

Inhibition of 2-nitropropane-induced cellular proliferation, DNA synthesis and histopathological changes by melatonin

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Abstract

OBJECTIVES: 2-Nitropropane (2-NP) is mutagenic in a number of short-term mutagenicity assays *in vitro* and *in vivo*, and is a potent hepatocarcinogen in rats. Many studies have determined that differences in the metabolism and disposition of the chemicals that produce mutagenicity were not responsible for their observed carcinogenic differences, but that carcinogenicity correlated with the ability of the respective isomer to induce cell proliferation in the target organ.

METHODS: Three groups of male rats (control, 2-NP-treated [4 mmol/kg] and 2-NP + melatonin [10 mg/kg]) were used in the current study. Cell proliferation was quantitated by incorporation of ³H-thymidine, detected by autoradiography, into newly synthesized DNA. Histopathological study was carried out to investigate the morphological changes.

RESULTS: Twenty four hours after 2-NP administration, there was an increase in the labelling index (LI) and grain count per labelled nucleus (GC/N) in the hepatocytes of 2-NP-injected rats versus those of control animals. The increase was 69.5% in LI and 29.4% in GC/N. Melatonin treatment, 30 minutes preceding 2-NP, reduced the increase in LI (44.4%) and GC/N (20.7%) when compared with 2-NP-treated rats. Histopathology revealed multiple focal areas of necrosis in the liver following 2-NP injection. In the lung, there was a mucinous degeneration of the bronchial epithelium. Melatonin treatment restored the histopathological changes in both the liver and lung and they are more or less normal.

CONCLUSION: Overall, these results seem to indicate that the stimulatory effect of 2-NP on the cellular proliferation and the rate of DNA synthesis in the liver may be one of mechanisms by which the carcinogen induces its carcinogenic action. Also, melatonin treatment strongly protects the studied organs against the toxic effect of 2-NP.

ABBREVIATIONS:

•OH	(hydroxyl radical)
LOO•	(peroxyl radical)
O ₂ • ⁻	(superoxide anion)
NO	(nitric oxide)
ONOO ⁻	(peroxynitrite anion)

Introduction

2-Nitropropane (2-NP) has been widely used in printing inks, adhesives, rocket propellants, paint and varnish removers, as a gasoline additive, and in the manufacture of nitrocellulose and chlorinated rubber [1]. They also reported that occupational exposure to 2-NP occurs principally in industrial construction, highway maintenance, ship building, furniture manufacture, and plastic production. The principal target organ of 2-NP toxicity is the liver. Following i.p. injection, 2-NP and its carbon-containing metabolites are concentrated initially in fat and subsequently in bone marrow, adrenal glands and other internal organs [2,3]. It is thought that generation of reactive oxygen species via the metabolism of 2-NP-nitronate to acetone and nitrite plays an important role for the carcinogenic effect [4].

It was hypothesized that the high rate of DNA synthesis might be related to the high incidence of tumors in liver, lung and other organs induced by exposure to carcinogens [5,6]. However, there is no evidence that the DNA synthesis rate is directly related to carcinogenesis. It was suggested that the cell cycle-dependent variation in susceptibility to hepatocarcinogenesis might be due to the efficient removal of potentially carcinogenic lesions from DNA. Additionally, it was reported that the activity of O⁶-methylguanine methyltransferase, which can repair alkylated bases in DNA, might be increased in the early S phase but be rapidly exhausted [7]. An increase in cell proliferation was observed following exposure to 2-NP [8,9]. These results indicate that cell proliferation may be requisite for expression of chemical-induced mutagenicity *in vivo* and thereby accommodate expression of carcinogenicity.

The main secretory product of the pineal gland, melatonin, has been shown to function as a free radical scavenger and antioxidant [10,11]. It is also an inhibitor of the cell cycle, thereby increasing the number of MCF-7 cells in G₁ and reducing the percentage of cells in S phase two-fold [12]. El-Sokkary [13] reported that melatonin has an anticancer function in the mammary glands and liver when it was injected after the chemical carcinogen 9,10-dimethyl-1,2-benzanthracene (DMBA) at different periods. In addition, melatonin exerted an inhibitory effect on the rate of DNA synthesis and the rate of proliferative activity as well as it prolonged the cell cycle duration after DMBA administration.

