A Comparison of the Protective Effect of Pyridoxine and N-Acetylcysteine in Paracetamol Induced Hepatotoxicity in Rats

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I N T R O D U C T I O N

The liver is highly susceptible to numerous toxicants including drugs [1]. Drug induced liver injury is one of the factors responsible for acute liver failure. Drug-induced hepatotoxicity accounts for 5% of all hospital admissions. It is the leading cause of 50% of all cases of acute liver failure [2]. Around 11% of acute liver failure patients in the US are associated with idiosyncratic drug induced liver injuries [3]. Antidepressants, antipsychotics, antibiotics, anti-TB, herbal and nutritional supplements, non-steroidal anti-inflammatory, paracetamol, and others, might result in hepatotoxicity [4-8]. Paracetamol overdose is the top cause of sudden liver failure in the United States, Australia, and New Zealand [9-11]. In the United States and Europe, paracetamol induced hepatotoxicity remains the primary cause of acute liver failure, resulting in over 300,000 hospitalizations each year and accounting for up to 42% of all cases of acute liver failure [12, 13]. According to the National Poison Data System's annual report in 2012, substance poisoning-related deaths were primarily caused by paracetamol [14]. The major molecule responsible for liver injury is not paracetamol itself, but rather a highly reactive metabolite NAPQI produced after metabolism. In the liver, paracetamol (85-90%) is metabolized and eliminated via glucuronidation or sulfation. Only a small part (2%) is excreted unchanged, while the remainder (10%) is metabolized by the cytochrome p450 system, specifically CYP2E1, into the reactive metabolite NAPQI. Normally, NAPQI is speedily transformed into harmless.

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metabolites by glutathione. In cases of glutathione depletion, such as paracetamol overdose, persistent alcohol intake, and starvation, NAPQI can lead to liver injury [15]. It has been reported that NAPQI can damage the liver in two main ways. First, it can alter the liver’s natural immune response. Secondly, it can produce protein conjugates that interfere with sulfhydryl groups, leading to mitochondrial dysfunction and cellular death [16, 17]. N-acetylcysteine is a well-known and documented substance used to reverse the toxic effects of paracetamol [18-20]. However, some studies have also reported the hepatoprotective effect of pyridoxine by preventing the depletion of GSH reservoir and lipid peroxidation [21]. Currently, no literature is available that indicates the combined use of pyridoxine and NAC as antidotes for the management of paracetamol induced liver injury.

The primary objectives of the current study were to evaluate the potential synergistic hepatoprotective effects of these two antidotes.

METHODS

Paracetamol was gifted by Legacy Pharmaceutical, located in Industrial State, Peshawar. N-acetyl cysteine, Pyridoxine, and 5,5-dithionitrobenzoic acid (DTNB) were procured from Sigma Aldrich (USA). Sulphosalicylic acid, disodium hydrogen phosphate, and sodium dihydrogen phosphate were purchased from Merck Company (Germany). Meanwhile, Roche Diagnostic (Switzerland) provided the liver biochemistry assay kits for ALT, AST, and total bilirubin. Healthy male Wistar rats weighing 200 ± 20 grams were acquired from Pakistan Council of Scientific and Industrial Research, Peshawar. The experiments were conducted according to all applicable ethical standards. The study registration number is KMU/IRBE/5th meeting/2023/9879-11. After 14 days of acclimatization period, the animals were randomly divided into five groups each having 6 rats. Animals grouping and the drug dose description shown in table 1.

### Table 1: Animals grouping and the drug dose description

<table>
<thead>
<tr>
<th>Animal groups</th>
<th>Drugs and doses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>Control group; received no drug</td>
</tr>
<tr>
<td>Group 2</td>
<td>Paracetamol 650 mg/Kg as a single dose (oral)</td>
</tr>
<tr>
<td>Group 3</td>
<td>Paracetamol 650 mg/Kg and 300 mg/kg N-acetylcysteine (oral)</td>
</tr>
<tr>
<td>Group 4</td>
<td>Paracetamol 650 mg/Kg and 200 mg/kg pyridoxine (oral)</td>
</tr>
<tr>
<td>Group 5</td>
<td>Paracetamol 650 mg/Kg, 300 mg/kg N-acetylcysteine, and 200 mg/kg pyridoxine (oral)</td>
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</table>

