Study of Potential Role of Estrogen Receptors and Cyclooxygenase-2 in the Pathogenesis of Induced Hepatocellular Carcinoma in Female Rats

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Abstract

Objective: Increased incidence of liver cancer is a challenge, prompting attempts to understand the pathogenesis of this disease in order at least to stop its progression. In early studies, the influence of estrogens has been linked for the increased risk of hepatocellular carcinoma as a sequel of oral contraceptive application. Therefore, the aim of the present work is to test the possible effects of tamoxifen (estrogen receptor modulator) in the pathogenesis of induced HCC in female rats.

Material and Methods: Twenty four female rats were involved in the current study weighting 170-240 grams. These rats were sectioned into the subsequent groups: Group 1: (Control group) which represented the placebo group, Group II: (HCC group) represented the untreated induced hepatocellular carcinoma (HCC) group, Group III: (HCC + tamoxifen) represented the induced HCC group supplemented with tamoxifen (estrogen receptor modulator). Among parameters evaluated in the current study were the serum levels of a-fetoprotein, estrogen. Also hepatic tissues were screened for assessment of gene expression of estrogen receptor and COX-2.

Results: Supplementation with tamoxifen had been associated with significant decrease in COX-2 gene expression in contrast to estrogen receptor gene expression which was significantly increased. This is consequently was reflected in the attenuation of histopathological state as well as biochemical analysis as regards decreasing serum level of AFP (tumor marker).

Conclusion: Tamoxifen may be beneficial in the early stages of the disease but its prolonged administration has to be focused in the further studies.

Key Words: Hepatocellular carcinoma - Estrogen – COX-2.

Introduction

HEPATOCELLULAR carcinoma (HCC) is one of the most common cancers. Mortality rate from this disease have been increased in recent years [1].

Many epidemiologic aspects make a distinction of hepatocellular carcinoma including diversity within racial groups, geographic areas, and the existence of variant potentially-preventable-hazard factors [2].

Investigating molecular pathways is crucial for apprehension of hepato-carcinogenesis [3]. In early studies, the effect of estrogens has been linked for the increased incidence of hepatic cancer as a sequel of oral contraceptive administration [4]. Recent advances however, in molecular research revealed that sex hormones play a significant role in the physiology of various organs in addition to the reproductive organs [5].

It has been observed that estrogens play a crucial role in both sexes including biological influences in the central nervous systems cardiovascular and immune systems [6].

Physiological actions of estrogens are accomplished via two nuclear receptor, ER-α and ER-β, that convey signals into output of variety of specific proteins that have multiple influences at different organs [7].

Environmental contaminants and certain plant ingredient also have the ability to bind to estrogen
receptors (ERs) such as polycyclic aromatic hydrocarbons-pesticides [8,9].

These environmental contaminants are now believed to be an endocrine disorganizer that consequently led to the development of several cancers as endometrial and breast cancers [10,11].

Cyclooxygenase-2 (COX-2) is an enzyme responsible for production of prostaglandin and its subsequent molecules as thromboxanes A2. Various stimuli, as growth factors, hormones and inflammatory mediators increase expression of COX-2 and the output of its activity are suggested to be employed in carcinogenesis by enhancing cellular proliferation, invasion, inhibiting apoptosis, stimulating angiogenesis and suppressing immunity toward malignant cells [12].

Material and Methods

Animals were purchased from the Animal House, Faculty of Medicine, Cairo University during 2014. 24 female rats, 10-12 weeks old, weighing 170 to 240 grams were settled in wire-mesh cages at steady temperature and dark/light cycles. They had free inlet to food and water. The rats were then randomized into three groups:

Group I (n=8): (Control group): Represented the vehicle-treated rats.

Group II (n=8): (HCC group) represented the untreated induced HCC group.

Group III (n=8): (HCC + tamoxifen) represented the induced HCC group which was provided with the selective estrogen receptor modulator "tamoxifen" (tamoxifen tablets, AstraZeneca UK). Each tablet which is equivalent to 10 mg tamoxifen, was diluted in olive oil (2mg/ml) and then orally administered at a dose of 10mg/kg/day throughout the duration of the study (Dias et al., 2013).

