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AN AUTORADIOGRAPHIC STUDY OF CELLULAR PROLIFERATON, DNA SYNTHESIS AND CELL CYCLE VARIABILITY IN THE RAT LIVER CAUSED BY PHENOBARBITAL-INDUCED OXIDATIVE STRESS: THE PROTECTIVE ROLE OF MELATONIN

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Abstract: The protective effect of melatonin against phenobarbital-induced oxidative stress in the rat liver was measured based on lipid peroxidation levels (malondialedyde and 4-hydroxyalkenals). Cellular proliferation, DNA synthesis and cell cycle duration were quantitated by the incorporation of ^{3}H-thymidine, detected by autoradiography, into newly synthesized DNA. Two experiments were carried out in this study, each on four equal-sized groups of male rats (control, melatonin [10 mg/kg], phenobabital [20 mg/kg] and phenobarbital plus melatonin). Experiment I was designed to study the proliferative activity and rate of DNA synthesis, and measure the levels of lipid peroxidation, while experiment II was for cell cycle time determination. Relative to the controls, the phenobarbital-treated rats showed a significant increase ($P < 0.01$) in the lipid peroxidation levels (30.7%), labelling index (69.4%) and rate of DNA synthesis (37.8%), and a decrease in the cell cycle time. Administering melatonin to the phenobarbital-treated rats significantly reduced ($P < 0.01$) the lipid peroxidation levels (23.5%), labelling index (38.2%) and rate of DNA synthesis (29.0%), and increased the cell cycle time. These results seem to indicate that the stimulatory effect of phenobarbital on the oxidized lipids, proliferative activity, kinetics of DNA synthesis and cell cycle time alteration in the liver may be one of the mechanisms by which the non-genotoxic mitogen induces its carcinogenic action. Furthermore, melatonin displayed powerful protection against the toxic effect of phenobarbital.

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Abbreviations used: GC/N – grain count per labelled nucleus; 4-HAD – 4-hydroxyalkenals; LI – labelling index; LPO – lipid peroxidation; MDA – malondialdehyde; PB – phenobarbital; ROS – reactive oxygen species

Key words: Phenobarbital, Melatonin, Lipid peroxidation, Cell proliferation, DNA synthesis, Cell cycle

INTRODUCTION

Phenobarbital (PB) is a non-genotoxic barbiturate, used for many years as a liver tumor promoter in rodents [1] and an anti-epilepsy drug in humans [2]. The biochemical effects of PB are exceedingly pleotropic, being both context-and strain-dependent. *In vivo*, PB-mediated events do not occur uniformly throughout the liver, but are mainly restricted to the pericentral region of the liver lobules [3] and transient hyperplasia [4]. A typical response of hepatocytes to tumor promoters *in vivo* is an increase in S-phase DNA synthesis [3, 5]. Pericentral hypertrophy is maintained throughout the exposure period to PB. By contrast, DNA synthesis is transient, peaking after 3 days and returning to basal levels after one week [6].

Not surprisingly, chemicals that can induce gene mutations or gross chromosomal aberrations through direct interaction with the DNA commonly exhibit carcinogenic activity [7]. Mitogenic, non-genotoxic carcinogens induce tumors in rodents without interacting directly with the DNA. These agents are capable of inducing hyperplastic cell proliferation in the absence of necrosis [8], and have also been shown to inhibit apoptotic cell death [9]. Genetic alterations are believed to be induced by oxidative stress and by spontaneous errors in DNA replication and repair during continual cell replication [8, 10].

Cell proliferation is one of many essential factors involved in non-genotoxic carcinogenesis [11]. There are 2 groups of chemicals that induce liver cell proliferation: with one, cell loss precedes cell proliferation, while with the other; there is no cell loss prior to hyperplasia [12]. The former group consists of a variety of necrogenic agents, including carbon tetrachloride and chloroform. The latter group consists of chemicals capable of inducing direct hyperplasia in the liver, and includes cyproterone acetate (CPA), nafenopin (NAF), phenobarbital (PB) and .-hexachlorocyclohexane (.-HCH). These agents, often called mitogens, overcome operating growth-controlling mechanisms. Liver mitogens, such as CPA, PB, NAF and .-HCH, inhibit apoptosis [13]. This disruption of the balance between cell proliferation and apoptosis may contribute to the mechanism of hepatocarcinogenesis, promoting tumors [9, 14].

PB has been observed to enhance the formation of reactive oxygen species (ROS) in neoplastic rat liver nodules [15, 16]. There is substantial evidence for the role of ROS in many of the effects of PB [17, 18], possibly via the uncoupling of cytochrome P450 [19]. Previous studies [20, 21] have shown the involvement of ROS in many models of tumor promotion, which may in part be mediated by an influence on kinases in the signal transduction pathways triggered by epidermal growth factor [22, 23].

