INFERRING THE EFFICACY AND SAFETY OF GREEN TEA WATER EXTRACT AFTER PROLONGED CONSUMPTION AGAINST EPIRUBICIN-INDUCED HEPATOTOXICITY IN MICE: A HISTOLOGICAL STUDY.

Mona Ramadan Alshathly.
Biology Department, King Abdulaziz University, Jeddah, Saudi Arabia.

Manuscript Info

Manuscript History:
Received: 15 May 2016
Final Accepted: 22 June 2016
Published Online: July 2016

Key words:
Epirubicin, Green Tea, Mice, Hepatotoxicity, Histology.

*Corresponding Author
Mona Ramadan Alshathly.

Abstract

Hepatotoxicity is a well-known complication of anticancer agent epirubicin, as a result of oxidative stress. One of the most commonly consumed herbal extracts with antioxidant properties is green tea. The objective of this study was to evaluate the effectiveness and safety of prolonged consumption of green tea water extract against epirubicin-induced hepatotoxicity in mice. One hundred adult female mice were divided into control (GI), treated with access to water (GIIa), treated with access to green tea water extract (GIIb) and untreated with access to green tea water extract only (GIII). Epirubicin was administrated every three weeks for eight cycles. Livers from five mice were taken from each group after one, three, four, six and eight cycles. The GIIa group showed hydropic degeneration, apoptosis and pyknotic nuclei from early cycles of administration and continued to be observed until marked necrosis and haemorrhage were observed after the 6th cycle. In contrast, the GIIb group showed good protective profile against epirubicin-induced histological changes observed in GIIa group until the 4th cycle where frequent presence of changes such as binucleated cells and inflammations were observed. By the end of the 6th cycle, severe histological changes were observed including marked necrosis followed by exponential increased of mortalities. The GIII group started showing histological changes after the 6th cycle. Although consumption of green tea ameliorated the epirubicin-induced changes, prolonged consumption introduced a potential pro-oxidant property when combined with epirubicin. This suggests that green tea anti-oxidant and pro-oxidant properties are interchangeable depending on the duration of consumption.

Introduction: -
Epirubicin (EPI) is the 4'-epimer of doxorubicin (DOX), both well-known and studied anticancer drugs from the anthracycline family. They have very similar pharmacologic profiles being metabolized in the liver and eliminated through the bile. Nevertheless, at similar doses, EPI appears to have a better side-effect profile than DOX (Mouridsen et al., 1990). The main mechanism of EPI antitumor effect is through intercalation of DNA and generation of free radical including reactive oxygen species (ROS) (Gianni et al., 1983). This could infer the more favourable toxicity profile of EPI compared to DOX.

Epirubicin has been commonly used solely and in combination against various types of cancer, especially parenchymal organs cancer in vivo such as breast, gastric, lymphoma, cardiac, liver (Di-Wen et al., 2016; Jänicek, 2000; Lopez et al., 1984; Sasu et al., 2015; Sha et al., 2012; Štěrba et al., 2012) and to a certain extent small-cell lung cancer (Jacot et al., 2012). The most common side effects are alopecia, nausea/vomiting, cardiotoxicity, hepatotoxicity, leukopenia, and stomatitis (Bonadonna et al., 1993; Cersosimo and Hong, 1986). However, side effects post-EPI treatment are considered less compared to other anthracyclines (Robert, 1993).
A recent study suggests that EPI-loaded liver-targeted drug delivery system could effectively inhibit the growth of liver tumours in situ and potentially reduce the systemic side effects (Di-Wen et al., 2016). Alas, EPI is known to be an irritant drug that is given mainly by intravenous injection (IV) causing extensive tissue damage and blistering if escapes from the vein (Doellman et al., 2009).

