CHAPTER 7

ANTICANCER STUDIES OF SURFACE-MODIFIED PACLITAXEL ATTACHED HYDROXYAPATITE AND TITANIUM DIOXIDE NANOPARTICLES

7.1 INTRODUCTION

Hepatocellular carcinoma (HCC) is the most frequent primary malignancy of the liver, and it accounts for as many as one million deaths worldwide in a year. In some parts of the world it is the most common form of internal malignancy and the most common cause of death from cancer (Jemal et al 2005). Well-known risk factors of HCC include hepatitis B virus (HBV), hepatitis C virus (HCV), aflatoxins, alcohol and oral contraceptives. Smoking, androgenic steroids and diabetes mellitus are also suspected risk factors (Chang et al 2006). One of the well-known biological characteristics of liver cancer is the high rate of recurrences in the remaining liver after complete resection of the primary tumor. Three years after resection, the incidence of recurrences of HCC in the remnant liver is as high as 70%. This makes liver cancer one of the most dreaded amongst all cancers (Yamamoto et al 1996).

Morbidity and mortality in HCC are primarily the result of the aggressive local spread, rather than the occurrence of distant metastasis. Treatments of HCC are conventionally divided into curative and palliative. Surgical resection is the major curative technique, but it is very limited for patients with multiple or metastatic tumors. Therefore, it is of great
importance to search for effective chemotherapeutic agents to improve the survival rate of patients with advanced or recurrent HCC after surgical treatment (Qin and Tang 2002). Chemoprevention by definition is the means of cancer management in which the occurrence of the disease can be entirely prevented, slowed or reversed by the administration of one (or) more naturally occurring and/or synthetic compounds called as anticarcinogens. Two forms of chemopreservation exist: one general population chemoprevention entails efforts at providing measures applicable to large population groups; the other, targeted chemoprevention, is directed towards individuals at increased risk of cancer in a specific tissue (Wattenberg 1992). Paclitaxel is prescribed to treat various cancers including hepato carcinoma (Lin et al 2000, Kang et al 2000).

The liver is one of the most important organs in energy metabolism. Most apolipoproteins, endogeneous lipids, and lipoproteins are synthesized in the liver, and depend on the integrity of the cellular functions of this organ. Under normal physiological conditions, the liver ensures homeostasis of lipid and lipoprotein metabolism. Hepatocellular carcinoma impairs this process leading to alterations in the lipid and lipoprotein patterns (Jiang et al 2006). A marker is synthesized by the tumor and released into the circulation, but it also may be produced by normal tissues in response to an invasion by cancer cells (Thangaraju et al 1998). A variety of substances, including enzymes, hormones, antigens, and proteins may be considered as tumor markers. An analyses of tumor markers can be used as an indicator of tumor response to therapy. Sensitive and specific liver cancer marker enzymes are used as indicators of liver injury. Analysis of these marker enzymes reflects the mechanisms of cellular damage and the subsequent release of proteins and extracellular turnover (Thirunavukkarasu and Sakthisekaran 2003).

Nitrosamines are compounds formed by the combination of amines and nitrates or nitrites. Studies have shown that nitrosamines can be formed in
the gastric juice of the human stomach by a process commonly referred to as endogenous nitrosation. The bacteria in the mouth chemically reduce the nitrate found in many vegetables to nitrite, which in turn can form nitrosating agents. Many foods that contain amines can react with these nitrosating agents in the acidic environment of the stomach to form nitrosamines (Jakszyn and Gonzalez 2006). Diethylnitrosamine (DEN), a hepatocarcinogen, is known to cause perturbations in the nuclear enzymes involved in DNA repair/replication, and is a carcinogen known to induce liver cancer in animal models. Investigations have provided evidence that N-nitrosamines cause a wide range of tumors in all animal species and these compounds are considered to be a serious human health hazard. Human exposure to preformed N-nitrosamines occurs through the diet, in certain occupational settings, and through the use of tobacco products, cosmetics, phamaceutical products and agricultural chemicals. It has been reported that DEN, after its metabolic biotransformation produces the promutagenic adducts, O6-ethyl deoxy guanosine and O4- and O6-ethyl deoxy thymidine that may initiate liver carcinogenesis (Ramakrishnan et al 2006).

