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Induction of hepatocellular carcinoma in mice and the role of melatonin

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Abstract 2-Nitropropane (2-NP) is suggested to be a human carcinogen, a genotoxicant and hepatocarcinogen in rodents. The present study was designed to evaluate the role of melatonin (Mel) as an anticancerous agent in hepatocellular carcinoma (HCC) induced by 2-NP. Forty eight male Swiss albino mice were divided into 5 groups. Group I, II and III served as controls. Group IV was injected IP with 2-NP every other day for 14 weeks. Group V was injected IP with melatonin 30 min prior to 2-NP injection. Mice were sacrificed after 4, 8 and 14 weeks. Histopathological results showed poorly differentiated HCC; pleomorphic atypical hepatocytes after 8 weeks of receiving 2-NP. Proliferating streaks and cords of malignant hepatocytes were prominent after 14 weeks of 2-NP injection. (Mel + 2-NP) treatment revealed histological features more or less similar to normal control with dilated sinusoids, evidence of apoptosis, eosinophilic cytoplasm, few large necrotic cells, microsteatosis and well defined Kupffer cells. Histochemically, group IV showed continuous decrease in catalase activity and very weak activity was reached after 14 weeks. In group V, marked and moderate catalase activity was observed after 4 and 8 weeks, respectively as well as marked diffused reaction after 14 weeks. Immunohistochemically, PCNA and VEGF expressions were significantly increased in group IV and this increment was time dependant. Decreased numbers of PCNA and VEGF expressions in group V were seen as compared with their counterparts. It was concluded that 2-NP was a hepatocellular carcinogen in mice and melatonin exerted its anticancerous role through its antiproliferative and antiangiogenic effects.

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Introduction

Hepatocellular carcinoma (HCC) is a primary liver cancer that arises from hepatocytes, the major cell type in the liver. It is the most common primary hepatic tumor and the fifth most common cancer worldwide, in addition to being the third most common cause of cancer mortality which affected

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approximately one million people around the world every year (Motola-Kuba et al., 2006 and El-Serag and Rudolph, 2007).

Chemical carcinogens played an important role in the etiology of HCCs (Zhang, 2010). Many recognized human carcinogens were occupational carcinogens (Siemiatycki et al., 2004). Nitroalkanes were used widely in the chemical industry for synthesizing common reagents, they were considered to be toxic and carcinogenic (Mijatovic, 2008). One of these nitroalkanes was 2-nitropropane (2-NP). It was widely used as an industrial solvent in many industries (Sakano et al., 2001). The liver was thought to be the target organ of 2-NP toxicity which caused cell proliferation (El-Sokkary, 2002). It was hepatocarcinogenic in rats (Nakayama et al., 2006; Mijatovic, 2008) and an oxidative stress inducing agent in mice (Cabelof et al., 2002). The liver damage induced by 2-NP was associated with oxidative damage and lipid peroxidation (Borges et al., 2006). 2-NP induced DNA damage in the liver tissue of mice (Unnikrishnan et al., 2009) by radicals generated via hydrogen peroxide species (Sakano et al., 2001).

Catalase is an antioxidant enzyme found in peroxisome, plays a significant role in protection against oxidative stress by the decomposition of hydrogen peroxide (H_2O_2) (Martín-Renedo et al., 2008; Ekanayake et al., 2008). This reaction was important in the liver and kidney cells, where the peroxisomes detoxified different toxic substances entering the blood (Alberts et al., 2002).

The vascular endothelial growth factors (VEGFs) are a family of proteins which have a pivotal role in regulating tumor angiogenesis (Shibuya and Claesson-Welsh, 2006). VEGF is a potent cytokine that binds to specific receptors on the endothelial cells lining blood vessels, triggering the signaling cascade which led to the formation of new capillaries (angiogenesis) (Stefanini et al., 2009). Vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) are two of the major factors among many factors involved in angiogenesis (Gjini, 2005).

