Comparative Histopathological & Immunohistochemical Studies between Melatonin and Grape-Seed Extract in Treating Hepatocellular Carcinoma

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Abstract: High levels of alpha-fetoprotein (AFP) are believed to be strongly suggestive of hepatocellular carcinoma (HCC) that is the fifth most frequent cancer and the third common cause of cancer related mortality in the world. AFP was studied immunohistochemically in addition to histopathology to delight the possible cure of melatonin (mel) or grape-seed extract (GSE) in induced HCC by two different carcinogens. Seventy five male albino mice were divided into six groups; normal group (n=5), experimental control group (n=10), experimental groups (n=30) and experimental treated groups (n=30). Histopathologically, the induced HCC by diethylnitrosamine (DEN) (8 w) was faster than 2-nitropropane (NP) (14 w). Malignant foci of HCC were manifested through cords of hyperchromatic malignant cells. However melatonin ameliorated these liver changes and GSE exhibited similar role but with a lesser extent. Immunohistochemically, the expression of AFP supported the superior effect of melatonin in HCC treatment. A significant value (1.20±0.77) (p=0.002) was recorded post mel treatment in comparison either with HCC (2.6±1.12) or with GSE (1.87±0.74) (p=0.063). In conclusion: DEN induced HCC in mice faster than 2-NP and melatonin exhibited strong cure than GSE in HCC.


Keywords: HCC, Mice, AFP, NP, Melatonin, Grape-Seed Extract, Immunohistochemistry

1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most frequent cancer and the third most common cause of cancer related mortality in the world (Jemal et al., 2011).

Alpha-fetoprotein (AFP) is a glycoprotein normally produced in large quantities during embryonic life in the foetal yolk sac and liver (Yap & Peh 1991). AFP is a well known representative tumor marker of HCC (Arrigoni et al., 1988). High levels of AFP are believed to be strongly suggestive of HCC (Tremolda et al., 1989). Greater than 70% of HCC patients have high serum concentration of AFP because of the tumor secretion. Elevation of AFP level up to pathological range in adults correlates with the appearance of several malignancies such as HCC and chronic liver disease (Jawed et al., 2009).

Melatonin (mel.) has been demonstrated to inhibit tumor development under both in vivo and in vitro conditions (Sririnivasam et al., 2011). There is widespread agreement that melatonin is a useful nontoxic molecule (Korkmaz et al., 2009).

The focus of cancer research in recent years has been shifting towards the isolation and characterization of potential chemopreventive agents present in fruits and vegetables (Kaur et al., 2006).

Grapes (Vitis vinifera) are one of the most widely consumed fruits in the world (Sharma et al., 2004). Grape seeds extract (GSE) is a complex mixture containing gallic acid (GA), catechin (C), epicatechin (EC) and several oligomers (Procyanidins) of C and/ EC, some of which are esterified to GA (Veluri et al., 2006). GA is a naturally available polyphenol, possess strong antioxidant activity with a capacity to inhibit the formation of tumors in several cancer models (Sharma et al., 2004).

GA treatment significantly reduced the levels of AFP, which revealed the anti-tumor effect of the compound against HCC (Ramakrishnan et al., 2007).

GSE has been shown to act as a potent scavenger of both reactive oxygen and nitrogen species. Furthermore it has also been shown that GSE significantly inhibits the activities of free-radical producing enzymes in biological systems because of its health benefits, particularly the strong antioxidant activity (Sundaram et al., 2008).

Aim of the Work

The current study was designed to compare between the possible role of melatonin and grape-seed extract in treating HCC induced by two different carcinogens histopathologically and immunohistochemically.

2. Materials and Methods

Seventy five male albino mice, three months old (30 ± 10 g) were used in this study and divided into the following groups:

Group I: 5 mice were served as normal group.
**Group II:** 10 mice were served as experimental control group.

Half of them were injected I.P. with 1ml of 10mg melatonin/kg body weight every other day for 14 weeks (Ravindra et al., 2006). The second half were received orally GSE (200 mg/kg bw/day) for 8 weeks (Singh et al., 2004).

**Group III:** 15 mice were injected I.P. with 1ml of 200 mg 2-nitropropane/kg bw every other day for 14 weeks (Borges et al., 2006).

**Group IV:** 15 mice were injected I.P. with DEN (100 mg/kg bw 5 days/week for 8 weeks (Subramaniyan et al., 2012).

**Group V:** 15 mice were injected with melatonin 30 minutes prior to 2-nitropropane for 14 weeks.

**Group VI:** 15 mice were received GSE (for 2 weeks) then (DEN) for 8 weeks.

All animals were sacrificed at the end of the experiment and the livers were prepared for the following parameters:

1. Histopathological study: sections of 5 µm were processed for H&E stains.
2. Immunohistochemical study for the detection of AFP, by using peroxidase antiperoxidase technique (Imoto et al., 1985).

**Statistical Analysis**

Data for (HCC, mel + 2-NP, and GSE + DEN) in relation to AFP intensity were expressed as mean, standard deviation (SD) and significantly using Mann Whitney test.

**3. Results**

**Histopathological Results:**

H&E stains showed the normal hepatocytes of group I with tightly packed and pink staining cytoplasm. The nuclei were round in shape and containing prominent nucleoli, the majority of hepatocytes were mononucleated but some binucleated hepatocytes were found (Fig. 1).

Examination of liver sections of group II which received either melatonin, or GSE showed no notable alternation as compared with normal histological structure except few microstatisis (Fig. 2) or few necrotic cells (Fig. 3) respectively.

