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The renoprotective efficacy of melatonin against dimethylbenz[a]anthracene induced changes after long term exposure in Mice

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Abstract

To evaluate the Renoprotective efficacy of melatonin against long term exposure induced changes of dimethylbenz(a)anthracene (DMBA), cell proliferation and DNA synthesis was determined in the epithelial cells of cortical and medullary renal tubules in female and male mice applying quantitative autoradiographic analysis and using ^{3}H thymidine as a radioactive label. A total of 30 male and female adult albino mice were divided into 3 groups, each of 10 individuals: control (group C), DMBA exposed (group D) and DMBA/melatonin exposed (group D+M) mice. In female mice, long term exposure for 150 days to a single injection of DMBA (10mg/ 100g b.w.) stimulated the incorporation rate of ^{3}H thymidine into the epithelium of cortical renal tubules by 6774% compared to control. The number of grains over labeled nuclei was reduced by 57.1%. Daily injected with melatonin (100 µg/ 100g b.w.) during the last 60 days of exposure to a single dose of DMBA attenuated cell division rate of the epithelial cells by 80% compared to group D, but remained 1275% higher than that of group C. The mean grain count over labeled nuclei was increased by 59.5% compared to group D, but remained 31.5% lower than that of control. In medullary portion of the renal tubules, DMBA induced changes were less pronounced than that in the cortical area. The cell division was stimulated by 833% compared to control and remained 8.1x lower than the percentage increase in the cortical part. The mean grain count over labeled nuclei was reduced by 40.4% compared to group C. Daily injected with melatonin (100 µg/ 100g b.w.) during the last 60 days of exposure to a single dose of DMBA reduced the mitotic cell division by 83.9% compared to group D and thereby showing a similar effect as in the cortical part of the renal tubules. Compared to group C, the ^{3}H labeling index remained 50% higher. In the cortical portion, the comparable value was 1275% higher than that of control. The mean grain count over labeled nuclei was increased by 26.2% compared to group D and remained 24.8% lower than in group C.

Keywords**:** dimethylbenz(a)anthracene, cell proliferation, kidney, melatonin, mice

1 Introduction

All living organisms are permanently exposed to an environment contaminated with an increasing number of pollutants. Such foreign compounds enter the mammalian body and can be excreted into the bile, feces, and expired air or urine. With the exception of exhalation, the ease with which these chemicals are eliminated from the body largely depends on their water solubility. Lipophilic compounds present in the excretory fluids are generally reabsorbed and concentrate thereby in the body. However, a number of biochemical processes are available that can convert lipophilic compounds to more water-soluble metabolites. This process is called biotransformation and is usually enzymatic in nature. The end-result of this reaction is the formation of metabolites that are chemically distinct from the parent compounds. These metabolites are usually more hydrophilic than the parent compounds and therefore, are less likely to be reabsorbed in the excretory fluids and thus excreted in urine (Lock and Reed, 1998).

The mammalian kidney possesses most of the common biotransformation enzymes and thus participates largely in the body's metabolism of xenobiotic components.

Nowadays; it is being increasingly recognized that elucidations of renal excretory mechanisms are important in understanding the mechanism of nephrotoxicity of certain compounds, (Grantham and Chonko, 1991;Grundemann et al., 1991; Krishna and Klotz, 1994).

Polycyclic aromatic hydrocarbons(PAH)are widely distributed in our environment and form a major class of environmental carcinogens implicated in various types of cancer diseases (Higgins et al., 1961).Dimethylbenz(-a)anthracene (DMBA), a member of the PAH family, is a potent procarcinogen and potent cancer inducing agent applied in many animal models(Huggins et al., 1961).

During the course of DMBA's metabolism, a number of unstable and reactive intermediates are formed affecting a large range of biological reactions. They increase mutation rate, alter cellular membrane composition, structural proteins, detoxifying enzymes and cellular signaling proteins (Marnett et al., 2003). Thereby, the procarcinogen DMBA turns into a carcinogen.

Melatonin (N-acetyl-5-methoxytryptamine) has been known to be a chemo-preventive agent in in-vivo studies and experimental animal models. (Mirunalini et al., 2010; El-Bakry et al., 2014;Ferreira et al., 2014). Moreover, it regulates antioxidant enzymes (Rogriquez et al., 2004).