Previous findings suggest that melatonin, both at physiological and pharmacological levels [24, 25], functions as an antioxidant and reduces

oxidative damage *in vivo*. Melatonin has been tested for its efficacy in reducing biomolecular damage in a large range of experimental conditions where free radicals are believed to be involved [26-31]. The beneficial effects of melatonin likely relate to its direct detoxification of free radicals, its indirect antioxidative actions, and its ability to preserve an efficient oxygen metabolism in the mitochondria, and possibly to other activities. These processes are facilitated by the ability of the indoleamine to be readily absorbed and to cross all morphophysiological borders and enter all parts of all cells.

This study was designed to investigate whether this non-genotoxic chemical exerts its carcinogenic effect in part by oxidative stress-induced lipid peroxidation, cellular proliferation, DNA synthesis and/or cell cycle time alteration, and whether melatonin has any protective role against its action. Autoradiography was used for the quantitative determination of the proliferative activity, rate of DNA synthesis and cell cycle time.

MATERIAL AND METHODS

Animals

One hundred seventy two adult male Sprague-Dawley rats (average weight about 150 g) were purchased from the Assiut University Joint Animal Breeding for use in this study. All the rats were kept under the same laboratory conditions of temperature (22 ± 2ºC) and light (12:12 light:dark cycle) and were given free access to standard laboratory chow and tap water. The appropriate animal care committee of Assiut University approved the protocol of the experiment.

Chemicals

Sodium Phenobarbital was purchased from Aldrich Chemical, Inc. (Milwaukee, WI) and melatonin was donated by Helsinn Chemicals SA (Biasca, Switzerland). Tritiated thymidine, ([^{3}H]TdR, specific activity 6.7 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Kodak NTB2 emulsion, Kodak D-19 developer and Kodak fixer were purchased from Eastman Kodak (Rochester, NY). An LPO-586 kit was purchased from Cayman Chemical (Ann Arbor, MI). All the other chemicals were of the highest quality available. Melatonin was dissolved in a small amount of ethanol before being diluted with saline solution. The final concentration of ethanol in the melatonin solution was < 1%. PB was dissolved in distilled water.

Experimental design

Experiment I

This experiment was designed to study proliferative activity and the rate of DNA synthesis and measure the levels of lipid peroxidation. The animals were divided into 4 groups of 7 rats each. The first group served as a control and was subcutaneously given the vehicle alone (0.9% NaCl solution containing 0.5% ethanol). The second group was subcutaneously given melatonin (10 mg/kg).

The third group was subcutaneously given PB (20 mg/kg). The fourth group was given a similar dose of PB, injected 30 minutes after a subcutaneous injection of melatonin (10 mg/kg). The administrations were repeated daily for 21 days 2 hours before lights out.

Experiment II

This experiment was designed to study the cell cycle durations. A total of 144 rats were used in this experiment. The animals were divided into 4 groups of 48 rats each, and treated as in experiment I. To obtain the percentage of labeled mitosis and the cell cycle durations, 3 rats from each group were killed at intervals of 6 h over a 72 h period (6, 12, 18, 24, 30, 36, 42, 48, 54, 60, 66 and 72 h).

Autoradiographic studies of the cell cycle phases

The durations of the cell cycle phases were calculated according to Sadava [33]. Twenty four hours after the last treatments, the remaining rats in all the groups were given a subcutaneous injection of 1 μCi/gm b.w tritiated thymidine (^{3}H-methyl thymidine). The animals were killed 2 h after the injection of the radioactive precursor for the estimation of the proliferative activity and the rate of DNA synthesis and at intervals for calculation of the cell cycle durations. 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 for 18 days in the dark at 4ºC. Thereafter, they were developed in Kodak D-19 developer for 3 min and fixed in Kodak fixer for 5 min at 15ºC. All the developed autoradiographs were stained with Harris's haematoxylin and eosin. 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 hepatocytes were counted in the liver parenchyma. The ^{3}H-labelling index (LI) was expressed as the percentage of hepatocytes that were labelled. 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 respectively represent the kinetics of proliferation and the rate of DNA synthesis [32].

Measurement of lipid peroxidation

Malondialdehyde (MDA) and 4-hydroxyalkenal (4-HDA) concentrations are considered to be an index of the lipid peroxidation. The colorimetric kit mentioned above was used to determine the levels of oxidized lipids. Just prior to the assay, liver samples were homogenized in ice-cold 50 mM Tris buffer (pH 7.4, 10% w/v) using an ultra-Turrax T25b homogenizer. Supernatants were prepared by centrifugation at 10,000 g for 10 min; these were used to measure the MDA and 4-HDA levels. Protein levels were measured using bovine albumin as the standard.

Statistical analysis

The data is presented as the arithmetic means ± SEM. Statistical analyses were performed using an analysis of variance (ANOVA) followed by the Student-Newman-Keuls t-test. $P < 0.05$ was considered significant. The percentage inhibition or stimulation was calculated from the mean values of the oxidized lipid levels, labelling index and grain count.

