Influence of small doses of various drug vehicles on acetaminophen-induced liver injury

Tomislav Kelava, Ivan Ćavar, and Filip Čulo

Abstract: The biological effects of drug vehicles are often overlooked, often leading to artifacts in acetaminophen-induced liver injury assessment. Therefore, we decided to investigate the effect of dimethylsulfoxide, dimethylformamide, propylene glycol, ethanol, and Tween 20 on acetaminophen-induced liver injury. C57BL/6 male mice received a particular drug vehicle (0.6 or 0.2 mL/kg, i.p.) 30 min before acetaminophen administration (300 mg/kg, i.p.). Control mice received vehicle alone. Liver injury was assessed by measuring the concentration of alanine aminotransferase in plasma and observing histopathological changes. The level of reduced glutathione (GSH) was assessed by measuring total nonprotein hepatic sulfhydrils. Dimethylsulfoxide and dimethylformamide (at both doses) almost completely abolished acetaminophen toxicity. The higher dose of propylene glycol (0.6 mL/kg) was markedly protective, but the lower dose (0.2 mL/kg) was only slightly protective. These solvents also reduced acetaminophen-induced GSH depletion. Dimethylformamide was protective when given 2 h before or 1 h after acetaminophen administration, but was ineffective if given 2.5 h after acetaminophen. Ethanol at the higher dose (0.6 mL/kg) was partially protective, whereas ethanol at the lower dose (0.2 mL/kg) as well as Tween 20 at any dose had no influence. None of the vehicles (0.6 mL/kg) was hepatotoxic per se, and none of them was protective in a model of liver injury caused by D-galactosamine and lipopolysaccharide.

Key words: acetaminophen, dimethylsulfoxide, dimethylformamide, propylene glycol, ethanol, Tween 20, liver injury.

Résumé : Les effets biologiques des excipients sont souvent négligés, ce qui peut causer des artéfacts dans l'évaluation d'une lésion hépatique induite par l'acétaminophène. Nous avons donc décidé d'examiner l'effet du diméthylsulfoxyde, du diméthylformamide, du propylèneglycol, de l'éthanol et du Tween 20 sur ce type de lésion. Des souris mâles C57BL/6 ont reçu un excipient spécifique (0,6 ou 0,2 mL/kg, i.p.) 30 min avant l'administration d'acétaminophène (300 mg/kg, i.p.). Les souris témoins n'ont reçu que l'excipient. La lésion hépatique a été évaluée en tenant compte de la concentration d'alanine aminotransférase dans le plasma et des modifications histopathologiques. Le taux de glutathion réduit (GSH) a été déterminé en mesurant les sulfhydriles hépatiques non protéiques totaux. Le diméthylsulfoxyde et le diméthylformamide (aux deux doses) ont presque totalement supprimé la toxicité de l'acétaminophène. Le propylèneglycol a eu un effet protecteur significatif à la dose élevée (0,6 mL/kg), mais un effet négligeable à la faible la dose (0,2 mL/kg). Ces solvants ont aussi réduit la déplétion de GSH induite par l'acétaminophène. Le diméthylformamide a eu un effet protecteur lorsqu'il a été administré 2 h avant et 1 h après l'acétaminophène, mais n'a eu aucun effet lorsqu'il a été administré 2,5 h après l'acétaminophène. L'éthanol a eu un effet protecteur partiel à la forte dose, mais aucun à la faible dose. Le Tween 20 n'a pas eu d'effet. Aucun des excipients (0,6 mL/kg) n'a été hépatotoxique et aucun n'a eu un effet protecteur dans un modèle de lésion hépatique causée par la D-galactosamine et le lipopolysaccharide.

Mots-clés : acétaminophène, diméthylsulfoxyde, diméthylformamide, propylèneglycol, éthanol, Tween 20, lésion hépatique.

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Introduction

Acetaminophen (APAP) is a widely used analgesic and antipyretic drug that is considered safe at therapeutic doses. Overdose of APAP causes liver damage and for the last 2 decades is the leading cause of acute hepatic failure in the Western world (Lee 2004; Norris et al. 2008). The toxicity of APAP is initiated by formation of a reactive metabolite, *N*-acetyl-*p*-benzoquinoneimine (NAPQI), by liver microsomal enzymes; CYP1A2 and CYP2E1 seem to be the most important isoforms in mice (in humans, CYP2E1 and CYP3A4 appear to be the most important isoforms). NAPQI first depletes glutathione (GSH) and then covalently binds to cellular proteins (Mitchell et al. 1973; Nelson 1982; Zaher et al. 1998; Laine et al. 2009). APAP-induced liver injury (AILI) in mice is the most extensively used model for studying mechanisms of drug-induced liver injury (Gunawan and Kaplowitz 2007; Masson et al. 2008).