The present study aims to investigate the renoprotective efficacy of melatonin against DMBA induced stimulation on cell proliferation rate and DNA synthesis in renal epithelial cells of mice after long term exposure to the polycyclic aromatic hydrocarbon .

2 Materials and Methods

Animals

30 adult male and female Swiss albino mice aged 90 ± 2 days and weighing 24 - 26g were used in this study. All animals were kept under the same conditions of an artificial light-dark cycle (12h-12h), temperature (22 ± 2^{o}C), and humidity (32± 5). They were given standard food and water ad libitum.

Chemicals

9,10-dimethyl-1,2-benzanthracene (DMBA)[Sigma Chemical Co., St. Louis, Mo] was dissolved in corn oil at concentration of 50 mg/ml. Melatonin [Sigma Co.] was dissolved in a few drops of absolute ethanol and diluted with distilled water to a concentration of 500 µg/ml. Tritiated thymidine, ^{3}H-methyl thymidine [New England Nuclear, Boston, Mass.] with a specific activity of 6.7 Ci/ mmol served as a radiolabelled tracer. Photo emulsion NTB2 [Eastman Kodak, Rochester, New York] in combination with Kodak D-76 developer and Kodak Fixer were used for the preparation of autoradiographs.

Experimental Design

Experimental male and female mice were separately divided into 3 groups, 10 animals each.

Group C: Control animals were injected subcutaneously with a single injection of corn oil (0.2ml/ 100g b.w.) and thereafter with a daily injection of distilled water (0.2ml/ 100g b.w.) for 149 days and served as control.

Group D: Animals were injected subcutaneously with a single dose of DMBA (10mg/ 100g b.w.) and thereafter with a daily injection of distilled water (0.2ml/ 100g b.w.) for 149 days.

Group D+M: Animals were exposed for 150 days to a single dose DMBA alone. During the last 60 days of exposure, melatonin(100 µg/ 100g b.w.) was additionally injected every day at 4.00 pm, 2h before the end of the light cycle.

Labeling

The day after the last melatonin injection, 5 animals of each group were injected subcutaneously with tritiated thymidine (^{3}H-methyl thymidine) at a dose of 1µCi/g b.w. and a specific activity of 6.7 Ci/ mmol. To minimize any possible diurnal influence, animals were injected at a fixed time (16.00h) and sacrificed two hours later by cervical dislocation according to the ethical criteria for animal care and treatment.

Tissues Preparation

Whole kidneys were removed, dissected, fixed in 10% neutral formalin and washed in running water to remove unbound ^{3}H-thymidine. Specimens were further routinely processed for paraffin embedding, sectioned at 5 µm and stained with hematoxylin and eosin.

Scoring

A total of 2,500 labeled and unlabeled epithelial cells of the cortical and medullary renal tubules were scored for each animal and the number of silver grains over labeled nuclei recorded. Cells showing 5 or more silver grains over the nucleus were considered to be labeled in the S-phase of the cell cycle.

^{3}H-labelling Index (LI)

The ^{3}H-labelling index was expressed as the number of labeled cells per 100 cells:

$$LI = \frac{\text{Number of labeled cells}}{\text{Number of total cells}} X\ 100$$

Mean Grain Count (GC)

The grain count per labeled nucleus was determined from the number of silver grains over labeled nuclei divided by the total number of labeled cells in the population.

$$G C = \frac{\text{Number of silver grains over labeled nuclei}}{\text{Number of total labeled cells}}$$

3 Results

According to the location within the nephron, the tubules of the cortex and that of the medulla were considered as two separate populations of renal tubules.

Female mice

The percentage of labeled epidermal cells and the grain density over labeled nuclei in the cortical portion of renal tubules are shown in table 1.

