Original article

Apoptosis associated inhibition of DEN-induced hepatocellular carcinogenesis by ellagic acid in experimental rats

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A B S T R A C T
Ellagic acid (EA), a natural polyphenol, has showed a wide variety of biological activities which make it a good candidate for the treatment of many oxidative stress-mediated diseases. The present study is aimed to evaluate its therapeutic potential by estimating the levels of lipid peroxidation and assaying activities of various marker enzymes in DEN-induced liver cancer bearing rats. The daily oral administration of EA (30 mg/kg bwt) to liver cancer bearing rats demonstrated a significant (P<0.05) decline in lipid peroxidation, key marker enzyme (AST, ALT, ALP, LDH, γ-GT and 5’NT) levels and increase in enzymic antioxidants (SOD, CAT, GPx, GR and GST) status. Hematoxylin and eosin (HE) staining and scanning electron microscope (SEM) analysis suggesting that maintenance of cell structure and integrity and also modulation of nucleic acids thereby exhibiting antitumor potential of EA in liver cancer bearing rats. Further, EA administration attenuated the agryrophilic nucleolar organizing regions (AgNORs) and increases cell death execution through the activation of Caspase-3. Thus, the modulatory effects of EA on attenuating the lipid peroxidation, AgNORs, and downregulation of key marker enzyme activities and upregulation of total protein content, enzymic antioxidants, and caspase-3 afford an assurance for treatment of liver cancer in the future.

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1. Introduction

Hepatocellular carcinoma (HCC), a highly aggressive form of solid tumor, has been increasing in South East Asia [1]. Primary hepatocellular carcinoma (HCC) is one of the most frequently occurring forms of a solid tumor. It exhibits a high prevalence with 620,000 cases per year reported worldwide of which more than 80% of cases are reported from China, Africa and South East Asia[2]. It is highly aggressive, as shown by the mortality of 595,000 cases per year that nearly matches the incidence of this tumor type [3]. HCC presents with limited therapeutic options. Hence, a thorough understanding of the biological bases of this malignancy might suggest new strategies for effective treatment.

Diethylnitrosamine (DEN), one of the most important environmental carcinogens, which is known to cause perturbations in the nuclear enzymes involved in DNA repair/replication and is normally used as a carcinogen to induce liver cancer in animal models [4]. N-nitroso compounds are known hepatocarcinogenic agents and have been implicated in the etiology of several human cancers [5]. These compounds are considered to be effective health hazards to man.

One approach to control liver cancer is chemoprevention—when disease is prevented, slowed or reversed substantially by the administration of one or more non-toxic naturally occurring or synthetic agents. In this regard, recently naturally occurring polyphenols are receiving increased attention because of their promising efficacy in several cancer models [6]. EA is one of such naturally occurring compounds present in many plant foods such as carrot, tomato, strawberry and blueberry [7]. It has been documented that EA possess anti-oxidative activities such as scavenging free radicals and chelating metal ions [8].

A marker is synthesized by the tumor and released into the circulation, but it may be produced by normal tissues in response to invasion by cancer cells [9]. A variety of substances, including enzymes, hormones, and proteins can be considered as tumor markers. Analysis 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 mechanisms of cellular damage and subsequent release of proteins and extracellular turnover [10]. Lipid peroxidation generates a complex variety of products, many of which are reactive electrophiles; some of these react with protein and DNA and as a result are toxic and mutagenic [11]. Taking the above into account, our present study was carried out to assess the efficacy of EA on DEN-induced HCC in experimental rats.

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2. Materials and method

2.1. Animals

Male wistar albino rats, weighing 150–180 g, procured from the Small Animal Breeding Centre, Agricultural University, Mannuthy, Kerala were used. Animals were acclimatized under standard laboratory conditions at 25 ± 2 °C and normal photoperiod (12 h light: dark cycle). The animals were fed with standard rat chow and water ad libitum. The food was withdrawn 18–24 h before the experiment. The care and use of laboratory animals were done according to the guidelines of the Council Directive CPCSEA, India (No: 659/02a) about Good Laboratory Practice (GLP) on animal experimentation. All animal experiments were performed in the laboratory according to the ethical guidelines suggested by the Institutional Animal Ethics Committee (IAEC).