The current study was designed to investigate the efficacy of melatonin as an antioxidant and anticancer in reducing the cellular proliferation and histopathological changes induced by 2-NP in rats. Also to demonstrate a mechanism likely to mediate the prevention of cellular degeneration by melatonin.

Material and Methods

Animals and treatments: 30 adult male Sprague-Dawley rats weighing 120 ± 10g, purchased from Assiut University Joint Animal Breeding, were used in this study. All rats were kept under the same laboratory conditions of temperature [22 ± 2 °C], lighting (12:12 light:dark cycle) and they were given free access to standard laboratory chow and tap water. The animals were divided into 3 groups 10 rats each. The first group served as control and was given olive oil and saline solution. The second group was given a single intraperitoneal injection of 2-NP with a dose of 4 mmol/kg body weight. The third group was given a similar injection of 2-NP, which preceded, by 30 minutes, by an intraperitoneal injection of melatonin (10 mg/kg body weight).

Chemicals: 2-NP was purchased from Aldrich chemical, Inc. (Milwaukee, WI) and melatonin was a gift from Helsinn Chemicals SA (Biasca Switzerland). Tritiated thymidine, ([³H]TdR) specific activity 6.7 Ci/mmol, was purchased from New England Nuclear (Boston, Mass.). Kodak NTB₂ emulsion, Kodak D-19 developer and Kodak fixer were purchased from Eastman Kodak (Rochester, New York). All other chemicals were of the highest quality available. Melatonin was dissolved in a small amount of ethanol before being diluted with saline. The final concentration of ethanol in the melatonin solution < 1%. 2-NP was dissolved in olive oil.

Autoradiography: 24 hours after the administration of 2-NP, 6 rats from each group were sacrificed. 2 hours before sacrificing, the rats received a subcutaneous injection of 1 μ Ci/gm body weight tritiated thymidine (³H-methyl thymidine). Portions from the liver were fixed in 10% neutral buffered formalin and 5 μm thick paraffin sections were prepared. The deparaffinized sections were dipped in Kodak NTB₂ emulsion (diluted 1:1 with distilled water) and kept 20 days in the dark at 4°C. Thereafter, they were developed in Kodak D-19 developer for 3 minutes and fixed in Kodak fixer for 5 minutes at 15 °C. A cell was scored as being labelled when it showed 5 or more grains over its nucleus.

Quantitation of autoradiographs: A total of 10,000 labelled and unlabelled hepatocytes were counted in the liver parenchyma. The ³H-labelling index (LI) was expressed as the percentage of labelled hepatocytes. The grain count per labelled nucleus (GC/N) was evaluated by dividing the total number of silver grains over the labelled nuclei by the total number of labelled cells. The LI and GC/N represent the kinetics of proliferation and the rate of DNA synthesis, respectively [14,15].

Histopathological Examination: 4 rats from each group were sacrificed concomitantly with the previous rats and specimens of liver and lung were removed, fixed, embedded in paraffin, sectioned and stained with haematoxylin and eosin.

Results

The distribution of labelled hepatocytes and the number of grains over the labelled nuclei in the liver of control rats are shown in *Figure 1-A*. In rats which were given 2-NP only, the number of labelled hepatocytes and grains over the labelled nuclei were increased

versus control animals as shown in *Figure 1-B*. The quantitative results are presented in *Figure 2*. These results reveal a significant increase in the mean values of LI and GC/N ($P < 0.01$) in 2-NP-treated rats versus control animals. In 2-NP-treated animals, both the LI and GC/N were increased by 69.5% and 29.4%, respectively, when compared to those in controls. In rats which were given 2-NP and preceded by melatonin administration, the number of labelled hepatocytes and grains over the labelled nuclei were decreased versus 2-NP-treated rats (*Figure 1-C*). The reduction in LI was significant ($P < 0.01$) by 44.4% while in the GC/N was 20.7% ($P < 0.05$).