In the cortical portion of renal tubules of control animals, 0.04 ± 0.01% of all epithelial cells were ^{3}H thymidine labeled. The mean grain count per labelled nucleus was 24.42 ± 0.10. Exposure of animals to a single dose of DMBA for 150 days (group D), remarkably increased the number of labelled cell nuclei compared with that of control mice (figs. 1 and 2). The incorporation rate of ^{3}H thymidine increased by 6774% to 2.75 ± 1.75%. Moreover, epithelial cells with ^{3}H labeled nucleus were arranged in small clusters. The grain count per labeled nucleus was decreased by 57.1% to 10.48 ± 2.50. Daily injection with melatonin during the last 60 days of DMBA exposure attenuated the cell proliferation rate to 0.55 ± 0.19%, a reduction by 80% compared to group D. Compared to control it remained 1275%higher. The mean grain count over labeled nuclei was increased by 59.5% to 16.72 ± 4.96 grains compared to group D, but remained31.2% lower than that of control (figs 3, 7 and 8) . In the medullary portion, a single injection of DMBA (group D) increased the cell replication by 833.3% from 0.06 ± 0.02% to 0.56 ± 0.50%. Moreover, epithelial cells with ^{3}H labeled nuclei were arranged in small aggregations (figs. 4 and 5). The incorporation of ^{3}H thymidine into a single cell nucleus was reduced from 28.82 ± 2.67 to 17.18 ± .1.13, a reduction by 40.4%. Daily injection with melatonin for the last 60 days of the experiment (group D+M) attenuated cell division rate by 83.9% to 0.09 ± 0.04%, reaching thereby nearly the level of control animals. The number of grains over labeled nuclei was increased compared to group D by 26.2% to 21.68 ± 7.83 and remained thereby just 24.8% below the control value (figs 6, 9 and 10).

Male mice

In male mice neither the cell proliferating effect of DMBA nor the attenuating impact of melatonin could be determined as all animals were dead before the end of the experimental period. The effect of treatments on mean grain count over labeled nuclei was also not measured.

4 Discussion

Xenobiotic agents are polluting increasingly the environment and enter accordingly the living organisms. In mammals, the primary route of excretion of xenobiotic compounds from the body is via the kidneys. To be excreted with the urine, lipophilic compounds, such as the PAH dimethylbenz[a]anthracene (DMBA), must be converted to more water-soluble metabolites in an enzymatic process called biotransformation (Lock and Reed, 1998). During this transformation carcinogenic intermediate metabolites are produced and affect a large range of biological reactions. They attack cellular DNA, causing cell toxicity and induce damage in many enzymes involved in DNA repair. Moreover, the metabolic by-products increase the mutation rate in oncogenes, alter cellular membrane composition, structural proteins, metabolic or detoxifying enzymes and cellular signaling proteins. Free radicals that are produced, are involved in tumor promotion by either direct chemical reaction or alteration of cellular metabolic process and their scavengers representing inhibitors at different stages of carcinogenesis (Shimadaand Fujii-Kuriyama,2004; Baird et al., 2005; Marnett et al., 2003; Shimada, 2006; Sharma and Paliwal, 2013).

In the present study, exposure of female mice to a single injection of DMBA (10mg/ 100g b.w.) for 150 days highly increased the cell proliferation of endothelial cells in both portions of the renal tubules, but not to the same extend. In the cortical tubules the percentage of incorporation of^{3}H-thymidine into the endothelium was 8.1 x higher than in the medullary portion. Comparable results were obtained in female mice exposed to DMBA for shorter time periods (Semmler and Aref, 2015 a).

Moreover, the duration of the exposure times to the agent (61, 120 and 150days) affected significantly the cell division rate. The longer the experimental period, the higher was the cell proliferation rate.

The DNA synthesis, measured as grain count per labeled nucleus, accordingly varied with the exposure time. In the medullary portion, the number of grains per nucleus increased with experimental duration, while in the cortical part, it varied insignificantly . This proves that the epithelial cells of the cortical renal tubules are much more sensitive to the impact of DMBA exposure than the medullary ones (Semmler and Aref, 2015 a).This is due to the fact that the enzymes of the cytochrome P450 family (CYP1A1 and 1B1), which are central to the metabolic activation of PAHs to epoxide intermediates, are not evenly distributed along the renal tubules, but are mainly localized within the proximal tubules. These enzymes convert DMBA with the aid of epoxide hydrolase to diol-epoxides, the ultimate carcinogens (Lock and Reed, 1998; Shimada and Fujii-Kuriyama, 2004).