Reactive oxygen species (ROS) are important inducers of both tissue injury and DNA damage. DEN induces oxidative stress possibly due to the generation of ROS, which are capable of initiating peroxidative damage to the cell (Bansal et al 2005). DEN is bio transformed by mixed-function cytochrome P-450 dependent monooxidase systems, and its metabolic activation is reported to be responsible for the onset of the toxic effects (Zimmerman 1993). Intermediate reactive compounds originating from the bioactivation of DEN are known to form covalent bonds with important cell constituents, thus inducing the onset of mutations, cancer, and necrosis (Schmitt et al 1993). Microsomal activation of DEN involves cytochrome P450 2E1. Hence compounds that selectively activate cytochrome P450 systems are being widely used by several investigators to induce
hepatocellular carcinoma in experimental animals. Enhancement of DEN initiated carcinogenesis could be done by promoters such as Phenobarbital (PB) which transforms DEN initiated cells to foci and to hepatocellular carcinoma. Phenobarbital has been found to be one of the most efficacious tumor promoting agents in the liver (Gayathri et al 2010).

This chapter deals with the anticancer studies of surface-modified PAC attached HAp and TiO$_2$ nanoparticles in DEN induced hepatocarcinoma animal models.

7.2 EXPERIMENT

7.2.1 Animals

Healthy male wistar albino rats (approximately 150-180 g body weight) aged 6 weeks were procured from Tamil Nadu Veterinary and Animal Science University, Chennai, India. The animals were housed in polypropylene cages with a maximum of three animals per cage and placed in a ventilated, temperature-controlled room. The standard conditions were supplied and maintained at 293 ±2 K room temperature, 60 ± 10% relative humidity and 12 h light/ dark cycle. The cages contained autoclaved paddy husk as bedding that was replaced on a bi-weekly basis. The commercial pellet diet and distilled water for rats were available ad libitum. The experimental protocol involving the animals was carefully reviewed and approved (111/PHARMA/SCRI/8 JULY 2011) by Institutional Animal Ethics Committee of Siddha Central Research Institute (SCRI), Chennai, India. The studies also were carried out at SCRI in compliance with the guidelines of the Committee for the Purpose of Control and Supervision of Experimental Animals (CPCSEA), India. Animals were acclimated to this environment for five days prior to treatment.
7.2.2 Experimental Design

Rats were randomly divided into 7 groups with 6 animals in each group. The experimental design and treatment protocol were as follows: Group 1 Rats, served as normal control were given normal saline. Group 2 to Group 7 animals were given DEN in saline as single i.p dose (200 mg/Kg body wt). After a recovery period of 2 weeks, phenobarbital at a concentration of 0.05% was given in drinking water for up to 14 successive weeks to promote the cancer. After the induction period, Group 2 animals were treated as negative control. Group 3 animals were given PAC at a dose of 50 mg/Kg BW in DMSO. Group 4 animals were treated with HAp-PEG-FA nanoparticles at a dose of 50 mg/Kg BW in PBS. Group 5 animals were treated with HAp-PEG-FA-PAC nanoparticles at a dose of 50 mg/Kg BW in PBS. Group 6 animals were treated with TiO$_2$-PEG-FA nanoparticles at a dose of 50 mg/Kg BW in PBS. Group 7 animals were treated with TiO$_2$-PEG-FA-PAC nanoparticles at a dose of 50 mg/Kg BW in PBS. Paclitaxel injection and nanoparticle suspensions were given by Intraperitonial injection five times at 2-days interval for 10 days. On completion of the treatment, the animals were sacrificed by cervical dislocation and necropsied for the gross evaluation of the various organs. The necropsy also included careful and consistent dissection of liver.

7.2.3 Blood Biomarker Assay

Blood samples were collected via the ocular vein. The serum was obtained by centrifugation of the whole blood at 3000 rpm for 15 min. Liver function was evaluated based on the serum levels of alkaline phosphatase (ALP), alanine aminotransferase (ALT) and aspartate aminotransferase (AST), total bilirubin, direct bilirubin, total protein, albumin, gamma glutamyl transferase (GGT), α feto protein (AFP). These biochemical parameters were
determined by an automated biochemical analyzer (Bayer RA-50 Chemistry Analyser).

7.2.4 Hematological Parameters Determination

Blood samples were collected in tubes containing EDTA as anticoagulant. Red blood corpuscles (RBC), white blood corpuscles (WBC), hemoglobin (HGB), platelets (PLT), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), Red cell distribution width (RDW), mean platelet volume (MPV), platelet distribution width (PDW) were measured using a haematology auto analyzer (BC 2800VET).

7.2.5 Histopathological Examination

After sacrifice, liver and kidney were removed and placed into buffered formalin. For pathological studies, all histopathological tests were performed using standard laboratory procedures. The tissues were embedded in paraffin blocks, then sliced into 5 µm in thickness and placed onto glass slides. After hematoxylin–eosin (HE) staining, the slides were observed and the photos were taken using optical microscope. The results were analyzed with the help of veterinary pathologist.