RESULTS

The level of lipid peroxidation (LPO) in the liver homogenates of rats that received vehicle injection alone (control) was 0.62 ± 0.01 (Fig. 1). Melatonin by itself did not change the basal level of LPO, yielding 0.61 ± 0.01. In PB-treated rats, the level of liver LPO was increased to 0.90 ± 0.03. This increase was greater than 31% and statistically significant ($P < 0.01$) relative to the values for the control animals. In the PB plus melatonin group, the level of LPO was reduced to 0.69 ± 0.01. This reduction was 24% and statistically significant ($P < 0.01$) relative to the values for the PB-treated animals. Thus, the administration of melatonin in combination with PB significantly inhibited the lipid damage caused by PB.

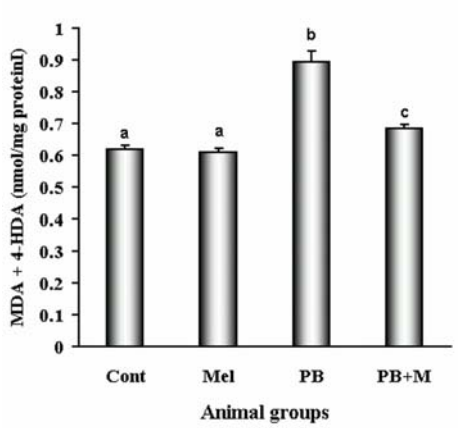

Fig. 1. Changes in the levels of lipid peroxidation products (malondialdehyde [MDA] and 4-hydroxyalkenals [4-HDA]) in the liver homogenates of the control and variously treated groups of rats. Groups with different letters differ significantly, while those with the same letters do not differ significantly. The values are the means ± SEMs.

The distribution of labelled hepatocytes and the number of grains over the labelled nuclei in the livers of the control, melatonin, PB and PB plus melatonin animals are shown in Fig. 2. The quantitative results are presented in Fig. 3. These results revealed a significant increase ($P < 0.01$) in the mean values of the labelling indices and the density of grains over the labelled nuclei in PB-treated rats relative to those for the controls. The increase was respectively 69% and 38% in the labelling index and grain count. In rats which received PB plus

melatonin, the number of labelled hepatocytes and the density of grains over the labelled nuclei were decreased relative to those for the PB-treated rats. Statistically, the reduction was significant ($P < 0.01$) by 38% and 29%, respectively. Again, melatonin by itself did not change the proliferative activity or the rate of DNA synthesis when compared to the control animals.

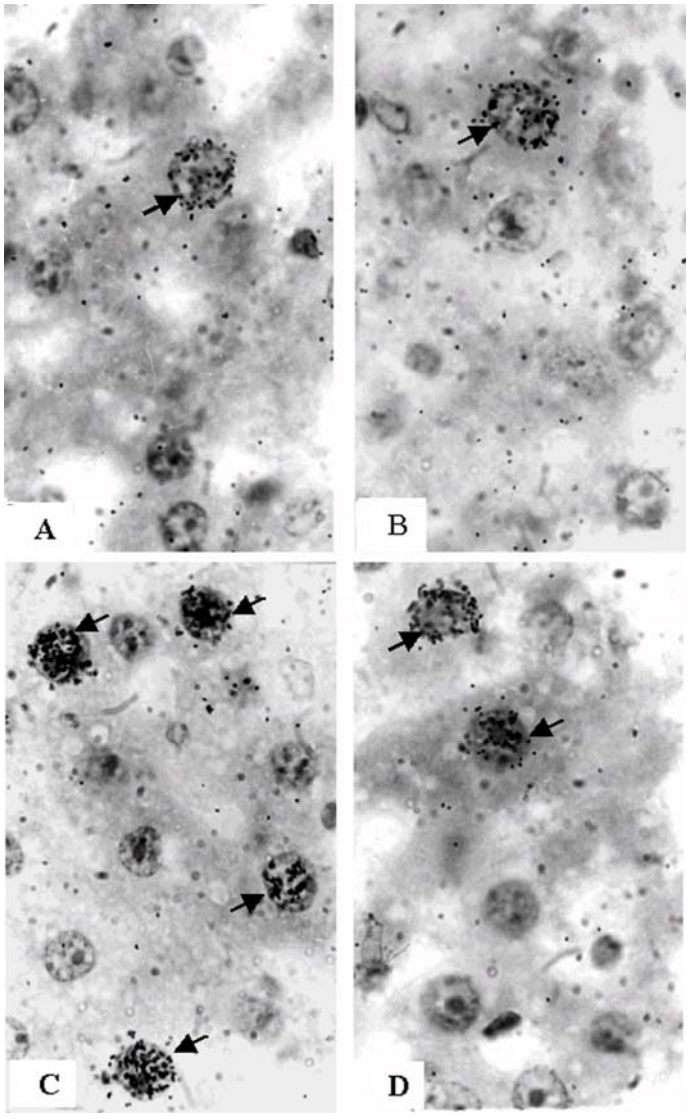

Fig. 2. Sections of rat livers showing the distribution of labelled cells (arrows) and the density of grains over the labelled nuclei. A – Control group. B – Melatonin group. C – Phenobarbital-treated group and D – Phenobarbital+melatonin group. H&E (x 1000).