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Some of the major biases in AILI investigations have occurred because the drug vehicles had biological effects per se, particularly dimethylsulphoxide (DMSO). For example, El-Hassan et al. (2003) reported a protective effect of the pancaspase inhibitor Z-VAD-fmk against APAP toxicity in mice. For controls, they used the cathepsin inhibitor benzyloxycarbonyl-Phe-Ala-fluoromethylketone (Z-FA-fmk) and achieved no protection with this compound. However, they did not add DMSO to Z-FA-fmk, which was used to dilute Z-VAD-fmk (El-Hassan et al. 2003). Subsequently, it was shown that DMSO and not Z-VAD-fmk protected mice from APAP (Jaeschke et al. 2006). Likewise, it was reported that natural killer cells have a pathogenic role in AILI (Liu et al. 2004), but recently it was shown that this happens only when DMSO is used to help dissolve APAP (Masson et al. 2008).

Commonly used vehicles in AILI experiments include DMSO (El-Hassan et al. 2003), ethanol (ETOH), corn oil (Donthamsetty et al. 2008), olive oil (Jodynis-Liebert et al. 2005), Tween 20 (Renić et al. 1993), Tween 80 (Kamanaka et al. 2003), propylene glycol (PG) (Oz and Chen 2008), and methylcellulose (Harrill et al. 2009). They are used for 2 purposes: (1) to facilitate solution of some other substance being investigated for its effect on AILI and (or) (2) to help dissolve APAP itself; in the latter case PG is frequently used. Despite its obvious importance, the influence of vehicles on AILI is rarely investigated. So far, it has been shown that DMSO, PG, and ETOH, if injected 15–30 min before or 1–2 h after APAP administration, reduce its toxicity, most probably by inhibition of cytochrome P450, which converts APAP to NAPQI. However, most of these investigations were conducted for doses of vehicles larger than 1 mL/kg (Hughes et al. 1991; Thomsen et al. 1995; Lee et al. 1999), which are 5–10 times higher than doses usually used in AILI experiments. A notable exception is DMSO, for which an effective dose-dependent study was conducted showing a strong protective effect even when DMSO was administered at a dose of 0.2 mL/kg (Yoon et al. 2006). The effects of various vehicles, to our knowledge, have not been compared.

Therefore, we decided to compare the effects of the 3 most frequently used solvents (DMSO, PG, and ETOH) on AILI, as well as to investigate the effects of Tween 20 and dimethylformamide (DMF), whose influence in this model have not been investigated. The main object of our study was to show which vehicles at doses of 0.2 and 0.6 mL/kg change the intensity of AILI and which do not. Besides common parameters of APAP toxicity, such as serum aminotransferase levels and liver pathohistology, the influence of APAP on GSH content in liver and microsomal enzyme activity was also investigated.

Materials and methods

Animals

C57BL/6 mice were raised in an animal colony unit at the Department of Physiology, School of Medicine, University of Zagreb, Zagreb, Croatia. Male mice aged 12–16 weeks and weighing 20–25 g were used in all experiments. The cages were stored in rooms with a 12 h light period from 0600 to 1800, a temperature of 21 ± 2 °C, and a relative humidity of 50% ± 5%. The cages were sanitized twice weekly. All mice were given free access to tap water and standard mouse chow diet (Diete Standard 4 RF21 Mucedola S.r.i., Milan, Italy). All animal protocols followed the guidelines of and were approved by the Ethics Committee of the School of Medicine, University of Zagreb.

Chemicals

Pure APAP was a kind gift from the Belupo Pharmaceuticals and Cosmetics (Koprivnica, Croatia). Trichloroacetic acid, ETOH (96%), DMSO, PG, DMF, and phenobarbitone were purchased from Kemika, Zagreb, Croatia. Reduced L-glutathione, D-galactosamine (GalN), bovine serum albumin, and 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) were purchased from Sigma–Aldrich (St. Louis, Mo., USA).

APAP-induced liver injury

A total of 5 experiments were conducted. In all experiments mice were fasted overnight (for 16 h). The next morning, mice were randomly divided into groups (n = 6–8 animals per group) and received saline, DMSO, DMF, PG, or Tween 20. All solvents were diluted in saline to a final volume of 0.2 mL per animal and injected intraperitoneally at 0.6 mL/kg in the first experiment and 0.2 mL/kg in the second experiment. Thirty minutes later, APAP (i.p. at 300 mg/kg, in a volume of approximately 0.5 mL per animal), dissolved in warm saline (37 °C) under mild magnetic stirring, was administered. Six groups of animals received only vehicle (i.e., without APAP). Two types of control groups were included: mice that received saline before APAP administration and mice given saline alone.