Induction of hepatocellular carcinoma: This was accomplished via a single intra-peritoneal injection of 200mg/kg body weight diethylnitrosamine. This was followed by a single weekly subcutaneous injection of 3ml/kg body weight carbon–tetrachloride (CCl4) for 6 weeks [13].

Preparation of diethylnitrosamine (the initiator of carcinogenesis): Was purchased from Sigma-Aldrich Egypt, number C 1900 in a solution form and was given diluted with castor oil [13].

Preparation of carbon-tetrachloride (CCl4) (the propagator of DNA damage): Was purchased from Sigma-Aldrich Egypt, number C 1900.

Biochemical measurements: Blood samples were centrifugated and serum levels of alphafetoprotein, and estrogen were assayed by ELISA. The animals were then victimized by cervical dislocation; their livers were dissected and divided into two samples for histopathological assessment using hematoxylin and Eosin (H&E) stain, Masson's trichrome stain, Periodic acid Schiff stain. The other sample for biochemical screening of liver tissues for evauation of gene expression of estrogen receptor and COX-2 (qPCR) [14].

Statistical methods: The data was analyzed using statistical package SPSS version 16. Data were evaluated by using (ANOVA). Differences of p-values <0.05 were considered significant [15].

Results

Upon induction of HCC, it has been shown that serum levels of AFP were significantly elevated in group II (induced HCC group) compared to group I (control group) as observed in Table (1) and Fig. (1). Otherwise, serum levels of estrogen showed significant decrease in group II (induced HCC group) compared to group I (control group) as observed in Table (1) and Fig. (1). The present study also reported a significant decrease in ER gene expression in group II (induced HCC group) compared to group I (control group) as observed in Table (1) and Fig. (1). However, COX-2 gene expression showed significant increase in group II (induced HCC group) compared to group I (control group) as observed in Table (2) and Fig. (2).

As observed in Table (3) and Fig. (3), serum levels of AFP showed significant decrease in group III (induced HCC group supplied with tamoxifen) compared to group II (induced HCC group). In addition, the present results revealed that serum levels of estrogen showed significant increase in group III (induced HCC group supplied with tamoxifen) compared to group II (induced HCC group). Moreover, as seen in Table (4) and Fig. (4), ER gene expression were significantly elevated in group III (induced HCC group supplied with tamoxifen) compared to group II (induced HCC group). Interestingly, COX-2 gene expression showed significant decrease in group III (induced HCC group supplied with tamoxifen) compared to group II (induced HCC group) Table (4) and Fig. (4).
Table (1): Comparison of serum levels of AFP, estrogen among group I (control group) and group II (induced HCC group).

<table>
<thead>
<tr>
<th></th>
<th>Control group</th>
<th>HCC group</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFP (pg/ml)</td>
<td>0.38±0.11</td>
<td>1.23±0.43</td>
<td>0.001</td>
</tr>
<tr>
<td>Estrogen (pg/ml)</td>
<td>18.80±3.18</td>
<td>10.23±1.94</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Values are represented as mean ± SD.

*: Statistically significant compared to corresponding value in control group (p<0.05).

Fig. (1): Comparison of serum levels of AFP, estrogen among group I (control group) and group II (induced HCC group).

Table (2): Comparison of ER and COX-2 gene expression among group I (control group) and group II (induced HCC group).

<table>
<thead>
<tr>
<th></th>
<th>Control group</th>
<th>HCC group</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER</td>
<td>4.40±0.96</td>
<td>1.73±0.49</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>COX-2</td>
<td>0.02±0.01</td>
<td>0.18±0.05</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Values are represented as mean ± SD.

*: Statistically significant compared to corresponding value in control group (p<0.05).

Fig. (2): Comparison of ER and COX-2 gene expression among group I (control group) and group II (induced HCC group).