7.2.6 Statistical Analysis

For statistical analysis, each of the experimental value was compared with its corresponding control. Results were expressed as mean ± standard deviation (SD). The significance of difference between the observations of control and exposed groups was analysed using Student’s t-test. Difference showing P<0.05 was considered significant.
7.3 RESULTS AND DISCUSSIONS
7.3.1 Biochemical Chemical Analysis

The activities of ALT, AST, ALP, total bilirubin, direct bilirubin, total protein, albumin, AFP and GGT are shown in Table 7.1. A significant increase in the levels of AST, ALT, ALP, GGT, AFP, total bilirubin and Direct Bilirubin and a significant decrease in the level of total protein and albumin in the serum was observed in group 2, as compared with group 1. Significant decrease in the level of the tumor markers AST, ALT, ALP, GGT, AFP, total bilirubin and direct bilirubin and significant increase in the level of total protein and albumin was demonstrated in the animals in group 3 through group 7 as compared with group 2. Group 5, treated with HAp-PEG-FA-PAC nanoparticles, treated group 5 showed a significant decrease in the level of AST, ALT, ALP, GGT, AFP, total bilirubin, direct bilirubin and significant increase in the level of total protein and albumin enzymes as compared group 2. HAp-PEG-FA treated group 4 shows a pattern similar to that of group 5, when compared to group 2. But even with the change in the group 4 tumor markers, they are still below the level of those shown by the HAp-PEG-FA-PAC treated group 5. This shows that the anticancer activity of HAp-PEG-FA-PAC nanoparticles is higher than that of the HAp-PEG-FA nanoparticles.

During carcinogenesis, some enzymes can be used as a biochemical indicators of tumor response to therapy (Thirunavukkarasu and Sakthisekaran 2003). Hepatospecific enzymes were activated when hepatocellular damage gave rise to abnormalities of liver function, and these enzymes are remarkably increased in HCC. AST, ALT and ALP activities in blood serum are generally accepted as an index of liver damage and this tendency is also known to be distinct in rodents (Ha et al 2001). Cellular damage exhibits good correlation with the enzyme leakage (Sherawat and Sultana 2006). Serum AST, ALT,
ALP, GGT, bilirubin and AFP are the most sensitive markers employed in the diagnosis of hepatic damage (Sallie et al 1991).

**Table 7.1  Effect of HAp-PEG-FA-PAC and TiO$_2$-PEG-FA-PAC on biochemical parameters of the control and the experimental group of animals**

<table>
<thead>
<tr>
<th>Groups</th>
<th>AST (U/L)</th>
<th>ALT (U/L)</th>
<th>ALP (U/L)</th>
<th>Total Bilirubin (mg%)</th>
<th>Direct Bilirubin (mg%)</th>
<th>Total Protein (g%)</th>
<th>Alb (g%)</th>
<th>GGT (U/L)</th>
<th>AFP (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 (Positive control)</td>
<td>155.66 ± 8.59</td>
<td>72.83 ± 15.35</td>
<td>221 ± 4.58</td>
<td>0.51 ± 0.04</td>
<td>0.33 ± 0.06</td>
<td>7.98 ± 0.89</td>
<td>3.51 ± 0.30</td>
<td>2.37 ± 0.45</td>
<td>0.51 ± 0.10</td>
</tr>
<tr>
<td>Group 2 (DEN induced negative control)</td>
<td>280.00 ± 9.02</td>
<td>138 ± 7.67</td>
<td>398.50 ± 5.21</td>
<td>0.93 ± 0.16</td>
<td>0.65 ± 0.10</td>
<td>3.18 ± 0.29</td>
<td>2.50 ± 0.32</td>
<td>4.5 ± 1.09</td>
<td>0.98 ± 0.12</td>
</tr>
<tr>
<td>Group 3 (Pure paclitaxel)</td>
<td>206.83 ± 4.89</td>
<td>94.83 ± 6.76</td>
<td>291.83 ± 4.47</td>
<td>0.68 ± 0.05</td>
<td>0.41 ± 0.17</td>
<td>6.91 ± 0.88</td>
<td>3.08 ± 0.29</td>
<td>2.12 ± 1.23</td>
<td>0.69 ± 0.13</td>
</tr>
<tr>
<td>Group 4 (HAp-PEG-FA)</td>
<td>230 ± 11.33</td>
<td>116.16 ± 9.57</td>
<td>312.16 ± 4.3</td>
<td>0.81 ± 0.08</td>
<td>0.48 ± 0.04</td>
<td>5.53 ± 1.21</td>
<td>2.68 ± 0.49</td>
<td>3.11 ± 0.21</td>
<td>0.72 ± 0.12</td>
</tr>
<tr>
<td>Group 5 (HAp-PEG-FA-PAC)</td>
<td>163.33 ± 10.30*</td>
<td>74.83 ± 16.99*</td>
<td>240.5 ± 3.72*</td>
<td>0.59 ± 0.09*</td>
<td>0.34 ± 0.08*</td>
<td>7.46 ± 0.88*</td>
<td>3.33 ± 0.34*</td>
<td>1.76 ± 0.35*</td>
<td>0.60 ± 0.14*</td>
</tr>
<tr>
<td>Group 6 (TiO$_2$-PEG-FA)</td>
<td>220.16 ± 2.44</td>
<td>105.33 ± 1.87</td>
<td>305.34 ± 4.30</td>
<td>0.75 ± 0.09</td>
<td>0.50 ± 0.09</td>
<td>5.62 ± 0.45</td>
<td>2.76 ± 0.13</td>
<td>2.75 ± 0.30</td>
<td>0.72 ± 0.09</td>
</tr>
<tr>
<td>Group 7 (TiO$_2$-PEG-FA-PAC)</td>
<td>158.33* ± 0.09</td>
<td>70.33* ± 1.45</td>
<td>224 ± 3.10*</td>
<td>0.54 ± 0.06*</td>
<td>0.38 ± 0.07*</td>
<td>7.25 ± 0.29*</td>
<td>3.36 ± 0.53*</td>
<td>2.30 ± 0.42*</td>
<td>0.58 ± 0.08*</td>
</tr>
</tbody>
</table>

