Effect of Diet Intake Imbalance in Hepatocellular Carcinoma Progression

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Abstract

Incidence of hepatocellular carcinoma (HCC) has increased sharply in the last 10 years, with an especially high incidence in Egypt. This study was conducted to evaluate the impact of unbalanced diets on liver tumor through investigation of some biochemical mediators/pathways implicated in the pathogenesis of HCC. Male albino mice were divided into two major groups: Control group and Hepatocellular carcinoma (HCC) group; each group was further divided into four subgroups according to received diet: high fat (HF), low fat (LF), high carbohydrate (HC), and low carbohydrate (LC) groups. The results indicated that induction of HCC in mice showed marked body weight loss. Liver sections of HCC groups showed malignant giant cells and strong expression of p53. HCC mice groups kept on HF and LC diets showed the lowest survival rate, a significant increase in glucose-6-phosphate dehydrogenase (G6PDH), aldolase, and citrate synthase activities, a significant increase in serum E-cadherin as well as a significant decrease in insulin-like growth factor-1 (IGF-1) compared with LF diet. These results suggest that the molecular pathogenesis of HCC in mice correlates reduction of serum IGF-1 and elevated serum E-cadherin accompanied by reprogrammed metabolic profile shifted towards increased glycolysis and lipogenesis. These pathogenic changes were enhanced by over-consumption of carbohydrates, fats, and proteins, whereas dietary fat restriction could have a protective/ameliorative effect against the incidence of HCC.

Keywords: hepatocellular carcinoma, fat-diet, carbohydrate-diet, IGF-1, E-cadherin, G6PDH, citrate synthase, aldolase, p53 protein

1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and the third leading cause of cancer-related mortality after lung and stomach cancers. Recent studies revealed increased incidence of HCC in Egypt over the last decade (El-Zayadi et al., 2010). Different genes have been implicated in hepatocarcinogenesis including genes involved in growth inhibition and apoptosis (e.g. tumor suppressor gene; p53), and genes responsible for cell-cell interaction and signal transduction (Coleman, 2003).

Development of HCC is a multi-stage process. In the molecular aspect, dysregulation of pleiotropic growth factors (e.g. insulin-like growth factor type-1, transforming growth factor-beta, and epidermal growth factor), adhesive molecules (e.g. epithelial cadherin; E-cadherin), and metabolic pathways represents a central pro-tumorigenic principle in human hepatocarcinogenesis (Wu & Zhu, 2011).

Different genes have been implicated in hepatocarcinogenesis including genes involved in growth inhibition and apoptosis (e.g. tumor suppressor gene; p53), and genes responsible for cell-cell interaction and signal transduction. Mutations in the p53 tumor suppressor gene are among the most common alterations which play an important role in either initiation or progression of HCC (Sharpless & DePinho, 2002).

Nutrition is thought to have a central role in the development of cancer, it was found that diet low in fruits and vegetables and high in red meat was positively associated with risk for malignancies, including prostate, colon, and breast cancer (Ferguson, 2010). The great interest during last decade in diet and human cancer derives from the large variations in rates of specific cancers among countries; such observations indicate the importance of potentially modifiable factors in the cause and prevention of cancer. Evidence from longer-term randomized...
trials indicates that excessive caloric intake from fat and carbohydrates similarly lead to weight gain leading to obesity, insulin resistance, diabetes, and hepatic steatosis which are the most important known risk factors for HCC (Key, 2011). HCC is thought to develop through a continuous transition of liver pathologies, which begin with steatosis and proceed through hepatitis, fibrosis and cirrhosis, and end with benign liver tumors and HCC (Hill-Baskin et al., 2009).

Dietary nutrients may also cause hepatic injury through pathways that do not involve the development or progression of hepatic steatosis. Carbohydrates, proteins, and lipids are all extensively metabolized in the liver and it is conceivable that they may influence the progression of chronic liver disease, either positively or negatively. In hepatitis B virus transgenic mice, a diet low in animal protein was associated with decreased liver injury and decreased incidence of HCC (Ioannou, Morrow, Connole, & Lee, 2009). In the presence of oxidative stress, dietary cholesterol may be oxidized in the liver to oxysterols, which can induce cell damage and malignant transformation (Ioannou, Morrow, Connole, & Lee, 2009).

The present study was conducted to investigate the impact of unbalanced diets on liver tumor through investigation of some biochemical mediators/pathways implicated in pathogenesis of HCC in mice.

2. Materials and Methods

2.1 Animals and Diets

Twenty hundred and eighty male albino mice were utilized in this study, 15-30g each. Mice were purchased from the animal house of Giza Institute of Ophthalmology, Cairo, Egypt. Mice were weighed and housed in wire cages for two weeks under identical environmental conditions for adaptation, and allowed free access to balanced laboratory diet and water ad libitum (Gebhardt & Thomas, 2002). Animals were handled according to ethics and guidelines of animal approval committee. After acclimatization period, mice were weighed and randomly divided into two major groups: Group 1: control group (80 mice) and Group 2: hepatocellular carcinoma (HCC) group (200 mice). Each group was further divided according to the received diet into 4 equal subgroups: high fat (HF), low fat (LF), high carbohydrate (HC), and low carbohydrate (LC) groups. Mice of both groups (1 and 2) were maintained on the assigned diet for 10 weeks whereas HCC induction was started after the 2nd week in mice of group 2.