The doses of 0.2 and 0.6 mL/kg were selected by searching the literature and manufacturers' data on the solubility of various substances, which were investigated previously for their effect on AILI, and solubility of APAP. It was found that effective doses of many substances, as well as APAP, can be dissolved with doses of solvents within this range. The data about solvents' toxicity were also examined; LD_{50} for DMSO, DMF, PG, ETOH, and Tween 20 in mice is reported to be 12.6, 6.2, 9.3, 4, and 2.4 mL/kg (i.p.), respectively (Bartsch et al. 1976). It was also recommended not to use doses higher than a quarter of LD_{50} for pharmacological and toxicological investigations, which gives 0.6 mL/kg as an upper limit for Tween 20. The preliminary toxicological study was also conducted for the dose of 0.6 mL/kg, and neither of the vehicles used caused toxicity in mice.

In a majority of published experiments, APAP and investigated substance were both injected intraperitoneally. To exclude the effect of the same administration route and possible interference of chemicals, we conducted the third experiment, which was almost identical to the first experiment except that APAP was now administered per os, by gastric lavage.

In the fourth experiment, saline or DMF (0.6 mL/kg, i.p) was injected 2 h before or 1, 2.5, and 4 h after APAP administration.

In the fifth experiment, mice were given phenobarbitone (300 mg/L) in drinking water for 7 days prior to APAP administration, to induce liver microsomal enzymes. DMSO (0.2 mL/kg, i.p.) or saline was injected 30 min before APAP administration.

In all experiments food was returned 4 h after APAP administration, and after an additional 3 h mice were anesthetized with Avertin; blood was collected by puncture of the medial eye angle with heparinized glass capillary tubes, and the livers were removed for analyses.

It is important to note that the doses of vehicles were adjusted to be equal in millilitres per kilogram (not in mg/kg or mol/kg). This was done deliberately, because the volume of the solvent is what is important when addition of a solvent is being considered.

Hepatic injury induced by GalN and lipopolysaccharide (LPS)

Mice were injected simultaneously with GalN (300 mg/kg, i.p.) and LPS (15 μg/kg, i.p.). Saline, DMSO, DMF, ETOH, PG, or Tween 20 was injected (0.6 mL/kg, i.p.) 30 min before GalN plus LPS treatment.

Evaluation of liver injury

Alanine aminotransferase (ALT) levels were determined from plasma by standard laboratory techniques in a clinical diagnostic laboratory. A portion of the liver was fixed in 10% neutral formalin, processed by histological techniques, stained with hematoxylin–eosin, and examined for morphological evidence of liver injury.

Total hepatic nonprotein sulfhydryl content determination

GSH level was assessed by measuring total hepatic nonprotein sulfhydrils, using the method of Ellman (Ellman 1959). A portion (approximately 100 mg) of liver was homogenized in Tris–HCl buffer (25 mmol/L, pH 7.4), and 0.1 mL of the homogenate was mixed with 0.2 mL of 5% trichloroacetic acid, followed by centrifugation at 860*g* for 10 min. Supernatant was mixed with DTNB. The absorbance was determined at 412 nm. The absorbance was compared with a standard curve prepared using 6 different GSH concentrations. From the rest of the homogenate, the protein concentrations were determined using the Bradford method, using bovine serum albumin as the standard (Bradford 1976), and GSH content was calculated in nanomoles per milligram of protein.

Measurement of phenobarbitone sleeping time

Influence on duration of phenobarbitone sleeping time was determined as an in vivo measure of liver microsomal enzyme activity (Piel et al. 1969; Chan et al. 2009). Mice (n = 4 per group) were injected with saline, DMSO, DMF, PG, ETOH, or Tween 20 (0.6 mL/kg), and after 30 min they received phenobarbitone (50 mg/kg, i.p.). The sleeping time was defined as the sleep-time period from the loss of the righting reflex to its complete recovery (Piel et al. 1969; Henderson et al. 2003; Chan et al. 2009).

Statistical analysis

Data are presented as means ± SE. Statistical significance was evaluated by one-way ANOVA followed by Dunnett's multiple comparison tests. Results were considered statistically significant at $p < 0.05$.

Table 1. Effects of DMSO, DMF, ETOH, PG, and Tween 20, given at a dose of 0.6 mL/kg, on APAP-induced liver injury.