The higher sensitivity of the cortical portion of the renal tubules to DMBA is also proved by the formation of early phases of tumorgenesis. Aggregations of epithelial cells with tritiated thymidine incorporation into the nucleus were found only in this part of the kidney.

The most common type of kidney cancer is the renal cell carcinoma (RCC). It originates from the cortical renal epithelium and accounts in humans for 80 to 85 percent of malignant kidney tumors (Motzer et al., 1996; Cohen and McGovern, 2005).

In the development of renal-cell carcinoma from normal renal epithelium, alterations in genes are involved that participate directly in controlling the cell cycle. These genes are the retinoblastoma (*Rb*) gene, the p53 tumor-suppressor gene and the *ras* gene family. The proteins of the later gene family are involved in communicating signals from outside the cell to the nucleus (signal transduction). Alterations in other cell-cycle genes, such as *Rb* and the cyclin-dependent kinase inhibitors p21 and p16 are infrequent. Increased expression of growth factors or their receptors may also enhance cellular replication in renal-cell carcinoma (Motzer et al., 1996).

Renal-cell carcinoma occurs nearly twice as often in men as in women (Kosary and McLaughlin, 1993). This might be a possible cause of death of all male mice before the end of

Table 1: ^{3}H-labelling indices and grain counts/labelled nucleus in cortical and medullary portions of renal tubules of female mice after long term exposure (150 days); C = control, D = single dose injection of DMBA (10mg/100g b.w); D+M = single dose injection of DMBA (10mg/ 100g b.w.) and concomitant daily injection of melatonin (0.2ml/ 100g b.w.) for the last 60 days of DMBA exposure.

	Cortical portion			**Medullary portion**		
Groups / Measurements	**C**	**D**	**D+M**	**C**	**D**	**D+M**
^{3}H-labelling index (%)	0.04 ±0.01	2.75 ± 1.75	0.55 ± 0.19	0.06 ± 0.02	0.56 ± 0.50	0.09 ± 0.04
changes between groups		+6775%	-80% +1275%		+833%	-83.9 + 50%
grain count / labelled nucleus	24.42 ± 0.10	10.48 ± 2.50	16.72±4.96	28.82 ±2.67	17.18 ± 1.13	21.68 ± 7.83
changes between groups		-57.1%	+59.5% -31.2%		–40.4%	+26.2% -24.9%

Data are given as mean ± SE;

the experiment as described in the present study. Shorter exposure times (61 and 120 days) to the same doses of DMBA did not have such an effect on males (Semmler and Aref, 2015 a).

In general, the impact of the PAH on cell proliferation was in male mice less pronounced than on females. Moreover, in male mice, the cortical and medullary portion of the renal tubules did not differ in the rate of mitotic cell division for given exposure times (Semmler and Aref, 2015 a).This proves that gender specific differences exist in levels of expression and catalytic activities of a variety of enzymes that activate and/or detoxify PAHs. These phenomena are thought to be critical in understanding the basis of individual differences in response to PAHs(Lock and Reed, 1998). In the present study, daily injection of female mice with melatonin (100 µg/ 100g b.w.) for the last 60 days of 150 days exposure to DMBA attenuated the cell proliferation rate in both distinct portions of the renal tubules by nearly identical percentage compared to the group exposed to DMBA alone. Identical effects were observed in female animals exposed for 120 days to DMBA alone and concomitantly to melatonin for the last 30 days (Semmler und Aref, 2015 a).

In conclusion, it could be stated that: 1.) independent from the exposure period to DMBA and 2.) independent from the onset and/or duration of melatonin injection, the pineal hormone revealed its renoprotective effect against the deleterious impact of the polycyclic aromatic hydrocarbon DMBA. Such effects include a wide range of metabolic processes such as antioxidant ;antproliferative and oncostatic activity. Cell growth and cell cycle distribution are also affected.