The composition of different diets was illustrated in Tables 1. The caloric equivalent of each diet was calculated on the basis that upon oxidation of metabolic fuels, 1.0 g of carbohydrate yields 4 calories, 1.0 g of fat yields 9 calories and 1.0 g of protein yields 4 calories (Gebhardt & Thomas, 2002). The diets were prepared weekly; the ingredients were mixed and formed into dough with water, rolled into pellets and stored at 4 ºC to minimize oxidation and rancidity.

2.2 Induction of HCC

For induction of hepatocellular carcinoma 200 mg/kg of diethylnitrosamine (DEN) (Sigma-Aldrich Inc. USA) was injected i.p. as a single dose (Kushida, Kamendulis, Peat, & Klaunig, 2011). After 14 days the mice were subjected to i.p. injection of thioacetamide (TAA) (Sigma-Aldrich Inc. USA) 100 mg/kg twice per week for four weeks (Novosyadlyy, Dargel, & Scharf, 2005). Then the mice were left for further two weeks without any treatment.

2.3 Sample Collection

At the end of experiment (10 weeks), mice were weighed, anaesthetized by ether, and blood was collected by cardiac puncture. The survival rate within each group was calculated as number of live animals after 10 weeks/number of animals at the start of experiment × 100 (Cosetti, Yu, & Schantz, 2008). Blood samples were centrifuged for 12 min at 3000 rpm, 4 ºC with cooling centrifuge (Sigma 3K15, Germany). The serum was divided into four portions; one portion was used for immediate determination of enzyme activity of glucose-6-phosphate dehydrogenase (G6PDH), and other portions were stored at -20 ºC until used for biochemical analysis of aldolase activity, IGF-1 and E-cadherin.

Liver was dissected, washed twice with ice cold saline, dried on clean paper towels, and weighed. Relative liver weight was calculated as liver weight (g)/final body weight (g) × 100. Liver was minced quickly and divided into two portions. One portion of liver was kept in 10% formalin for histopathological examination and immunohistochemical staining of p53. The second portion was kept frozen in liquid nitrogen at -80 ºC till determination of citrate synthase activity.

2.4 Serum Analysis

Insulin-like growth factor type-1 and E-cadherin were determined by mouse IGF-1 and mouse E-cadherin
ELISA kits, respectively, according to manufacturer instructions. The ELISA kits were purchased from Boster Biological Technology, Ltd. (China). The enzyme activities of G6PDH (Tian, Pignatare, & Stanton, 1994) and aldolase (Pinto, Kaplan, & Van Dreal, 1969) were determined by measurement of the rate of absorbance change at 340 nm using kits obtained from Randox Laboratories Ltd Company (England).

2.5 Determination of Liver Citrate Synthase Activity

Liver extract was prepared according to Morgunov and Srere (1998). To 50 mg liver, Cellytic MT reagent (Sigma-Aldrich Inc. USA) was added in the ratio of 1:20 w/v and protease inhibitor cocktail (Sigma-Aldrich Inc. USA) was added in the ratio of 20:1 w/v. The mixture was homogenized under cooling, centrifuged at 15,000 × g for 10 min at 4 °C, and the protein containing supernatant was separated and used for determination of citrate synthase activity. The reaction mixture contains 100 μL 1.01 mM dithionitro benzoic acid, 25 μL 10% Triton X-100, 50 μL 10 mM oxalacetate, 25 μL 12.2 mM acetyl CoA, and 790 μL redistilled water. 20 μL of supernatant was added, mixed carefully and incubated for 10 min at 30 °C. The yellow product 5-thio-2-nitrobenzoic acid was measured spectrophotometrically at 412 nm (Trounce, Kim, Jun, & Wallace, 1996). The protein content was determined according to Fleury and Eberhard (1951) using kits obtained from Biodiagnostics Co. Ltd. (Egypt) and citrate synthase activity was expressed as μmol/min/mg protein.

2.6 Histopathology

Liver sections were prepared (3-5 μm thick) and stained with hematoxylin and eosin (H&E). The sections were investigated under light microscope (Leica, Switzerland) using image analysis system under magnification × 400. Liver sections were investigated by a pathologist. Hepatocytes were seen with blue nuclei and pink to red cytoplasm.

2.7 Immunohistochemical Detection of P53 Protein

p53 Protein was detected by immunostaining of liver sections prepared from formalin-fixed, paraffin-embedded liver, using an Invitrogen kit (Histostain™-SP Kit). The kit utilizes the labeled streptavidin-biotin (LAB-SA) staining methodology. The slides were investigated with light microscopy (Leica, Switzerland) by a pathologist for number of positive cells and color intensity (Jadali et al., 2011). Strongly p53-stained cells (++++) are those showing nuclei/cytoplasm with dark brown color and highest number of apoptotic bodies or figures. Moderately-p53 stained cells (++) are those showing intermediate golden brown color and modest number of apoptotic bodies or figures. Weakly-p53 stained cells (+) are those showing light brown color and least number of apoptotic bodies or figures.