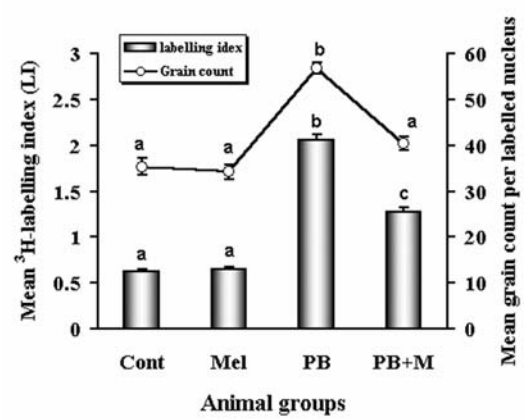

Fig. 3. Mean ^{3}H-labelling indices and grain counts per labeled nucleus in the hepatocytes of the control and variously treated groups of rats. Groups with different letters differ significantly; those with the same letters do not differ significantly. The values are the means ± SEMs.

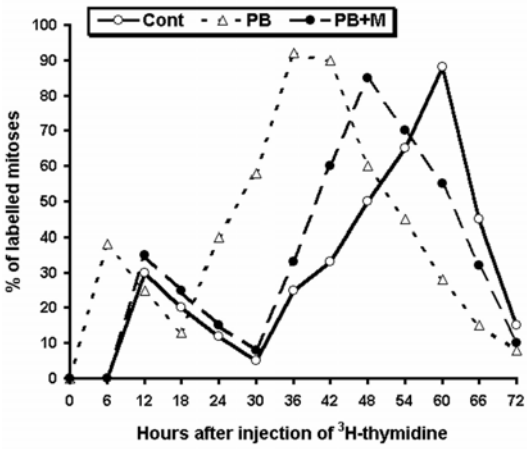

Fig. 4. Curves of the percentages of labeled mitoses obtained from the liver parenchyma (hepatocytes) of the control and variously treated groups of rats.

Tab. 1. Cell cycle phases and their durations in the liver parenchyma (hepatocytes) of control, phenobarbital and phenobarbital+melatonin-treated rats.

Treatments / Phases	Control	Phenobarbital	Phenobarbital + Mel
C	48 h	30 h	42 h
G_2+M	3.5 h	3.5 h	3.5 h
S	15 h	9 h	12 h
G_1	30 h	18 h	21 h

C = cell cycle time; G2 = G2-phase time (post-DNA duplication); S = S-phase time (DNA synthesis).

The curves for the percentage of labelled mitoses and the duration of the cell cycle obtained from the hepatocytes of the control, PB and PB plus melatonin-trated animals are shown in Fig. 4 and Tab. 1. The duration of the cell cycle in the hepatocytes of PB-treated animals was shorter than for the controls. Melatonin administration prolonged the cell cycle duration relative to that for the PB-treated rats. The melatonin group was not included in the cell cycle time calculations because there were no differences between the control and melatonin groups, as indicated in the previous parameters.

DISCUSSION

In response to PB, rodents display multiple changes that might contribute to the transient increase in hepatocellular proliferation, which, if not attenuated as occurs in normal hepatocytes, may contribute to tumor promotion. The multiplicity of changes make it difficult to delineate the critical initial interactions of PB in hepatocytes responsible for this response. It was previously shown that PB can enhance epidermal growth factor-induced S phase DNA synthesis in primary cultures of rat hepatocytes [34].

In this study, the levels of lipid peroxidation end products (MDA and 4-HDA) were significantly elevated by PB administration. Various studies have shown that reactive oxygen species have a role in the effects of PB [17, 25]. Imaoka *et al.* [16] found that PB induced lipid peroxidation via the production of hydroxyl radicals. Bock *et al.* [36] reported that PB is a strong inducer of cytochrome P450. The induction of the microsomal mixed function oxidase system including P450 and the subsequent increased production of oxygen intermediates may play a role in the toxicity associated with inducing agents. The increased lipid peroxidation levels after PB administration may play a role in the carcinogenic promotion of this reagent.

The results of this study revealed a significant increase in the proliferative activity and the rate of DNA synthesis in the liver parenchyma (hepatocytes) after PB administration. There was also a remarkable shortening of the cell cycle in the hepatocytes of PB-treated animals. In agreement with this result, Murkofsky *et al.* [37] reported that PB treatment induced cell proliferation in the rat liver. A study by Kinoshita *et al.* [38] revealed correlative changes in oxidative stress, proliferation, apoptosis, and DNA damage and repair, which occur in the rat liver during continuous PB exposure. Kaufmann *et al.* examined the proliferative effects of PB *in vitro* using cultures of putative initiated hepatocytes [39]. They stated that when PB was present in the cultures, the hepatocytes exhibited a labelling index of about 50% by the third day, and this level of labelling was preserved for up to 2 weeks. By contrast, very few hepatocytes were found to synthesize DNA in the absence of PB.

