradiation procedure:

Rats were exposed to 0.20 Gy (0.685 rad/sec) for whole body gamma irradiation in Cesium 137 Gamma Cell-40 unit (Atomic Energy of Canada Ltd, Ottawa, Ontario, Canada), installed in the "National Centre for Radiation Research and Technology (NCRRT)", Atomic Energy Authority, Cairo, Egypt.

Experimental protocol:

The experimental animals were divided into seven groups; then these groups were divided into three subgroups, each comprising of six animals. Control group (1), normal rats received standard diet and drinking water; HCC group (2), rats were orally administrated NDEA at a dose of 20 mg/kg (5 times/week) for 8 weeks, followed by 10 mg/kg (5 times/week) for 9 weeks [12]; group (3), rats bearing HCC were exposed to a single low dose of 0.20 Gy of γ-irradiation [13]; group (4), rats bearing HCC were intravenously injected with 10 mg/kg of CA-4DP [14]; group (5), rats bearing HCC were injected with CA-4DP 24 h post exposure to whole body γ- irradiation; group (6), normal rats injected with CA-4DP; group (7), normal rats exposed to whole body γ- irradiation. Random rats were sacrificed after 3, 5, 9, 13, and 17 weeks. Histopathological examination of liver tissues was performed to ensure the formation of HCC. At the end of the experiment period all groups were anesthetized and sacrificed after 6, 24, and 48 hours post CA-4DP treatment.

Histopathological examination:

Liver samples were taken from rats in different groups and fixed in 10% formal saline for 24 h. Washing was done in tap water then serial dilutions of alcohol (methyl, ethyl and absolute ethyl) were used for dehydration. Then, Specimens were cleared in xylene and embedded in paraffin at 56 °C in hot air oven for 24 h. Paraffin wax tissue blocks were prepared for sectioning at 4 microns thickness by sledge microtome. Subsequently, the obtained tissue sections were collected on glass slides, deparaffinized, stained with hematoxylin and eosin and examined by the light electric microscope.

3. Results

Morphological Examination:

The surface of the liver in the control group was soft in texture with an evident gloss (Fig. 1A). In contrast, in NDEA group it became abnormal. The surface of the liver gradually roughened in early carcinogenesis (1-9 weeks) (Fig. 1B). In the intermediate carcinogenesis (10-13 weeks) the liver surface became completely roughened, and had many lesions (Fig. 1C). Finally, in the late carcinogenesis (13-17weeks) the surface of the liver was covered with multiple small and large nodules (Fig. 1D).

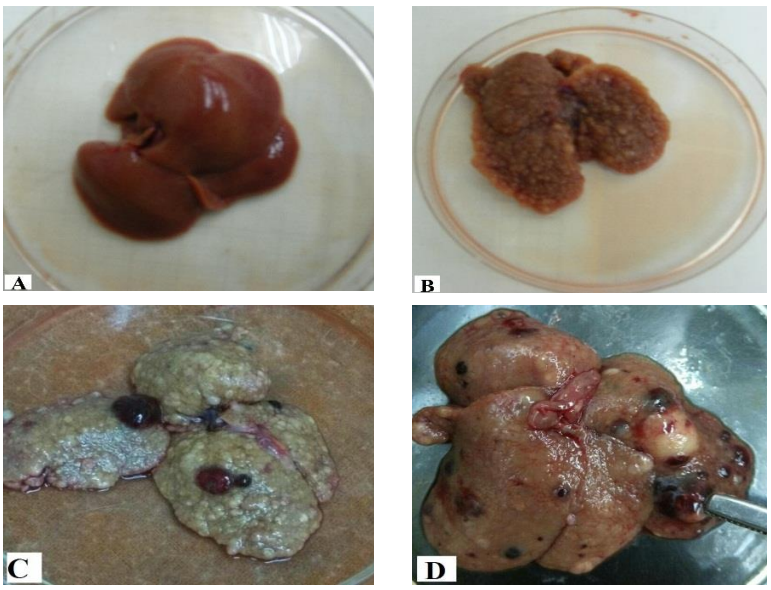

Fig (1): Changes observed in the liver morphology during the development of HCC by NDEA. (A) Normal group, (B) Early carcinogenesis, (C) Intermediate carcinogenesis, and (D) Late carcinogenesis.

Histopathological investigation:

Liver sections from control group revealed a normal histological structure of the central vein (CV) and hepatocytes (H) (Fig. 2A). Meanwhile, oral administration of NDEA for 3 weeks induced the proliferation of oval cells and retention of lipids within the hepatocytes which resulted

in hepatic steatosis (Fig. 2B). In addition, after 5 weeks, abnormal changes in hepatocytes shape, size, and organization was observed (Fig. 2C). Also, a marked proliferation of oval cells which function as tumor progenitors was noticed 9 weeks following NDEA treatment (Fig. 2D). Moreover, multiple focal anaplastic hepatocytes were detected in liver sections at the 13th week (Fig. 2E). Furthermore, at the end of the 17th week, loss of architecture, focal extravasated red blood cells, hyperplastic cystic dilatation in the bile ducts, fibrosis, and variegated forms of hepatocellular carcinoma were recorded (Fig. 2F).

