Studies on the Chemopreventive Potential of Melatonin on 7,12-dimethylbenz(a)anthracene Induced Mammary Carcinogenesis in Rats

Sankaran Mirunalini, Kandhan Karthishwaran, Ganesan Dhamodharan and Mohan Shalini

Department of Biochemistry and Biotechnology, Faculty of Science, Annamalai University, Tamil Nadu, India.

Abstract: Chemoprevention using drugs is a promising approach to control the mortality and occurrence of cancer that accounts for millions of death worldwide. Melatonin (N-acetyl-5-methoxytryptamine), a hormone of the pineal gland produced from the amino acid tryptophan in minute quantities has been known to be a chemopreventive, anticancer agent in vitro and in vivo studies. DMBA (7, 12-dimethylbenz(a)anthracene) has been shown to act via generation of reactive oxygen species (ROS) as tumor promoter and also involved in the generation of free radicals. Many natural and synthetic compounds exhibit potent cancer chemopreventive property as observed in the last few decades. Most of them are known to exert their effects by quenching reactive oxygen, inhibiting lipid peroxidation. DMBA is a potent carcinogenic agent that induces mammary cancer. Here we evaluate the chemopreventive potential of melatonin on experimental animal models, female albino Wistar rats were randomized into four groups. Group I animals served as control without any treatment. Group II animals received a dose of 5mg/kg body weight of DMBA orally at weekly intervals for one month. Group III animals received a daily intraperitoneal administration of melatonin 5mg/ml per animals for 15 days prior to the first oral administration of DMBA and continued for a month and Group IV animals received a daily intraperitoneal administration of melatonin 5mg/ml beginning the next day after first DMBA administration for one month. At the end of the experiment the rats were killed, blood, liver, kidney and mammary tissues were taken for biochemical and histological studies. As a marker for liver function, the activity of gamma glutamyl transpeptidase (GGT) was measured in the serum. To assess the lipid peroxidation and the antioxidant status in tissues, the levels of thiobarbituric acid reactive substances (TBARS), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), reduced glutathione (GSH), ascorbic acid (vitamin C) and α–tocopherol (vitamin E) were measured. DMBA administration inhibited body weight and enhanced macroscopically detectable tumors with a increase in serum GGT and TBARS levels. DMBA treatment decreased both enzymic and non-enzymic antioxidant status. Melatonin administration significantly curtailed tumor development and counteracted all the biochemical effects. This indicates that melatonin should be considered as an adjuvant drug in the treatment of neoplastic disease.

Key words: melatonin, mammary carcinogenesis, gamma glutamyl transpeptidase, antioxidants, lipid peroxidation

INTRODUCTION

7, 12-dimethylbenz(a)anthracene (DMBA) is a potent carcinogenic polycyclic aromatic hydrocarbon (PAH) used to induce mammary cancers in animal models. DMBA consistently produces carcinomas in animal models. Sources of PAHs are widely distributed in our environment and are implicated in various types of cancer. Enzymatic activation of PAHs leads to the generation of active oxygen species such as peroxides and superoxide anion radicals, which induce oxidative stress in the form of lipid peroxidation. The consequences of the damage initiated by these metabolic by products affect a large range of biological reactions, like increases in mutation rate, alteration of cellular membrane composition, structural proteins, metabolic, detoxifying enzymes and cellular signaling proteins. DNA damage induced by oxidative stress and/or deficient DNA repair may have etiological or prognostic role in cancer. Free radicals play an important role in tumor promotion by direct chemical reaction or alteration of cellular metabolic process and their scavengers represent inhibitors at different stages of carcinogenesis.
Despite abundant information about its etiopathogenesis and early detection, effective therapeutic modalities for patients with advanced stages of the disease are still needed. Accumulating evidence derived from laboratory studies and study cohorts drawn from the general population have led to the search for "chemoprotective" agents to attenuate the risk of breast cancer based on the observation that most human cancer are associated with a long period of latency. Chemoprevention using drugs is a promising approach to control the mortality and occurrence of cancer that accounts millions of death World Wide\(^4\). Melatonin (N-acetyl-5-methoxytryptamine) a hormone of the pineal gland produced from the amino acid tryptophan in minute quantities has been known to be a chemopreventive agent in \textit{in vivo} studies and experimental animal models. It is soluble in lipids and in aqueous environments and is also reported as a highly efficient free radical scavenger and act as a strong general antioxidant\(^6\). Moreover Melatonin acts on the target cells either directly on G-protein receptors, such as MT1R, MT2R and MT3R that modulate several intracellular messengers such as cAMP, cGMP and ca\(^{2+}\)|. In addition to its potential on anti tumor activity melatonin has proved to modulate the effects of cancer chemotherapy, by enhancing its therapeutic efficacy and reducing its toxicity. The increase chemotherapeutic efficacy by melatonin may depend on two main mechanisms, namely prevention of chemotherapy-induced lymphocyte damage and antioxidant effect, which has been proved to amplify cytotoxic actions of the chemotherapeutic agents against cancer cells\(^7\). However, the clinical results available at present, with melatonin and chemotherapy in the treatment of human neoplasm are generally limited. Considering the potential antitumor/antioxidant activity of melatonin, the present investigation was carried out to study on the chemopreventive potential of melatonin against 7,12-dimethylbenz(a)anthracene induced mammary carcinogenesis in female albino Wistar rats.

