Effect of Propranolol in Hepatic Blood Flow: A Way to Encounter Phenytoin Induced Hepatotoxicity in Albino Rabbits

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ABSTRACT
Phenytoin has proved to be the most effective anti-seizure drug to treat the partial and tonic clonic seizure. However; hepatotoxicity associated with the Phenytoin (PHY) is of great concern in clinical settings. Contrarywise, Propranolol (PRL) is reported to possess the significant hepatoprotective effects owing to the reduced blood flow. Therefore, the present study was aimed to evaluate the hepatotoxicity of PHY alone and in combination with PRL in healthy male rabbits. Post treatment level of various liver enzymes including alanine transaminase (ALT), alkaline phosphatase (ALP), gamma-glutamyltransferase (γ-GT) and bilirubin (BRB) were measured and compared with the normal values. Additionally, histological examination was done through micrometry and scanning electron microscopy (SEM) to estimate the intensity of hepatotoxicity induced by PHY and PHY with PRL. Animals received PHY alone treatment showed a significant elevation of liver enzymes than control and combination. Conversely, the lower level of serum ALT, ALP, γGT and BRB were found when administered PRL with PHY (p<0.005). Prominent change in hepatic cells structure and diameter of nucleus were observed (p<0.05). Severe hepatic damage reflected by necrosis, hemorrhage and dilation of sinusoids, inflammation with dilation and congestion of portal vein were obvious in PHY treated group. Combination treatment of PHY with PRL showed normal liver function and identical tissue pattern as control group exhibited. It is concluded that the PRL actually reduces the blood flow to the liver consequently, supply of noxious substance is blocked. Henceforth, the PRL would provide the beneficial effects when co-administered with the hepato-toxic drugs.

INTRODUCTION
Epilepsy is a neurologic disorder affected millions of people worldwide and considerable cause of morbidity and mortality. The prevalence of epilepsy is relatively higher in developing countries than the developed countries. The predicted efficacy of antiepileptic drugs has to be weighed against potential adverse effects, while at the same time considering the risks associated with withholding of treatment (Schmidt and Schachter, 2014). Phenytoin, carbamazepine and valproate are utilized to be the first line antiepileptic drugs. It is extensively documented that Phenytoin (PHY) has been found to be highly effective in management of partial and tonic clonic seizure. It is also prescribed in neuropathic pains and cardiac arrhythmias especially in digitalis induced arrhythmias. Beyond these treatment benefits unfortunately such aromatic antiepileptic drugs are prone to cause hepatic injuries (Vidaurre et al., 2017). PHY is almost 95% metabolized in the liver while only 5% is excreted via kidney. It belongs to the aromatic antiepileptic category, often associated with hepatotoxicity usually manifested as idiosyncratic reaction. Symptoms of hepatotoxicity are usually appeared within first six weeks of the therapy (Curry et al., 2018; Patocka et al., 2020). Common warning signs are often include pyrexia,
skin irritation, lymphadenopathy, hepatomegaly, anorexia and myalgias or joint pain. Hospitalized patients may develop jaundice, periorbital and facial edema. Alteration in liver function parameters related with the PHY hepatotoxicity are elevation in serum aminotransferase, lactate dehydrogenase, alkaline phosphatase, bilirubin and prothrombin time (Smythe and Umstead, 1989; Misra et al., 2013). Although the exact mechanism of hepatotoxicity is still unknown but probably the immune related hypersensitivity reaction would lead to an oxidative stress, resulting in liver damage (Santos et al., 2008; Kump et al., 2012). PHY induced hepatotoxicity was manifested as cholestasis with hepatitis, granulomatous hepatitis and acute hepatitis (Cadman and Featherstone, 2003; Diwan et al., 1993; Mahendra and Hendra, 2011).

Drug induced hepatotoxicity is now become a global burning issue especially when drugs are to be administered for long duration (Yoon et al., 2016; Kaplowitz, 2004). Various herbal formulation and vitamins are studied to evaluate their effects in PHY induced hepatotoxic animal models (Sasaki et al., 2013; Owoeye et al., 2015; Saraswathy et al., 2015b).

PRL is a non-selective beta adrenergic receptors antagonist, excellently reduce the portal hypertension and bleeding from esophageal varices. The effectiveness of drug in such disorders is due to the decrease hepatic blood flow (Tursi, 2010). It is therefore, hypothesized that the reduction in blood flow to hepatic portal system by PRL would be responsible to subside the toxic effect of PHY to liver.