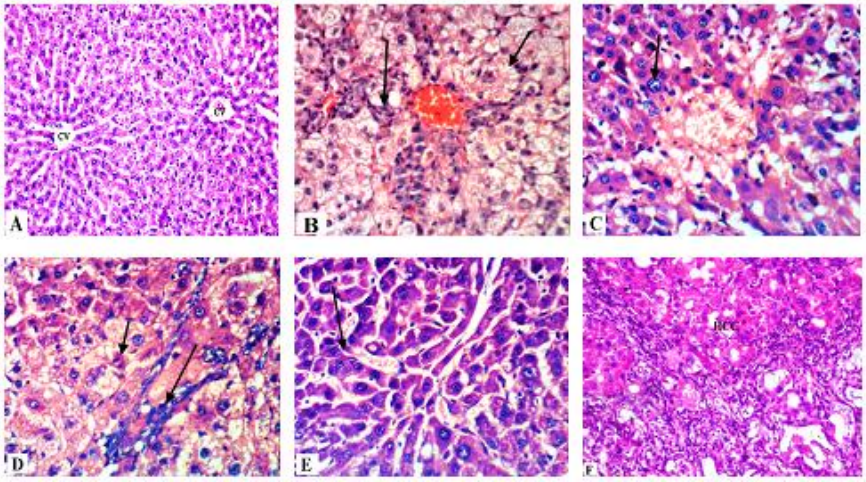

Fig (2): Photomicrographs of sections in liver tissue of male rat stained with H&E.
A: Section in liver from control group (Mag. 200X). B: Section in liver from NDEA group after 3 weeks showing steatosis (small arrow) and marked proliferation of oval cells (large arrow) (Mag. 400X). C: Section in liver from NDEA group after 5 weeks showing dysplastic hepatocytes and karyomegally of hepatocytes nuclei (arrow) (Mag. 400X). D: Section in liver from NDEA group after 9 weeks showing mitotic figure of hepatocytes (small arrow) and marked proliferation of oval cells (large arrow) (Mag. 400X). E: Section in liver from NDEA group after 13 weeks showing anaplastic hepatocytes (arrow) (Mag. 400X). F: Section in liver from NDEA group after 17 weeks showing hepatocellular carcinoma (HCC) (Mag. 200X).

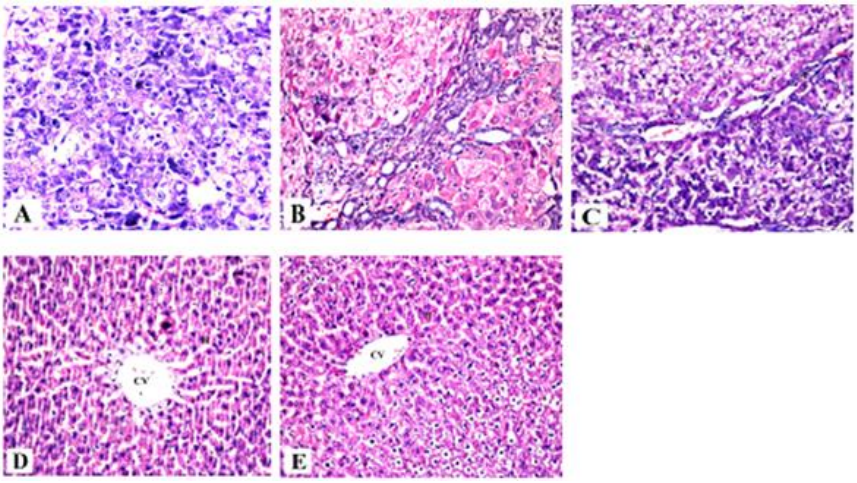

Fig (3): Photomicrographs of sections in the liver tissue after 6 h from CA-4DP injection.
A: Section in the liver from HCC group exposed to LDR showing degeneration of hepatocytes (H&E, 200X). B: Sections in the liver from HCC group treated with CA-4DP showing fibrosis dividing the degenerated and necrosed hepatocytes into lobules (H&E, 200X). C: Section in the liver from HCC group exposed to LDR and treated with CA-4DP showing fibrosis and degeneration of hepatocytes (H&E, 200X). D: Section in the liver from LDR group showing no histopathological alteration in the structure of the central vein and surrounding hepatocytes in the parenchyma (H&E, 200X). E: Sections in the liver from CA-4DP group showing no histopathological alteration in the structure of the central vein and surrounding hepatocytes in the parenchyma (H&E, 200X).