Table (3): Comparison of serum levels of AFP, estrogen among group II (induced HCC group) and group III (induced HCC group supplied with tamoxifen).

<table>
<thead>
<tr>
<th></th>
<th>HCC group</th>
<th>HCC + tamoxifen group</th>
<th>p-value</th>
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<tbody>
<tr>
<td>AFP (pg/ml)</td>
<td>1.23±0.43</td>
<td>0.67±0.14</td>
<td>0.008</td>
</tr>
<tr>
<td>Estrogen (pg/ml)</td>
<td>10.23±1.94</td>
<td>13.09±1.72</td>
<td>&lt;0.007</td>
</tr>
</tbody>
</table>

Values are represented as mean ± SD.

*: Statistically significant compared to corresponding value in HCC group (p<0.05).

Fig. (3): Comparison of serum levels of AFP, estrogen and among group II (induced HCC group) and group III (induced HCC group supplied with tamoxifen).

Table (4): Comparison of ER, and COX-2 gene expression among group II (induced HCC group) and group III (induced HCC group supplied with tamoxifen).

<table>
<thead>
<tr>
<th></th>
<th>HCC group</th>
<th>HCC + tamoxifen group</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER</td>
<td>1.73±0.49</td>
<td>2.93±0.45</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>COX-2</td>
<td>0.18±0.05</td>
<td>0.04±0.01</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Values are represented as mean ± SD.

*: Statistically significant compared to corresponding value in HCC group (p<0.05).

Fig. (4): Comparison of ER and COX-2 gene expression among group II (induced HCC group) and group III (induced HCC group supplied with tamoxifen).
Histopathological Results

Fig. (5): Section in the hepatic tissues in group I (control group), displaying normal liver layout, normal hepatic cell and portal tract appearance (H&E, x100).

Fig. (6): Section in the hepatic tissues in group I (control group), displaying normal hepatic layout, convenient glycogen content (Periodic acid schiff, x 200).

Fig. (7): Section in the hepatic tissues in group I (control group), displaying normal hepatic layout and normal portal tract appearance (Masson’s trichrome, x 100).

Fig. (8): Section in the hepatic tissues in group II (induced HCC group), displaying distorted liver layout associated with high grade of dysplastic changes in the form of dark nuclei, minimal dark cytoplasm and increased nuclear/cytoplasmic ratio (H & E, x 100).

Fig. (9): Section in the hepatic tissues in group II (induced HCC group), displaying reduced hepatocytic glycogen content with hydropic changes (Periodic acid schiff, x 200).
Fig. (10): Section in the hepatic tissues in group II (induced HCC group), displaying fibrous bands surround cirrhotic nodule (Masson’s trichrome, x 200).

Fig. (11): Section in the hepatic tissues in group III (induced HCC group supplied with tamoxifen), displaying maintained liver layout with mild cellular atypia, focal necrosis and infiltration of inflammatory cells in the portal tract (H & E, x 100).

Fig. (12): Section in the hepatic tissues in group III (induced HCC group supplied with tamoxifen), displaying maintained hepatic layout and convenient glycogen content (Periodic acid schiff, x 200).

Fig. (13): Section in the hepatic tissues in group III (induced HCC group supplied with tamoxifen), displaying normal layout and normal portal tract appearance (Masson’s trichrome, x 200).

Discussion

Hepatocellular carcinoma was achieved in the present study through administration of a genotoxic compound followed by a promotion phase in which carbon tetrachloride (CCl4) is used. Thus, the present findings recorded a significant elevation in the estimated levels of AFP (a tumor marker of HCC) in the main experimental group of HCC. Interestingly, it has been revealed through previous studies that AFP is related to size of malignant tumor [16-18].

Interestingly, Zhang et al. [19] observed a lower mortality rate in HCC patients that had AFP levels similar to healthy subjects. However, conflicting studies showed that for patients with small sized-tumors, the false negative results with AFP levels alone may be as high as 40% [20,21].