Based on the above mentioned facts, the current study is design to evaluate the extent of liver damage produced by the PHY. Additionally, the role of PRL in PHY induced injuries will be explored with the concurrent administration of PHY and PRL. The liver strength will be assessed by various enzymatic and non-enzymatic parameters of liver function test including alanine transaminase (ALT), alkaline phosphatase (ALP) and gamma-glutamyltransferase (γ-GT) and bilirubin (BRB). The histo-pathological studies of liver will be performed using scanning electron microscopy of hepatic tissue to estimate the hepatic toxicity induced by the phenytoin and the repair of the hepatic injuries produced by PRL. Moreover, the micrometric analysis of liver by means of viable hepatocyte count and diameter and nucleus were also analyzed.

**MATERIALS AND METHODS**

PHY and PRL were obtained from Lisko Pakistan (Pvt) Ltd. Heamotoxalin and eosin (H and E) staining solution kit ALP, γ-GT, ALT and BRB standard kits and poly-1-lysine coated glass slides were purchased from Sigma–Aldrich, St. Louis, MD, USA. Hard paraffin wax and formalin were purchased from Merck.

**Animal model**

Healthy male rabbits were selected for the study due to identical human blood parameters (Feroz and Khan, 2013). Thirty six rabbits 1000-1200 g were purchased from the animal house of Baqai Medical University, Karachi, Pakistan. All rabbits were adapted from housing condition one week before experiments. Animals were kept in animal house of Baqai Medical University according to the standard protocol (Albus, 2012). All procedures of animal handling complied with international ARRIVE guidelines. Temperature was maintained to 25 ± 2 °C for 12 h light/dark cycle. Each rabbit was placed in a separate cage during the study period. Animal fed with laboratory standard balance diet and water ad libitum.

**Experimental design**

Total 36 animals were included in the study. Rabbits were divided into three groups normal saline, PHY and PRL having 12 animals in each one. Control group received normal saline orally only. In PHY treated group rabbits were treated with PHY 300mg/kg for 14 days as a single daily dose in form of oral solution through gastric gavage (Huang et al., 2004). In PHY+PRL treated group rabbits were received the PHY 300mg/kg and PRL 30mg/kg simultaneously once daily for 14 days in the form of oral solution via gastric tube (Huang et al., 1997; D’Amico et al., 2012).

Blood samples (5ml) were collected from the animals after 24 h of last administered dose. Serum was isolated after centrifugation at 4000 rpm for 8min (Parasuraman et al., 2010) and used for estimation of liver enzymes ALT, ALP and γ-GT and BRB by Automatic Hematology Analyzer by DRAWELL using standard kits within 2 h of serum separation.

**Histological studies**

Animals were sacrificed after 24 h of last administered dose and livers were delicately separated. Livers were flushed with sterile normal saline and finally fixed in 10% buffered formalin. The fixed tissues were processed embedding for paraffin following the standard procedures (Alturkistani et al., 2016). Thick sections (5µm) of portions were cut and stained with haemotoxalin (H) and eosin (E) (Sharma and Jannmeda, 2013). The micrometric analysis of hepatic tissues was done by measuring the number of viable hepatocyte with their diameter and diameter of...
nucleus using Olympus BX40 bright field microscope camera (AmScope) and Toup View software (v3.7.7934).

Scanning electron microscopy (SEM)
The sectioned hepatic tissue were dehydrated and mounted on specimen stub with the help of electro-conductive double sided adhesive tape. The sample was coated with gold to observe under electron microscope (Murtey and Ramasamy, 2016).

Statistical analysis
The quantitative results were statically evaluated by statistical package for social sciences SPSS software (version 21). Findings of PHY treated and PHY+PRL treated group treated were compared with control group by taking mean and standard errors using one way analysis of variation ANOVA, considering the p<0.05 is significant for any response.

RESULTS

Gross toxicity
The animals of PHY treated group were unhealthy and less reactive to external stimuli as compared to control group, where all individuals were found to be alive. There were no major architectural abnormality observed in the liver of PHY treated group animals rather only a slight increase in liver size. The color of livers was observed to be dull brown and dark brown respectively for treated and control group without the adhesion of fatty tissues and abnormal contracture. Upon post treatment analysis, the animals of phenytoin + propranolol treated group were active, healthy and well-responsive to external stimuli. All the animals of this set were alive but less active than control group. The liver of PHY+PRL treated group animals had regular architecture having smooth surface and normal reddish brown color without any adhesion and contracture.