Vehicle (0.6 mL/kg)	APAP (mg/kg)	ALT (U/L)	GSH (nmol/mg protein)
Saline	300	6747.5±2290.4	9.8±1.26
DMSO	300	178.6±96.9*	53.2±1.7*
DMF	300	135±47.7*	56.1±1.0*
ETOH	300	4633.8±2066.4	19.6±5.1
PG	300	441.4±317.8*	46.2±5.7*
Tween 20	300	5268.6±2512.6	9.4±2.5
Saline	—	20±1.5	64.9±0.5
DMSO	—	30.7±5.7	65.8±0.9
DMF	—	27±1.5	57.1±0.28†
ETOH	—	32.7±5.2	63.8±1.3
PG	—	28.3±4.4	65.1±0.7
Tween 20	—	37.3±7.1	64.5±2.4

Note: Mice were treated with DMSO, DMF, ETOH, PG, Tween 20, or saline intraperitoneally 30 min before APAP administration (300 mg/kg, i.p.) or only with DMSO, DMF, ETOH, PG, Tween 20, or saline. GSH and ALT levels were determined 7 h after APAP. Each value represents the mean ± SE of 6–8 mice. GSH content was measured as total hepatic nonprotein sulfhydryl content. *, significantly different from mice treated with saline before APAP administration (ANOVA followed by Dunnett's multiple comparison test, $p < 0.05$); †, significantly different from mice given only saline (ANOVA followed by Dunnett's multiple comparison test, $p < 0.05$). APAP, acetaminophen; ALT, alanine aminotransferase; GSH, reduced glutathione; DMSO, dimethylsulphoxide; DMF, dimethylformamide; ETOH, ethanol; PG, propylene glycol.

Results

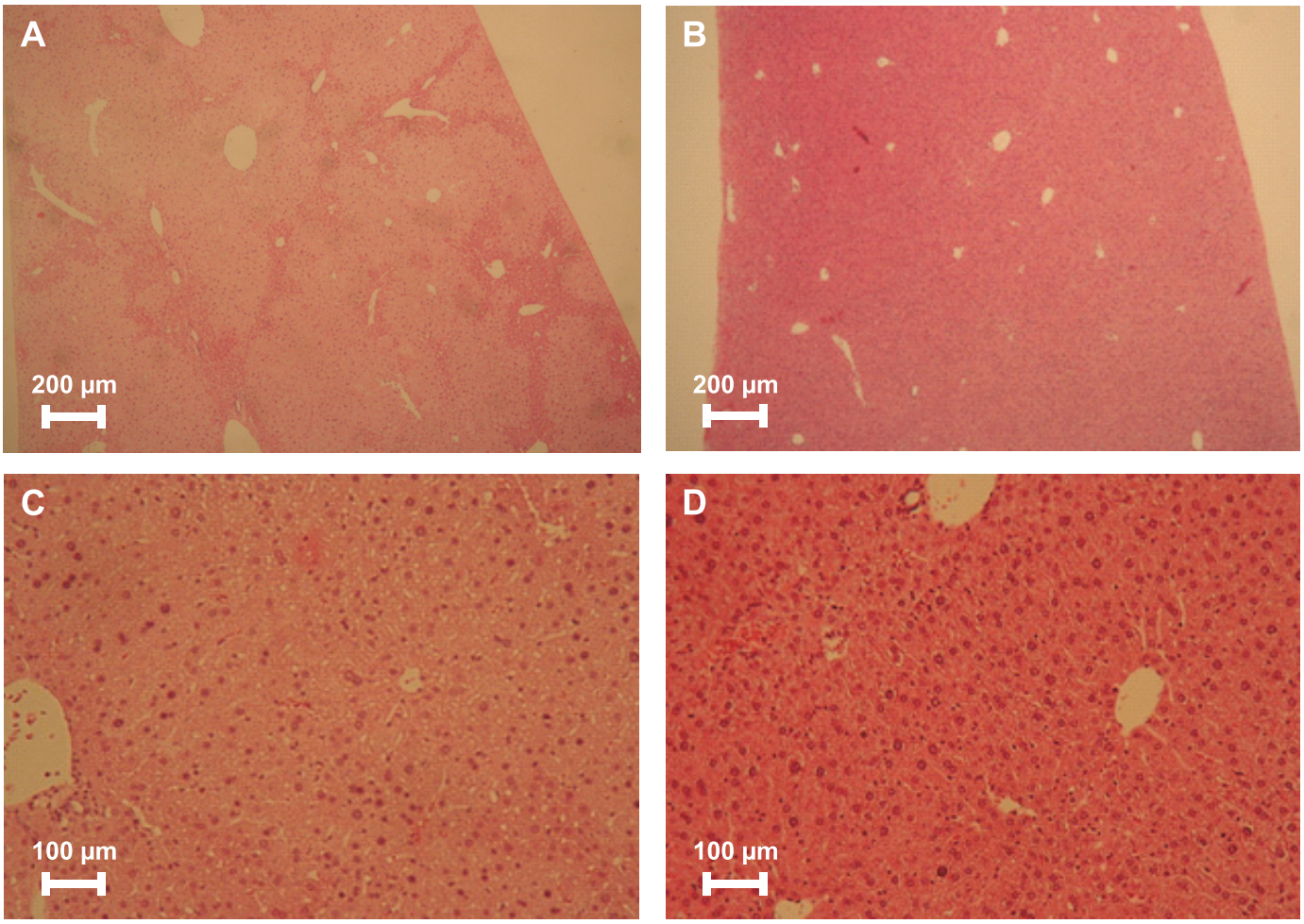
Effects of drug vehicles on APAP-induced liver injury