2.2. Hepatocarcinogenesis initiation using diethylnitrosamine (DEN)

The experimental hepatocarcinogenesis was induced by using DEN (Sigma, USA). DEN is the most important environmental carcinogen among nitrosamines and primarily induces tumors of liver [12]. The presence of nitrosamines and their precursors in human environment, together with the possibility of their endogenous formation in human body from ingested secondary amines and nitrates, have led to the suggestions of their potential involvement in HCC [13]. It is now widely used as a standard experimental model for HCC [14].

2.3. Experimental design

The experimental animals were divided into four groups, each group containing six animals, analyzed for a total experimental period of 16 weeks as follows:

- group 1: normal control rats fed with standard rat chow and pure drinking water;
- group 2 (EA alone): rats were orally given EA (30 mg/kg body weight) in the form of aqueous suspension daily once a day for 16 weeks. This dose of EA is set based on the effective dosage fixation studies;
- group 3: rats were induced with DEN (0.01%) alone in drinking water for 16 weeks;
- group 4: rats were administered DEN (0.01%) in drinking water for the first 10 weeks followed by post-treatment with EA as in group 2 for the remaining 6 weeks.

At the end of 16 weeks, experimental rats (n = 6 per group) were sacrificed. The liver tissues were collected and homogenized by a Teflon® homogenizer in phosphate buffered saline, pH 7.4 and samples were stored at −80 °C for further assays.

2.4. Biochemical studies

The protein content was estimated by the method of Lowry et al. [15] using bovine serum albumin (BSA) as standard. The DNA was estimated by the method of Burton [16] and RNA was estimated by the method of Rawal et al. [17]. The macromolecular damage such as LPO was estimated by the method of Ohkawa et al. [18]. The activities of aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH) were estimated by the method of King [19–21]. Gamma-glutamyl transpeptidase (γ-GT) was estimated by the method of Rosali and Rau [22]. 5’-nucleotidase (5’NT) was estimated by the method of Luly et al. [23], and the activities of the antioxidant enzymes superoxide dismutase (SOD) Misra and Fridovich [24], catalase (CAT) Sinha [25], glutathione peroxidase (GPx) Rotruck et al. [26], glutathione transferase (GST) Habig et al. [27], and glutathione reductase (GR) Maron et al. [28] were estimated in liver.

2.5. Histopathological studies

A portion of the liver was cut into two to three pieces of approximately 6 mm³ sizes and fixed in phosphate buffered 10% formaldehyde solution. After embedding in paraffin wax, thin sections of 5 μm thickness of liver tissues were cut and stained with haematoxylin–eosin. The thin sections of liver were made into permanent slides and examined [29] under high-resolution microscope with photographic facility and photomicrographs were taken. For scanning electron microscope (SEM), the intact liver samples were prepared according to methods described previously reported [30].

2.6. Argyrophilic nucleolar organizing regions (AgNORstaining)

AgNOR staining was performed according to the method of Ploton et al. [31]. The liver sections were dewaxed in xylene and rehydrated through decreasing concentrations of ethanol to distilled deionized water. The AgNOR solution was freshly prepared by dissolving gelatin at a concentration of 2 g/dl in 1 g/dl aqueous formic acid. This solution was added to 50 g/dl aqueous silver nitrate solution (1:2, v/v). This final solution was then immediately poured on to the slides, which were left in the dark at room temperature for 45 min. The silver colloid was washed from the section with deionized water and the sections were dehydrated through a graded series of ethanol to xylene. For quantification, a mean of 10 different areas of sections were chosen to determine the homogeneous AgNOR quantification throughout all groups and at least 500 cells were counted, the AgNOR dots were easily identified as black points within the nuclei.

2.7. Immunohistochemistry

Immunohistochemical staining was carried out following the method of Ramakrishnan et al. [32]. The tissue sections were deparaffinized in two changes of xylene at 60 °C for 20 min each and hydrated through a graded series of alcohol, the slides were incubated in a citrate buffer (pH 6.0) for three cycles of 5 min each in a microwave oven for antigen retrieval. The sections were then allowed to cool to room temperature and then rinsed with 1 × Tris-buffered saline (TBS), and treated with 0.3% H₂O₂ in methanol for 10 min to block endogenous peroxidase activity. Non-specific binding was blocked with 3% BSA at room temperature for 1 h. The sections were then incubated with caspase-3 (Santa Cruz Biotechnology, USA) rabbit polyclonal antibody at a dilution of 1:500 at 4 °C overnight. The slides were washed with TBS and then incubated with anti-rabbit HRP-labeled secondary antibody (Genei, Bangalore, India) at a dilution 1:5000 for 1 h in room temperature. The peroxidase activity was visualized by treating the slides with 3,3’-diamino benzidine tetrahydrochloride (SRL, Mumbai, India), the slides were counterstained with Meyer’s hematoxylin. Negative controls were incubated with TBS instead of primary antibodies. Quantitative analysis was made in a blinded manner under a light microscope. Each section was examined at high magnification (100 ×).