In contrast, HCC group exposed to LDR displayed a degeneration in hepatocytes, fibrosis with inflammatory cells infiltration (Figures 3A, 4A); focal necrosis, as well as, dysplastic hepatocytes (Fig. 5A). Moreover, HCC group

treated with CA-4DP showed a marked degeneration in the hepatocytes, fibrosis dividing the necrosed hepatocytes into lobules (Figures 3B and 4B), congested blood vessels, scattered foci of tumor tissue necrosis and inflammatory cells infiltration (Fig. 5B).

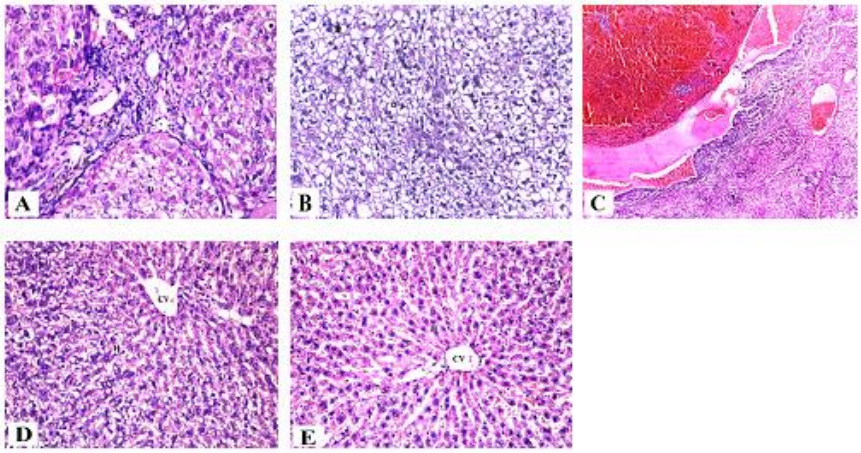

Fig (4): Photomicrographs of sections in the liver tissue after 24 h from CA-4DP injection.
A: Section in the liver from HCC group exposed to LDR showing fibrosis, inflammatory cells infiltration and degeneration in hepatocytes (H&E, 200X). B: Sections in the liver from HCC group treated with CA-4DP showing a marked degeneration in hepatocytes (H&E, 200X). C: Section in the liver from HCC group exposed to LDR and treated with CA-4DP showing markedly congested blood vessels and extensive bleeding in tumor tissue (H&E, 100X). D: Section in the liver from LDR group showing no histopathological alteration in the structure of the central vein and surrounding hepatocytes in the parenchyma (H&E, 200X). E: Sections in the liver from CA-4DP group showing no histopathological alteration in the structure of the central vein and surrounding hepatocytes in the parenchyma (H&E, 200X).

Furthermore, HCC group exposed to LDR 24 h pre CA-4DP injection displayed degeneration in hepatocytes and fibrosis (Fig. 3C), a marked congestion in the blood vessels (Fig. 4C), as well as, extensive areas of tumor tissue necrosis (Fig. 5C). Interestingly, following 48 h no sign for neoplastic activity was detected. On the other hand, liver sections from LDR group (Figures 3D, 4D, 5D) and CA-4DP group (Figures 3E, 4E, 5E) revealed no histopathological alteration in the histological structure of the central vein and surrounding hepatocytes in the parenchyma.