Due to the intense toxicity profile, limited dosage and accompanying side effects, alternative and herbal treatment of cancer have been widely researched recently. The focus-concerned herbal extracts with antioxidant activity to ameliorate the drug’s effect. One of the most commonly consumed beverages is green tea (GT), prepared from the dried leaves of the plant Camellia sinensis, has been studied extensively for its cancer preventive effects in many different experimental systems, including animal models (Clark and You, 2006; Hou et al., 2004; Ju et al., 2007; Khan et al., 2006). A study showed that powdered GT extract reconstituted in distilled water offers protection against DOX-induced cardiotoxicity via reduction of oxidative stress (Patil and Balaraman, 2011). This protection property is attributed to green tea catechins (GTC), the main metabolites in GT (Quiles et al., 2002). They possess outstanding antioxidant and free radical scavenging properties (Scott et al., 1993; Toda, 2011) and include (−)−epigallocatechin-3-gallate (EGCG) which comprises more than 50% of the mass of all GTCs (Nagle et al., 2006). Furthermore, EGCG has been implicated with chemoprevention and anticancer properties (Azam et al., 2004) alas, high dosage of EGCG was reported to cause toxicity of rat liver mitochondria and hepatocytes (Kucera et al., 2015) as well as severe hepatic necrosis due to increased oxidative stress and lipid peroxidation in mice (Lambert et al., 2010).

The present study aimed to shed the light on the possible protective effect of GT water extract against EPI-induced hepatotoxicity in mice and evaluate its safety upon prolonged consumption in uncontrolled dose using histopathological evaluation of liver parenchyma.

Material and Methods:-

Materials:-

Ellence® Pharmorubicin (the trade name of EPI) was purchased in the form of powder package 10 mg from Pfizer Company (Shanghai, China). Dried leaves of GT was purchased from local market in Jeddah, Saudi Arabia. All other chemicals were purchased from Sigma–Aldrich (St. Louis, Missouri, United States).

Epirubicin preparation:-

For IV injections, packages of 10 mg epirubicin was dissolved in 5 ml of normal saline for a final concentration of 2mg/ml according to manufacturer’s instructions.

Green tea preparation:-

The GT water extract was prepared by adding 100 ml boiling water to 2 g of GT leaves, cooled down to room temperature, filtered and put in drinking bottle for mice (Jiang et al., 2001). Adjusted daily water intake for mice was reported to be 5.7±0.2 ml/30 g body weight (Bachmanov et al., 2002).

Animals:-

One hundred adult Albino female mice (BALB/C) strain were purchased from animal unit of King Fahd Medical Research Centre at King Abdulaziz University, Jeddah, Saudi Arabia. Animal weight ranged between 18-22 g. Each five mice were put in a plastic cage and left one week for accommodation (Temperature 22 °C, Humidity 35-50% and light/dark cycle of 12:12 hours). Animal care and treatment were carried accord to rules provided by the ethical committee for research.

Animal grouping and dosage:-

Dosage was chosen according to previous studies (Meir et al., 2003; Pujol et al., 1997; Viens et al., 2001) and adjusted for mice (Paget and Barnes, 1964). Animals were divided into four groups as follows: GI (n=25) served as control, were given 0.9% normal saline IV via tail vein, and had food and Ad libitum access to water. GII (n=50) were injected with EPI via tail vein by standard dose of 90mg/m2 which equal 11.7mg/kg body weight. The dose was given every three weeks through eight cycles (“A prospective randomized phase III trial comparing combination chemotherapy with cyclophosphamide, fluorouracil, and either doxorubicin or epirubicin. French Epirubicin Study Group,” 1988; Eksborg et al., 1992). Those animals were further divided into two sub-groups, GIIα (n=25) were allowed Ad libitum access to water and GIIb (n=25) were allowed Ad libitum access to GT water extract. GIII (n=25) were untreated animals with Ad libitum access to GT water extract only.
Five animals from each group were euthanized after one, three, four, six and eight cycles. Heart was perfused with normal saline followed by 10% neutral buffered formalin to insure perfect organ fixation. The liver was dissected carefully, cut into small pieces (1 cm²) then re-fixed in 10% neutral buffered formalin for subsequent paraffin processing. Five micron thick sections were stained by Haematoxylin and Eosin (H&E) (Bancroft and Layton, 2008). Stained sections were examined using light microscope connected to digital camera (OLYMPUS, United States). Photographs from all groups were compared in regards to hepatocytes and sinusoidal changes in both central vein (CV) and portal area regions.