**MATERIALS AND METHODS**

**Animals:** Female rats of the Wistar albino strain (120 days old) weighing 100g, were procured from Tamilnadu veterinary science institute, Chennai, India, were used. The animals (six per group) were fed with standard pellet diet (Hindustan Lever Limited, Mumbai, India) and water \textit{ad libitum} throughout the experimental period and replenished daily. The animals were housed in well ventilated large polypropylene cages under controlled conditions of light (12 hr light/12 hr dark), humidity (50%) and ambient temperature (30\(\pm\)2\(^\circ\) C). The animals used in the present study were kept in accordance with the guidelines of National Institute of Nutrition, ICMR, Hyderabad, India and by the Animal Ethical Committee, Annamalai University (Reg. No. 486/160/1999 CPCSEA).

**Chemicals:** 7, 12-dimethylbenz(a)anthracene was purchased from Sigma Chemical Company, St.Louis, MO,USA. Melatonin was obtained from Sisco Research Laboratories, Mumbai, India. All other chemicals including solvents used in the study were of high purity and of analytical grade.

**Experimental Design:** Group I animals served as control without any treatment. To induce mammary carcinogenesis, rats received 5mg/kg body weight of DMBA in 1 mL corn oil orally once in a week with a total dose of 20 mg/kg body weight for 4 weeks. Corn oil is used as 'promoter of carcinogenesis in this animal model\(^6\). Melatonin was dissolved in ethanol which had previously been diluted 20 times with Phosphate buffered saline (PBS) (Group II). Group III animals were received a daily intraperitoneal administration of melatonin 5mg/mL per animal per day for 15 days before the first oral administration of DMBA and continued for a month. Group IV rats received melatonin (5mg/mL) beginning the next day after first DMBA administration for one month\(^8\).

Animals were monitored everyday. After the termination of the experiment, all animals were anesthetized with 24 mg/kg ketamine chloride and sacrificed by cervical dislocation after an overnight fast. The liver, kidney and mammary tissues were dissected out, weighed, cleared off blood and immediately transferred to beakers containing ice-cold solution. A 10% tissue homogenate were prepared using appropriate buffer. Another aliquot of tissues were stored in 10% formalin for histopathological studies. The tissues were sectioned using microtome, dehydrated in alcohol, embedded in paraffin, sectioned and stained with hematoxylin and eosin. Serum was collected from the blood after centrifugation at 1000g for 15 minutes for various biochemical estimation.

**Biochemical Assays:** As marker enzyme for liver function in serum, the activity of gamma glutamyl transferase (GGT) (expressed as IU/L plasma), was assessed\(^9\). To assess lipid peroxidation and antioxidant status in liver, kidney and mammary tissues, the levels of thiobarbituric acid reactive substances (TBARS) (expressed as nmol/100g tissue\(^10\)) and GSH levels (expressed as mg/100mg tissue\(^12\)) were measured. In addition, the activity of superoxide dismutase (SOD)\(^13\), catalase (CAT)\(^14\) glutathione peroxidase (GPx)\(^15\), ascorbic acid (vitamin C)\(^16\) and \(\alpha\)-tocopherol (vitamin E)\(^17\) were also estimated. Enzyme activity was
expressed as units as follows: SOD (enzyme required for 50% inhibition of nitroblue tetrazolium reduction mm/mg protein), CAT (µmol of hydrogen peroxide utilized/min/mg protein), GPx activity (µmol of GSH utilized/min/mg protein), vitamin C (mmol/gm wet tissue) and vitamin E (mmol/mg protein).