Animals were administered the single dose of all experimental drugs and closely monitored for a week. After 1 week all the rats were anaesthetized with ketamine and blood samples (3 mL) were collected using gel tubes for further analysis. Subsequently, the liver of each rat was separated and washed with an ice-cooled saline solution. One section of each liver was frozen and stored at a temperature of -80°C to prepare liver homogenates, while the remaining sections were treated with 10% formalin for histopathologic examination. The blood samples from all groups were collected for serum preparation. Serum was separated through centrifugation at 1000–2000 g for 10 minutes, and then carefully stored at -80 °C for further analysis of biochemical parameters by commercially available test kits [22]. Liver homogenate preparation involved carefully washing the liver with a solution of ice-cold normal saline containing 0.9% sodium chloride to ensure proper cleanliness and sterility. Then, 10% tissue homogenate was prepared utilizing PBS. Subsequently, the homogenate was subjected to centrifugation at 1008 g for 20 minutes at a controlled temperature of 4°C to ensure optimal results. After centrifugation, the supernatant layer of homogenate was removed and stored in the freezer for further analysis [23]. Biochemical assays were conducted for the determination of ALT, AST, bilirubin and GSH levels. For ALT determination, 1.0 mL of alanine α-KG substrate was added to test tubes for treated and control samples and incubated at 37°C. After 30 minutes, 0.2 mL of serum was added, followed by 1.0 mL of color reagent after another 30 minutes. The mixture was incubated for 20 minutes, and 10 mL of 0.40 N sodium hydroxide solution was added, mixed, and left for five more minutes. Finally, the absorbance was read at 505 nm using water as a reference [24]. For AST determination, 1.0 mL of aspartate substrate was taken into test tubes for the exposed and control samples and incubated at 37°C. After warming at 37°C, 0.2 mL of serum was added to each test tube and left to incubate for 60 minutes. Next, 1.0 mL of color reagent was added and left for 20 minutes. A sodium hydroxide solution was added, and the mixture was left for another 5 minutes. The absorbance was read at 505 nm, using water as a reference for the calibration curve [25]. Total bilirubin levels were determined by coupling with 3,5-dichlorophenyl diazonium in a strongly acidic medium using a suitable solubilizing agent. This reaction produces azobilirubin and the intensity of the resulting red azo dye is directly proportional to the amount of total bilirubin present. This photometric method was used to determine the total bilirubin level[26].

Ellman’s reagent was used to determine the nonprotein sulphydryl contents to estimate the GSH level in the liver. 4% sulphosalicylic acid and liver homogenate were taken 1 mL each in a tube and mixed well. The mixture was left for an hour at 4°C before being centrifuged at 1200 g for 15 minutes at 4°C, and the supernatant was collected. To 100 μL of supernatant, 2.7 mL of PBS (0.1 M pH 8.0) and 0.2 mL of
Ellman’s reagent (0.01 M) were added. The samples were mixed well by shaking them. The solution’s absorbance was measured against a reagent blank with no homogenate within five minutes of adding DTNB at 412 nm. 1.36 × 104 M−1 cm−1 MEC was used to determine GSH content in μmole/g tissue [27]. Histological evaluation was conducted by fixing slices of the liver tissues that reflect the organ was obtained and fixed in 10% Formalin. Ethanol was used to dehydrate the fixed tissues of rats. To get rid of the ethanol and make it easier to infiltrate molten paraffin wax at 55°C, the tissue was then passed through a solution of xylene. They were then immersed in the Paraffin wax block after that. The rotary microtome was used to cut 6-micron-thick paraffin sections, which was then placed on spotless glass slides. Hematoxylin and Eosin (H&E) stain was used to finish staining the sections. A light microscope was used to examine the stained slides as photomicrographs of the tissue samples were taken. Statistical analysis was performed using a one-way ANOVA test followed Tukey post hoc test was performed in this study. For properly distributed variables, the resulting data were shown as a mean standard deviation (SD), as well as in graphical and tabular formats. The data analysis was conducted using a p-value of 0.05 or below.

RESULTS

The effect of NAC and pyridoxine on body weight was evaluated, and the percentage change in body weight of rats is summarized in Table 2. Paracetamol has caused a significant decrease in body weight as compared to the normal group. On the other hand, the animals treated with NAC and pyridoxine alone and in combination exhibited an increase in body weight compared to the paracetamol group.

Table 2: Effect of paracetamol, pyridoxine, and N-acetylcysteine on the weight. All values represent mean SD.