* P < 0.05 not significant for control; *P < 0.05 significant from control.
The role of transamination in biological systems is well known. It is apparent from transaminase substrate, i.e. α-ketoglutaric, oxaloacetic and pyruvic acids on one hand and glutaric and aspartic acids on the other hand, that transamination is concerned with the interconversion of highly important metabolites. Elevated aminotransferase activity levels were observed in cancer bearing animals. Transaminase becomes gradually more pronounced towards the terminus, which indicates the severity of an advanced cancer condition. The increase in the activities of these enzymes in serum might be due to the leakage of these cytosolic enzymes into the in circulatory system resulting from hepatocellular damage during DEN administration. This is indicative of the onset of hepatocellular damage due to liver dysfunction and disturbance of the biosynthesis of these enzymes, with alteration in the permeability of liver membrane.

These enzymes are more unique and changes in their activities reflect the effect of the proliferation of cells with growth potential. But the enzyme’s metabolic turnover is dramatically different from those of normal cells. The rise in their activities is shown to be in good correlation with the number of transformed cells in cancer conditions. The measurement of concentrations and activities of these biochemical markers and enzymes in the plasma plays a significant role in disease investigation and diagnosis (Malomo 2000). There was a good correlation between the activities of these enzymes and tumor volume during therapy. Rocchi et al (1997) reported that there is an increase in the levels of these transaminases activity in serum of HCC patients. In concurrence with the above findings an elevated serum aminotransferase activities were observed in animals bearing HCC.

ALT, which is mainly produced in the hepatocytes, is more specific for liver injury (Thomson 1998). It has been reported that ALT is generally increased in situations where there is damage to the liver cell membrane
(Schumann et al 2002). Thus, when the liver is injured, the levels of ALT in plasma usually rise. Alkaline phosphatase (ALP) is involved in transport of metabolites across cell membrane, protein synthesis, secretory activities and glycogen metabolism. Alkaline phosphatase is a membrane bound enzyme and its alteration is likely to affect the membrane permeability and produce derangement in the transport of metabolites. ALP was noticed in the serum and liver of hepatoma - bearing animals (Patel et al 1994). The rise in the activity of ALP in cancer bearing animals may be due to the disturbance in the secretory activity or in transport of metabolites, or may be due to altered synthesis of certain enzymes in these conditions. ALP is used as a specific tumor marker during diagnosis in the early detection of cancer (Kobayashi and Kawakubo 1994).

Elevation of alkaline phosphatase is one of the signs, suggesting space-occupying lesions in the liver. An increased activity of ALP was seen in blood serum and also in the liver of animals with HCC. This may be due to the disturbance in secretory activity or due to altered gene expression under these conditions. The development of a tumor results in tissue damage that lead to the release of ALP into circulation (Iqbal et al 2004) and this enzyme level has been elevated in the blood serum and liver tissue of the tumor-bearing animals. This elevation is significantly suppressed by the surface-modified and surface-modified PAC attached HAp and TiO$_2$ nanoparticles.