**Statistical Analysis:** Statistical analysis of results was performed and data were presented as mean ± S.D. Differences between groups were assessed by analysis of variance (ANOVA) using SPSS software package followed by DMRT. A value of P<0.05 was considered to indicate a significant differences between groups.

**Results:** Figure 1 presents the effect of melatonin on carcinogen-induced changes in the body weight of control and experimental animals. DMBA brought about a significant impairment in body growth, which was partially counteracted by the administration of melatonin.

Macroscopically detectable tumors developed in the DMBA-treated rats, an effect prevented by melatonin administration. Histopathological examination of the liver in group II shows mononuclear inflammatory infiltrate around portal triad, kidney shows glomeruli with cellular proliferation and tubular epithelial damage and mammary tissues shows highly invasive tumor cells surrounding the residual and expanding into adjacent stroma were seen. These histopathological features were greatly reduced in rats treated with DMBA plus melatonin. The tissues of melatonin pretreated or control rats did not differ in morphological features (Fig.2 (i), (ii), (iii).

Figures and tables summarize the effect of melatonin on carcinogen-induced changes on liver-related enzyme, lipid peroxidation and antioxidant status. As shown in Fig. 3, the parameter of liver damage, i.e., serum GGT activity, exhibited comparable changes after experimental manipulation. DMBA treatment augmented them, while melatonin partially counteracted the effect of DMBA. In all the three tissues liver, kidney and mammary tissues, DMBA brought about a significant increase of TBARS levels (P<0.05) when compared to control animals. The effect was almost fully and partially counteracted by melatonin in groups III and IV (fig. 4). Activities of enzymic and non-enzymic antioxidants in liver, kidney and mammary tissues were augmented during carcinogenesis, an effect fully reversed by melatonin in group III and partially in group IV (tables 1, 2 and 3).

**Discussion:** Chemoprevention involving the use of synthetic or natural products to inhibit or reverse the carcinogenic process is an effect approach to control cancer (18, 19) 7,12-dimethylbenz(a)anthracene, a poly cyclic aromatic hydrocarbon (PAH) is widely present in our environment and are implicated in various types of cancer. Sources of PAHs include industrial and domestic oil furnaces, gasoline and diesel engines.[1]

In the present study, DMBA-treated rats exhibited a significant inhibition of body growth, macroscopically detectable tumors, with increased levels of serum GGT. DMBA treatment increased TBARS levels in liver, kidney and mammary tissues, while the activities of SOD, CAT, GPx, vitamin C, vitamin E were decreased. The generation of reactive oxygen species (ROS) and the peroxidation of membrane lipids are well associated with the initiation of carcinogenesis affecting the normal biochemical process, which further leads to the reduction of body weight.[20,21]. These results agree with the hypothesis that the administration of DMBA to rats brought changes in body weight and enzyme activities which may serve as markers to evaluate the chemopreventive role of compounds of a potential clinical use.

Melatonin is a highly conserved naturally occurring molecules that is present virtually in all organism tested from bacteria to mammals. However, it is a nutrient and has been identified in bananas, tomatoes, cucumbers and beetroots[22]. The present result indicates that its administration significantly curtailed mammary tumor development and counteracted all the biochemical effects of DMBA. Several studies reported in the literature support the efficacy of melatonin to prevent the development of mammary cancer. For example, melatonin, in vivo, prevents the promotion and growth of spontaneous or chemically induced mammary tumors in rodents, whereas in vitro melatonin inhibits breast cancer cell proliferation and invasiveness. Moreover, melatonin hypothesis suggests that decreased melatonin production may increase breast adenocarcinomas had lower nocturnal plasma levels of melatonin than healthy women.

There are several mechanisms by which melatonin can exert its oncostatic actions in rat mammary gland: (a) by its direct action on the mammary tissues and on the activation of estrogen receptor for DNA binding[23]; (b) by enhancing its free radical scavenging /antioxidant activity[24]; (c) by enhancing immune mechanisms in body[25]; (d) by reducing the uptake of key factors for tumor growth and tumor growth signaling molecules (e.g. linoleic acid)[26]; Additionally, however melatonin kills neoplastic cells in vivo via the induction of apoptotic process preventing apoptosis in normal cells[27].