**Results:**

**Histological structure of control mice liver:**

The control mice liver showed histological structure similar to what was previously described in literature. Hepatic lobules are ill-defined and can be only marked by the presence of CV and the peripherally located portal regions containing branches of portal vein (PV), hepatic artery and bile duct while surrounded by scanty connective tissue. Hepatocytes are arranged in regular cords radiating from the CV, have slightly basophilic cytoplasm, and rounded vesicular nuclei of uniform size. Few cells are bi-nucleated and separated by thin walled blood sinusoids lined by flat endothelial cells. Von Kupffer cell nuclei are occasionally seen projecting into sinusoidal lumina (Fig. 1).

![Fig. 1](image1.png)

**Fig. 1:** Sections of mice liver from the control group (GI). a. Central vein (CV) region with regular hepatocytes cord (black arrows) separated by thin walled blood sinusoids lined by flat endothelial cells (dotted arrows). b. Portal region showing bile duct (BD) and part of portal vein (PV) with hepatocytes cord (black arrows) and blood sinusoids (dotted arrows) (H&E; x10).

**Effect of EPI administration in different cycles on histological features in mice liver (GIIa):**

**Histological structure of mice liver after 1st cycle:**

One cycle post-EPI administration, the mice liver showed swollen hepatocytes compressing or obliterating the blood sinusoids. Loss of cellular out lines and hydropic degeneration (unstained vacuolated cytoplasm) were observed. Nuclei appeared deformed and pyknotic or even absent (Fig. 2a). Some cells showed shrunken, deeply stained acidophilic cytoplasm and frequent bi-nucleated cell were observed (Fig. 2b).

![Fig. 2](image2.png)

**Fig. 2:** Sections of mice liver (GIIa) after 1st cycle of EPI administration. a. Swollen hepatocytes with unstained cytoplasmic regions (black arrows) and some with deformed, decreased or karyolytic nuclei (dotted arrows). b.
Hepatocytes with shrunken cells with pyknotic nuclei (white arrows) and bi-nucleated cells (black arrows) (H&E; a (x10), b (x40)).

**Histological structure of mice liver after 3rd cycle:**
Continuous administration of EPI for the 3rd cycle resulted in marked ballooning of hepatocytes, which showed hydropic degeneration. The nuclei lost its vesicular appearance become small and deeply stained. Foci of cell necrosis with residual cell debris could be seen (Fig. 3).

Fig. 3: Sections of mice liver (GIIa) after 3rd cycle of EPI administration. a. Marked hydropic degeneration resulted in vacuolation or unstained cytoplasm (black arrow). Focal area of degenerated cells (star) is also present. b. Higher magnification showing hepatocytes with pyknotic nuclei and the vacuolated cytoplasm (black arrows) or with karyolytic nuclei (dotted arrows). CV, central vein (H&E; a (x10), b (x40)).

**Histological structure of mice liver after 4th cycle:**
After four cycles of EPI administration, showed marked swelling of hepatocyte around CV obliterating hepatic sinusoids (Fig 4). The cytoplasm of cells is unstained while the nuclei are small and dark. Hepatocytes near PV showed loss of normal hepatocyte arrangement and vacuolation of cytoplasm. Furthermore, giant cells and lymphocyte aggregation around PV were observed. In some cases, apoptosis and nuclear pleomorphism were observed.

Fig. 4: Sections of mice liver (GIIa) after 4th cycle of EPI administration. a. Marked swelling of hepatocytes (black arrows) around the central vein (CV) obliterating hepatic sinusoids. b. Hepatocytes near portal vein (PV) showing loss of normal hepatocytes arrangement and vacuolation of cytoplasm (black arrows), giant cells (Thick black arrows) and lymphocyte aggregation around PV (stars). c. Hepatocytes with apoptosis (white arrows) or nuclear pleomorphism (black arrows) (H&E; x10).
Histological Changes of mice liver after 6th cycle:
Variations in hepatocytes within this cycle were markedly observed. Some hepatocytes had marked loss of normal lobular architecture, ill-defined borders and pyknotic degenerated nuclei with CV showed congestion. Few hepatocytes had nuclear pleomorphism and prominent Von Kupffer cell nuclei. Hepatocytes with unstained cytoplasmic regions, bi-nucleation nuclear inclusion and karyomegaly were also observed (Fig. 5).