Microscopic examination of liver from control rats revealed no histopathological changes (*Fig. 3-A*). In the rats which were administered with 4 mmol/kg of 2-NP, microscopic examination of the liver showed multiple focal areas of necrosis. These necrotic changes always observed at the periphery of the hepatic lobules adjacent to the portal area (*Fig. 3-B*). The nuclei of these necrotic hepatocytes showed pyknosis and karyorrhexis, the cytoplasm was coagulated and slightly acidophilic. In the vicinity of the necrotic area the remaining hepatic tissue manifested diffuse severe vacuolar degeneration. Mild cellular reactions mostly of lymphocytic type were constantly seen in the vicinity of the necrotic and degenerated hepatic tissue. In some cases, necroses of individual hepatocytes were observed in between degenerated hepatic cells (apoptosis). In all cases, necrotic changes were massive and extend to involve a relatively abundant amount of hepatic tissue with prominent pyknosis and karyorrhexis of the nucleus. Some rats showed lymphoid and fibroblastic cellular reaction in the portal tract. The blood vessels of the portal tract and sinusoid were congested and contained abundant amount of leukocytes and sometimes filled with serous fluid.

Examination of the liver of rats administered with 2-NP and treated with melatonin (10 mg/kg) revealed that changes were mostly of mild degenerative type (vacuolar degenerative). Necrotic changes were rarely detected and only involve few number or groups of hepatic cells (*Fig. 3-C*). The cellular and vascular reaction was mostly observed in the area of the portal tract. Histopathological examination of the lung of control rats revealed no detectable changes (*Fig. 4-A*). Histopathological lesions of the lung were not so severe as those detected in the liver. They were confined to the blood vessels and the lining epithelium of the bronchial system. Microscopic examination of the lung of 2-NP

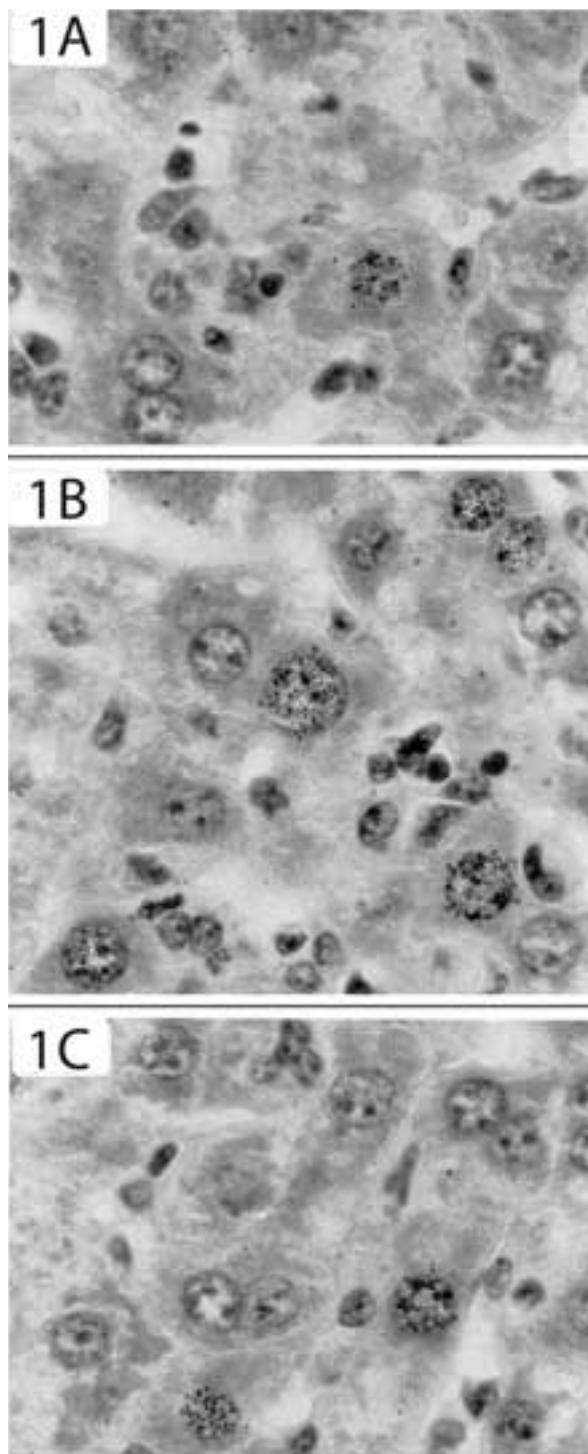
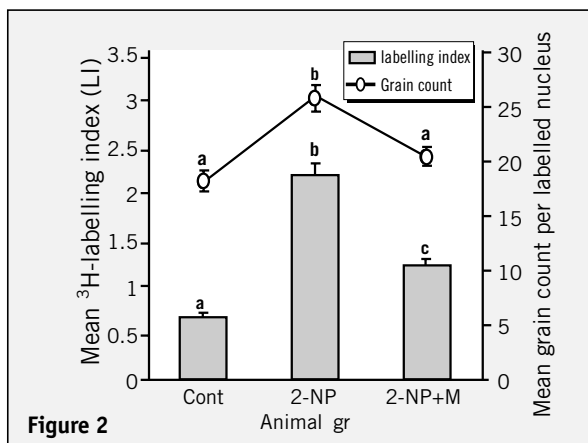


