Potentiation of Acetaminophen Hepatotoxicity and Mortality by Doxapram in Mice

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Whether a single dose of doxapram (DOP) can modulate the acute toxicity and the hepatotoxicity induced by acetaminophen (AA) was examined. Pretreatment with DOP (40 mg/kg, i.p.) 30 min prior to the administration of AA dose-dependently potentiated the lethality of AA in both native mice and mice fasted for 18 h, and the potentiating activity was greater in fasted mice than in native mice. The hepatotoxicity of AA was assessed by plasma transaminases activity (glutamyl oxaloacetic transaminase, GOT; glutamyl pyruvic transaminase, GPT) and the amount of plasma lipid peroxides at 6, 12, 18, 24, 36 and 48 h after the administration of AA and histopathological examination of liver sections at 24 h after the administration of AA. DOP (40 mg/kg, i.p.) did not increase the plasma transaminase activity or the lipid peroxides level significantly, whereas AA administration to DOP-treated animals produced earlier maximal elevation of transaminase and lipid peroxide values compared to AA alone. These findings indicate that mortality and hepatotoxicity of AA is potentiated by DOP in mice.

Key words doxapram; acetaminophen; acute toxicity; hepatotoxicity

Since acetaminophen (AA) is included in many cold remedies as an analgesic or antipyretic, and frequently used over a long term, there are numerous opportunities for concomitant use with other drugs. Therefore, to evaluate the interaction between AA and other drug is important in using AA more safely.

AA is known to cause hepatic necrosis in man and experimental animals. At normal doses, AA is metabolized primarily to its glucuoric acid and sulphate ester conjugates, whereas at much higher doses sulphation is saturated and more of the AA is metabolized by cytochrome P-450 to N-acetyl-p-benzoquinone imine which normally reacts with glutathione. However, after over-dosage, intracellular glutathione becomes depleted and N-acetyl-p-benzoquinone imine may induce toxicity either by binding covalently to cellular macromolecules or by causing an oxidative stress and oxidation of protein thiol. Furthermore, toxic metabolites of AA are produced by the cytochrome P-450 system. Some isozymes of the enzyme seem to produce greater quantities of toxic metabolites than others, so their induction might lead to unexpected increases in the toxicity of the drug. Inhibitors of cytochrome P-450 generally decrease toxic metabolite formation and thus might be used to prevent AA hepatotoxicity.

Doxapram (DOP) is an agent that produces marked respiratory stimulation and pressor effects with a wider margin of safety than other compounds. Therefore, it is a drug used to stimulate ventilation in both animals and humans. Recently, we have shown that DOP is bound to liver microsomes and is a potent inhibitor of mixed-function oxidative metabolism in mice and rats. Surprisingly, however, we have found that DOP acts synergistically with AA on the mortality and hepatotoxicity of AA in mice. In this paper, we report the augmentation of mortality and hepatotoxicity of AA by DOP in mice.

MATERIALS AND METHODS

Male ddY mice (Japan SLC, Hamamatsu) were acclimated for at least 1 week in a room with a 12 h light/12 h dark cycle. Animals weighed 27 to 30 g (6 weeks old) at the time of AA administration. Experiments were performed according to a strict schedule because of known circadian variations in tissue glutathione. Between 9:30 and 10:00 A.M., mice were given AA dissolved in 20% Tween 80 in normal saline 30 min after DOP dissolved in 0.9% saline or saline. After the indicated time the animals were sacrificed by decapitation, and then samples for serum enzyme assay were collected from the neck veins.

Acute mortality was recorded over the 72 h after the intraperitoneal injection of AA as described previously. LD50 values were determined by plotting the logarithm of the AA dose versus the mortality of the treated mice. Serum glutamyl oxaloacetic transaminase (GOT) and glutamyl pyruvic transaminase (GPT) were monitored with a commercial kit from Wako Pure Chemical Co., Ltd. (Tokyo, Japan).

Plasma lipid peroxide was determined by the method previously described. Liver samples for liver examination were taken from the anterior portion of the left lateral lobe. Paraffin sections were prepared after fixation with 10% formalin in phosphate buffered saline, and were stained with hematoxylin and cosin.