Fig. 5: Sections of mice liver (GIIa) after 6th cycle of EPI administration. a. Most hepatocytes showing ill-defined borders (dotted arrows) and pyknotic nuclei (star). Central veins (CV) showed congestion (black arrows). b. Hepatocytes with dark cytoplasm and small pyknotic nuclei (white arrows). c. Hepatocytes with nuclear pleomorphism and bi-nucleated cells (dotted arrows) or karyomegaly (thick black arrow). Von kuppfer cell nuclei were prominent (black arrows). d. Hepatocytes showing unstained cytoplasmic regions (thin black arrows), karyomegaly (thick black arrow) and nuclear inclusions (white arrow) (H&E; a-b (x10), c-d (x40)).

Histological changes of mice liver after 8th cycle:
Livers from this cycle showed marked lobular disruption with hydropic degeneration. Some showed massive necrosis and haemorrhage. Inflammatory cell aggregation near portal area was also observed (Fig. 6).

Fig. 6: Sections of mice liver (GIIa) after 8th cycle of EPI administration. a. In the bile duct (BD) region, marked hydropic degeneration of hepatocytes (black arrows) were observed. b. Multiple regions of massive necrosis (white arrows) and haemorrhage (black arrows). c. Inflammatory cells (white stars) around dilated portal vein (PV) and bile duct (BD). Notice the desquamated cells (black star) within the PV. CV, central vein (H&E; x10).
Effect of GT consumption on mice liver post-EPI administration (GIIb):

After 1<sup>st</sup> cycle:-
Hydropic degeneration observed in EPI-treated group (GIIa) was markedly absent. Hepatocytes arrangement and histological features looked similar to control (Fig. 7).

![Fig. 7](image)

**Fig. 7:** Sections of mice liver (GIIb) after 1<sup>st</sup> cycle of EPI administration and Ad libitum access of GT water extract. Central vein (CV) and elongated bile duct (BD) are shown with noticeable absence of hydropic degeneration. Hepatocytes arrangement and histology features seemed normal and comparable to control group (black arrows) (H&E; x10).

After 3<sup>rd</sup> cycle:-
The liver of this cycle showed continued histopathological protective profile with numerous presence of bi-nucleated hepatocytes (Fig. 8).

![Fig. 8](image)

**Fig. 8:** Sections of mice liver (GIIb) after 3<sup>rd</sup> cycle of EPI administration and Ad libitum access of GT water extract. Central vein (CV) region showing protection profile from EPI-induced changes where hepatocytes arrangement and histological features looked similar to normal although, bi-nucleated cells are numerous (black arrows) (H&E; x10).

After 4<sup>th</sup> cycle:-
Green tea potential protection against EPI induced hepatic changes was still observed with less vacuolation of hepatocytes. In addition, inflammatory changes near portal triad and presence of giant cells were less frequent and few apoptotic cells were observed (Fig. 9).
Fig. 9:- Sections of mice liver (GIIb) after 4\textsuperscript{th} cycle of EPI administration and Ad libitum access of GT water extract. a. Moderate decrease in cell vacuolation (black arrows) although hepatocytes still appeared swollen compressing sinusoids and few apoptotic cells were observed (white arrow). b. Hepatocytes showing decreased inflammatory cells (star) around bile duct (BD) and portal vein (PV). Hepatocytes looked less vacuolated (black arrows) while few degenerated giant cells were observed (white arrow). CV, central vein (H&E; x10).

After 6\textsuperscript{th} cycle:-
Continuous similarities with 4\textsuperscript{th} cycle were observed in the 6\textsuperscript{th} cycle as well. Potential protection was observed in liver of some animals where necrotic foci and haemorrhagic regions were less frequent. Apoptotic cells and cells with ill-defined borders were still observed. Congestion of central and portal vessels were still evident (Fig. 10).

Fig. 10:- Sections of mice liver (GIIb) after 6\textsuperscript{th} cycle of EPI administration and Ad libitum access of GT water extract. a. Hepatocytes still showing ill-defined outlines with pyknotic nuclei (black arrows). b. Hepatocytes showing decreased frequency of nuclear pleomorphism and nuclear inclusion (black arrows) and slight congestion of central vein (CV). PV, portal vein (H&E; x10).