GGT is found in all cells of the body except myocytes. Particularly high concentration of GGT is found in hepatocytes and the kidney. It is involved in the transport of amino acids and peptides into cells (Hanigan and Pitot 1985). GGT has been shown to play an important role in the metabolism of foreign substances, and during cell growth and differentiation (Thusu et al 1991). It is overexpressed in tumor cells (Bailey et al 2001). Experimental studies have shown that GGT was strikingly activated during the course of
Chemical carcinogens may initiate some systematic effects that induce GGT synthesis (Vanisree and Shyamaladevi 1998). This elevation reflects the progress of carcinogenesis, since its activity correlates with tumor growth rate, differentiation and survival of the host (Koss and Greengurd 1982); in concurrence with above findings, there was an increase in the levels of GGT in the serum and liver of animals bearing HCC. This elevation indicates the basic tumor burden, and treatment with surface-modified and surface-modified PAC attached HAp and TiO$_2$ nanoparticles decreased the elevation of the level of this enzyme.

α - fetoprotein (AFP) is a molecule that carries the onco-fetal specificity Tumor marker. AFP is a serum protein that is detected in elevated concentration in conditions like HCC. AFP is a serum protein similar in size, structure and amino acid composition to serum albumin, but it is detectable only in minute amounts in the serum of normal adults. Elevated serum concentrations of this protein can be observed in adults exposed to hepatotoxic agents (or) hepatocarcinogens and are frequently associated with HCC. It is a 72 KDa α-1 globulin with an uncertain biological function. It is synthesized during embryonic life by fetal yolk sac, liver and intestinal tract. AFP has high specificity for hepatocarcinoma. Its serum concentration can be used to confirm hepatocarcinoma and for the diagnosis of tumor response to therapy. More than 90% of patients with hepatic cancer have increased serum AFP levels (Maideen et al 2012). Elevation of serum AFP levels has been reported in several diseases including HCC (Abeleb 1986). AFP is most extensively used in the diagnosis of HCC (Banker 2003). In our study there was also an increased level of AFP in the DEN administered animals confirming the presence of HCC. Significantly, the treatment by and surface-
modified and surface-modified PAC attached HAp and TiO$_2$ nanoparticles significantly reduced the elevation of AFP. This indicates a positive prognosis.

Albumin is a major protein formed by the liver and together with the total protein level in the blood, reflects the protein function of the liver (Benoit et al 2000). The albumin and protein levels have decreased in group 2. The albumin level of animals in group 2 decreased when compared to group 1. Group 5 to group 7 in this study were treated with nanoparticles and show an increased albumin level. The decrease in albumin level is not a large one because a decrease in plasma albumin is usually not seen early in hepatocarcinogenesis (Cheesbrough 1998). This could also be due to the long half life of albumin, which has been reported to be around 20 days (Halsted and Halsted 1991).

7.3.2 Hematological Analysis

The hematological parameter analysis of the animals were given in Table 2. In the group 2 animals the values of WBC, MCV, MCH, MCHC, RDW, MPV and PDW have increased compared to group 1. The values of RBC, PCV hemoglobin and platelets have decreased compared to group 1 animals. In the group 4 to group 7 animals, a significant decrease in the values of WBC, MCV, MCH, MCHC, RDW, MPV and PDW and significant increase in RBC, PCV, hemoglobin and platelets compared to group 2, have been noted due to the treatment with surface-modified and surface-modified PAC attached HAp and TiO$_2$ nanoparticles.
Table 7.2 Effect of HAp-PEG-FA-PAC and TiO$_2$-PEG-FA-PAC on hematological parameters of control and experimental group of animals