Arora *et al.* [40] found that oval cell proliferation and cholangiocellular lesions were produced in PB-treated rats. Weghorst and Klaung [41] reported that male mice exposed to PB exhibited an increased level of DNA synthesis in normal

hepatocytes but not in preneoplastic foci. Female mice administered with PB demonstrated significant increases in the levels of DNA synthesis in both preneoplastic and normal hepatocytes relative to non-PB treated females and PB-treated males. Debiec-Rychter and Wang [42] reported that PB stimulated DNA synthesis and increased the labelling index, but did not induce hyperplasia in urothelial cells.

In reference to the hypothesis that carcinogenesis or tumor promotion by xenobiotics may result at least in part from the increased cell turnover elicited by the chronic cytotoxicity of these chemicals, some authors suggested that PB induced cellular proliferation and DNA synthesis by increased cell turnover [41-43]. They reported that the increased cell turnover may be histopathological evidence of increased mitotic figures, regenerative hyperplasia and increased labelling indices with tritiated thymidine autoradiography.

Many mitogenic, non-genotoxic chemicals may exert their carcinogenic effect in part by altering cell-cycle checkpoints [44]. Cell-cycle checkpoints function to maintain genomic integrity [45]. They act as surveillance systems capable of recognizing genetic alterations or situations that may lead to genetic damage. They are capable of inducing signals that allow the cell to respond appropriately to DNA damage. If this checkpoint function is lost, DNA repair systems may not have time to remove potentially mutagenic or clastogenic DNA lesions before they are fixed during DNA replication and mitosis [46]. Loss of checkpoint responses can, therefore, lead to genomic instability [47, 48], which plays an integral role in neoplastic transformation [49].

Gonzales *et al.* [44] evaluated the ability of PB to abrogate the checkpoint responses to DNA damage in mouse hepatocytes. Lower G_1/S ratios in cells treated with PB suggested that PB delayed and attenuated the G_1 checkpoint response to DNA damage. Thus, it is believed that the abrogation of checkpoint controls may play an important mechanistic role in shortening the cell cycle duration time obtained in this study.

In this study, melatonin administration to PB-treated rats significantly reduced the levels of lipid peroxidation end products. The discovery that melatonin acts as a direct free radical scavenger [50] and as an indirect antioxidant via its stimulatory actions on antioxidative enzymes [29, 51] greatly increased interest in the use of this agent in the experimental and clinical setting. Its potential utility in humans is supported by its very low toxicity [24], its availability in a pure form, and the fact that it is inexpensive. Melatonin activities that have been identified include its ability to directly neutralize a number of toxic reactants and stimulate antioxidative enzymes.

Beyond its antioxidant activities, melatonin has been tested for and successfully used in other clinical situations. It was initially taken by transmeridian travelers to quell the severity of jet lag [52], and thereafter became popular as a sleep-promoting agent [53]. Interest in its use in the suppression of growth of certain cancer types is supported by the experimental and clinical observations of a number of scientists [26, 27, 54].

In this study, melatonin administration to PB-treated animals significantly inhibited the proliferative activity and the kinetics of the DNA synthesis of the hepatocytes. Also, melatonin increased the duration of the cell cycle compared to PB-treated animals. Melatonin exerted a direct antiproliferative effect on MCF-7 human breast cancer cells *in vitro* [55] and on hepatocytes [27]. Persengiev and Kyurkchiev [56] reported that melatonin has a powerful inhibitory effect on lymphoid cell proliferation.

Melatonin increases the population doubling time in cultures with a high proliferative rate, but does not modify the doubling time length of slowly proliferating cells, which is already slow [58]. In addition, it has long been known that proliferation and differentiation appear to have a reciprocal relationship [57]. A study by Crespo *et al.* [58] demonstrated that melatonin allows human breast cancer cells to achieve a greater differentiation, suggesting that this indoleamine delays the entry of MCF-7 cells into mitosis.

The results for cell cycle duration in this study concur with those of Jayat and Ratinaud [59], who reported that the duration of the S and G_2 phases are characteristic for particular cell types and usually do not show much intrapopulation variation. However, the duration of G_1 is extremely variable. Therefore, our results suggest that melatonin acts specifically in the cell cycle at G_0/G_1, increasing the length of this part of the cell cycle and, as a consequence, augmenting the fraction of cells in this phase of the cell cycle and delaying the entrance of cells into the S-phase. Cos *et al.* [60] suggested that melatonin could target the cell nucleus to control the cell proliferation by modulating cell cycle kinetics. Thus, it is known that melatonin delays the progression of the MCF-7 cells from G_0/G_1 to the DNA synthesis phase of the cell cycle.

In conclusion, the PB-mediated generation of reactive oxygen substances and induction of oxidative stress may be responsible for lipid peroxidation, cellular proliferation, DNA synthesis and cell cycle time shortening. At the same time, the results support the hypothesis that melatonin antioxidative ability and the inhibitory effect of cellular proliferation could be mediated at the nuclear level through regulatory effects on DNA synthesis and cell cycle kinetics.