2.8 Statistical Analysis

Analysis of data was performed with Statistical Package for Social Science (SPSS) version 17. Data are presented as mean ± SEM. Comparison between the studied groups was performed with one-way ANOVA (F-testing). Non-parametric correlation between variables was evaluated using Spearman's correlation coefficient. P < 0.05 was considered statistically significant.
Table 1. Composition of different diets (Judge et al., 2008; Srinivasan et al., 2008)

<table>
<thead>
<tr>
<th>Diet composition (g)</th>
<th>Balanced Diet (BD)</th>
<th>High Fat Diet (HF)</th>
<th>Low Fat Diet (LF)</th>
<th>High Carbohydrate Diet (HC)</th>
<th>Low Carbohydrate Diet (LC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yellow corn</td>
<td>540</td>
<td>540</td>
<td>540</td>
<td>700</td>
<td>170</td>
</tr>
<tr>
<td>Casein</td>
<td>370</td>
<td>270</td>
<td>400</td>
<td>190</td>
<td>750</td>
</tr>
<tr>
<td>Corn oil</td>
<td>60</td>
<td>160</td>
<td>30</td>
<td>80</td>
<td>50</td>
</tr>
<tr>
<td>Cellulose</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Dicalcium phosphate</td>
<td>16</td>
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<tr>
<td>Calcium carbonate</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>*Vitamins &amp; trace minerals</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
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<tr>
<td>Sodium bicarbonate</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Antibiotic &amp; antifungal</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>L-lysine HCl</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>DL-methionine</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Total weight (g)</td>
<td>1000</td>
<td>1000</td>
<td>1000</td>
<td>1000</td>
<td>1000</td>
</tr>
</tbody>
</table>

Caloric equivalent (kcal/g diet) | 4.18 | 4.7 | 4.03 | 4.28 | 4.13 |

% of total energy

<table>
<thead>
<tr>
<th>Carbohydrates</th>
<th>54</th>
<th>54</th>
<th>54</th>
<th>70</th>
<th>17</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fat</td>
<td>6</td>
<td>16</td>
<td>3</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>Protein</td>
<td>37.2</td>
<td>27.2</td>
<td>40.2</td>
<td>19.2</td>
<td>75.2</td>
</tr>
</tbody>
</table>

*Vitamins and trace minerals were obtained as Premix®, a product of Pharmamix Company, Cairo, Egypt. Other components were obtained from El-Gibali Company, Tanta, Egypt.

3. Results

3.1 Results of Survival Rate, Body Weight, and Relative Liver Weight

Figure 1 demonstrates that the survival rate decreased markedly in HCC mice compared to their corresponding control groups. The survival rate in both control and HCC groups showed nearly the same descending order as follows: LF-group > HC-group > LC-group ≥ HF-group.

As illustrated in Figure 2 LF and LC diets produced a marked body weight loss in control groups in contrast to HF and HC diets which produced weight gain. The relative liver weight in HF-HCC group was significantly higher than that obtained in each of HF-control, LF-HCC, and HC-HCC groups (Figure 3).

3.2 Effects of Different Diets and HCC on IGF-1, E-Cadherin

The induction of hepatocellular carcinoma in mice groups kept on different diets showed a significant decrease in serum IGF-1 and a significant increase in serum E-cadherin compared with corresponding control groups (Table 2). Within the HCC groups, LF and HC diets produced a significant increase in IGF-1 and significant decrease in E-cadherin versus each of HF and LC diets.

3.3 Effects of Different Diets and HCC on Some Metabolic Enzymes

The enzyme activities of G6PDH, aldolase, and citrate synthase were enhanced in HCC groups versus their corresponding controls (Table 3). HF-HCC showed a significant increase in G6PDH activity compared to each
of LF-HCC and HC-HCC groups. LF-HCC showed a significant decrease in aldolase activity as well as citrate synthase activity compared to each of HF-HCC and LC-HCC groups (Table 3). Within control groups; HF-control group showed a significant decrease in G6PDH, aldolase, and citrate synthase activities compared to LF-control group. LC-control group showed a significant decrease in G6PDH and aldolase activities and a significant increase in citrate synthase activity compared to HC-control group (Table 3).

3.4 Correlation Study

Table 4 demonstrated that p53 expression showed a significant negative correlation with IGF-1 and a significant positive correlation with each of E-cadherin, G6PDH activity, aldolase activity, and citrate synthase activity.