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Group 4</th>
<th>Group 5</th>
<th>Group 6</th>
<th>Group 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC (X10$^9$ /L)</td>
<td>8.36 ± 0.12</td>
<td>17.96 ± 0.78</td>
<td>10.82 ± 0.51</td>
<td>12.34 ± 0.87</td>
<td>9.55 ± 0.74</td>
<td>13.34 ± 0.87</td>
<td>6.55 ± 0.15$^a$</td>
</tr>
<tr>
<td>RBC (X10$^{12}$ /L)</td>
<td>9.17 ± 0.4</td>
<td>5.65 ± 0.41</td>
<td>7.98 ± 0.32</td>
<td>6.56 ± 0.21</td>
<td>8.23 ± 0.56</td>
<td>6.18 ± 0.71</td>
<td>9.13 ± 1.39$^a$</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>15.25 ± 0.89</td>
<td>8.62 ± 0.57</td>
<td>13.06 ± 0.45</td>
<td>10.1 ± 0.29</td>
<td>14.26 ± 0.29</td>
<td>10.55 ± 1.11</td>
<td>15.06 ± 1.39</td>
</tr>
<tr>
<td>Platelet (X109 /L)</td>
<td>164.83 ± 1.32</td>
<td>81.10 ± 2.60</td>
<td>135.08 ± 1.56</td>
<td>129.78 ± 1.23</td>
<td>158.54 ± 1.35</td>
<td>118.50 ± 3.67</td>
<td>167.16 ± 1.23$^a$</td>
</tr>
<tr>
<td>PCV (%)</td>
<td>45.88 ± 1.21</td>
<td>33.36 ± 2.60</td>
<td>38.78 ± 0.34</td>
<td>36.91 ± 0.33</td>
<td>43.98 ± 1.55</td>
<td>35.18 ± 1.71</td>
<td>45.46 ± 1.89$^a$</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>50.11 ± 2.11</td>
<td>65.22 ± 1.34</td>
<td>53.11 ± 1.29</td>
<td>55.23 ± 2.87</td>
<td>51.23 ± 0.21</td>
<td>56.41 ± 1.47</td>
<td>51.85 ± 1.81$^a$</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>16.61 ± 0.81</td>
<td>25.58 ± 0.53</td>
<td>18.41 ± 1.29</td>
<td>20.45 ± 0.78</td>
<td>17.65 ± 0.64</td>
<td>19.45 ± 0.43</td>
<td>17.41 ± 0.38$^a$</td>
</tr>
<tr>
<td>MCHC (g/dL)</td>
<td>33.25 ± 0.45</td>
<td>42.78 ± 0.89</td>
<td>37.39 ± 0.87</td>
<td>40.23 ± 0.32</td>
<td>34.87 ± 0.53</td>
<td>39.89 ± 0.12</td>
<td>35.78 ± 0.78$^a$</td>
</tr>
<tr>
<td>RDW (%)</td>
<td>11.88 ± 0.97</td>
<td>17.01 ± 0.96</td>
<td>13.29 ± 0.59</td>
<td>14.44 ± 0.23</td>
<td>12.61 ± 1.01</td>
<td>13.63 ± 0.62</td>
<td>11.91 ± 1.01$^a$</td>
</tr>
<tr>
<td>MPV (fL)</td>
<td>6.09 ± 0.28</td>
<td>9.7 ± 0.36</td>
<td>6.99 ± 0.34</td>
<td>7.94 ± 0.45</td>
<td>6.86 ± 0.56</td>
<td>7.34 ± 0.76</td>
<td>6.11 ± 0.12$^a$</td>
</tr>
<tr>
<td>PDW (%)</td>
<td>15.01 ± 0.20</td>
<td>17.65 ± 0.25</td>
<td>15.99 ± 0.23</td>
<td>16.23 ± 0.98</td>
<td>15.08 ± 0.31</td>
<td>16.65 ± 0.38</td>
<td>15.1 ± 0.26$^a$</td>
</tr>
</tbody>
</table>

* P < 0.05 not significant for control; $^a$P < 0.05 significant from control.

Group 1- Positive control, Group 2- DEN induced negative control, Group 3- paclitaxel treated, Group 4- HAP-PEG-FA treated, Group 5- HAP-PEG-FA-PAC, Group 6- TiO$_2$-PEG-FA, Group 7- TiO$_2$-PEG-FA-PAC.

White blood cells (WBC) are important parameters in liver damage. When the liver cell become damaged, the immune system of the body would respond to this damage and cause inflammation inside the liver. The production of WBC will increase due to inflammation. This would cause the
liver to enlarge in size. The tumor can cause the liver cells around them to become damaged and this can cause the inflammation and an increase in WBC.

Cancer may decrease production of red blood cells and platelets by impacting the organs that affect blood count, such as the kidneys, bone marrow and spleen. Production of red blood cells (erythropoiesis) occurs in the bone marrow and is regulated in a series of specific steps. One of the important enzymes regulating this process is called erythropoietin (Epo). The majority of Epo is produced and released by the kidneys, and a smaller portion is released by the liver. Packed cell volume (PCV), the amount of cells in the blood, is expressed as a percentage of the total volume of blood. The decrease in PCV is mainly due to the decrease in RBC and due to liver disorder.