Hepatic biomarkers
The mean serum levels of ALT, ALP and γ–GT and BRB are given in Table I. The statistical analysis showed that there was significant increase in the levels of all hepatic biomarkers in PHY treated group when compared to control. These biomarkers were also found to be increased in PHY and PRL treated group than control group. However; the level of ALT, ALP and γ–GT and BRB were significantly reduced in PHY+PRL treated group in comparison to the means of PHY treated group.

Histological analysis
H and E stained sections of liver of control group animals showed normal hepatic lobules with radial arrangement of hepatocytes and central vein. Portal triad was appeared to be normal with portal vein, hepatic artery and one or two bile ducts. The sinusoids were seemed to be lined by Kupffer cells and endothelial cells (Fig. 1A, B, C). The morphological examination of the H and E stained liver sections of PHY treated rabbits’ liver showed moderately disturbed but intact hepatic cord with dilated sinusoids especially in periportal area. Necrotic patches and hemorrhagic sinusoids were also found in various fields. The central vein was seem to be congested and markedly dilated. Moderate to severe mononuclear cell infiltrations were also observed in portal tract with dilated and congested portal vein. Neutrophils and plasma cells were noticed within the inflammatory area (Fig. 1D, E, F, G).

Table I. Effect of propranolol on liver function and structure parameters of albino rabbits with phenytoin induced hepatotoxicity.

<table>
<thead>
<tr>
<th>Parameters (Units)</th>
<th>Control (n=12)</th>
<th>Phenytoin (n=12)</th>
<th>Phenytoin+Propranolol (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT (IU/L)</td>
<td>40.80 ±1.14</td>
<td>128±3.07*</td>
<td>6.75±1.55**</td>
</tr>
<tr>
<td>ALP (IU/L)</td>
<td>42.60±2.79</td>
<td>114.8±3.61*</td>
<td>55.1±1.34**</td>
</tr>
<tr>
<td>γ GT (IU/L)</td>
<td>8.00 ± 0.93</td>
<td>24.2±1.29*</td>
<td>14.60±1.08**</td>
</tr>
<tr>
<td>Total BRB (µmol/L)</td>
<td>8.52±1.03</td>
<td>35.98±3.32*</td>
<td>21.51±2.82**</td>
</tr>
<tr>
<td>Hepatocyte count (cell/reticule)</td>
<td>21.70±1.13</td>
<td>15.27±2.02*</td>
<td>20.00±1.10</td>
</tr>
<tr>
<td>Hepatocyte diameter (µm)</td>
<td>13.90±0.33</td>
<td>11.98±1.86*</td>
<td>13.24±1.14**</td>
</tr>
<tr>
<td>Nuclear diameter (µm)</td>
<td>5.89±0.07</td>
<td>4.27±0.52*</td>
<td>5.65±0.35**</td>
</tr>
</tbody>
</table>

Results values are expressed in Mean±SEM. ALT, alanine transaminase; ALP, alkaline phosphatase; γ-GT, gamma-glutamyltransferase; BRB, bilirubin.

*Significant when Phenytoin compared with control. **Significant when Phenytoin+propranolol compared with control.
Fig. 1. Effect of propranolol on histological structure of phenytoin-treated liver of albino rabbits.

A, Photomicrograph of 5 micron thick hematoxylin and eosin (H and E) stained paraffin section from liver of rabbits of control group showing normal central vein (CV) and radiating hepatic architecture and normal sinusoidal spaces (arrow) (100X). B, showing normal portal vein (PV) and bile duct (BD), normal sinusoidal spaces (arrow) and binucleated hepatocytes (star) (400X). C, showing normal hexagonal hepatocytes, kupffers cells (thick arrow) (1000X). D, PHY treated group showing moderately disturbed hepatic cords with dilated sinusoids especially in periportal area. The central vein (CV) was also markedly dilated (100X). E, showing necrotic patches (N), polymorphonuclear cell infiltration (thick arrow) especially within portal tract and hemorrhagic sinusoids (thin arrow) 100X. F, illustrating that the portal tract was severely inflamed with mononuclear cells with dilated and congested portal vein (PV) (400X). G, pointing out the inflammatory patch. Plasma cells and neutrophils were observed within the patch (thick arrow) (1000X). H, PHY and PRL treated group showing almost normal hepatic lobules with normal uninfamed central vein (CV) (100X). I, showing small number of inflammatory cells in portal tract. The diameter of portal vein (PV) mildly increased (400X). J, explaining that the hepatic cells were normal with granular cytoplasm and nucleus with nucleolus 1000X.