3.5 Histopathological and Immunohistochemical Results

Histopathological investigations revealed that liver sections from HF-control group showed some inflammation, steatosis, and slight pleomorphism (different nuclear sizes) Figure 4a. On the other hand, LF-control group (Figure 4b) showed only slight inflammation. Liver sections of HC-control group (Figure 4c) showed cytoplasmic clearance (due to increased glycogen content) and those of LC-control group (Figure 4d) showed inflammation and congestion. HF-HCC mice group showed hepatic steatosis and increased number of malignant cells (Figure 4e). Malignant cells are giant and considerably larger than their neighbors, characterized by extremely hyperchromatic (darkly stained) and large nuclei. The nuclear-cytoplasmatic ratio is increased. The chromatin is coarse and clumped (Figure 4e). HF-HCC group also showed inflammation around the central vein (Figure 4f). Liver sections from LF-HCC mice (Figure 4f) and LC-HCC mice (Figure 4g) showed malignant cells. HC-HCC mice group showed malignant cells in the liver but with increased clearance due to increased glycogen contents (Figure 4h).

Liver sections from control groups showed either weak expression (+) of p53 (LF-control, Figure 5a) or moderate expression (+++) of p53 (HF-control, HC-control, and LC-control, Figures 5b-5d). On the other hand Immunohistochemical staining of liver sections from all HCC mice groups showed strong expression (+++) of p53 (Figures 5e-5h).

Table 2. Effect of different diets and hepatocellular carcinoma on serum levels of IGF-1 and E-cadherin in mice groups

<table>
<thead>
<tr>
<th>Group</th>
<th>HF (ng/mL)</th>
<th>LF (ng/mL)</th>
<th>HC (ng/mL)</th>
<th>LC (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>Control Group 6</td>
<td>36.39 ± 0.63</td>
<td>34.35* ± 0.55</td>
<td>34.68 ± 0.42</td>
</tr>
<tr>
<td>(n)</td>
<td>HCC Group 6</td>
<td>23.98* ± 0.99</td>
<td>30.58* ± 0.36</td>
<td>29.55* ± 0.65</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Control Group 6</td>
<td>36.39 ± 0.63</td>
<td>34.35* ± 0.55</td>
<td>34.68 ± 0.42</td>
</tr>
<tr>
<td>(ng/mL)</td>
<td>HCC Group 6</td>
<td>23.98* ± 0.99</td>
<td>30.58* ± 0.36</td>
<td>29.55* ± 0.65</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>Control Group 6</td>
<td>6.10 ± 0.35</td>
<td>5.69 ± 0.30</td>
<td>5.49 ± 0.34</td>
</tr>
<tr>
<td>(ng/mL)</td>
<td>HCC Group 6</td>
<td>9.64* ± 0.11</td>
<td>7.08* ± 0.43</td>
<td>7.24* ± 0.38</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. HCC: hepatocellular carcinoma, HF: high fat diet, LF: low fat diet, HC: high carbohydrate diet, LC: low carbohydrate diet, IGF-1: insulin-like growth factor type-1. *: Significant vs corresponding control group, a: Significant vs HF-control, b: Significant vs HF-HCC, c: Significant vs LF-HCC, d: Significant vs HC-HCC.
Table 3. Effect of different diets and hepatocellular carcinoma on some metabolic enzymes

<table>
<thead>
<tr>
<th>Group</th>
<th>HF</th>
<th>LF</th>
<th>HC</th>
<th>LC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>6</td>
<td>11</td>
<td>10</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>9</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>Serum G6PDH activity (mU/mL)</td>
<td>3.31 ± 0.60</td>
<td>5.32a ± 0.66</td>
<td>7.13a ± 0.52</td>
<td>3.36bc ± 0.53</td>
</tr>
<tr>
<td>HCC Group</td>
<td>9.35* ± 0.38</td>
<td>7.28*±d ± 0.52</td>
<td>7.48d ± 0.52</td>
<td>8.24* ± 0.46</td>
</tr>
<tr>
<td>Serum Aldolase activity (U/L)</td>
<td>0.39 ± 0.07</td>
<td>0.77a ± 0.04</td>
<td>0.82a ± 0.05</td>
<td>0.36bc ± 0.05</td>
</tr>
<tr>
<td>HCC Group</td>
<td>1.35* ± 0.08</td>
<td>1.03*±d ± 0.05</td>
<td>1.19*± 0.04</td>
<td>1.24*± ± 0.06</td>
</tr>
<tr>
<td>Liver CS activity (µmol/min/mg protein)</td>
<td>0.58 ± 0.09</td>
<td>2.97a ± 0.44</td>
<td>0.86 ± 0.05</td>
<td>3.13b ± 0.22</td>
</tr>
<tr>
<td>HCC Group</td>
<td>6.62* ± 0.65</td>
<td>3.38d ± 0.31</td>
<td>3.71*±d ± 0.15</td>
<td>4.98*±df ± 0.16</td>
</tr>
</tbody>
</table>


Table 4. Correlation of p53 with other parameters in control and hepatocellular carcinoma mice groups maintained on different diets

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Correlation with p53</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-1 (ng/mL)</td>
<td>-0.752*</td>
</tr>
<tr>
<td>E-cadherin (ng/mL)</td>
<td>0.640*</td>
</tr>
<tr>
<td>G6PDH activity (mU/mL)</td>
<td>0.555*</td>
</tr>
<tr>
<td>Aldolase activity (U/L)</td>
<td>0.717*</td>
</tr>
<tr>
<td>Liver citrate synthase activity (µmol/min/mg protein)</td>
<td>0.510*</td>
</tr>
</tbody>
</table>

Score of p53 according to Jadali et al. (2011), weak expression (+):1, moderate expression (++):2, strong expression (+++):3. IGF-1: insulin-like growth factor type-I, r: Spearman's correlation coefficient, *: Significant at P < 0.01, n = 62.