Moreover, Li and his colleagues [22] observed a higher survival rate in HCC patients with a lower AFP levels and those patients may get a benefit from surgical interference in contrast to those patients with higher AFP levels. Thus, based on these results, the fundamental role of AFP in the mortality of HCC patients and its pathological role in progression of HCC have to be focused.

The significant elevation estimated by the current results in COX-2 gene expression in response to induction of HCC is in accordance with a previous report that documented increased cyclooxygenase-2 (COX-2) expression in human HCC [23]. This highlighted the fact that hepatocellular carcinoma originate following chronic inflammatory hepatic diseases [24].

In fact, cumulative evidence suggests that products of inflammation that result from activation of COX-2, might be involved in the pathogenesis of HCC. Cyclooxygenase-2 (COX-2) has also been suggested to be linked with colorectal tumors [25], prostatic carcinoma [26], breast, gastric cancers [27,28] and pancreatic tumors [29].

Evidence indicates that targeting COX-2 may have a beneficial value to exert anticancer influences in variant forms of tumors [30].

Cyclooxygenase-2 (COX-2) may also have a role in HCC differentiation grade as demonstrated
by Bae et al. [23]. Furthermore, Leng et al. [31] revealed that COX-2 expression has a fundamental role in the survival of HCC cells. Therefore increased expression of COX-2 conveys a growth advantage for HCC cells. That study also showed that celecoxib (COX-2 inhibitors) induces apoptosis in HCC.

In concept of the role of estradiol and their receptor in the sequence of disease, previous studies showed that their roles in HCC are opposite [32]. However, based on recent discoveries, the protective influence of estrogens in HCC has been superimposed on its oncogenic effects [33].

Interestingly, in the present study, it was observed that upon induction of HCC in female rats, the serum levels of estrogens were disrupted in the form of a decrease in the serum estrogen levels. That finding prompted us towards thinking that it is not only sex hormones that affect the disease but also the disease in itself might affect the serum levels of sex hormones.

However, conflicting results from recent studies revealed higher levels of serum estradiol in HCC patients with liver cirrhosis [34] suggesting that, this hormonal disturbance might be embraced in hepatocarcinogenesis. Tanaka et al. [35] earlier explained this conflicting finding to be associated with the severity of cirrhosis within HCC patients.

It has long been shown that in animal experiments that estradiol can restrain chemical induced hepato-carcinogenesis in animals via estrogen receptors (ERs) which, may be embraced in the attenuation of malignant mutation of preneoplastic hepatic cells as confirmed by Shimizu et al. [36]. In fact, Naugler et al. [37] explained the lower incidence of HCC in females via anti-inflammatory role of estrogens. They postulated that after DEN administration, estrogen attenuate Kupffer cells (KCs) production of of IL-6 that is involved in the pathogenesis of HCC.

Furthermore, increases serum estrogen levels during pregnancy exert a beneficial effect against development of HCC [38]. On the other hand, oophorectomy is considered as a risk factor for HCC, suggesting that female sex hormones including progesterone or estrogens may be protective against HCC [38]. It has also been revealed that prior to carcinogenic events, administration of estrogens, is suggested to protect the liver, and this explain lower incidence of HCC in females [39].

In the present study, supplementation with tamoxifen had been associated with significant decrease in COX-2 gene expression in contrast to estrogen receptor gene expression which was significantly increased that was reflected in the attenuation of histopathological state as well as biochemical analysis as regards decreasing serum level of AFP (tumor marker). This improvement was depicted consequently in the serum levels of sex hormones as regards increasing the serum levels of estrogen.

The protective effects of tamoxifen through its efficacy on estrogen receptors are in accordance with a previous clinical trial that used tamoxifen to treat selected group of HCC patients that express wild-type ER and revealed reducing in the size of tumor [40].

Furthermore, previous work done by Gelmann [41], revealed that tamoxifen can act without expression of ER. However, a previous clinical trial observed no survival improvement in HCC patients following the administration of high-dose of tamoxifen as reported by Chow et al. [42]. A possible explanation for the negative result may be improper selection according to expression of ER. On the other hand, tamoxifen could be only effectual in case of the existence of variant estrogen receptors (vER) [43].