The H and E stained liver sections of PHY and PRL treated rabbit livers showed mildly dilated hepatocytes with normal hepatic cords. The central vein was observed normal and inflammatory cells were almost absent in pericentral area. Few inflammatory cells was observed as well in portal tracts and diameter of portal vein was also slightly increased. The hepatocytes was appeared normal with granular cytoplasm around nucleus. Hepatocytes were present as normal with darkly stained nucleus (Fig. 1H, I, J).

The mean values of viable hepatocyte count, diameter and nuclear diameter were provided in Table I. The results showed that the viable hepatocyte counts were prominently reduced in both PHY groups when compared to other sets of animals (PHY treated group and in PHY+PRL treated group). Likewise, the diameters of hepatocyte were also noticeable decreased in PHY treated group while observed to be almost equal in control group and PHY+PRL treated group. Similar results were observed in case of nuclear diameter. The nuclear diameters were also reduced significantly in PHY treated group as compared to control and PHY+PRL treated group.
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Scanning electron microscopy (SEM)

SEM micrograph of surface structure of section of liver of control rabbits showed normal hepatic cords with regular polyhyderal structure of hepatocytes. Hepatocytes were separated by normal sinusoidal spaces as shown in Figure 2A.

SEM of liver section of PHY treated rabbits showed noticeably distorted hepatic cords and sinusoidal dilations. Bleb formations on the membrane of hepatocytes were also observed, with the perforation of liver surfaces as illustrated in Figure 2B.

Scanning electron microscopy of liver section of rabbits of PHY+PRL treated group illustrated partial restoration of hepatic architecture. Blebs formation and round shaped hepatocytes were also reduced. The sinusoids were found to be dilated slightly as shown in Figure 2C.

DISCUSSION

Phenytoin being an aromatic antiepileptic drug (AED) is used to treat variety of seizures and neuropathic pains. It is also prescribed in treatment of arrhythmias especially digoxin induced rhythm failures. Beyond benefits, PHY has potential to induce the hepatic injury owing to hypersensitivity or cholestatic pattern. PHY and other AEDs due to their prospective hepatotoxicity may lead to severe manifestations resulting in liver transplantation or even death. Oxides are formed upon PHY metabolism consequently, leading to the substantial hepatotoxicity either cytotoxic or cholestatic. Till date, no treatment is available, once the PHY hepatotoxicity has been developed (Björnsson, 2008) rather drug withdrawal or treatment discontinuation. Liver dialysis with molecular adsorbent recirculating system device is also often used to treat patients with PHY toxicity (Sen et al., 2003).

In this study, PHY was administered alone or in combination of PRL to evaluate the role of PRL in PHY induced hepatotoxicity. No kind of macroscopic damage was observed in the rabbits of PHY treated group. Only animals of PHY treated group were found to be lethargic and tired as compared to other group sets (PHY treated group and in PHY+PRL treated group). However, animals who received the combination of both drugs were comparatively active. Their livers were also appeared in normal reddish brown color without any unusual sticking.

The mean serum levels of γ GT, ALT, ALP, and total BRB were determined to assess the extent of liver injuries. Table I showed that the serum levels of mentioned hepatic parameters were prominently higher in PHY treated group animals as compared to control group, reflecting significant PHY induced hepatotoxicity. Sasaki and co-workers (Sasaki et al., 2013) had established a mouse model of PHY induced hepatotoxicity and concluded that PHY would surely elevate the serum ALT level by altering an innate immune response. Such elevation of serum levels of all standard hepatic functions (ALT, ALP, γGT and BRB) in PHY treated animals were also reported in past during preclinical and clinical study trials (Altuntas et al., 2003; Hussein et al., 2013; Saraswathy et al., 2015a). Conversely, hepatic enzymes were considerably reduced in animals of PHY+PRL treated group. The comparison among various treatment groups is expressed in terms of mean differences and found a significant variation between PHY treated group and combination of PHY and PRL treated animal groups. On the basis of findings, the role of PRL has proved in reduction of the PHY induced hepatic function elevations. However, PRL being a hepato-protective agent could not be capable to fully reverse the PHY induced values to the normal ranges. In portal biliopathy and biliary obstructive disorders, PRL...
is reported to decrease the hepatic biomarkers (Koshy, 2006; Fizanne et al., 2008). Utilization of some vitamins including B12, C, folic acid and other hepato-protective herbal drugs had investigated by researchers to assess their function against hepatic parameters when administered concurrently with PHY (Imosemi et al., 2010; Ekaidem et al., 2007).