Figure 1. Survival rate in the studied groups. HCC: hepatocellular carcinoma, HF: high fat diet, LF: low fat diet, HC: high carbohydrate diet, LC: low carbohydrate diet.
Figure 2. Body weight difference of control and HCC mice groups maintained on different diets. Values are mean± SEM, n=7-11. HCC: hepatocellular carcinoma, HF: high fat diet, LF: low fat diet, HC: high carbohydrate diet, LC: low carbohydrate diet. a: Significant vs HF-control, b: Significant vs HC-control

Figure 3. Relative liver weights of control and HCC mice groups maintained on different diets. Values are mean± SEM, n=7-11. HCC: hepatocellular carcinoma, HF: high fat diet, LF: low fat diet, HC: high carbohydrate diet, LC: low carbohydrate diet. a: Significant vs HF-control, b: Significant vs HF-HCC

Figure 4a. Histopathology of liver section from HF-control group showing some inflammation (INF) close to the vein. Abnormal cells have different size and shapes (pleomorphism) and show chromatin clumping (CC). Steatosis (S) could be also seen in the sections (H&E x 400). HF: high fat diet

Figure 4b. Histopathology of liver section from LF-control group revealing normal cells with some inflammatory cells (arrow) close to peripheral zone (H&E x 400). LF: low fat diet
Figure 4c. Histopathology of liver section from HC-control group showing normal cells with cytoplasmic clearance (C) and apoptotic bodies (AB) (H&E x 400). HC: high carbohydrate diet

Figure 4d. Histopathology of liver section from LC-control group revealing normal cells with inflammation in the peripheral area (INF), and congestion in the vein in the central area (arrow) (H&E x 400). LC: low carbohydrate diet

Figure 4e1. Histopathology of liver section from HF-HCC group revealing malignant giant cells (MC) with increased nuclear-cytoplasmic ratio. Chromatin clumping (CC), cytoplasmic dissociation (CD), steatosis (S) and a huge number of apoptotic bodies (AB) are also seen (H&E x 400). HF: high fat diet, HCC: hepatocellular carcinoma

Figure 4e2. Histopathology of liver section from HF-HCC group revealing giant tumor cells (MC), with inflammation around the central vein (arrow) (H&E x 400). HF: high fat diet, HCC: hepatocellular carcinoma

Figure 4f. Histopathology of liver section from LF-HCC group revealing tumor cells with intra-nuclear inclusion (NI), pleomorphism and chromatin clumping (CC) (H&E x 400). LF: low fat diet, HCC: hepatocellular carcinoma

Figure 4g. Histopathology of liver section from LC-HCC group revealing malignant cells (MC) with pleomorphism and hyperchromatic cells (arrow). A large number of apoptotic bodies (AB) and increased areas of necrosis (N) could be seen (H&E x 400). LC: low carbohydrate diet, HCC: hepatocellular carcinoma
Figure 4h. Histopathology of liver section from HC-HCC group revealing tumor cells with pleomorphism, cytoplasmic clearance (arrow) and chromatin clumping (CC) (H&E x 400). HC: high carbohydrate diet, HCC: hepatocellular carcinoma.

Figure 5a. Immunostaining of p53 in liver section from LF-control group showing very weak (+) p53 expressing cells (arrow) (LAB-SA x 400). LF: low fat diet.

Figure 5b. Immunostaining of p53 in liver section from HF-control group showing moderate cytoplasmic (++) p53 expressing cells (arrow) with moderate number of apoptotic bodies (AB) (LAB-SA x 400). HF: high fat diet.

Figure 5c. Immunostaining of p53 in liver section from HC-control group showing moderate cytoplasmic expression (++) of p53 (arrow) with areas of clearance (C) (LAB-SA x 400). HC: high carbohydrate diet.

Figure 5d. Immunostaining of p53 in liver section from LC-control group showing moderate cytoplasmic expression (++) of p53 (arrow) with moderate number of apoptotic bodies (LAB-SA x 400). LC: low carbohydrate diet.
4. Discussion

Diet and cancer are associated; it has been estimated that 35 percent of cancer deaths may be related to dietary factors. Almost all cancers (80% - 90%) are caused by environmental factors, and of these, 30 - 40% of cancers are directly linked to the diet (Ferguson, 2010). Although many dietary recommendations have been proposed to reduce the risk of cancer, few have significant supporting scientific evidence (Ferguson, 2010).