REFERENCES

1. Peraino, C., Fry, R.J.M. and Staffeldt, E. Enhancement of spontaneous hepatic tumorigenesis in C3H mice by dietary Phenobarbital. **J. Natl. Cancer Inst.** 51 (1973) 1349-1350.
2. Clemmesen, J. and Hjalgrim-Jensen, S. Is Phenobarbital carcinogenic? A follow-up of 8078 epileptics. **Ecotoxicol. Environ. Safety** 1 (1978) 457-470.
3. Jirtle, R.L., Meyer, S.A. and Brockenbrough, J.S. Liver tumor promoter Phenobarbital: a biphasic modulator or hepatocyte proliferation. **Prog. Clin. Biol. Res.** 369 (1991) 209-216.

4. Tsai, W.H. and DeAngelo, A.B. Responsiveness hepatocytes from dichloroacetic acid or phenobarbital treated mice to growth factors in primary cultures. **Cancer Lett.** 99 (1996) 177-183.
5. Jones, H.B., Clarke, N.A. and Barras, N.C. Phenobarbital-induced hepatocellular proliferation: anti-bromodeoxyuridine and anti-proliferating cell nuclear antigen immunocytochemistry. **J. Histochem. Cytochem.** 41 (1993) 21-27.
6. Peraino, C., Fry, R.J.M., Staffeldt, E. and Christopher, J.P. Comparative enhancing effects of Phenobarbital, amobarbital, diphenylhydantoin and dichlorodiphenyl-trichloroethane on 2-acetylaminofluorene induced hepatic tumourigenesis in rat. **Cancer Res.** 35 (1975) 2884-2890.
7. Shelby, M.D. and Zieger, E. Activity of human carcinogens in the salmonella and rodent bone-marrow cytogenetics tests. **Mutat. Res.** 234 (1990) 257-261.
8. Butterworth, B.E., Popp, J.A., Conolly, R.B. and Goldsworthy, T.L. Chemically induced cell proliferation in carcinogenesis. in: **Mechanisms of Carcinogenesis in Risk Identification. Scientific Publications** (Vainio, H., Magee, P.N., McGregor, D.B.and McMichael, A.J. Eds.), Lyon, France, 1992, 279-305.
9. Schulte-Hermann, R., Bursch, W., Grasl-Kraupp, B., Torok, L., Ellinger, A. and Mullauer, L. Role of active cell death (apoptosis) in multi-stage carcinogenesis. **Toxicol. Lett**. 82/83 (1995) 143-148.
10. Butterworth, B.E., Conolly, R.B. and Morgan, K.T. A strategy for establishing mode of action of chemical carcinogens as a guide for approaches to risk assessments. **Cancer Lett.** 93 (1995) 129-146.
11. Melnick, R. and Huff, J. Liver carcinogenesis is not a predicted outcome of chemically induced hepatocyte proliferation. **Toxicol. Indust. Health** 9 (1993) 415-438.
12. Columbano, A., Ledda-Columbano, G., Coni, P., Pichiri-Coni, G., Curto, M. and Pani, P. Chemically induced cell proliferation and carcinogenesis: Differential effect of compensatory cell proliferation and mitogen-induced direct hyperplasia on hepatocarcinogenesis in the rat. in: **Chemically Induced Cell Proliferation: Implications for Risk Assessment** (Butterworth, B., Slaga, T., Farland, W. and McClain, M. Eds.), Wiley-Liss, New York. 1991, 217-225.
13. Bursch, W., Fesus, L. and Schulte-Hermann, R. Apoptosis ('Programmed' cell death) and its relevance in liver injury and carcinogenesis. in: **Tissue-Specific Toxicity Biochemical Mechanisms** (Dekant, W. and Neumann, H. Eds.), Academic Press, London. 1992, 95-115.
14. Goldsworthy, T., Fransson-Steen, R. and Maronpot, R. Importance of and approaches to quantification of hepatocyte apoptosis. **Toxicol. Pathol.** 24 (1996) 24-35.
15. Stark, A.A., Russell, J.J., Langenbach, R., Pagano, D.A., Zeiger, E. and Huberman, E. Localization of oxidative damage by a glutathione-γ-glutamyl transpeptidase system in neoplastic lesions in sections of livers from carcinogen-treated rats. **Carcinogenesis** 15 (1994) 343-348.
16. Imaoka, S., Osada, M., Minamiyama, Y., Yukimura, T., Toyokuni, S., Takemura, S., Hiroi, T. and Funae, Y. Role of Phenobarbital-induced