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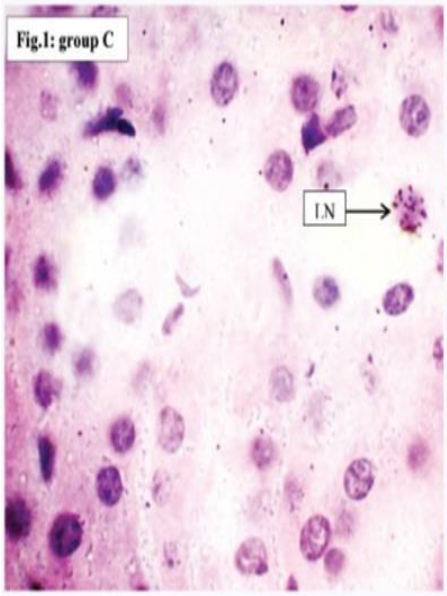
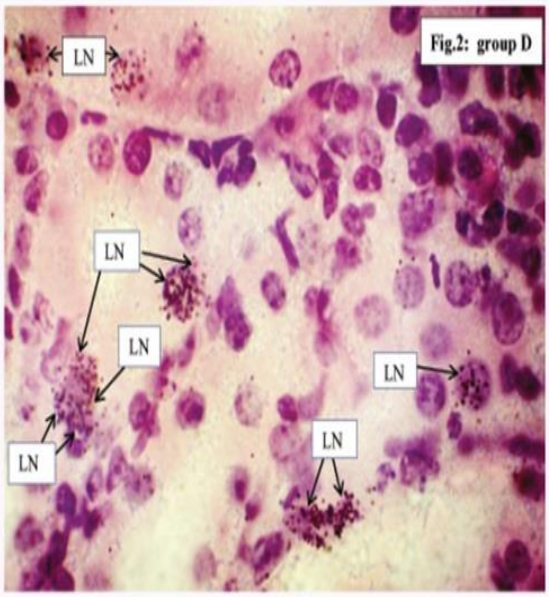
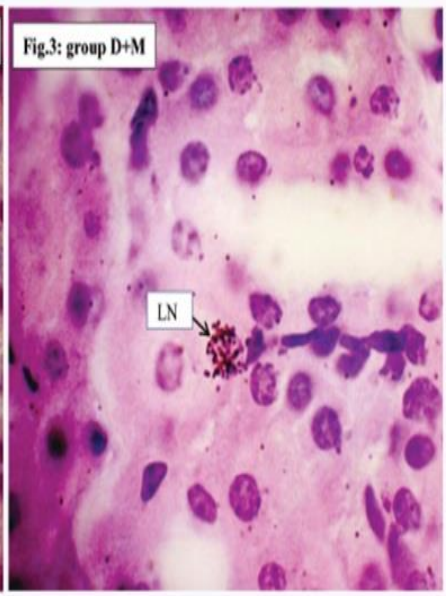
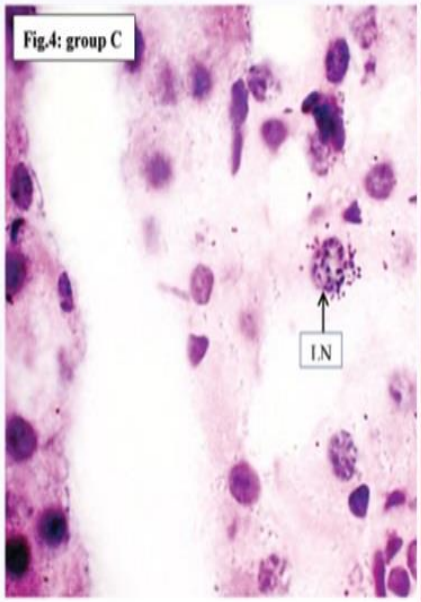
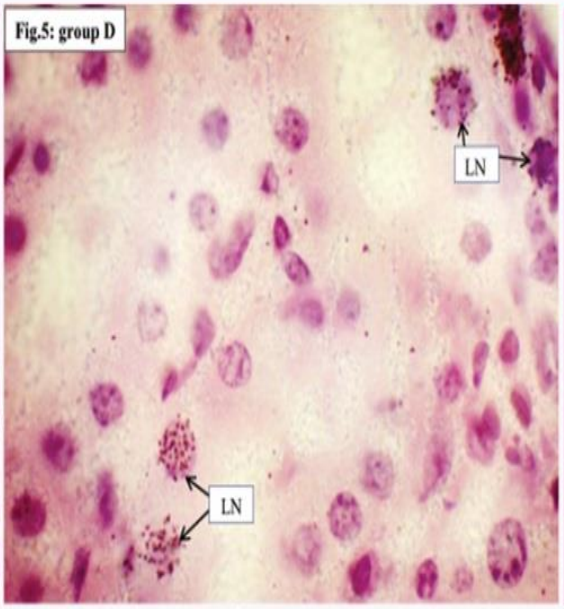