Our results indicated that weight gain in HF-control group was significantly greater than in the HC-control group. The high energy density of fatty food (9 Kcal/g), compared with low energy density of carbohydrate or protein (4 Kcal/g), results in less satiating effect for the fatty food, and thus, promotes passive over-consumption of fatty food and subsequent weight gain (Swinburn, Caterson, Seidell, & James, 2004). On the other hand, dietary restriction of fat (LF-control group) and carbohydrates (LC-control group) resulted in significant body weight loss compared to HF- and HC-control groups. Our findings were in agreement with Swinburn, Caterson, Seidell, and James (2004), who reported that consuming high fat and high carbohydrate diet was directly linked to increased weight gain and obesity.

Recent studies demonstrated that consumption of high-carbohydrate diets showed an increased energy expenditure and glucose oxidation. Excess glucose is used to load glycogen stores to saturation and the rest converted to fat by de novo lipogenesis. Since glucose is preferentially oxidized, less fat will be oxidized, leaving an excess that will be deposited as body fat stores leading to increase in body weight (Manuel-y-Keenoy & Perez-Gallardo, 2012). Histopathology of HC-control group support the metabolic profile of this group through presence of cytoplasmic clearance which was due to increased glycogen stores. On the other hand
increasing proteins at the expense of carbohydrate in LC diet in the present work lead to increased lipolysis and subsequent weight reduction.

Hepatocellular carcinoma was induced in mice in the present work by DEN/TAA and was associated with loss of body weight and increase of relative liver weight, which are considered common features of HCC (Bialecki & Di Bisceglie, 2005). Indeed, early stage HCC was evidenced by the histopathological results, which indicated neoplastic manifestations in livers of all HCC mice groups and were in line with those recorded by Roncalli, Terracciano, Di Tommaso, David, and Colombo (2011).

The induction of HCC mice groups herein was associated with strong expression of mutant form of p53. Activated p53 functions as a transcription factor to regulate the expression of many different downstream genes, which products are implicated in cell cycle arrest, DNA repair, or apoptosis. The function of p53 is tightly controlled by Mouse double minute 2 homolog (Mdm2), which is an E3 ubiquitin ligase implicated in the inactivation of the tumor suppressor by accelerating its nuclear export to cytoplasm and degradation by the 26S proteasome (Michael & Oren, 2002). Therefore, the strong expression of p53 in all HCC subgroups could be explained on the basis that the mutant p53 protein expressed in HCC often do not induce Mdm2, and is thus able to accumulate at very high concentration. This leads to the accumulation of unfolded proteins, which initiates transcriptional and translational-signaling pathways known as the unfolded protein response (UPR), leading to up-regulation of the expression of p53 (Sharpless & DePinho, 2002).

Within the control groups, HF-, HC-, and LC-diets induced greater expression of p53 than LF-diet. These results could be interpreted by the finding that increased consumption of macronutrients such as fats, carbohydrates, and proteins would lead to increased production of free radicals and oxidative stress, which is considered one of the causes of mutant p53 protein formation (Sharpless & DePinho, 2002).

The IGF pathway has highly conserved function in mammals and plays a critical role in energy metabolism and cell renewal in response to nutrients. IGF pathway promotes cell proliferation, migration and transformation into malignant clone (Wu & Zhu, 2011). In the current study serum level of IGF-1 was significantly reduced in all HCC mice subgroups compared to their corresponding controls. Our results were in agreement with other reports demonstrating low IGF-1 levels in HCC patients (Ibrahim, Attia, Rabea, & El-Gayar, 2013). Reduced IGF-1 could be due to the increased oxidative damage in cirrhosis and HCC, leading to increased damage of parenchymal liver cell and decrease in IGF-1 synthesis (Ibrahim, Attia, Rabea, & El-Gayar, 2013). Another explanation was provided by Mazziotti et al. (2002), who proposed that IGF-1 was low in HCC patients because of reduced ability of growth hormone to stimulate IGF-1 synthesis due to either a reduction of growth hormone receptors number in the diseased liver or a post receptor defect. Low circulating IGF-1 levels in HCC may be also derived from an inhibitory effect by some tumor cytokines, like transforming growth factor- beta and platelet-derived growth factor (Mazziotti et al., 2002). Our data showed that HF- and LC-HCC mice groups reported the lowest levels of IGF-1, indicating that HF and LC diets could participate in the progression of HCC, which was supported by the significant negative correlation between p53 expression and IGF-1.

In the present work, HF-control group reported the highest serum level of IGF-1 compared to other control groups, moreover, liver sections of HF-and LC-control groups showed inflammation, congestion (LC-control group), steatosis, and pleomorphism (HF-control group). Such pathological changes indicate the harmful effect of high fat and low carbohydrate diets to the liver as they may precede hepatocarcinogenesis (Hill-Baskin et al., 2009). The significant increase in IGF-1 serum level in HF-control group compared with LF-control group was in agreement with Holmes, Pollak, Willett, and Hankinson (2002), who documented that the high-fat diet, especially saturated fat of animal origin, has a significant positive association with serum IGF-1 level.