Statistical significance was assessed by Student's t-test in the analysis of serum enzyme activities.

RESULTS

Mortality Deaths resulting from the administration of AA (500 mg/kg, intraperitoneally) and DOP (40 mg/kg, intraperitoneally) at various time intervals were recorded throughout the 72 h after AA administration (Table 1). These results indicate that the mortality of mice treated with DOP and AA simultaneously or treated with AA subsequent to DOP administration is higher than that of mice treated with DOP given after the AA. The maximum effect on AA mortality (60%) occurred with 30 min pretreatment with DOX

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prior to AA administration.

The ability of various doses of DOP to affect the toxicity of AA was also studied in native mice and mice fasted for 18 h. The effect of DOP (5, 10, 20, 30 or 40 mg/kg, intraperitoneally) pretreatment on the mortality caused by various doses of AA is shown in Fig. 1. The LD₅₀ of AA was 820 mg/kg (intraperitoneally). As seen in the figure, the pretreatment with DOP increased the lethality of AA dose-dependently in native mice.

Furthermore, the effect of DOP on the AA mortality was investigated in mice that were fasted overnight to reduce glutathione content, thereby both AA metabolism and toxicity were accelerated. Under these conditions, pretreatment with DOP increased the mortality of AA dose-dependently.

The LD₅₀ of AA were 825 and 680 mg/kg (i.p.) in native and fasted mice, respectively. The LD₅₀ of AA were 805, 780, 760 and 670 mg/kg when combined with 5, 10, 20 and 40 mg/kg of DOP, respectively, in native mice. Also, the LD₅₀ of AA were 670, 510, 470, and 408 mg/kg when combined with 5, 10, 20 and 40 mg/kg of DOP, respectively, in fasted mice. DOP induced leftward shifts in the dose-response curves for AA. The slopes of the lethal responses to AA in DOP-treated animals were approximately parallel to the

slope of the lethal responses to AA alone. The effect of DOX was greater in fasted mice than in native mice.

**Hepatic lesion** Furthermore, the results also suggest increased hepatotoxicity when mice were given DOP 30 min prior to administration of AA. The effect of pretreatment with DOP on AA hepatotoxicity assessed by plasma GOT and lipid peroxide is shown in Figs. 2 and 3.

The top panel in the figure depicts the plasma GOT in mice pretreated with DOP 30 min before receiving AA (400 mg/kg, i.p.). DOP alone did not increase plasma GOT levels significantly (data not shown). AA alone, depending upon the time-course employed, increased GOT levels significantly. However, AA administration to DOP-treated animals produced an earlier maximal elevation of GOT activity and an increase in GOT activity, compared to results in AA alone, indicating a significant enhancement of liver injury. The response curve for GPT activity by administration to DOP-treated animals was similar to GOT activity (data not

![Graph](image1)

**Table 1. Time Course of the Potentiating Effect of DOP on AA Mortality**

<table>
<thead>
<tr>
<th>Drugs (mg/kg, i.p.)</th>
<th>Time of administration with respect to DOP (40 mg/kg, i.p.)</th>
<th>No. of deaths</th>
<th>Total percent mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA 700</td>
<td>Exp. 1, Exp. 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>2/15, 1/10</td>
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<tr>
<td>-12</td>
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<tr>
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<td>36</td>
<td></td>
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<tr>
<td>-0.5</td>
<td>8/15, 7/10</td>
<td>60</td>
<td></td>
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<tr>
<td>Simultaneously</td>
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</tr>
<tr>
<td>+0.5</td>
<td>4/15, 3/10</td>
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</tr>
<tr>
<td>+1</td>
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<tr>
<td>+6</td>
<td>2/15, 1/10</td>
<td>12</td>
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</tbody>
</table>

Mice were given AA (700 mg/kg, i.p.) at various times before or after DOP (40 mg/kg, i.p.) administration. Each group consisted of 10—15 animals. Mortality of mice was observed 72 h after the injection of AA.