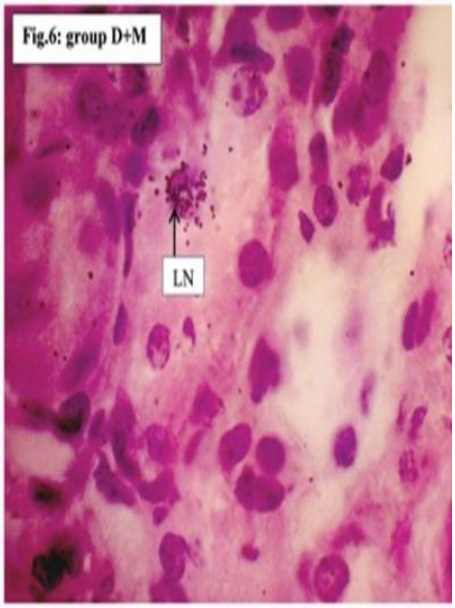




Figs. (1,2,3): Autoradiograms of ^{3}H-thymidine labelled kidney of female mice stained with H&E. showing the distribution of labelled nuclei (LN) in cortical portion of renal tubules after long term exposure (150 days). 1- Control mice (C), 2 - Exposed to a single dose of DMBA for 150 days (D) and 3- Exposed to a single dose of DMBA for 150 days and concomitantly to a daily injection of melatonin for last 60 days (D+M). X 1300







Figs. (4,5,6): Autoradiograms of ^{3}H-thymidine labelled kidney of female mice stained with H&E. showing the distribution of labelled nuclei (LN) in medullary portion of renal tubules after long term exposure. 4-control mice (C), 5-Exposed to a single dose of DMBA for 150 days (D) and 6- Exposed to a single dose of DMBA for 150 days and concomitantly to a daily injection of melatonin for last 60 days (D+M). X 1300

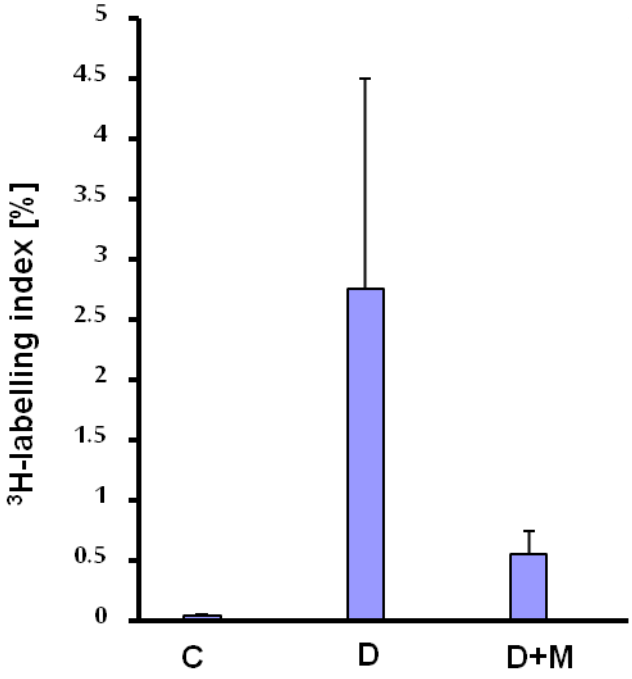


Fig. 7: ^{3}H-labelling indices [%] (± S.E) in the cortical portion of renal tubules of control (C), DMBA-treated (D) and DMBA- melatonin-treated (D+M) female mice after long term exposure.