Proliferating cell nuclear antigen (PCNA), a cell cycle marker protein, functions as a sliding clamp during DNA replication (Bowman et al., 2004 and Freudenthal et al., 2008). PCNA presented in mammalian cells as a double homotrimeric protein (Freudenthal et al., 2008). PCNA is involved in protein–protein interactions (Maga and Hubscher, 2003) and in the DNA damage tolerance pathway known as post-replication repair (Lehmann and Fuchs, 2006).

Melatonin (*N*-acetyl-5-methoxytryptamine) is the main secretory product of the pineal gland in mammals (Tan et al., 2007), and is also produced in many organs and tissues such as the retina, the gastrointestinal tract, skin, bone marrow and 1ymphocytes (Pandi-Perumal et al., 2006). Melatonin is a powerful scavenger of reactive oxygen species (ROS), as well as being a stimulator of the antioxidant enzymes: superoxide dismutase, glutathione peroxidase, and catalases, that led to a decrease in DNA damage (Blask et al., 2002).

The present study was designed to evaluate the role of 2-nitropropane (2-NP) in HCC induction in mice and the role of melatonin as an anti-cancerous agent through the following parameters: histopathological, histochemical and immunohistochemical studies.

Materials and methods

This study was carried out in accordance with the protocol of Laboratory Animal Unit of Medical Research Institute, Alexandria University concerning guiding principles for biomedical research involving animals.

The present study was carried out on forty eight male Swiss albino mice, three month old and weighing 30 ± 10 g. They were obtained from the Laboratory Animal Unit, Medical Research Institute, Alexandria University. They were kept under the same laboratory conditions of 20–25 °C and 12 h light–dark cycle and given free access to standard food and water.

They were then randomly divided into the following groups:

Group I: Normal control group (6 mice).
Group II: Experimental control group (6 mice) was injected IP with 1 ml of 10 mg melatonin (Amoun Co. Egypt)/kg BW every other day (Ravindra et al., 2006) at 2 pm for 14 weeks. Melatonin (Mel.) was dissolved in ethanol before being diluted with saline making the final concentration of ethanol in melatonin < 1% (El-Sokkary et al., 2007).
Group III: Solvent control group (6 mice) was injected IP with 1 ml of olive oil every other day for 14 weeks.
Group IV: Experimental group (15 mice) was injected IP with 1 ml of 200 mg 2-NP (Sigma–Aldrich Chemie Co.), dissolved in olive oil, /kg body weight (Borges et al., 2006) every other day for 14 weeks.
Group V: Experimental treated group (15 mice) was injected IP with 1 ml of 10 mg melatonin/kg body weight every other day 30 min prior to 2-NP injection for 14 weeks.

Two mice from each control group and 4 mice from each treated group were sacrificed after 4, 8 and 14 weeks from the beginning of the experiment. Liver was dissected out and the right lobe was cut into 2–4 mm^3 pieces which were either fixed in 10% neutral buffered formalin for 24 h or used as frozen cryostat sections. Then the following studies were performed:

Histopathological study

Formalin fixed specimens were processed and prepared to get blocks. Sections 3 μm thick were cut and stained with hematoxylin and eosin (H&E) for histopathological investigations (Bancroft and Stevens, 2002).

Histochemical study

3,3-Diaminobenzidine (DAB) method was used for studying catalase, 10 m-thick cryostat sections were fixed in acetone at 4 °C for 5 min, incubated in buffered DAB solution (Without hydrogen peroxide) for 1 min at room temperature, and then rinsed in distilled water. The sections were incubated in DAB solution with hydrogen peroxide for 15 min, washed in 3 changes of distilled water. Reaction was intensified in 0.1 M phosphate buffer (pH 7.2) for 5 min, washed in distilled water for 5 min and counterstained with toluidine blue, then dried and mounted with glycerin gel (Kiernan, 2001).The control reaction was performed through omitting the specific substrate.