Red cell distribution width (RDW) is a measurement of the variation in the size of the red blood cells. Red blood cells help carry oxygen in the blood. A high RDW (over 14.5%) means that the red blood cells vary a lot in size. There are many possible reasons why the RDW level can be too high. This value tells how consistent in size the red blood cells are. Newly made cells (reticulocytes) are larger than iron deficient cells. In liver disorder condition, there is a reduction in RBC. So, there will be only newly produced RBC. This leads to the increase in RDW value.

A comparison is made between RDW and the mean corpuscular volume (MCV). The MCV is the average amount of space occupied by each red blood cell. When both the RDW and MCV levels are increased, the main cause is liver disease. The liver is the main organ in the body responsible for filtering (removing) harmful chemical substances, producing important chemicals for the body, and other important functions. Another cause of high
RDW & MCV levels is hemolytic anemia. Hemolytic anemia is a condition in which the red blood cells are destroyed earlier than they should be.

An increased mean corpuscular volume (MCV) is followed by hepatocellular damage. The actual mechanism by which alcohol causes an increase in MCV appears to include the direct toxic effect of alcohol on red blood cells, folic acid deficiency and hepatic damage. Mean platelet volume (MPV) is one of the most widely used surrogate markers of platelet function. It has been shown to increase in liver damage. Decrease in the platelet count is another important reason for the increase in MPV. Thrombocytopenia is a common complication in liver disorder. Reductions in the level or activity of the hematopoietic growth factor thrombopoietin (TPO) may also play a role. MPV has an inverse, non-linear relation with platelet count, while platelet volume heterogeneity has a direct, non-linear relation with MPV. Bone marrow is not a usual site of metastases in HCC. For this reason higher MPV values in HCC cannot be explained by the invasion and destruction of the bone tissue matrix by tumor cells (Kurt et al 2012).

Interleukin-6 (IL-6) is a broad spectrum cytokine that exhibits potent effects on megakaryocytic maturation. When used in combination with the early acting cytokine IL-3, IL-6 is synergistic, promoting increased growth of megakaryocytic and early hematopoietic progenitor cells. IL-6 is capable of progressively augmenting platelet diameter. This observation suggests that the cytokine (directly or indirectly) modifies terminal maturation of megakaryocytes. The substantial proportion of platelets may achieve extremely large size due to the effect of IL-6. Circulating levels of IL-6 increase markedly during development and progression of tumors of different cancer types, like liver, breast, pancreas, lung, ovary and prostate. High concentration of this cytokine may explain the higher MPV values in HCC. Platelets in the HCC patients have been suggested to store more brain-
derived-neurotropic factor (BDNF) than in the normal. BDNF is a novel functional protein that could promote tumor cell growth in a rat HCC model. It was also demonstrated that BDNF mRNA was over-expressed in human HCC tumor tissue compared with that in non-tumorous tissue and in cirrhotic or normal liver tissues (Kurt et al 2010). Platelet distribution width (PDW) is a measurement of the degree at which the platelets vary in size. The PDW increases in liver damage.

The mature red blood cell (also known as an erythrocyte) carries oxygen attached to the iron in hemoglobin. The number of red blood cells decreases in liver damage and since hemoglobin is the most abundant protein found within the red blood cell, it is found to decrease as were. Hemoglobin must be evaluated with the hematocrit (HCT), RBC, and the RBC indices (MCV). The hematocrit represents the volume of red blood cells in 100ml of blood. Low hematocrit and hemoglobin usually indicate the decreased production, excessive loss, or destruction of the red blood cells. The amount of hemoglobin in a single red blood cell is indicated by the MCH. The conditions for the increase and decrease of MCH are the same as those for the MCV. The average hemoglobin concentration per unit volume (100 ml) of packed red cells is indicated by MCHC.

Thrombopoietin (TPO), a glycoprotein hormone produced mainly by the liver, regulates the production of platelets by the bone marrow. Liver damage causes a decrease in thrombopoietin. This causes reduced platelet counts in the blood, the threshold for thrombocytosis. This in turn stimulates tumor growth and the continuation of the cycle. Platelets may function as a fuel depot for tumors by providing them with growth factors, which were found not only in the blood, but also in the tumor's microenvironment, in the tumor bed, and in ascites. The hematological parameters which are increased due to hepatic damage have decreased significantly in nanoparticle treated
groups. The hematological parameters which are decreased due to hepatic damage have increased significantly in nanoparticle treated groups. This shows the anticancer activity of the surface-modified and surface-modified PAC attached HAp and TiO$_2$ nanoparticles.