Gamma glutamyl transferase activities in serum are indexes of liver damage. Elevated activities of GGT observed in the DMBA-treated group are indicative of DMBA- induced hepatic damage and subsequent leakage of these enzymes into circulation[28]. GGT act
Table 1: Effect of melatonin on enzymic and non-enzymic antioxidants in the liver of control and experimental animals

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group I Control</th>
<th>Group II DMBA</th>
<th>Group III Mel + DMBA</th>
<th>Group IV DMBA + Mel</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD</td>
<td>6.37 ± 0.43</td>
<td>3.80 ± 0.32</td>
<td>5.99 ± 0.47</td>
<td>4.92 ± 0.42</td>
</tr>
<tr>
<td>CAT</td>
<td>48.94 ± 4.73</td>
<td>24.17 ± 2.12</td>
<td>46.15 ± 2.02</td>
<td>32.45 ± 2.18</td>
</tr>
<tr>
<td>Gpx</td>
<td>5.90 ± 0.29</td>
<td>3.10 ± 0.21</td>
<td>5.67 ± 0.37</td>
<td>4.43 ± 0.28</td>
</tr>
<tr>
<td>GSH</td>
<td>6.98 ± 0.42</td>
<td>4.15 ± 0.21</td>
<td>6.15 ± 0.29</td>
<td>4.76 ± 0.23</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>3.50 ± 0.32</td>
<td>1.08 ± 0.09</td>
<td>3.05 ± 0.26</td>
<td>2.57 ± 0.19</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>4.79 ± 0.43</td>
<td>1.53 ± 0.15</td>
<td>4.52 ± 0.31</td>
<td>3.23 ± 0.25</td>
</tr>
</tbody>
</table>

Table 2: Effect of melatonin on enzymic and non-enzymic antioxidants in the kidney of control and experimental animals

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group I Control</th>
<th>Group II DMBA</th>
<th>Group III Mel + DMBA</th>
<th>Group IV DMBA + Mel</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD</td>
<td>5.63 ± 0.39</td>
<td>2.12 ± 0.19</td>
<td>5.29 ± 0.32</td>
<td>3.75 ± 0.24</td>
</tr>
<tr>
<td>CAT</td>
<td>40.72 ± 3.86</td>
<td>17.25 ± 1.14</td>
<td>35.80 ± 2.81</td>
<td>23.42 ± 1.82</td>
</tr>
<tr>
<td>Gpx</td>
<td>3.64 ± 0.27</td>
<td>0.75 ± 0.06</td>
<td>3.43 ± 0.24</td>
<td>1.82 ± 0.16</td>
</tr>
<tr>
<td>GSH</td>
<td>4.65 ± 0.41</td>
<td>1.69 ± 0.08</td>
<td>3.69 ± 0.31</td>
<td>2.18 ± 0.21</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>1.72 ± 0.11</td>
<td>0.25 ± 0.01</td>
<td>1.65 ± 0.11</td>
<td>0.86 ± 0.05</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>2.17 ± 0.18</td>
<td>0.56 ± 0.03</td>
<td>1.96 ± 0.15</td>
<td>0.98 ± 0.05</td>
</tr>
</tbody>
</table>

Table 3: Effect of melatonin on enzymic and non-enzymic antioxidants in the mammary tissues of control and experimental animals

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group I Control</th>
<th>Group II DMBA</th>
<th>Group III Mel + DMBA</th>
<th>Group IV DMBA + Mel</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD</td>
<td>10.61 ± 0.95</td>
<td>6.25 ± 0.44</td>
<td>10.05 ± 0.76</td>
<td>8.00 ± 0.56</td>
</tr>
<tr>
<td>CAT</td>
<td>60.00 ± 3.74</td>
<td>30.00 ± 2.73</td>
<td>55.00 ± 4.73</td>
<td>46.33 ± 3.47</td>
</tr>
<tr>
<td>Gpx</td>
<td>8.50 ± 0.68</td>
<td>5.80 ± 0.32</td>
<td>8.01 ± 0.63</td>
<td>6.92 ± 0.46</td>
</tr>
<tr>
<td>GSH</td>
<td>8.48 ± 0.75</td>
<td>5.90 ± 0.49</td>
<td>8.10 ± 0.62</td>
<td>7.00 ± 0.47</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>3.24 ± 0.31</td>
<td>1.42 ± 0.09</td>
<td>3.20 ± 0.29</td>
<td>2.50 ± 0.23</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>5.25 ± 0.46</td>
<td>3.10 ± 0.23</td>
<td>5.10 ± 0.36</td>
<td>4.10 ± 0.29</td>
</tr>
</tbody>
</table>

Fig. 1: Effect of melatonin on body weight of control and experimental animals
Fig. 2(i): Histopathological examination of liver tissues of control and experimental animals

Fig. 2(ii): Histopathological examination of kidney tissues of control and experimental animals
as useful markers for hepatic metastasis from breast and colon primaries. Melatonin administration to DMBA-treated animals significantly decreased GGT activities indicating a significant cytoprotective effect on the hepatocytes[29].