Cancer progression is a multi-step process in which some adhesion molecules play a pivotal role in the development of recurrent, invasive, and distant metastasis. E-cadherin is an epithelial cell adhesion molecule that helps establish and maintain intercellular connections. Loss of E-cadherin function is a critical factor in the initial stages of cancer invasion, and is associated with poor prognosis in a variety of epithelial carcinomas including HCC (Conacci-Sorrell et al., 2003).

Our results showed a significant increase in serum concentration of E-cadherin in all HCC subgroups compared to corresponding control groups. It could be suggested that E-cadherin dysfunction in tumor cells was partly mediated by the degradative action of proteases secreted from these cells. Soluble E-cadherin with a molecular weight of about 80kDa remarkably increased in the circulation and it can reasonably be derived from proteolytic digests of the cell-surface E-cadherin (Shen, Hirsch, Sasiela, & Wu, 2008). Our findings were in agreement with Soyama et al. (2008), who demonstrated a significant increase in E-cadherin in HCC patients. Moreover, a
significant increase in serum E-cadherin was observed in HF- and LC-HCC groups compared to LF- and HC-HCC groups, indicating that HF and LC diets could increase the progression of HCC towards malignancy more than HC diet due to loss of E-cadherin function. The significant positive correlation between p53 and E-cadherin supports our findings that the increased expression of p53 accompanied by increased serum E-cadherin level was associated with increased progression of HCC to the worse.

Cancer cells have nutritional requirements that are different from normal cells. They need to take up, generate and use nutrients differently in order to divide and grow rapidly. They accomplish this change by accumulating stable mutations in genes that are key regulators of metabolism (Brahimi-Horn, Chiche, & Pouyssegur, 2007). The metabolic profile of all HCC subgroups in the present work indicated significant increase in G6PDH activity (except HC-HCC), aldolase activity, and citrate synthase activity (except LF-HCC) compared to corresponding control groups. Our data were supported by other previous findings; Frederiks et al. (2008) demonstrated that chemically induced HCC in rats was characterized by increased G6PDH activity. Sharma, Lakshmi, Chitra, Lakshmi, and Pharm (2011) reported an increased aldolase activity in rats with induced HCC. Schlichtholz et al. (2005) reported a significant increase in citrate synthase activity in human pancreatic cancer.

Glycolysis is the most favorable pathway that promotes the invasion and metastasis of tumor cells. Aldolase activity in the present study was increased in HCC mice subgroups indicating enhanced rate of glycolysis. Cancer cells require to continuously produce ATP and cofactors (NAD+, NADPH) in order to satisfy their need for synthesis of great quantities of macromolecules and lipids to proliferate and build new cells. Therefore; these cells consume glucose in excessive manner. Enhanced activity of G6PDH in HCC groups can supply NADPH for fatty acid biosynthesis, whereas the insignificant increase in G6PDH activity in HC-HCC group compared to HC-control group could be explained on the basis that control mice fed HC diet showed enhanced lipogenesis and increased G6PDH activity.

In addition, increased G6PDH activity in HCC provides reducing power for regeneration of reduced glutathione (GSH) and other detoxification processes. Thus, increasing intracellular GSH levels and the activation of the redox-sensitive transcription factor; nuclear factor-κB (NF-κB), could play a major role in the proliferation of tumor cells, invasion, angiogenesis and metastasis (Lou & Kaplowitz, 2007).

In cancer cell, citrate synthase activity is elevated, condensing acetyl CoA and oxaloacetate (OAA); thus, citrate increases and ketone bodies decrease. Consequently, decreased ketone bodies formation will stop stimulating pyruvate carboxylase. Hence, pyruvate is processed by lactate dehydrogenase, increasing the lactate released by cancer cell, and NAD+ required for glycolysis (Icard, Poulain, & Lincet, 2012).

Enhanced activity of G6PDH in HCC groups can supply NADPH for fatty acid biosynthesis, whereas the insignificant increase in G6PDH activity in HC-HCC group compared to HC-control group could be explained on the basis that control mice fed HC diet showed enhanced lipogenesis and increased G6PDH activity.

In the present study, HF- and LC-HCC groups showed increased activities of G6PDH, aldolase, and citrate synthase compared to LF- and HC-HCC groups, meanwhile, LF-HCC group showed the lowest activities of G6PDH, aldolase, and citrate synthase compared with other HCC subgroups. These findings could be attributed to the fact that HF and LC diets enhance progression of HCC to the worse through increased glycolysis and lipogenesis required by cancer cells (Brahimi-Horn, Chiche, & Pouyssegur, 2007). Our histopathological findings supported the biochemical data and indicated that excess fat intake contributed to progression of HCC in mice by inducing inflammation and steatosis. On the other hand, low fat diet may retard progression of HCC in mice by suppressing the activities of G6PDH, aldolase, and citrate synthase.