7.3.3 **Histopathological Examination**

Histopathological examination of liver sections from control group 1 animals revealed normal architecture, and cells with granulated cytoplasm and small uniform nuclei (Figure 7.1A). Liver sections from group 2 animals (Figure 7.1B) revealed the loss of architecture, and showed a tendency to spread by intrahepatic veins, both hepatic and portal with significant tumor thrombi within portal vessels (indicated by arrows). The histologic appearance of the HCC in group 2 is also extremely variegated. The disarrangement of normal hepatic cells with intense centrilobular necrosis was observed: the central veins surrounded by extensive necrosis and inflammatory infiltrate (indicated by circle), with clusters of hepatocyte necrosis. The histologic patterns were varied. The tumor cells are growing in nests and thick cords that are separated from one another by thin walled sinusoids.

Cytologically, the tumor cells bear some resemblance to normal hepatocytes, but they are slightly larger. Group 3 (Figure 7.1C) animals showed a reduction in the sinusoidal spaces and an absence of granules in the cytoplasm. Further, dysplastic and premalignant state of hepatocytes were not observed in this group and it showed a pattern of recovery. The group 4 (Figure 7.1D) and group 6 (Figure 7.1F) animals treated with HAp-PEG-FA and TiO$_2$-PEG-FA nanoparticles showed few neoplastically transformed cells, and a mild degeneration of hepatocytes (indicated by arrows), maintaining near normal architecture. Group 5 (Figure 7.1E) and group 7 (Figure 7.1G)
animals treated with HAp-PEG-FA-PAC and TiO$_2$-PEG-FA-PAC nanoparticles showed normal liver histology with unremarkable central veins, a hepatic lobule with few scattered foci of inflammation, no evidence of necrosis or cirrhosis, and no reactive atypia. There are no pathological changes in the liver tissue of rats comparable with that of the normal control rats.

Figure 7.1 Histopathological analysis of liver A) Control B) DEN induced C) Paclitaxel treated D) HAp-PEG-FA E) HAp-PEG-FA-PAC F) TiO$_2$-PEG-FA G) TiO$_2$-PEG-FA-PAC

The biochemical findings are supported by histopathological observations of the liver. The histopathological patterns of liver injury observed in rats treated with DEN was found to be more pronounced than those of normal rats, indicating hepatocellular damage during DEN administration. In contrast, the liver sections of rats treated with HAp-PEG-FA and TiO$_2$-PEG-FA nanoparticles showed improved hepatocellular architecture with signs of recovery, indicating a protective effect of these
nanoparticles, similar to that of the paclitaxel treatment. Further, individual treatments of HAp-PEG-FA-PAC and TiO$_2$-PEG-FA-PAC were shown to be non-toxic because they no change in the biochemical parameters or in the pathological observations.

The animals in group 5 treated with surface-modified PAC attached HAp nanoparticles show higher anti-cancer activity than the animals in group 3 which are treated with PAC standard. PAC has a higher anticancer activity than surface-modified HAp nanoparticles. All the biochemical and hematological parameters shows that the anticancer activity of surface-modified PAC attached HAp nanoparticles were higher than surface-modified HAp nanoparticles and PAC standard.

The animals in group 7 treated with surface-modified PAC attached TiO$_2$ nanoparticles show higher anti-cancer activity than the animals in group 3 which are treated with PAC standard. PAC has a higher anticancer activity than surface-modified TiO$_2$ nanoparticles. All the biochemical and hematological parameters shows that the anticancer activity of surface-modified PAC attached TiO$_2$ nanoparticles were higher than surface-modified TiO$_2$ nanoparticles and PAC standard.

The increase in the anticancer activity of the group 5 and group 7 surface-modified PAC attached HAp and TiO$_2$ nanoparticles over the group 3 PAC standard is due to the targeting of PAC to the liver cancer cells. The surface modification with FA targets PAC to FR in liver cancer cells. The dose of PAC in the targeted system is less than the dose of PAC standard. The surface modification increases the accumulation of PAC attached nanoparticles in the cancer cells. This increases the drug concentration at the cancer site and reduces the amount of the drug at non-cancerous sites, leading to increased anti-cancer activity with less toxic effect.
7.4 CONCLUSIONS

Anticancer activity of the surface-modified and surface-modified PAC attached HAp and TiO$_2$ nanoparticles has shown anticancer activity in DEN induced hepatocarcinoma. Biochemical and hematological parameters show that the anticancer activity of the surface-modified PAC attached HAp and TiO$_2$ nanoparticles are higher than the anticancer activity of pure PAC. Although surface-modified nanoparticles without PAC have anticancer activity, it is less than that of pure PAC. The higher anticancer activity of surface-modified PAC attached HAp and TiO$_2$ nanoparticles than pure PAC may be because of the precise targeting of PAC to the cancer cells only.