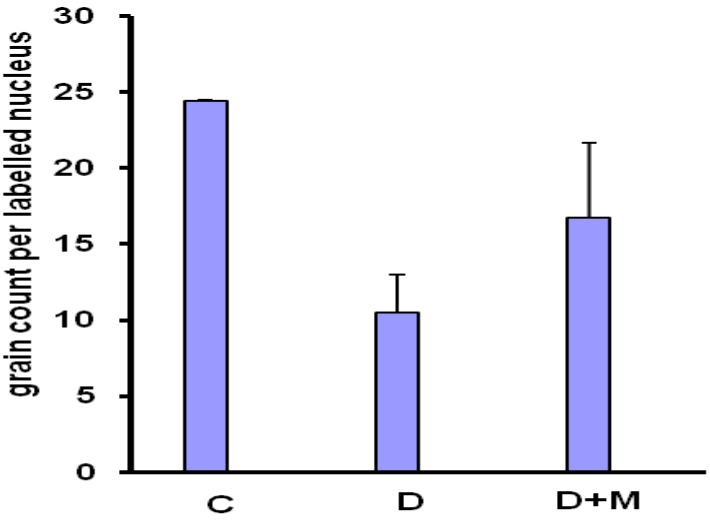


Fig. 8: grain count per labelled nucleus (±S.E) in the cotical portion of renal tubules of control (C), DMBA-terated(D) and DMBA-melatonin-treated (D+M) female mice after long term exposure.

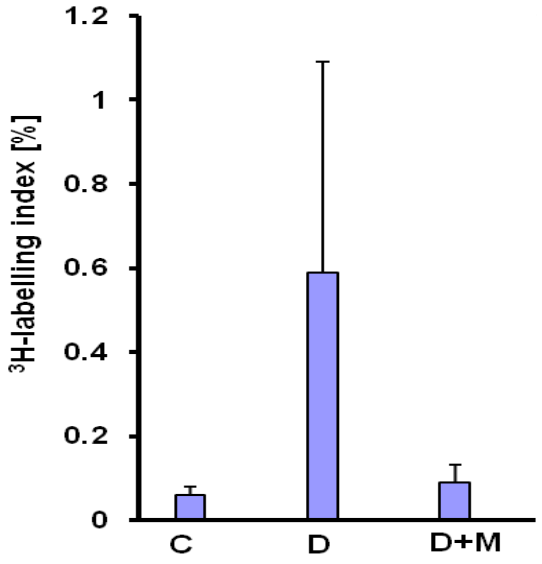


Fig.9: ^{3}H-labelling indices [%] (± S.E) in the medullary portion of renal tubules of control (C), DMBA-treated (D) and DMBA-melatonin-treated (D+M) female mice after long term exposure.

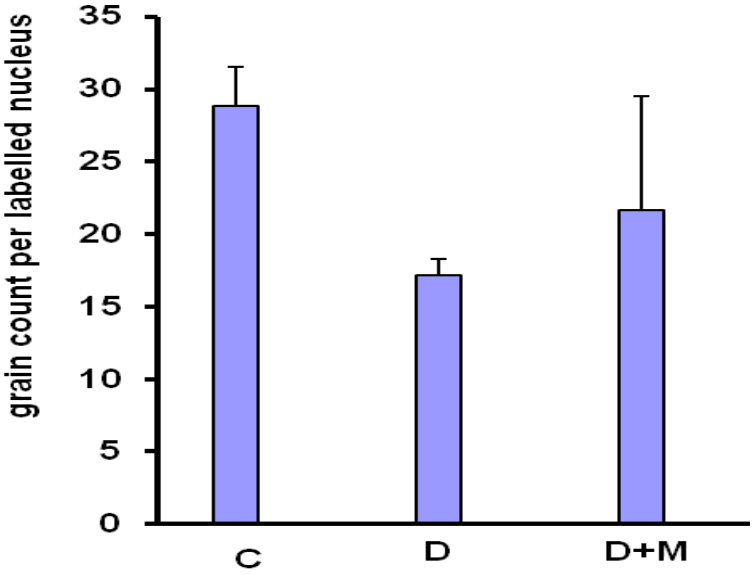


Fig. 10: grain count per labelled nucleus (± S.E) in the medullary portion of renal tubules of control (C), DMBA-terated (D) and DMBA-melatonin-treated (D+M) female mice after long term exposure.

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