cytochrome P450s as a source of active oxygen species in DNA-oxidation. **Cancer Lett.** 203 (2004) 117-125.
17. Diez-Fernandez, C., Sanz, N., Alvarez, A.M., Wolf, A. and Cascales, M. The effect of non-genotoxic carcinogens, phenobarbital and clofibrate, on the relationship between reactive oxygen species, antioxidant enzyme expression and apoptosis. **Carcinogenesis** 19 (1998) 1715-1722.
18. Venditti, R., Daniele, C.M., Deleo, T. and DiMeo, S. Effect of phenobarbital treatment on characteristics determining susceptibility to oxidants of homogenates, mitochondria and microsomes from rat liver. **Cell Physiol. Biochem.** 8 (1998) 328-338.
19. Aniya, Y., Shimoji, M. and Naito, A. Increase in liver microsomal glutathione-S-transferase activity by phenobarbital treatment of rats: possible involvement of oxidative activation via cytochrome P450. **Biochem. Pharmacol.** 46 (1993) 1741-1747.
20. Saintot, M., Astre, C., Pujol, H. and Gerber, M. Tumor progerssion and oxidant antioxidant status. **Carcinogenesis** 17 (1996) 1267-1271.
21. Wolfle, D. and, Marquardt, H. Antioxidants inhibit the enhancement of malignant cell transformation induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin, **Carcinogenesis** 17 (1996) 1273-1278.
22. Whisler, R.L., Goyette, M.A., Grants, J.S. and Newhouse, Y.G. Sublethal levels of oxidant stress stimulate multiple serine/threonine kinases and suppress protein phosphatases in jurkat T cells. **Arch. Biochem. Biophys.** 319 (1995) 23-25.
23. Kass, G.E.N.,. Free radical induced changes in cell signal transduction. in: Free radical Toxicology (Wallace, K.B. Ed.), Taylor and Francis, Washington, DC, 1997, 349-374.
24. Reiter, R.J. Oxidative damage in the central nervous system: Protection by melatonin. **Prog. Neurobiol.** 56 (1998) 359-384.
25. Reiter, R.J. Melatonin: Lowering the high price of free radicals. **News Physiol. Sci.** 15 (2000) 246-250.
26. El-Sokkary, G.H. Melatonin protects against oxidative stress induced by the kidney arcinogen KBrO3. **Neuroendocrinol. Lett.** 21 (2000) 461-468.
27. El-Sokkary, G.H. Inhibition of 2-nitropropane-induced cellular proliferation, DNA synthesis and histopathological changes by melatonin. **Neuroendocrinol. Lett.** 23 (2002) 335-340.
28. El-Sokkary, G.H.; Reiter, R.J. and Abdel Ghaffar, S.Kh. Melatonin supplementation restores cellular proliferation and DNA synthesis in the splenic and thymic lymphocytes of old rats. **Neuroendocrinol. Lett.** 24 (2003) 215-223.
29. El-Sokkary, G.H., Abdel-Rahman, G.H. and Kamel, E.S. Melatonin protects against lead-induced hepatic and renal toxicity in male rats. **Toxicology** 213 (2005) 25-33.
30. Abd-Elghaffar, S.Kh, El-Sokkary, G.H. and Sharkawy, A.A. Aluminium-induced neurotoxicity and oxidative damage in rabbits: Protective effect of melatonin. **Neuroendocinol. Lett.** 26 (2005) 609-616.

31. Martinez-Cruz, F., Osuna, C. and Guerrero, J.M. Mitochondrial damage induced by fetal hyperphenylalaniemia in the rat brain and liver: Its prevention by melatonin, vitamin E and vitamin C. **Neurosci. Lett.** 392 (2006) 1-4.
32. Hamatani, K., Kawahara, A. and Amano, M. Quantitative study of deoxycytidine incorporation in large and small lymphocytes of the mouse. **Cell Tissue Kinet.** 16 (1983) 557-570.
33. Sadava, D.E. Cell cycle. in: **Cell Biology, Organelle Structure and Function**. Jones and Bartlett Publishers, 1993, 442-496.
34. Jirtle, R.L. and Meyer, S.A. Liver tumor promotion: effect of phenobarbital on EGF and protein kinase C signal transduction and TGFβ1 expression. **Dig. Dis. Sci.** 36 (1991) 659-668.
35. Guppy, M.J., Wilton, J.C., Sharma, R. and Chipman, J.K. Modulation of phenobarbitone induced loss of gap junctional intercellular communication in hepatocyte couplets. **Carcinogenesis** 15 (1994) 1917-1921.
36. Bock, K.W., Lipp, H.P. and Bock-Hennig, B.S. Induction of drug-metabolizing enzymes by xenobiotics. **Xenobiotica** 20 (1990) 1101-1111.
37. Murkofsky, R.L., Glover, S.E., Miller, R.T., Popp, J.A. and Cattley, R.C. Effect of regeneration and hyperplasia on levels of DNA base oxidation in rat liver. **Cancer Lett.** 70 (1993) 51-56.
38. Kinoshita, A., Wanibuchi, H., Imaoka, S., Ogawa, M., Masuda, C., Morimura, K., Funae, Y. and Fukushima, S. Formation of 8-hydroxydeoxyguanosine and cell-cycle arrest in the rat liver via generation of oxidative stress by phenobarbital: association with expression profiles of p21 WAF1/Cip1, cyclin D1 and Ogg1. **Carcinogenesis** 23 (2002) 341-349.
39. Kaufmann, W.K., Ririe, D.G. and Kaufman, D.G. Phenobarbital-dependent proliferation of putative initiated rat hepatocytes. **Carcinigenesis** 9 (1988) 779-782.
40. Arora, V.K., Bhatia, A. and Sood, S.K. Synergistic promoter effect of diethylstilbesterol & phenobarbitone in diethylnitrosamine induced hepatic neoplasia in rats. **Indian J. Med. Res.** 90 (1989) 9-16.
41. Weghorst, C.M. and Klaunig, J.E. Phenobarbital promotion in diethylnitrosamine-initiated infant B6C3F1 mice. Influence of gender. **Carcinogenesis** 10 (1989) 609-612.
42. Debiec-Rychter, M. and Wang, C.Y. Induction of urinary bladder of the F344 rat. **Toxicol. Appl. Pharmacol.** 105 (1990) 345-349.
43. Morsi, G.A. Autoradiographic studies on the effect of sodium phenobarbital on the kinetics of DNA synthesis in the hepatocytes of male albino rat. **Egypt. J. Histol.** 14 (1991) 511-523.
44. Gonzales, J.A., Christensen, J.G., Preston, R.J., Goldsworthy, T.L., Tlsty, T.D. and Fox, T.R. Attenuation of G1 checkpoint function by the non-genotoxic carcinogen Phenobarbital. **Carcinogenesis** 19 (1998) 1173-1183.
45. Hartwell, L.H. and Weinhert, T.A. Checkpoints: Controls that ensure the order of cell cycle events. **Science** 246 (1989) 629-633.