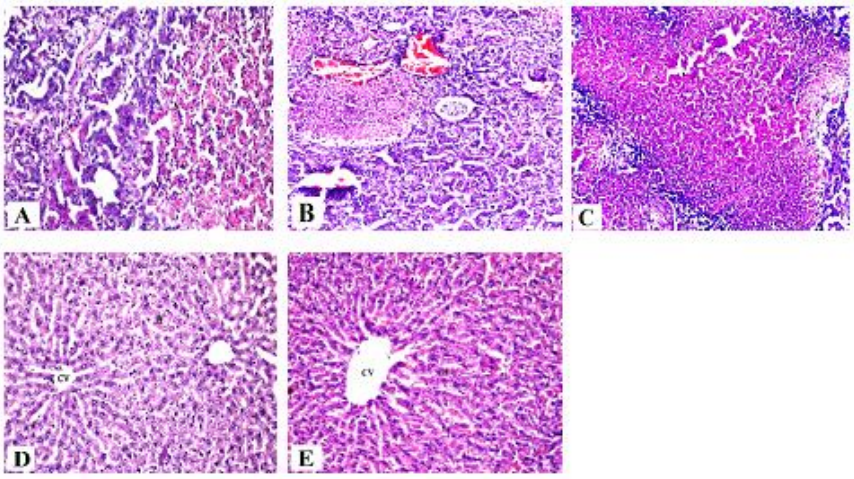

Fig (5): Photomicrographs of sections in the liver tissue after 48 h from CA-4DP injection.
A: Section in the liver from HCC group exposed to LDR showing focal necrosis and areas of dysplastic hepatocytes (H&E, 100X). B: Sections in the liver from HCC group treated with CA-4DP showing congested blood vessels, scattered foci of tumor tissue necrosis and focal inflammatory infiltration (H&E, 100X). C: Section in the liver from HCC group exposed to LDR and treated with CA-4DP showing wide areas of extensive necrosis in tumor tissue (H&E, 100X). D: Section in the liver from LDR group showing no histopathological alteration in the structure of the central vein and surrounding hepatocytes in the parenchyma (H&E, 200X). E: Sections in the liver from CA-4DP group showing no histopathological alteration in the structure of the central vein and surrounding hepatocytes in the parenchyma (H&E, 200X).

4. Discussion

N-nitrosodiethylamine has been used for decades in rodents to induce carcinogenesis. Our study showed that intravenous injection of CA-4DP in rats with HCC resulted in extensive changes in tumor histology consistent with necrosis and apoptosis. These results are consistent with [14, 15]. In addition, previous studies reported that CA-4 inhibited tumor cells growth and metastasis and retards tumor growth in many cancer models [16, 17, 18].

Iyer *et al*. [19] demonstrated that CA-4 acts as a potent inhibitor of cell proliferation leading to cell death via pathways rather than Caspase-3 induced apoptosis. However, CA-4P can't bind directly with β-tubulin. Stratford and Dennis [20] reported that shortly after administration, CA-4P was cleaved to its active form CA-4 that could bind with β-tubulin at or near the colchicine binding site and cause microtubule destabilization. In addition, CA-4 was reported to arrest cancer cells in the G2/M phase of the cell cycle by suppressing spindle dynamics, leading to mitotic arrest and cell death [21]. Moreover, Böhle *et al*. [22] and Nabha *et al*. [23] demonstrated that cancer cell death via mitotic catastrophe probably occurred due to metabolic breakdown. However, previous reports demonstrated that CA-4P alone is insufficient for tumor eradication because of evident peripheral residue of tumor cells that lead to recurrence [14, 24, 25]. Our study aimed to inspect whether the combination therapy of CA4-DP and LDR can produce an improved anticancer effect in the treatment of HCC induced by NDEA in rats with.

Findings from our study showed that exposure of HCC group to LDR resulted in tumor necrosis, these results are consistent with [26, 27]. They reported that immune stimulation by LDR could inhibit tumor growth and metastasis. Moreover, rats with HCC exposed to LDR 24 h before CA-4DP showed extensive bleeding and necrosis in wide areas in the tumor not only the core of the tumor. The anticancer effects of low dose radiation could be accomplished by enhancement of the immune functions not through directly killing tumor cells by radiation [28]. Additionally, Reissfelder *et al*. [29] reported that LDR abrogated the immune system inhibition by tumor cells via the downregulation of hypothalamus-pituitary-adrenal axis.

Previous animal studies have shown that LDR at a dose of (0.1 or 0.2) Gy suppressed tumor metastases in BALB/c mice. The anti-cancer effects of LDR could be abolished by the natural killer (NK)-suppressive anti-asialo GM1 antibody. These finding suggest that NK cells play an essential role in LDR's anti-cancer effect through activation the P38-MAPK pathway [13, 30]. In addition, many reports demonstrated that exposure to low dose X-rays or gamma radiation stimulate the immune response by increasing natural killer (NK) cells, dendritic cells, macrophages, T cells, mast cell activity [31, 32]. Also, LDR was reported to increase the expression of cell surface markers, on antigen-presenting cells (APCs) and T cells thereby stimulating the anti-cancer immunity and inhibit tumor progression [33]. Moreover, a single dose of LDR has been shown to reduce the T-regulatory cells (Tregs) that contribute in immune-evasion by cancer cells [28]. Furthermore, Kojima *et al*. [26] reported that LDR increased antibody secretion and enhanced the antibody-dependent cellular cytotoxicity response in tumor-bearing mice.

5. Conclusion

Our study concluded that treatment with CA-4DP or LDR alone only induced a modest tumor growth delay. The remained viable cells after treatment with CA-4DP are thought to survive because they derive nutrients and oxygen from the blood vessels in adjacent normal tissue which are not responsive to the CA-4DP. In contrast, exposure of HCC bearing rats to whole body LDR pre-CA-4DP led to eradication of residual tumor cells and augmented the antitumor effects of CA-4DP via activation of the immune responses.

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