In the first experiment, drug vehicles were given 30 min before APAP administration (300 mg/kg, i.p.). As shown in Table 1, DMSO, DMF, and PG at a dose of 0.6 mL/kg almost completely abolished APAP toxicity; ALT levels were 37, 45, and 15 times lower, respectively, than in the control group (which received saline before APAP administration). ETOH only slightly reduced ALT levels, and Tween 20 had no influence. APAP depleted GSH from liver, and DMF, DMSO, and PG reduced this depletion. The protective effect of drug vehicles was clearly visible in histopathological analysis (Fig. 1); centrilobular necrosis, which was seen in mice given saline (or Tween 20) before APAP administration (Fig. 1A, 1C), was almost absent in mice given DMF (Fig. 1B, 1D) or DMSO before APAP administration.

When given alone (i.e., without APAP treatment), none of the vehicles had a significant effect on the ALT level; that is, the concentration of ALT in plasma was similar to that in plasma from mice given saline. However, DMF slightly reduced the GSH level in comparison to that in mice given saline, while other vehicles had no such effect (Table 1).

At a dose of 0.2 mL/kg (second experiment), DMF and DMSO were similarly effective; ALT levels were, respectively, 15 and 18 times lower than in the control group. They also significantly inhibited GSH depletion. At this dose, PG also reduced ALT concentration and blocked GSH depletion, but not significantly. ETOH and Tween 20 had no influence. DMF alone had no influence on GSH levels (Table 2).

Fig. 1. Effects of drug vehicles on histopathological changes in liver. Hematoxylin–eosin stained liver sections. (A, C) Typical liver sections (A: 100× original magnification; C: 200× original magnification) from a mouse treated with saline 30 min before APAP administration (300 mg/kg) showing extensive necrosis of centrilobular hepatocytes. The ALT level measured in the plasma of this mouse was 6480 U/L. Liver sections of mice treated with Tween 20 before APAP administration looked similar. (B, D) Typical liver sections (B: 100× original magnification; D: 200× original magnification) from a mouse treated with dimethylformamide (0.6 mL/kg) 30 min before APAP administration (300 mg/kg) showing essentially normal histology. The ALT level measured in the plasma of this mouse was 150 U/L. Liver sections of mice treated with PG or DMSO (0.6 mL/kg) before APAP treatment looked similar.



Protective effect of drug vehicles was not dependent on the administration route

In previous experiments, both vehicle and APAP were injected intraperitoneally, with short intervals between the 2 injections, which might cause physical or chemical interference of the agents. To exclude this, we conducted an experiment in which APAP was administered per os by gastric lavage. As shown in Fig. 2, the effect of solvents on concentration of ALT in plasma was very similar to that when APAP was given intraperitoneally (comparison of data in Table 1 and Fig. 2).

Protective effect of DMF was dependent on time of DMF application

To examine the effect of time, DMF was given at various time points before or after APAP administration. DMF completely blocked APAP toxicity if given 2 h before APAP administration; it was also effective if given 1 h after APAP administration, but ineffective 2.5 or 4 h after APAP (Fig. 3).

Phenobarbitone pretreatment had no influence on protective effect of DMSO

It is known that phenobarbitone induces microsomal enzymes and therefore stimulates formation of a toxic metabolite of APAP, NAPQI (Belardinelli et al. 2008). Mice were pretreated for 7 days with phenobarbitone in drinking water (300 mg/L) and then received DMSO (0.2 mL/kg, i.p.) or saline prior to APAP (300 mg/kg, i.p.). As shown in Fig. 4, DMSO was also highly protective in this model (Fig. 4).