Figure 1: Sections of the liver stained with H&E showing the distribution of labelled hepatocytes and the density of grains over the labelled nuclei.

A: control rat, **B:** 2-NP-treated rat and **C:** melatonin and 2-NP-treated rat (X 1000).

Figure 2: Mean ^3H -labelling indices and grain count per labelled nucleus in the hepatocytes of control and different treated groups of rats. Groups with different letters differ significantly while those with the same letters do not differ significantly. Data are means \pm SEMs.



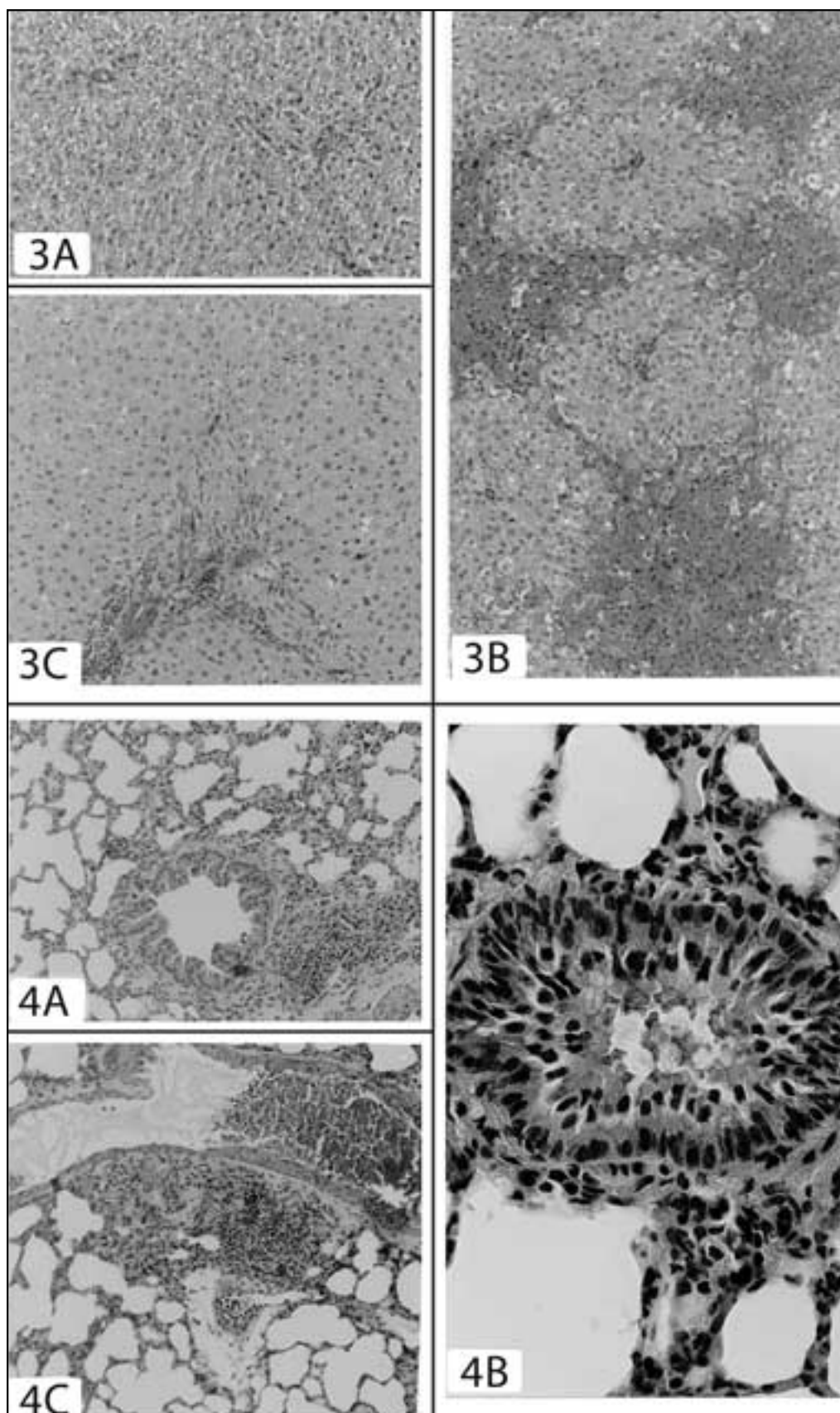


Figure 3. Sections of the liver stained with H&E showing:
A) Control rat with normal articulation of hepatocytes (X100).
B) 2-NP-treated rat with focal areas of necrosis which involve massive amount of hepatic tissue (X160).
C) Rat administered with 2-NP and treated with melatonin with mild degenerative nature (vacuolation) and more prominent cellular reaction (X160).

Figure 4. Sections of lung stained with H&E showing:
A) Control rat with the normal picture of alveolar wall, bronchi and peribronchial lymphoid tissue (X100).
B) 2-NP-treated rat with bronchial epithelium manifesting severe mucinous degeneration (X400).
C) Rat administered with 2-NP and treated with melatonin with blood vessels filled with serous fluid (X100).

administered rats revealed mucinous degeneration of the bronchial epithelium as shown in *Figure 4-B*. The wall of the bronchus was infiltrated with few lymphoid cells. Some of the bronchi showed proliferation of the bronchial epithelium and the interalveolar capillaries were severely congested. Hemorrhage and edema of the bronchi were observed. Large blood vessels were congested showed abundant population of leukocytes; some of them are thrombosed. Examination of the lung of rats treated with 10 mg/kg melatonin showed mild lymphoid cell reaction in between the alveoli along with a very mild congestion of the alveolar capillaries. Most of the lung tissues in this group are more or less normal (*Fig. 4-C*).