Reactive oxygen species generation is a major factor involved in all steps of carcinogenesis, i.e. initiation, promotion and progression[30]. Oxidant-antioxidant balance impacts the rate of cell proliferation and tumor cells generally display low levels of lipid peroxidation which in turn can stimulate cell division and promote tumor growth[31]. Oxidative stress induced due to the generation of free radicals and/or decreased antioxidant level in the target cell and tissues has been suggested to play an important role in carcinogenesis[32].
Free radicals are involved both in the initiation as well as promotion stage of tumorigenesis and their biochemical reactions in each stage of the metabolic process are associated with cancer development\(^{(33)}\). It is evident from the results that increased level of LPO was found in cancer bearing animals when compared to control group. On the contrary, reduced level of LPO was observed in melatonin treated animals indicating that it is a potent free radical scavenger.

Both physiological and pharmacological concentration of melatonin in vivo are and commonly effective in reducing total oxidative burden with in organisms\(^{(34)}\). During the process of neutralizing toxic reactants, melatonin protects proteins, lipids, mitochondrial DNA and nuclear DNA from oxidative damage\(^{(35)}\).

Antioxidants act as the primary line of defense against ROS and suggest their usefulness in eliminating the risk of oxidative damage induced during carcinogenesis. SOD and CAT acts as mutually supportive antioxidative enzymes, which provide protective defense against reactive oxygen species\(^{(36)}\). The present study reveals that the activity of SOD is depleted in the cancer-bearing animals, which may be due to altered antioxidant status caused by carcinogenesis. A similar result was observed for CAT in group II, which may be due to the utilization of antioxidant enzymes in the removal of \(\text{H}_2\text{O}_2\) by DMBA. GPx is an important defense enzyme which catalyses the oxidation of GSH to GSSG at the expense of \(\text{H}_2\text{O}_2\)\(^{(37)}\). Decreased GPx activity was also observed in cancerous conditions\(^{(38)}\). Our findings agree well with this observation.

The non-enzymatic antioxidant systems are the second line of defense against free radical damage. GSH and GSH depended enzymes are involved in scavenging the electrophilic moieties involve in the cancer initiation\(^{(39)}\). GSH serves as a marker for evaluation of oxidative stress and it act as an antioxidant at both intracellular and extra cellular levels\(^{(40)}\). We also observed decreased activities of GSH in cancer-bearing animals. Melatonin increased the GSH levels, which clearly suggest their antioxidant property. Decreased levels of water-soluble antioxidant in cancer-bearing animals may be due to the utilization of antioxidants to scavenge free radicals. Ascorbic acid is a good scavenger of free radicals and it protect cellular membranes their by preventing degenerative disease like cancer\(^{(41,42)}\). Decreased levels of water soluble antioxidants in cancer bearing animals may be due to the utilization antioxidants to scavenge free radicals. Vitamin C undergoes a synergistic interaction with tocopheroxyl radical in the regeneration of \(\alpha\)-tocopherol\(^{(43)}\). Vitamin E protects cell membranes from oxidative damage initiated by carcinogens. The free radical clearing capacity of vitamin E is due to the localization of an unpaired electron on its conjugated double bond.

Administration of melatonin significantly reversed the alteration to near normal level in cancer-bearing animals and substantially inhibited the breast tumor incidence or decrease in initiation of tumorigenesis in pretreated group. From the results it can be inferred that melatonin positively modulated antioxidant activity by quenching and detoxifying the free radicals induced by DMBA. It is worth emphasizing the protective role of melatonin against the side effects of chemo and/or radiotherapy. Although majority of the studies involved experimental animals, the results could suggest applicability of melatonin in humans. Further investigation on the anticancer mechanisms of melatonin remains to be carried out \textit{in vitro} studies.
REFERENCES