DMF and DMSO prolonged phenobarbitone sleeping time

Sleeping time was measured in mice injected with saline or drug vehicles (0.6 mL/kg) 30 min before phenobarbitone administration (50 mg/kg, i.p.). DMSO and DMF significantly increased phenobarbitone sleeping time. PG pro-

Table 2. Effects of DMSO, DMF, ETOH, PG, and Tween 20, given at a dose of 0.2 mL/kg, on APAP-induced liver injury.

Vehicle (0.2 mL/kg)	APAP (mg/kg)	ALT (U/L)	GSH (nmol/mg protein)
Saline	300	8767±1663	9.7±0.9
DMSO	300	585±397**	44.9±1.7**
DMF	300	470±188**	44±2.9**
ETOH	300	8991±2048	12.1±4.5
PG	300	5830±1242	17.2±5.5
Tween 20	300	8774±1762	11±2.2
Saline	—	25.6±1.6	67.5±1.8
DMSO	—	29±1.3	65.6±0.5
DMF	—	34.6±3.5	65.7±0.7
ETOH	—	24.6±1.9	65.8±1.9
PG	—	20.3±0.2	65.3±1.8
Tween 20	—	29.6±3.3	64.1±2.1

Note: Mice were treated with DMSO, DMF, ETOH, PG, Tween 20, or saline intraperitoneally 30 min before APAP administration (300 mg/kg, i.p.) or only with DMSO, DMF, ETOH, PG, Tween 20, or saline. GSH and ALT levels were determined 7 h after APAP. Each value represents the mean ± SE of 6 mice. GSH content was measured as total hepatic nonprotein sulfhydryl content. **, significantly different from mice treated with saline before APAP administration (ANOVA followed by Dunnett's multiple comparison test, $p < 0.01$). APAP, acetaminophen; ALT, alanine aminotransferase; GSH, reduced glutathione; DMSO, dimethylsulphoxide; DMF, dimethylformamide; ETOH, ethanol; PG, propylene glycol.

longed it only slightly, while Tween 20 and ETOH had no influence (Table 3).

Effects of drug vehicles on liver injury induced by GalN and LPS

Treatment of mice with GalN and LPS highly increased plasma ALT levels (Fig. 5). None of the vehicles had a significant effect on the concentration of ALT, indicating that they are not protective against liver injury induced by LPS plus GalN.

Discussion

Although vehicles are generally considered relatively inert, they can induce biological effects that are often overlooked. AILI is shown to be very sensitive to bias caused by solvents (Jaeschke et al. 2006; Masson et al. 2008). Our results show that DMSO and DMF greatly suppress AILI even at small doses (0.2 mL/kg), PG is somewhat less protective, and it seems that doses of ETOH (0.2 mL/kg) and Tween 20 (0.2 and 0.6 mL/kg) have no effect. None of the vehicles appears to be hepatotoxic itself at the above-mentioned doses. The efficacy of vehicles in vivo was not dependent on the route of administration (Fig. 2); that is, DMSO, DMF, and PG were protective to a similar extent regardless of whether APAP was administered orally or intraperitoneally. The most probable means of protection is interference with the liver microsomal metabolism of APAP. This is supported with our findings that PG, DMSO, and DMF inhibited APAP-induced GSH depletion and prolonged phenobarbitone sleeping time. Yoon et al. proposed that DMSO inhibited microsomal formation of

Fig. 2. Effects of DMSO, DMF, ETOH, PG, and Tween 20 on concentration of ALT in plasma, when APAP was given orally. Mice were treated with DMSO, DMF, ETOH, PG, Tween 20, or saline (0.6 mL/kg, i.p.) 30 min before APAP administration (300 mg/kg, per os). ALT levels were determined 7 h after APAP administration. Each value represents the mean ± SE of 6 mice. ** and ***, significantly different from mice treated with saline before APAP administration (ANOVA followed by Dunnett's multiple comparison test; $p < 0.01$ and $p < 0.001$, respectively). DMSO, dimethylsulphoxide; DMF, dimethylformamide; ETOH, ethanol; PG, propylene glycol; ALT, alanine aminotransferase; APAP, acetaminophen; GSH, reduced glutathione.