![Graph](image2)

**Fig. 1. Effect of DOP on Acute AA Mortality**

AA (700 or 500 mg/kg, i.p.) was injected 30 min after injection of saline and DOP of indicated dose (i.p.) in native (non-fasted) and fasted mice that were fasted for 18 h before receiving AA; water was available ad libitum. Each group consisted of 10 animals. See legend in Table 1 for the mortality and for analysis of the data.

**Fig. 2. Effect of DOP on the AA-Elevated Serum GOT Values in Mice**

Each group consisted of 5—6 animals. Left panel: GOT activity was estimated using standardized Wako Pure Chemical kits at the indicated time in mice pretreated with either saline (C, i.p.) or DOP (●, 40 mg/kg, i.p.), 30 min prior to receiving AA (400 mg/kg, i.p.). Right panel: AA (400 mg/kg, i.p.) was injected 30 min after injection of saline (i.p.) and DOP (5, 10, 20, 40, or 60 mg/kg, i.p.). Control received saline. GOT activity determined 12 h after the injection of AA. Significant difference (p<0.05) in GOT activity from AA alone is designated by an asterisk.
Fig. 3. Effect of DOP on the AA-Induced Lipid Peroxide Levels of Liver in Mice

Each group consisted of 5–6 animals. Left panel: hepatic lipid peroxide level was estimated using the method of thiobarbituric acid at the indicated time in mice pretreated with either saline (C, i.p.) or DOP (40 mg/kg, i.p.) 30 min prior to receiving AA (400 mg/kg, i.p.). Right panel: AA (400 mg/kg, i.p.) was injected 30 min after injection of saline (i.p.) and DOP (5, 10, 20, 40, or 60 mg/kg, i.p.). Control received saline. Hepatic lipid peroxide level determined 12 h after the injection of AA. Significant difference (p<0.05) in hepatic lipid peroxide from AA alone is designated by an asterisk.

Fig. 4. Histopathologic Findings in Mouse Liver 24 h after Treatment with DOP and/or AA

Mice were given AA (250 mg/kg, i.p.) 30 min after injection of saline and DOP (40 mg/kg, i.p.), and 24 h following AA administration, mice were decapitated. Control received saline. After fixation, mouse livers were routinely dehydrated and flat embedded in paraffin; sections were trimmed and cut to include all lobes of each liver, when possible. Sections 5 to 8 μm thick were stained with hematoxylin and eosin. A: control, B: AA alone, C: DOX + AA.

shown). This marked potentiation by DOP was further demonstrated when two of six animals pretreated with DOP died as a result of a subsequent 500 mg/kg dose of AA while all control mice tested at this dose survived.

Furthermore, Fig. 3 shows the effects of DOP pretreatment on AA liver injury as assessed by plasma lipid peroxide levels. The pattern of enhancement closely paralleled plasma GOT activity.

In addition, potentiation was also supported by histopathology studies. Twenty-four hours after AA (400 mg/kg, i.p.) injection, mice were sacrificed, the organs were examined, and various tissues were fixed. Gross changes were observed only in the livers. The livers of control or AA alone mice appeared pink with occasional areas of subpleural blood. In contrast, the livers of mice receiving the combination of DOP plus AA showed considerable damage by visual inspection.

The gross findings were consistent with the histological sections in the various treatment groups. Figure 4 illustrates the microscopic findings. The left panel shows the liver of a mouse used for saline control experiments (A). The microscopic appearance of livers from mice receiving AA alone showed a mild/moderate vacuolar degeneration and minimal necrosis (B). In contrast, in the livers of mice given DOP plus AA, there was marked centrilobular coagulative necrosis surrounded by hydropic changes (C). DOP alone did not produce any hepatic necrosis (data not shown).