Mortality in this group (GIIb) increased exponentially after the 7\textsuperscript{th} cycle with all the animals dead before the 8\textsuperscript{th} cycle. The liver showed marked necrosis and loss of normal texture (Fig. 11).
Fig.11: Sections of mice liver (GIIb) after 7th cycle of EPI administration and Ad libitum access of GT water extract. Massive necrosis and loss of hepatocytes (black arrows) located near portal vein (PV) were observed (H&E; x10).

Effect of GT consumption for eight cycles on histological changes in untreated mice liver (GIII):-
This group did not show significant changes on histological structure of mice liver until the 8th cycle. Bi-nucleated cells were frequent and nuclei with increased peripheral chromatin were observed (Fig. 12a, 12b, 12c). Furthermore, slight vacuolation of hepatocyte cytoplasm, darkly stained small sized deformed nuclei were observed (Fig. 12d).

Fig.12: Sections from mice liver (GIII) allowed Ad libitum access of GT water extract only. a. After one cycle, normal hepatocyte cords (black arrows), basophilic cytoplasm, few bi-nucleated cells (white arrows) and sinusoidal nuclei (dotted arrows) were observed. b. After three cycles, similarities to normal hepatocyte architecture are still observed with normal hepatocytes cords (black arrows), basophilic cytoplasm, few bi-nucleated cells (white arrows) and sinusoidal nuclei (dotted arrows). c. After six cycles, normal hepatocytes (black arrows), bi-nucleated cells (white arrows) and sinusoidal nuclei (dotted arrows) were still observed. d. After eight cycles, darkly stained, small sized, deformed nuclei and slight vacuolation of cytoplasm (black arrows) were observed. CV, central vein; PV, portal vein and BD, bile duct (H&E; x10).

Discussion:--
Throughout the eight cycles, the liver of the control group (GI) was similar to what was previously described in literature pertaining to normal histological structure. The uniformity of the nuclei, basophilic cytoplasm and the regular arrangement of the hepatocytes close to CV were observed in all the samples. Although the negative control group (GIII) showed similar histological structure compared to GI, significant changes were observed after the 8th cycle. Particularly, the frequent presence of bi-nucleated cells and increased peripheral chromatin of the nuclei. This might infer that prolonged consumption of GT might affect the normal hepatocytes.

After one cycle of EPI administration, treated group (GIIa) started showing swollen hepatocytes, loss of cellular outlines and hydropic degeneration, all signs of hepatotoxicity introduction. Furthermore, signs of programmed cell death (apoptosis) were also observed in the form of deformed and pyknotic nuclei (small and darkly stained due to chromatin condensation). The mechanism of inducing apoptosis by EPI was previously reported in non-small cell lung cancer and hepatica G2 cells via productions of ROS and reduction of scavenging enzymes (Ozkan and Fişkin, 2004). The acidification of the cytoplasm was also observed which indicates disruption of cellular signalling and membrane transport in both the endocytic and exocytic directions (Cosson et al., 1989) as well as disturbance of...
cellular homeostasis since the intracellular pH is an important parameter for cellular functions and membrane traffic. On the other hand, the treated group (GIIb) already started showing protective profile compared to (GIIa). The absence of hydropic degeneration was markedly observed and hepatocytes are more comparable to the control group (GI). Similar protective profile after DOX treatment was observed in a previous study where decreased lipid peroxidation induced by the drug increased the cellular concentration of glutathione which is needed to protect hepatocytes (Sugiyama and Sadzuka, 2016). Given that EPI is a derivative of DOX this mechanism could interpret the protective profile observed in this study.

After the 3<sup>rd</sup> and 4<sup>th</sup> cycles of continuous administration of EPI, the unceasing ballooning of hepatocytes in the treated group (GIIa) was still observed. The continuous chromatin condensation in the nuclei observed as darkening of the stain, which suggest progression of apoptosis in the cell appears to develop into cell necrosis with residual cell debris observed. Vacuolation of the cytoplasm, giant cells and lymphocyte aggregation around PV were also observed. This suggest that EPI induction of hepatotoxicity is efficient and increases with the continuous dose administration of EPI.