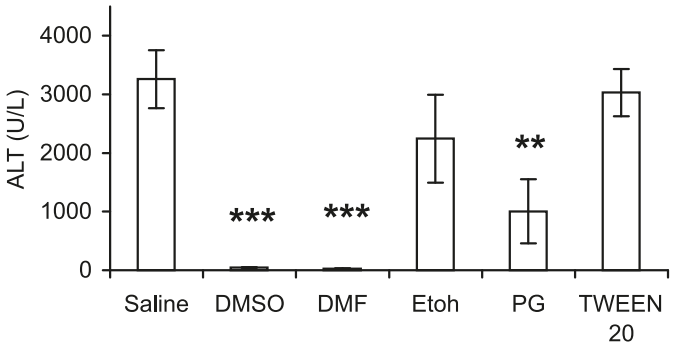
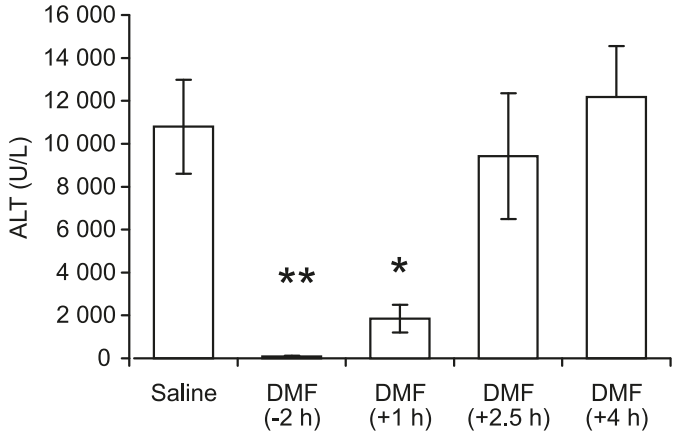


Fig. 3. Effect of varying time of DMF application on ALT plasma levels. Mice were treated with saline or DMF (0.6 mL/kg, i.p.) at indicated times prior to or after APAP (300 mg/kg, i.p.). ALT levels were determined 7 h after APAP. Each value represents the mean ± SE of 6 or 7 mice. * and **, significantly different from mice treated with saline and APAP (ANOVA followed by Dunnett's multiple comparison test; $p < 0.05$ and $p < 0.01$, respectively). DMF, dimethylformamide; ALT, alanine aminotransferase; APAP, acetaminophen.



NAPQI in a competitive manner (Yoon et al. 2006). Therefore, we hypothesized that an experimental model in which microsomal enzymes are induced prior to APAP administration could be less sensitive to DMSO. However, our results showed that mice that received phenobarbitone for 7 days in drinking water (Guarner et al. 1988; Belardinelli et al. 2008) were equally protected by DMSO. Aside from its effect on APAP metabolism, each of these vehicles can change several other parameters that play a role in AILI. For example, DMSO shows antioxidative activity (Jeffery et al. 1988; Dunphy et al. 2007), and several antioxidants

Fig. 4. Effect of DMSO on ALT levels in mice pretreated with phenobarbitone. Mice were pretreated for 7 days with phenobarbitone in drinking water (300 mg/L) and then treated with DMSO (0.2 mL/kg, i.p.) or saline prior to APAP (300 mg/kg, i.p.). ALT levels were determined 7 h after APAP administration. Each value represents the mean ± SE of 8 mice. **, significantly different from mice treated with saline before APAP administration (Student's *t* test, $p < 0.001$). DMSO, dimethylsulphoxide; ALT, alanine aminotransferase; APAP, acetaminophen.

Table 3. Effects of DMSO, DMF, ETOH, PG, and Tween 20 (0.6 mL/kg) on phenobarbitone sleeping time.

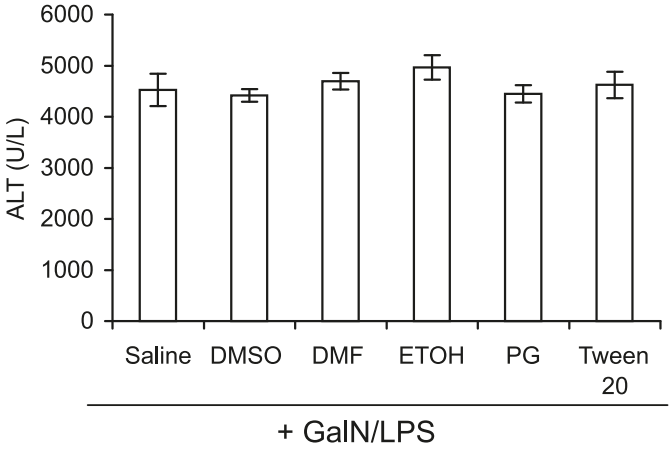
Vehicle (0.6 mL/kg)	Sleeping time (min)
Saline	28.5±5.8
DMSO	60±5*
DMF	55.5±5.4*
ETOH	27±7.2
PG	34.5±6.3
Tween 20	26.7±3.5

Note: Mice were treated with DMSO, DMF, ETOH, PG, Tween 20, or saline intraperitoneally 30 min prior to phenobarbitone treatment. Each value represents the mean ± SE of 4 mice. *, significantly different from mice treated with saline (ANOVA followed by Dunnett's multiple comparison test, $p < 0.05$). DMSO, dimethylsulphoxide; DMF, dimethylformamide; ETOH, ethanol; PG, propylene glycol.

protect against AILI (Marotta et al. 2009; Reisman et al. 2009). It also demonstrated adjuvant-like actions on the immune system (Kunze 1975; Yamasaki et al. 1988), which were the cause of bias in the previously mentioned natural killer cell investigation.