<table>
<thead>
<tr>
<th>Group</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Group 4</th>
<th>Group 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.01 ± 0.98</td>
<td>10 ± 0.14</td>
<td>3.35 ± 0.03</td>
<td>3.55 ± 0.37</td>
<td>3.25 ± 0.31</td>
</tr>
</tbody>
</table>

The effect of NAC and pyridoxine on ALT, AST and bilirubin are shown in Figure 1 and 2, and 3 respectively. When compared to the positive paracetamol group, animals treated with NAC and pyridoxine individually or in combination showed significant decrease in ALT, AST and bilirubin level (P < 0.001). There was seen no significant difference between NAC and pyridoxine in improving the liver functions. However, combination of NAC and pyridoxine has markedly decreased the level of ALT, and AST.

Figure 1: Effects of pyridoxine and NAC on the ALT levels of the rats treated with single dose of paracetamol

Figure 2: Effect of pyridoxine and NAC on AST levels in rats treated with single toxic dose of paracetamol

Figure 3: Effect of pyridoxine and NAC on total bilirubin in rats treated with single toxic dose of paracetamol

The effect of NAC and pyridoxine on glutathione is shown in Figure 4. The groups that received NAC and pyridoxine showed a significant (P < 0.001) increase in glutathione level.
as compared to the paracetamol group. There was seen no significant difference between NAC and pyridoxine in raising the glutathione level. Conversely, combination of NAC and pyridoxine produced less effect on serum glutathione level.

Figure 4: Effect of pyridoxine and NAC on glutathione levels in rats treated with hepatotoxic single dose of paracetamol

All values represent mean ± SD. Group 2 (Paracetamol 650 mg/kg), Group 3 (Paracetamol 650 mg/kg and N-acetyl cysteine 300 mg/kg), Group 4 (Paracetamol 650 mg/kg and Pyridoxine 200 mg/kg), Group 5 (Paracetamol 650 mg/kg and N-acetyl cysteine 300 mg/kg and Pyridoxine 200 mg/kg. **p > 0.001, ***p < 0.001, ns (not significant). The histopathological assessment of the control group displayed a healthy liver structure (Figure 5a). Conversely, the group that received the toxic dose of paracetamol showed moderate to severe changes, such as fatty alteration, ballooning deterioration, sinusoidal congestion, and infiltration of inflammatory cells (Figure 5b). On the other hand, the group that received paracetamol along with NAC showed nearly healthy structure with mild congested portal veins. Mild fatty changes and sinusoidal dilation was observed. There was no inflammation and hepatocyte ballooning compared to the group that received toxic dose of the paracetamol (Figure 5c). The group administered paracetamol in conjunction with pyridoxine revealed healthy hepatocytes and slight fatty changes. The findings demonstrated mild inflammation, moderate portal veins congestion (Figure 5d). The group that received a combination of paracetamol, NAC, and pyridoxine demonstrated no inflammation and mild sinusoidal dilation with moderately congested central and portal veins and fatty changes (Figure 5f). Histopathological alteration in liver treated with drugs. Severity is graded based on the percentage of the liver area impacted. 0: normal observations; +++ (Severe, ≥ 50%), ++ (Moderate, 25-50 %), + (Mild, ≤ 25%). a) normal morphology of control rat, (b) Paracetamol intoxicated group showing damaged liver morphology, (c) effect of NAC on paracetamol induced alterations in liver, (d) effect of paracetamol and pyridoxine, (e) effect of paracetamol, pyridoxine and NAC (table 3 and figure 5).