Discussion

2-NP, an industrial chemical [2] and component of cigarette smoke [16] is hepatocarcinogenic in rodents [17]. It is also genotoxic in a variety of test systems *in vitro*, including those of mutagenicity in bacteria [18] and unscheduled DNA synthesis in hepatocytes [19]. Furthermore, 2-NP has been reported to elicit serious manifestations of hepatotoxicity in occupationally exposed humans [20].

The mechanism by which 2-NP causes toxicity is only poorly understood. 2-NP exists in chemical equilibrium with its tautomer propane 2-nitronate (P2N) [21]. P2N is much more potently genotoxic than 2-NP [18,22] and probably responsible for the genotoxicity exerted by 2-NP [23]. Khol *et al.* [24] found that 2-NP and its nitronate generate a NO species in cells which may mediate, or contribute to, 2-NP genotoxicity. NO, which can exist in the biological micromilieu as NO[•], NO⁺ and/or NO⁻ is an important endogenous bioregulator, but also an environmental pollutant with genotoxic properties [25].

Kohl and Gescher [26] reported that P2N seems to be a pivotal part of the mechanism by which 2-NP causes its toxicity. They tested the hypothesis that the tautomeric equilibration is influenced by enzymes in the liver, the target organ of 2-NP toxicity. Therefore, the enzyme-catalysed increase in P2N generated from 2-NP is pertinent to the interpretation of 2-NP toxicity. By way of its anionic properties P2N, once generated, is likely to be trapped inside the cell allowing it to initiate the toxic lesion, whilst the lipophilic tautomer 2-NP would more easily permeate through membranes out of the cell.