46. Kaufmann, W.K. and Wilson, S.R. G1 arrest during cell cycle dependent clastogenesis in UV-irradiated human fibroblasts. **Mutat. Res.** 314 (1994) 67-76.
47. Livingstone, L.R., White, A., Sprouse, J., Livanos, E., Jacks, T. and Tlsty, T.D. Altered cell cycle arrest and gene amplification potential accompany loss of wild-type p53. **Cell** 70 (1992) 923-935.
48. White, A.E., Livanos, E.M. and Tlsty, T.D. Differential disruption of genomic integrity and cell cycle regulation in normal human fibroblasts by the HPV oncoproteins. **Genes Dev.** 8 (1994) 666-677.
49. Hartwell, L. Defects in a cell cycle checkpoint may be responsible for the genomic instability of cancer cells. **Cell** 71 (1992) 543-546.
50. Tan, D.X., Chen, L.D., Poeggeler, B., Manchester, L.C. and Reiter, R.J. Melatonin: a potent, endogenous hydroxyl radical scavenger. **Endocr. J.** 1 (1993) 57-60.
51. Reiter, R.J., Tan, D.X., Qi, W., Manchester, L.C., Karbownik, M. and Calvo, J.R. Pharmacology and physiology of melatonin in reduction of oxidative stress in vivo. **Biol. Signals Recept.** 9 (2000) 160-171.
52. Cardinali, D.P., Borfman, G. P., Liotta, G., Perez Floret, S., Albornoz, L.E., Cutrera, R.A., Batista, J. and Ortiga, G.B. A multifactorial approach employing melatonin to accelerate resynchronization of sleep-wake cycle after a 12 time-zone westerly transmeridian flight in elite soccer athletes. **J. Pineal Res.** 32 (2002) 41-46.
53. Cardinali, D.P. Clinical perspectives for the use of melatonin as a neuroprotective chronobiotic in Alzheimer's disease. **Aktual. Neurol.** 3 (2003) 188-204.
54. Blask, D.E., Sauer, L.A. and Dauchy, R.T. Melatonin as a chronobiotic/ anticancer agent. **Curr. Top. Med. Chem.** 2 (2002) 113-132.
55. Cos, S. and Sanchez-Barcelo, E.J. Melatonin inhibition of MCF-7 human breast cancer cells growth: influence of cell proliferation rate. **Cancer Lett.** 93 (1995) 207-212.
56. Persengiev, S.P. and Kyurkchiev, S. Selective effect of melatonin on the proliferation of lymphoid cells. **Int. J. Biochem.** 25 (1993) 441-444.
57. Malamud, D. The cell cycle and cancer. (Baserga, R. Ed.), Cold Spring Harbor, New York. 1971, 132-141.
58. Crespo, D., Fernandez-Viadero, C., Verduga, R., Ovejero, V. and Cos, S. Interaction between melatonin and estradiol on morphological and morphometric features of MCF-7 human breast cancer cells. **J. Pineal Res.** 16 (1994) 215-222.
59. Jayat, C. and Ratinaud, M.H. Cell cycle analysis by flow cytometry: Principles and Applications. **Biol. Cell** 78 (1993) 15-25.
60. Cos, S., Blask, D.E., Lemus-Wilson, A. and Hill, A.B. Effects of melatonin on the cell cycle kinetics and estrogen rescue of MCF-7 human breast cancer cells in culture. **J. Pineal Res.** 10 (1991) 36-43.