3. Results

Abnormalities in DNA content are associated with malignancy. It is an indicator of proliferating activity in tumor conditions. Hence, it is important to determine DNA content in cancer state. The effect
Table 1
Effect of ellagic acid (EA) on the levels of nucleic acid in liver of control and experimental animals.

<table>
<thead>
<tr>
<th>Parameters (mg/g wet tissue)</th>
<th>Control</th>
<th>EA alone</th>
<th>DEN</th>
<th>DEN + EA</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>5.78 ± 0.20</td>
<td>5.62 ± 0.20</td>
<td>9.83 ± 0.92&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.76 ± 0.32&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>RNA</td>
<td>3.36 ± 0.13</td>
<td>3.48 ± 0.14</td>
<td>6.12 ± 0.66&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.31 ± 0.28&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD (n = 6). Statistical significance P<0.05.
<sup>a</sup> Comparisons are made with group 1 (control).
<sup>b</sup> Comparisons are made with group 3 (DEN-induced).

Table 2
Effects of ellagic acid (EA) on serum enzymic antioxidant status in the liver of control and experimental group of rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>SOD</th>
<th>CAT</th>
<th>GPx</th>
<th>GR</th>
<th>GST</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.76 ± 0.14</td>
<td>6.78 ± 0.52</td>
<td>14.12 ± 1.08</td>
<td>11.32 ± 0.33</td>
<td>4.07 ± 0.17</td>
</tr>
<tr>
<td>EA alone</td>
<td>2.72 ± 0.13</td>
<td>6.70 ± 0.50</td>
<td>14.06 ± 0.99</td>
<td>11.28 ± 0.29</td>
<td>4.12 ± 0.16</td>
</tr>
<tr>
<td>NDEA</td>
<td>0.31 ± 0.37&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.08 ± 0.85&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.31 ± 0.72&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.39 ± 0.78&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.96 ± 0.43&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>EA + NDEA</td>
<td>2.68 ± 0.15&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.32 ± 0.47&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13.78 ± 1.12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.76 ± 0.37&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.89 ± 0.37&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± S.D. (n = 6). Statistical significance P<0.05. Activity is expressed as μmol of GSH Oxidized per min per mg of protein for GPx; units per min per mg of protein for GST; 50% inhibition of epinephrine auto oxidation for SOD; μmole Of hydrogen peroxide decomposed per min per mg of protein for CAT and μmole of NADPH oxidized/(min mg protein) for GR.
<sup>a</sup> Comparisons are made with group 1 (control).
<sup>b</sup> Comparisons are made with group 3 (NDEA-induced).

Table 3
Effects of ellagic acid (EA) on the activities of marker enzymes in the liver of control and experimental group of rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>AST</th>
<th>ALT</th>
<th>ALP</th>
<th>LDH</th>
<th>γ GT</th>
<th>5′ NT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.76 ± 0.12</td>
<td>12.83 ± 0.46</td>
<td>26.03 ± 0.86</td>
<td>2.01 ± 0.16</td>
<td>4.92 ± 0.15</td>
<td>2.78 ± 0.12</td>
</tr>
<tr>
<td>EA alone</td>
<td>2.72 ± 0.13</td>
<td>12.89 ± 0.47</td>
<td>26.07 ± 0.84</td>
<td>2.03 ± 0.15</td>
<td>4.90 ± 0.12</td>
<td>2.76 ± 0.13</td>
</tr>
<tr>
<td>DEN</td>
<td>7.83 ± 0.34&lt;sup&gt;a&lt;/sup&gt;</td>
<td>40.36 ± 0.79&lt;sup&gt;a&lt;/sup&gt;</td>
<td>41.36 ± 1.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.72 ± 0.45&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.36 ± 0.72&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.73 ± 0.52&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>EA + DEN</td>
<td>3.06 ± 0.18&lt;sup&gt;b&lt;/sup&gt;</td>
<td>16.16 ± 0.56&lt;sup&gt;b&lt;/sup&gt;</td>
<td>27.12 ± 0.79&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.97 ± 0.19&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.12 ± 0.19&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.82 ± 0.12&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD (n = 6). Statistical significance P<0.05. Activity is expressed as IU/L for AST, ALT, ALP, LDH, γGT and 5′NT.
<sup>a</sup> Comparisons are made with group 1 (control).
<sup>b</sup> Comparisons are made with group 3 (DEN-induced).