Table 3: Group observations overview

<table>
<thead>
<tr>
<th>Observations</th>
<th>Groups</th>
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<tbody>
<tr>
<td></td>
<td>a</td>
</tr>
<tr>
<td>Hepatocyte ballooning</td>
<td>0</td>
</tr>
<tr>
<td>Periportal inflammation</td>
<td>0</td>
</tr>
<tr>
<td>Portal inflammation</td>
<td>0</td>
</tr>
<tr>
<td>Portal vein dilation and congestion</td>
<td>0</td>
</tr>
<tr>
<td>Central vein dilation and congestion</td>
<td>0</td>
</tr>
<tr>
<td>Sinusoidal dilation</td>
<td>0</td>
</tr>
<tr>
<td>Steatosis (Fatty liver change)</td>
<td>0</td>
</tr>
</tbody>
</table>
serum are a reliable indicator of the degree of liver damage. Therefore, monitoring the concentration of these enzymes in the bloodstream can be an effective means of evaluating liver function and identifying potential health risks [18, 25]. The objective of the study is to comprehensively investigate the experimental model of liver injury in rats induced by an overdose of paracetamol. The treatment with NAC and pyridoxine significantly improved the paracetamol induced percent change in weight in our study. Our study confirms earlier research indicating that exposure to paracetamol leads to a significant increase in serum enzyme levels of ALT and AST, implying hepatic structural damage [26-29]. According to reports of previous studies, paracetamol causes liver damage by augmenting the generation of free radicals and diminishing the antioxidant level of the liver [30, 31]. However, administration of NAC and pyridoxine restores these enzyme levels to the normal range, indicating their hepatoprotective effects. Notably, administering NAC and pyridoxine together results in a significant reduction in enzyme levels. The results of our study indicated that pyridoxine may be an effective treatment for hepatic damage caused by paracetamol exposure. The ability to effectively conserve the normal hepatic physiological functions that have been unstable due to hepatotoxins is one of the most dependable standards for evaluating the quality of any hepatoprotective drug [32]. Serum total bilirubin levels are crucial for assessing liver function. Elevated levels may indicate hepatobiliary disease and severe disruption of hepatocellular function as well as erythrocyte degradation rate caused by liver damage from hepatotoxin exposure [33, 34]. However, our recent research suggests that the treatment regimen studied herein has a hepatoprotective effect, effectively restoring bilirubin levels to normal. The use of NAC and pyridoxine has led to a significant reduction in bilirubin levels which was in agreement with other studies, with the combination of these two drugs resulting in an even more significant decrease in erythrocyte degradation rate caused by liver damage from hepatotoxin exposure [27, 29]. Our findings support the potential of this treatment to counteract the negative impacts of hepatotoxin exposure on liver function. A high dose of NAPQI can harm the liver by depleting the GSH and protein thiol group [35]. Our body's defense system uses enzymes like super oxide dismutase (SOD) and catalase to prevent toxicity from free radicals. Paracetamol-induced hepatotoxicity can disturb reactive oxygen species (ROS) generation and antioxidant defense, resulting in oxidative stress and hepatic necrosis [36]. To maintain a healthy liver, an adequate level of GSH should be maintained. GSH, a nonenzymatic antioxidant, is an essential marker of tissue vulnerability to oxidative stress [37]. A study has associated the depletion of GSH with

**Figure 5:** Histopathological assessments results

**Discussion**

Paracetamol overdose can cause liver injury due to a highly reactive metabolite known as NAPQI. Elevated levels of hepatic transaminase enzymes, such as ALT and AST, in
increased toxicity to chemicals, including CCL4 and paracetamol [38]. Our study’s results demonstrate that the acute administration of paracetamol leads to oxidative stress in rat liver, as indicated by a significant decrease in hepatic GSH levels. However, the pretreatments with NAC and pyridoxine significantly increased the GSH level in paracetamol-treated rats, which was in agreement with the study conducted by Mazraati et al. [21]. When the drugs were given in combination, there was an increase in the GSH level, but it was not statistically significant. NAC serves as a powerful reductant of disulfide bonds, a potent scavenger of reactive oxygen species, and a crucial precursor for glutathione biosynthesis [39]. As for pyridoxine (Vitamin B6), its exact antioxidant mechanism is still a matter of debate, but it is believed to effectively remove nucleophiles and oxygen-derived free radicals, thereby effectively preventing oxidative stress. Furthermore, Vitamin B6, a coenzyme, plays a vital role in two special enzymatic reactions in the methylation cycle, which convert homocysteine into cystathionine and then into cysteine. This pathway synthesizes cysteine, which is necessary for the GSH synthesis [40]. Our study conclusively shows that pyridoxine and NAC were equally effective in preventing liver damage caused by paracetamol-induced ALT levels and oxidative stress. In addition, pyridoxine was observed to perform better in reducing AST and GSH. Based on these findings, it is evident that pyridoxine is a potential candidate for an effective antidote for paracetamol-induced hepatotoxicity. In our study, the biochemical results and histopathological findings of these drugs revealed protective effect either alone or in combination. In summary, pyridoxine and N-acetylcysteine have been found to have hepatoprotective effects and are beneficial in protecting the liver from oxidative damage and reducing inflammation.

**CONCLUSIONS**

This study evaluated the effects of pyridoxine and NAC on paracetamol-induced toxicity in rats, and the results of the study indicated that pyridoxine can be used as a potential hepatoprotective drug in paracetamol-induced hepatotoxicity. In combination with NAC, it showed protective effects in paracetamol-induced hepatotoxicity. However, no synergistic effects were observed in the study when given in combination with NAC.

**Authors Contribution**

Conceptualization: GB  
Methodology: AJ  
Formal analysis: GB, AJ  
Writing-review and editing: AJ, HS, HB  

All authors have read and agreed to the published version of the manuscript.

**Conflicts of Interest**

The authors declare no conflict of interest.

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**REFERENCES**