DISCUSSION

From the results of mortality, plasma enzymes, hepatic lipid peroxide and histopathology, it can be said that DOP pretreatment has the ability to potentiate AA-induced hepatotoxicity and mortality in mice. Plasma GOT activity and his-
tological examination was used as a marker of AA hepatotoxicity. Also, since AA causes oxidative stress and protein thiol oxidation, and lipid peroxidation, hepatic lipid peroxide was also used as a marker. The lethal event in the DOP-AA synergism appears to reflect a magnification of AA-induced hepatotoxicity. Namely, 30 min after application of DOP, the AA-induced elevations of serum activities of GOT and hepatic lipid peroxide (Figs. 2 and 3) were strikingly increased, and this potentiated effect of DOP on AA hepatotoxicity was supported by histological assessment (Fig. 4).

The mechanism for the observed effect is not clear. AA is metabolized by a cytochrome P-450-mediated enzyme system, and the reactive metabolites (N-acetyl-p-benzoquinone imine, etc.) produced are detoxified by binding to reduced glutathione, followed by excretion in the urine as mercapturic acid. When the production of the reactive metabolite is sufficiently large enough to deplete hepatic glutathione, the reactive metabolite can no longer be detoxified by this pathway and binds to the cellular macromolecules. This interaction of reactive metabolite with macromolecules has been postulated to lead eventually to irreversible cellular damage. Thus, the mechanism by which a compound potentiates AA hepatotoxicity is dependent on either increased reactive metabolite production or diminished reactive metabolite detoxification.

Pretreatment with some inducers of mixed-function oxidases, such as phenobarbital and ethanol, has been shown to potentiate AA-induced hepatotoxicity. It is unlikely that in the present study DOP enhanced AA-induced hepatotoxicity by increasing the production of the reactive metabolite due to enzyme induction, since DOP was pretreated 30 min prior to AA and enhanced hepatotoxicity was apparent after the treatment of AA. We previously reported that DOP inhibited drug metabolizing enzyme activities by inhibiting the binding of substrate to cytochrome P-450 in microsomal fractions of mouse liver cells. We also reported that DOP increased the binding of AA to hepatic cell membranes. In the presence of DOP, therefore, the production of a reactive metabolite of AA could be inhibited by competition resulting in the prevention of AA induced hepatotoxicity. Nevertheless, in the present study, AA-induced hepatotoxicity was potentiated in the presence of DOP. In an experimental using primary cultured mouse liver cells, an increase in AA-induced hepatotoxicity was also observed after pretreatment with DOP (data not shown).

Hinson et al. were of the view that, above all, additional factors like the organelle susceptibility may have a role to play in the mechanism of potentiation. Briefly, it was reported that the severity of AA-induced hepatotoxicity is dependent on the binding capacity of N-acetyl-p-benzoquinone imine to specific proteins in cytosols, microsomes or mitochondria of liver cells. Previous studies suggested a compensatory increase in the activities of enzymes involved in AA metabolism after the inhibition of drug metabolizing enzyme activities by DOP or increased binding of AA to specific proteins in liver organelle. Although no such attempt is made in the present study to probe into these factors, the results may serve as a model to understand the responses of AA interaction with DOP through mortality and liver histopathology as well as other parameters of hepatotoxicity.

Using this model with mice for further experiments dealing with the time-course of toxicity of AA following DOP pretreatment, species and organelle susceptibility studies are in progress to identify the additional factors responsible for the potentiation of AA toxicity by DOP. Since DOP increases the binding of AA to hepatic cell membranes, the influence of DOP on the binding of AA to hepatic cell membranes should be evaluated in detail. Furthermore, it was considered that the discrepancy between the time course of lipid peroxide levels, a parameter of increased AA-induced hepatic toxicity after DOP administration, and that of increased GOT activity should also be evaluated in detail.

The present observation is clinically important because DOP and AA are the most frequently utilized drugs in common respiratory stimulation preparations and analgesic and antipyretic preparations, respectively. These medicines may cause AA-induced hepatic injury at smaller doses of AA than those contained in pure AA tablets. Clinical use of DOP in combination with AA may cause a further increase. Careful observation for hepatotoxicity is recommended when AA and DOP are prescribed simultaneously. However, since the present study was performed on mice, further studies should be performed on humans to resolve the question of possible interaction in man.

In conclusion, AA-induced hepatotoxicity is increased by coadministration of DOP. The mechanism of this effect remains to be elucidated.

REFERENCES