of EA on the levels of nucleic acids (DNA and RNA) in control and experimental group of rats are shown in Table 1. In DEN-induced group 3 animals, the levels of nucleic acids were found to be significantly (P<0.05) increased when compared to control group of rats. Conversely, these elevated levels were significantly (P<0.05) decreased in EA treated group 4 animals when compared to group 3 animals.

Proteins play a significant role in the maintenance of cell structure and integrity. Abnormalities in protein content results in severe deformities in cancer conditions. The total protein content in liver of control and experimental animals are shown in Fig. 1. There was a significant decrease in total protein levels were observed in group 3 cancer bearing animals when compared to group 1 control group. On the other hand, the total protein content significantly increased (P<0.05) in liver of EA treated group 4 animals when compared to cancer bearing group 2 animals.

LPO is an important consequence of oxidative stress. The effect of EA on LPO in liver of control and experimental rats are given in Fig. 2. The levels of LPO were increased significantly (P<0.05) in DEN induced group 3 animals when compared to control group of animals. Whereas, they appeared to be neutralized to near normal in animals treated with EA.

Effect of EA on the enzymic antioxidant levels of control and experimental group of animals are shown in Table 2. Antioxidant levels decreased significantly (P<0.05) in DEN-induced group 3 animals, whereas they remained near normal level after EA administration. Thus, EA restored the changes by its antioxidant efficacy.

Effect of EA on the activities of pathophysiological key marker enzymes in the liver of control and experimental group of rats are given in Table 3. A significant (P<0.05) increase in the activities of these enzymes were noted in DEN-induced group 3 rats when compared with control group of rats. On the other hand, EA administration showed significant (P<0.05) reduction in the activities of these enzymes when compared with cancer bearing group 3 rats.

Histological examination of liver sections from control (Fig. 3A) and EA alone (Fig. 3B) animals revealed normal architecture and cells with granulated cytoplasm and uniform nuclei. DEN-induced (Fig. 3C) animals showed loss of architecture with granular cytoplasm and larger hypochromatic nuclei, whereas group 4 (Fig. 3D) animals treated with EA maintained near normal architecture with fewer neoplastically-transformed cells.

SEM of liver of control and EA alone (Fig. 4A and B) maintained a similar kind of architecture. Hepatocyte damage and membrane deformation were found in DEN-induced group 3 animals (Fig. 4C).
Conversely, ultimate change such as hepatocyte regeneration was noted in EA administered group 4 (Fig. 4D) animals. Fig. 5 shows the levels of AgNORs in the liver of control and experimental group of animals. Tumor-induced group 3 animals showed a significant increase ($P<0.05$) in the numbers of AgNOR nuclei when compared to control group of animals. EA treated group 4 animals showed significant decrease in the numbers of AgNOR nuclei when compared to cancer bearing animals of group 3.

Fig. 6 shows the effect of EA on the activation of caspase-3 in the liver of control and experimental group of animals by immuno histochemical staining. DEN-induced group 3 animals showed downregulation in the expression of caspase-3 when compared to control group of animals. However, EA treated group 4 animals showed upregulation in the expression of caspase-3 when compared to the tumor bearing animals of group 3.

4. Discussion

Nucleic acids play an important role during neoplastic transformation and the determination of DNA content was more meaningful with regard to biological and functional aspects of the tumor, because it is an index of proliferative activity in tumor conditions. Additionally DNA content is found to be an independent indicator of prognosis, since the size of the tumor often correlates well with the DNA content of tumor [34]. In the present study, increased level of DNA synthesis in cancer bearing animals may be due to the increased expression of the enzymes necessary for differentiated cell function. Increased RNA level in cancer bearing animals may be due to the increased DNA content, this lead
to an increased transcription and thereby elevated RNA content in cancerous condition. Contrarily, EA administration controlled the nucleic acid biosynthesis and exerts anticancer effect.