PHY is documented to cause variable degree of liver injuries presented as swelling of hepatocyte, dilated central vein, degeneration of hepatocytes, and congestion in periportal area, fatty disintegration and vacuolar degeneration. The present investigation showed the similar histologic patterns as reported in past studies (Saraswathy et al., 2010, 2015b). The livers of group B animals displayed dilation of sinusoidal spaces and disrupted hepatic cords especially seen in periportal area. This type of hepatic architectural damage was also endorsed by many researchers (García-Estrada et al., 2000; García-Estrada et al., 1990). The PHY treated rabbits’ livers also showed necrotic patches, portal vein dilation and congestion. The inflammatory spaces were filled with neutrophil and plasma cells. This was happened probably due to the PHY associated hypersensitivity reactions, hepatitis and cholestasis (Altuntas et al., 2003; Bosia et al., 1999). The livers of PHY+PRL treated group rabbits showed minor deviations in hepatic architecture when compared with control animals. It was observed that combined administration of PRL and PHY produced lesser degree of damage, when comparing the histology of PHY treated group alone. Henceforth, these outcomes advocate the beneficial effects of PRL in PHY induced liver injury. Non selective beta blocker, PRL is extensively utilized in the treatment of several fatal hepatic disorders including variceal bleeding and portal hypertension in cirrhosis. It has further proved its efficacy not only in animals but bring improvement in gastric and hepatic cirrhotic patients (Dib et al., 2006; Pizzuceta et al., 1989). PRL is actually responsible to reduce the portal pressure and so improve the hepatic histology in animal model of CLD (chronic liver disease) (D’Amico et al., 2012). PRL administration has expressed promising results in children who had CLD but the liver biopsy is not allowed due to hyperspleen. PRL administration is considered to facilitate the treatment of thrombocytopenia and decrease the size of spleen through vasoconstriction in splanic artery (Poddar et al., 2015).

In the current study, the micrometric analysis of H and E stained section of liver provide further authentication for the underlying research hypothesis. The viable hepatocyte count was significantly (P<0.05) reduced in PHY treated group rabbits liver having the reduced viability of liver. The shrinkage of the hepatocytes were noted by quantifying the diameter of hepatocytes. The diameter of PHY+PRL treated group is nearly similar to the control, demonstrating the restoration of hepatocytes. Etephon is responsible for the shrinkage of the cell that assessed by decrease in hepatocyte diameter (Bhadoria et al., 2015). Mean of nuclear diameter was also reduced in PHY treated group. Such mentioned changes were documented to produce by an ethanol (Rasheed et al., 2012). The nuclear diameters were improved in PHY+PRL treated group, indicating the reduction of cellular activity by PHY and improvement was seen by simultaneous administration of PHY and PRL.

SEM micrograph showed bleb formation on the membrane of hepatocytes with distortion of hepatic cords. Livers micrograph have confirmed the PHY induced hepatic damage as well. Contrawise, the micrograph of PHY+PRL treated group has illustrated that the hepatic cords were comparatively intact and blebs were almost absent. The micrograph showed preservation of hepatic architectures and nearly follow the pattern of control group animals.

The hepato-protective effects of PRL in acetaminophene induced hepatic necrosis was explored in an early research conducted 1974 (Gazzard et al., 1974). Recently the role of PRL in hepato-toxicity was also established in carbamazepine drug; prone to cause liver impairment in animals. PRL was found to successfully reduced such carbamazepine hepatic damages (Abrar et al., 2019). Findings of the current study and the past literature as well are sufficient to justify the role of propranolol in reduction of hepatotoxicity of PHY. Moreover, PHY develops hepato-toxicity possibly due to the reduction of supply of noxious agents to the liver and hepato-protection is achieved due to the antioxidant effect of propranolol. Physicians and clinicians must need to be vigilant during treatment as an early detection of liver injuries may reduce the severity/intensity of hepatic damage and so could safe patients from liver failures.

On the basis of current findings, authors recommended the concurrent administration of PHY and PRL for considerable reduction in PHY induced hepatotoxicity especially for long term treatments clinically. Nevertheless, additional advanced research on molecular and genetic aspects of hepatotoxicity is need to be conducted for clinical implementation of this study.

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IRB approval
The study is approved from the Ethical Committee of Baqai Medical University

Ethical statement
All procedures were approved by the institutional ethics committee for the care and use of animals.

Study approval
The study has been approved by the Board of Advanced Study Research (BASR) of Baqai Medical University, Karachi, Pakistan.

Statement of conflict of interest
There are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

REFERENCES


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