The anti-inflammatory effect of tamoxifen on COX-2 gene expression in the present study is in accordance with previous work done by Salman et al. [44] which revealed that tamoxifen produces anti-inflammatory influences in animal models by reducing COX-2 levels.

However, the present study revealed that administration of tamoxifen was associated with beneficial decrease in the inflammatory COX-2 gene expression during early hepatocarcinogenesis. On the other hand, previous reports revealed that long-term treatment with tamoxifen in the animal models has been revealed to be an enhancer in hepatocarcinogenesis [45,46].

The current study thus, adopts the hypothesis that tamoxifen, may be beneficial in the early stages of the disease but its prolonged administration may turn it into carcinogenic side.

The present study concluded that recognition of the importance of receptor status and the stage of HCC with regard to hormonal action will guide the design of new ongoing clinical and animal studies. In particular, this study may contribute to setting research priorities for clinical, translational and basic scientists to further explore the area of hormone therapy in HCC especially at the prema-
lignant condition in order at least to stop progression of this aggressive fatal disease. Finally, additional studies are warranted for determining the prognostic value of the COX-2 expression pattern and the chemo-preventive and therapeutic efficacy of some COX-2 inhibitors in HCC.

References


المختصر العربي

الهدف: زيادة الإصابة بسرطان الكبد يشكل تحدياً، مما يدفع للمحالات لفهم آلية حدوث هذا المرض وذلك على الأقل لوقف تقدمه. في الدراسات المبكرة، قد تم تأثير الاستروجين لزيادة خطر الإصابة بسرطان الكبد كنتيجة لاستخدام جريب لمنع الحمل. وذلك، فإن الهدف من هذا العمل هو اختيار الأثر المحتلة للتاموكسيفين (المعدل لمستقبلات الاستروجين) في آلية حدوث سرطان الخلايا الكبدية المحفز في إناث الفئران.

المواد المستخدمة والأساليب: تم تشارك أربعة وعشرين من إناث الفئران في الدراسة الحالية تتراوح أوزانهم من 170-240 غرام. تم تقسيم هذه الفئران إلى المجموعات الآتية: المجموعة الأولى: مجموعة الخلايا: مجموعة الفئران المُعالج (التفتيش بالخلايا الكبدية) تمثل مجموعة تم تحفيز سرطان الخلايا الكبدية بعد تلقلي علاج المجموعة الثانية: (سرطان الخلايا الكبدية + التاموكسيفين) تمثل مجموعة تم تحفيز سرطان الخلايا الكبدية بعد امتصاصها التاموكسيفين (المعدل لمستقبلات الاستروجين). من بين المعتدلين التي تم قياسها في المجموعات المدونة كان مستويات المصل من عامل البروتين الجنيني-دلا، الاستروجين. كما تم فحص نسبة الكبد لتقييم التعبير الجنيني لمستقبلات الاستروجين، ونسبة الأكسيدة الحلقية.

النتائج: إن مكملات التاموكسيفين قد ارتبطت مع انخفاض ملحوظ في التعبير الجنيني لإنزيمات الأكسيدة الحلقية على النقيض من التعبير الجنيني لاستروجين التي قد ازداد، والذي قد انعكس وبالتالي تخفيف الحالة الهيستوبولوحيولوجية وكذلك تحليل الكيمياء الحيوية فيما يتعلق في خفض مستوى المصل من عامل البروتين الجنيني-دلا (لادة الديم).

الخلاصة: قد يكون التاموكسيفين مفيداً في دراسة المرحلة المبكرة من المرض ولكن تناوله لفترات طويلة يحتاج إلى الانتباه على الضوء في المزيد من الدراسات.

الكلمات الرئيسية: سرطان الخلايا الكبدية، الاستروجين، إنزيمات الأكسيدة الحلقية.