The chemopreventive nature of EA against DEN-induced hepatocellular carcinogenesis was investigated in the current study. Proteins and its synthesis is an important phenomenon in normal as well as in cancer conditions. The highest rate of synthesis of tissue proteins and major protein mass of the organism is severely affected in cancer [33]. In the present study, a decrease in protein content was observed in cancer bearing animals; this may be due to the use of host protein for tumor growth. Conversely, EA administration increased the protein content which indicates that EA is involved in the maintenance of macromolecular structure.

Lipid peroxidation is one of the major mechanisms of cellular injury caused by free radicals [35]. Administration of DEN has been reported to generate lipid peroxidation products like MDA that may interact with various molecules leading to oxidative stress and carcinogenesis [36]. The level of LPO increases with the administration of DEN during hepatocarcinogenesis. This dynamic action may further lead to uncompromised production of free radicals overwhelming the cellular antioxidant defense [37]. From the present study, it is evident that increased level of LPO was found in cancer bearing animals. However, the administration of EA decreased the LPO level which may be due to the free radical scavenging activity of EA.

Antioxidant enzymes are a major strategy for protecting cells against a variety of endogenous and exogenous toxic compounds such as reactive oxygen species (ROS) and chemical carcinogens [41]. SOD, CAT and GPx are involved in the direct elimination of ROS [42]. SOD plays an important role in scavenging superoxide anion free radical and CAT catalyses conversion of hydrogen peroxide, powerful and potentially harmful oxidizing agent, to water and oxygen [43]. SOD acts as the first line of defense against superoxide radicals. The reduction in activity of these enzymes may be caused by the increase in radical production during NDEA metabolism [44]. In the present study, decreased activities of antioxidant enzymes were observed in cancer bearing animals; this may be due to the increased production of ROS by DEN metabolism. On the other hand, EA administration increased the activities of these enzymes; this may be due to the free radical scavenging potential of EA.

Liver damage caused by DEN generally reflects instability of liver cell metabolism which leads to distinctive changes in the serum enzyme activities. Serum transaminases, ALP, LDH and γ-GT are representative of liver function; their increased levels are indicators of liver damage. The elevation of ALT activity is repeatedly credited to hepatocellular damage and is usually accompanied by a rise in AST [38]. 5′NT is present at the bile canalicular and sinusoidal surface of plasma membrane of hepatocytes [39] and it is used as a diagnostic tool for liver injury [40]. In the present study, the elevation of these marker enzymes in group 3 animals may be correlated with the malignancy. Post-treatment with EA attenuated the elevated levels of these enzymes. Histopathological and SEM analysis confirm the membrane regeneration and it is suggested that EA involves in paraenchymal cell regeneration in liver.

**Fig. 4.** Representative photomicrograph of rat liver section under the scanning electron microscope exhibited morphological feature of (A) control, (B) EA alone, (C) DEN-induced→arrow indicates that morphological alteration in liver, (D) EA + DEN (post-treatment).
Thus, protecting membrane integrity and thereby decreasing the enzyme leakage.

Cell proliferation is thought to play an important role in several steps of the carcinogenic process. AgNORs are a set of nucleolar proteins that are necessary for ribosomal biogenesis. They can be demonstrated in formalin-fixed paraffin-embedded tissues by one step silverstaining, resulting black dots being termed as AgNORs. The amount of AgNOR proteins can be used as a marker of cell proliferation [45]. In the present study, EA administration significantly reduced the amount of AgNORs in cancer bearing group when compared to control group, this confirms the antiproliferative potential of EA.

The caspase family plays an important role in the regulation of apoptosis. Activation of caspase-3 requires the activation of initiator caspases, such as caspase-8 or caspase-9, in response to pro-apoptotic signals [46]. In the present study, EA
administration upregulated the expression of caspase-3; this may be due to the mitochondrial permeability and activation of procaspases and thereby execute cell death.

5. Conclusion

The results of the present study demonstrate that EA attenuates LPO, normalizes pathophysiological marker enzymes and nucleic acid levels, increases antioxidant status and protein content. Further, EA inhibits cell proliferation and induce apoptosis. Thus, the results of the present investigation have confirmed the efficacy of EA as an effective chemotherapeutic agent.

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

References