The metabolic profile of control groups differs from HCC groups. The results of HF-control group in the present work showed a significant decrease in G6PDH and citrate synthase activities compared to LF-control group which were consistent with the work of Gad, Ehssan, Ghiet, and Wahman (2010) and Gupta, Bomhoff, Swerdlow, and Geiger (2009), respectively. Excessive fat in the diet will lead to increased plasma concentrations of triglycerides and free fatty acids. The increased free fatty acids allows for increased uptake into hepatocytes, and increased triglycerides storage, leading to steatosis (Bradbury, 2006). Liver steatosis was evidenced in the present work in liver sections from HF-control group. According to Bonnard et al. (2008), the decrease in citrate synthase activity in HF fed mice could reflect initiation of mitochondrial dysfunction. It was suggested that an increase in fatty acid oxidation that is not matched by increased flux through downstream mitochondrial pathways results in an accumulation of incomplete fatty acid oxidation byproducts and reactive oxygen species (ROS), which increase damage of liver cells leading to progression of steatosis to fibrosis, cirrhosis, and finally may lead to HCC (Gupta, Bomhoff, Swerdlow, & Geiger, 2009).

Excessive dietary fat intake is known to decrease hepatic lipogenesis and glycolysis switching the energy production towards fatty acid oxidation (Dentin et al., 2005). As the provider of NADPH for fatty acid biosynthesis, G6PDH is one of the lipogenic enzymes; a high-fat diet inhibits G6PDH. The inhibition of G6PDH,
gene expression is induced by the presence of polyunsaturated fatty acid in the high-fat diet (Tao et al., 2002). Liver sections from HF-control group revealed also inflammation and increased apoptotic index. Accumulation of inflammatory cells plays a critical role in promoting obesity-related disorders, such as fatty liver disease, and is associated with release of pro-inflammatory cytokines that induce natural killer T cell apoptosis (Deng et al., 2009). Pleomorphism observed in liver sections from HF-control group is a sign of presence of pre-neoplastic cells, which indicate the possible transformation of steatosis toward malignancy (Roncalli, Terracciano, Di Tommaso, David, & Colombo, 2011).

Earlier studies showed that low-fat diet has beneficial effects on human health. Increased activity of aldolase in LF-control group evidenced in the present study could be explained on the basis that low-fat diet improves glucose tolerance through decreasing insulin resistance and increase glucose uptake by cells, leading to increased glycolysis and decreased plasma glucose levels (Hill-Baskin et al., 2009).

Our work indicated also that LC-control mice showed significant decrease in G6PDH and aldolase activities, but a significant increase in citrate synthase activity compared with HC-control group. Our results were in line with Manuel-y-Keenoy and Perez-Gallardo (2012) and Civitarese, Smith, and Ravussin (2007).

Ishii, Iizuka, Miller, and Uyeda (2004) reported that consumption of high-carbohydrate diets leads to increased mRNA expression, primarily through increased gene transcription for enzymes in the glycolytic and lipogenic pathways involved in converting glucose to fatty acids. Included among these enzymes is liver pyruvate kinase, acetyl-CoA carboxylase, and G6PDH (Salati & Amir-Ahmady, 2001). On the other hand the decreased activity of citrate synthase in HC-control group may reflect downregulation in the transcription genes of mitochondrial enzymes as a consequence of fat/carbohydrate overfeeding (Civitarese, Smith, & Ravussin, 2007).

Stepien and his colleagues (2011) revealed a decreased mRNA encoding glycolysis and lipogenesis enzymes in rats fed low-carbohydrate high protein diet. These results are in agreement with our findings of decreased G6PDH and aldolase activities and increased citrate synthase activity in LC-control group. Pogozelski, Arpaia, and Priore (2005) suggested that increased proteins at the expense of carbohydrate in diet leads to decreased glycolysis, which is compensated by an increase in fat breakdown to satisfy body needs of energy via formation of ketone bodies from fatty acids. Amino acids formed from protein catabolism together with fatty acids were utilized in gluconeogenesis. Thus decreased carbohydrates in diet have a glucose sparing effect via increased lipolysis and gluconeogenesis. It was also suggested that weight reduction resulted from consuming low-carbohydrate high protein diet was associated with elevated expression of transcription genes of mitochondrial enzymes and subsequent improvement of mitochondrial functions (Civitarese, Smith, and Ravussin, 2007).

Liver sections from LC-control group showed inflammation and high apoptotic index which may indicate chronic inflammatory liver disease caused by metabolic stress on liver due to excess protein in diet as recorded by Manninen (2004). It was also found that high protein consumption and excessive oxidation of amino acids lead to increased production of free radicals and consequently release of inflammatory cells which induce apoptosis (Gu, Shi, & Le, 2008).

5. Conclusion

Consumption of excess fat or less carbohydrates induced biochemical and pathological changes that could worsen the condition in cases of HCC. Food intake imbalance may not be by-itself a cancer causing agent, but may be a risk factor that impedes the liver cells and fosters the occurrence of HCC. Dietary restriction of fat was beneficial in improving the metabolic profile and mitochondrial function and reducing the availability of pathogenic mediators contributing to HCC.

References


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