In the present study, the proliferative activity in the hepatocytes was increased after 2-NP administration (69.5%) while the increase in DNA synthesis was 29.4% when compared to those of controls. As a reason for the high susceptibility of DNA in proliferating cells to damage, a series of conformational changes in chromosomal DNA in proliferation might be suspected because of the very high reactivity and short life of hydroxy radicals [27]. For instance, topoisomerases change the three-dimensional conformation of nuclear DNA of cells in proliferation for replication and mitosis [28,29]. The conformation of DNA might be changing continuously through all proliferation steps and be more relaxed than in G₀ cells, which are necessary for translation of limited

information in the genome only for steady-state maintenance. It is probable that the changing and relaxed formation of DNA is susceptible to attack by radicals.

In this study, severe macromorphological and micro-morphological changes were detected in the liver of the rats administered with a hepatocarcinogen 2-NP. These lesions include necrotic changes and degenerative changes of vacuolar and fat types. These results are in agreement with Hine *et al.* [30] and Adachi *et al.* [31]. The severity and diffuse of necrotic and degenerative process indicating the high susceptibility of rat for this toxin, it also considered an important indication for hepatotoxic effect of 2-NP [4,32]. Minimum histopathological and no macromorphological changes were detected in the rats treated with 10 mg/kg of melatonin. This indicate that such dose of melatonin may be effective in the prevention of hepatotoxicity induced by 2-NP. It has been postulated that melatonin scavenge or quench free radicals liberated from the effect of the intermediate product of 2-NP on the hepatocytes and thus preventing their damage.

Lung lesion consisting of vascular change, hemorrhage in the alveoli, mucinous degeneration in the bronchial epithelium and proliferation of the bronchial epithelium were detected in rats administered with 2-NP only. Such lesions indicated by toxic effect of 2-NP. In the rats that treated with 10 mg/kg of melatonin, minimal changes were detected and mainly of vascular reaction i.e. such dose may be effective to inhibit the histopathological changes induced in the lung after 2-NP administration. Lung lesion in rats administered with 2-NP only are less severe when compared to hepatic lesion in the same group suggesting that the liver is the target organ of this toxin. From these investigations, we can conclude that the dose of 10 mg/kg body weight of melatonin may be effective in the prevention of about 90% of pathological lesion induced by 2-NP in the liver and lung of Albino rats.

The pineal secretory melatonin was recently shown to scavenge or quench free radicals and reactive oxygen intermediates including $\cdot\text{OH}$, $\text{LOO}\cdot$, $\text{O}_2\cdot^-$, singlet oxygen and $\text{ONOO}\cdot^-$. [33]. Kim *et al.* [34] found that melatonin protected the lung and kidney from oxidative damage of 2-NP with an efficiency similar to that seen in the liver. These findings indicate that melatonin, as a free radical scavenger, readily enters cells in these organs and protects them from the toxic reactions of free radicals. Since melatonin also crosses all morphophysiological barriers, melatonin is apparently able to protect all cells in the organism from oxidative damage [35,36].

In the present investigation, melatonin treatment was inhibited the cellular proliferation and the rate of DNA synthesis by 44.4% and 20.7% respectively, when injected preceding the 2-NP. It was reported that melatonin inhibits cell proliferation in several tumors [37] and inhibits apoptosis in thymocytes [38]. It also an inhibitor of the cell cycle, thereby increasing the number of MCF-7 cells in G₁ and reducing the percentage of cells in S phase two-fold [12]. Mayo *et al.* [39] reported that melatonin is able to inhibit cell proliferation in undifferentiated neuronal cells (PC12), decreasing cell

number and the total amount of DNA, and the mRNA for the histone H₄, which are known to increase during DNA synthesis. Melatonin does not decrease the number of cells in nonproliferating PC12 cells, indicating that does not cause cell death. These findings support the hypothesis of a relationship between oxidative stress and regulation of the cell cycle. It was found that melatonin prevented oxidative stress induced by the kidney carcinogen KBrO₃ [40] and exhibited an efficient protective effect against aflatoxin B₁ [41].

The results of the present study demonstrate that 2-NP markedly stimulated the cellular proliferation and DNA synthesis in the liver as well as induced histopathological changes in the liver and lung and that melatonin effectively protects against 2-NP toxicity. These observations suggest that melatonin may find clinical application against a variety of toxins where cellular damage is a consequence of free radicals.

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