Compared to this treated group, continued histopathological protective profile was perceived in the treated group (GIIb) as well as the presence of some bi-nucleated hepatocytes. The decreased inflammatory changes near PV, which points toward GT being a good source for anti-oxidant herbal extract against EPI-induced hepatotoxicity. The 6<sup>th</sup> dose of EPI administration marked a cycle with numerous variations within each treated group. The (GIIa) showed continuous hepatotoxicity similar to the previous cycles with the addition of marked loss of normal lobular architecture, nuclear pleomorphism, bi-nucleation inclusions and karyomegaly (enlargement of the nuclei) observed. Nuclear pleomorphism, inclusions and karyomegaly were previously described in literature in cases of different forms of hepato- or nephrotoxicity (Baudrimont et al., 2001; Bokhari and Shaker Ali, 2008). Nuclear inclusion observed with EPI hepatotoxicity in the present study have been described previously with aging (Wenger et al. 2014), virus infection (Yang et al., 2011), alteration in cellular metabolism and exposure to toxic drugs (Takahashi et al., 2003; Uchihara et al., 2003). Though (GIIb) continued to have a protective profile and similarities with the control group (GI), apoptotic cells as well as congestion of central and portal vessels were observed.

After the 8<sup>th</sup> EPI administration, necrosis, haemorrhages and marked lobular disruption of hepatocytes were present more frequently in the treated group (GIIa). Previous study showed that the use of chemotherapeutic agents might induce injury to endothelial cells lining the sinuses, leading to subintimal thickening and extravasation of erythrocytes into the sub-endothelial space of Disse (perisinusoidal space) (Maor and Malnick, 2013). Similar mechanism may also cause the massive focal regions of necrosis and haemorrhage observed in the present study, which may result in ultimate liver failure and animal death.

On the contrary, mortalities in the treated group (GIIb) increased exponentially after the 7<sup>th</sup> cycle with all the animals dead before the 8<sup>th</sup> cycle. Marked degenerative changes on liver of surviving animals included necrosis and loss of normal texture. This suggests that the protective profile of GT was challenged by the prolonged consumption alongside the EPI administrations. The severity of the histological changes can be interoperated as the anti-oxidant properties of GT were reversed with pro-oxidant counter-response due to the prolonged consumption. Similar effect was observed in a previous study where GT increased DOX histological damage in tumours by one-to-seven folds compared to DOX treated only (Sadzuka et al., 1998). This enhanced activity of antitumor agents may provide possible new role of GT as a biochemical modulator.

A study have reported that EGCG from GT can interact with proteins, phospholipids in plasma and mitochondrial membranes resulting in severe oxidative stress that damages cellular integrity, disrupt nuclear and mitochondrial function and inducing cell death (Kim et al., 2000). Another study showed evidence that GTCs may be related to oxidative stress where such pro-oxidant effects appear to be responsible for the induction of apoptosis in tumour cells as a protective response against carcinogenic cells (Lambert and Elias, 2010). Such effect could be the cause of massive necrosis of mice liver parenchyma observed in the present study upon prolonged usage with subsequent liver failure and death of animals.

**Conclusion:**
Green tea extract through its anti-oxidant effect could provide a protective profile for mice liver from hepatic injury caused by EPI if given for a short duration and controlled dosage. Alas, prolonged consumption with continuous EPI administration may lead to a synergetic effect as anti-cancer and pro-oxidants will increase hepatotoxicity and
severe necrosis of liver parenchyma resulting in increased mortalities in animals. Further studies are needed to determine the anti-oxidant defence agents in the serum and liver homogenates of treated animals.

**List of abbreviations:**
- Bile duct (BD); Central vein (CV); Doxorubicin (DOX); (-)-epigallocatechin-3-gallate (EGCG); Epirubicin (EPI); Green tea (GT); Green tea catechins (GTC); Haematoxylin and Eosin (H&E); Intravenous injection (IV); Portal vein (PV); Reactive oxygen species (ROS)

**Ethical approval:**
All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

**Conflict of interest:**
The author declares no conflict of interest.

**Acknowledgement:**
The author would like to thank professor Soad Shaker Ali, faculty of medicine at King Abdulaziz University in Jeddah for providing valuable comments and reviewing the manuscript.

**References:**
42. Takahashi, J., Fujigasaki, H., Iwabuchi, K., Bruni, A.C., Uchihara, T., El Hachimi, K.H., Stevanin, G., Dürr, A., 1071