Groups III and IV showed HCC appearance with loss of hepatic architecture and having proliferating streaks and cords of malignant hepatocytes (Fig. 4). It is worth mentioning that there were no detectable histopathological difference between the HCC induced by 2-NP or HCC induced by DEN.

While liver sections in group V showed few large necrotic cells and microstatisis, atypical nuclei were still seen (Fig. 5).

Group VI: liver sections showed some apoptosis and few red blood corpuscles scattered in veins, the hepatic cords were more or less regular (Fig. 6).
II. Immunohistochemical Result:

The expression of AFP is well demonstrated as brown granules in the cytoplasm and arranged around cell membranes either of hepatocytes or sinusoids.

In the present study, the normal group revealed a weak positivity of AFP (Fig. 7).

Group II: the two parts of this group showed increased positivity of AFP when compared with group I (Fig. 8).

Group III & IV: showed intense positivity of AFP in most cells (Fig. 9).

Group V: weak positivity of AFP was also seen in this group more or less as control group (Fig. 10).

Group VI: showed moderate positivity of AFP (Fig. 11).

The overall changes in AFP reaction were summarized in table (1).
Figure (9): Paraffin section of mice liver (HCC) showing intense positivity of AFP in most cells. PAP method (bar = 50 µm)

Figure (10): Paraffin section of mice liver 14 weeks post (mel. + 2 – NP) showing weak positivity of AFP more or less as control group. PAP method (bar = 50 µm)

Figure (11): Liver section of mice post (GSE + DEN) showing moderate positivity of AFP. PAP method (bar = 50 µm)

Staining intensity of AFP was scored semiquantitatively on a scale of negative to intense positivity, negative (0), weakly positive (+1), moderately positive (+2), strong positive (+3) and intense positive (+4) (Table 1).

Table (2) illustrated mean and S.D of HCC group (2.60 ± 1.12), after melatonin treatment (1.20 ± 0.77) and after GSE (1.87 ± 0.74). HCC groups showed a significant increase in the AFP positivity while the experimental treated groups were significantly decreased with AFP positivity.

Table (1): Comparison between the different studied groups according to AFP

<table>
<thead>
<tr>
<th>No of mice</th>
<th>HCC group</th>
<th>Mel + 2NP group</th>
<th>GSE + DEN group</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>+3</td>
<td>+2</td>
<td>+2</td>
</tr>
<tr>
<td>2</td>
<td>+1</td>
<td>0</td>
<td>+2</td>
</tr>
<tr>
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<td>+1</td>
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<td>0</td>
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<tr>
<td>15</td>
<td>+1</td>
<td>+1</td>
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</tbody>
</table>

| Min.       | 1.0       | 0.0            | 1.0            |
| Max.       | 4.0       | 2.0            | 3.0            |
| Mean ± SD  | 2.60 ± 1.12| 1.20 ± 0.77 | 1.87 ± 0.74 |
| Median     | 3.0       | 1.0            | 2.0            |

<table>
<thead>
<tr>
<th>Intensity of AFP</th>
<th>HCC group</th>
<th>Mel + 2NP group</th>
<th>GSE + DEN group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0.0</td>
<td>3</td>
</tr>
<tr>
<td>+1</td>
<td>3</td>
<td>20.0</td>
<td>6</td>
</tr>
<tr>
<td>+2</td>
<td>4</td>
<td>26.7</td>
<td>6</td>
</tr>
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| Min. – Max. Mean ± SD Median | 1.0 – 4.0 | 0.0 – 2.0 | 1.0 – 3.0 | 2.60 ± 1.12 | 1.20 ± 0.77 | 1.87 ± 0.74 | 3.0 | 1.0 | 2.0 |

*p: p value for Mann Whitney test
*: Statistically significant at p ≤0.05
These novel findings show that melatonin by inducing cell death and cell cycle arrest, might be useful as adjuvant in hepatocarcinoma therapy (Martin et al., 2008).

Regarding GSE many studies have shown that it inhibited human breast carcinoma MCF-7, human lung cancer A-427 and human gastric cancer cell CRL-1739 growth (Veluri et al., 2006). Another studies also showed that, GSE inhibited cell proliferation, induced cell cycle arrest and induced apoptotic cycle in human breast carcinoma cells MDA-MB468 and prostate Du 145 carcinoma cells (Agarwal et al., 2000 and Agarwal et al., 2002). Several studies in mice showed that GSE inhibited chemically – induced lipid peroxidation, DNA fragmentation, and subsequent apoptosis (indicators of oxidative tissue damage) in a dose-dependent manner in hepatic and brain tissues (Bagchi et al., 1998).

Respecting AFP, there was shortage in literature which dealing with its immunohistochemical expression in HCC under the effect of mel. and perhaps, the present study may be the first one in this field.

Our present results showed that AFP was re-expressed in HCC induced by 2-NP while after melatonin treatment its concentration was weak (more or less as control group). In accordance to GSE treatment this positivity was moderate.

In accordance with our findings, (Ray et al., 2000) Reported that GSE administration was beneficial in preventing animal hepatic and renal toxicity. These authors studied the role of GSE in acetaminophene and other drug poisoning and attributed the protective effect of GSE to detoxification of cytotoxic free radicals or facilitation of DNA repair. Our results supported the role of phenolics to attenuate the alterations resulted through HCC induction by DEN. (Sundaram et al., 2008), (Kahkonen et al., 1999) The antioxidant activity of these compounds may be mainly due to their redox properties that allow them to act as reducing agents, hydrogen donators, and singlet oxygen quenchers. In conclusion: DEN induced HCC in mice faster than 2-NP and melatonin exhibited strong cure than GSE in HCC.

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**References:**