Because DMSO is so frequently a source of bias, we supposed that DMF might be a useful alternative (some of the substances that we intend to investigate in the future can be dissolved in both DMSO and DMF). However, DMF showed a protective effect similar to that of DMSO. There are no reports about the effect of DMF on oxidative stress or the immunological system. On the other hand, DMF shows anticoagulant-like effects (Imbriani et al. 1986), and some anticoagulants are able to protect from AILI (Ganey et al. 2007). Since this is the first report about the protective effect of DMF, we further investigated the effect of time of administration. It showed a typical pattern for substances that interfere with APAP metabolism, a very strong protection if given before or 1 h after APAP administration, but no protection if given 2.5 h after APAP administration,

Fig. 5. Effects of DMSO, DMF, ETOH, PG, and Tween 20 on ALT levels in a GalN plus LPS model of liver injury. Mice were treated with DMSO, DMF, ETOH, PG, Tween 20, or saline (0.6 mL/kg, i.p.) 30 min prior to GalN plus LPS administration. ALT levels were determined 7 h after GalN plus LPS administration. Each value represents the mean ± SE of 6 mice. DMSO, dimethylsulphoxide; DMF, dimethylformamide; ETOH, ethanol; PG, propylene glycol; ALT, alanine aminotransferase; GalN, D-galactosamine; LPS, lipopolysaccharide.



when biotransformation of APAP is essentially complete. DMF inhibits CYP2E1, most probably by suicidal enzyme inactivation (Tolando et al. 2001).

The protective effects of PG are well known; however, these were shown in experiments in which high doses of PG (2–4 mL/kg) were used (Hughes et al. 1991; Thomsen et al. 1995). Our results demonstrated that even small doses (0.2 mL/kg, and particularly 0.6 mL/kg) can suppress AILI. For ETOH, it is generally accepted that its chronic consumption increases AILI, but acute administration, together with or shortly before APAP administration, suppresses AILI (Prescott 2000). The dose of 0.2 mL/kg ETOH was not protective, and the dose of 0.6 mL/kg only slightly decreased ALT levels. PG and ETOH can reduce inflammatory responses and, opposite to the effect of DMSO, inhibit natural killer activity (Denning and Webster 1987; Oshiro et al. 1990; Chen et al. 2006; Pan et al. 2006).

Detergents such as Tween 20 and Tween 80 are also sometimes added to increase the solubility of lipophilic drugs in AILI experiments. It appears that Tween 20 at doses of 0.2 and 0.6 mL/kg has no influence on AILI or GSH content in the liver. There are no reports about its influence on the immunological response, oxidative stress, CYP2E1 activity, or coagulation system.

In another common model of liver injury, GalN plus LPS, none of the vehicles was protective or harmful. Interestingly, there are reports that show that DMSO is protective against GalN alone in rats. However, in those experiments the dose of DMSO was much higher (2.5 mL/kg) (Iida et al. 2007).

To avoid artifacts in AILI investigations it is advisable to use, when possible, pure saline or distilled water as a drug vehicle without adding any organic solvent or detergent. This is possible more frequently than is actually done. For example, 300 mg/kg of APAP, dissolved in warm saline under mild magnetic stirring, can be safely administered in

a volume of approximately 0.5 mL per mice, and this is more than enough to induce AILI. Because PG and DMSO suppress AILI when used as vehicles for APAP, the dose of APAP must be increased to 500 or even 750 mg/kg (Oz and Chen 2008) to cause similar liver damage. The result is an unnecessary increase in the APAP dose and application of a more biologically different organic solvent. Liu et al. reported that 300 mg/kg APAP is not enough to induce AILI in C57BL/6 mice and concluded that this strain was more resistant to APAP (Liu et al. 2004). Apparently, this was because DMSO was added to the APAP vehicle. In our experience, the 300 mg/kg dose of APAP induces severe hepatitis in CBA or BALB/c mice and as shown by Harill et al. in many other mouse strains (Harrill et al. 2009). When substances with low solubility in water are investigated for their effect on AILI, the control groups should be formed more carefully. Typical investigations include a treatment group, in which the substance of interest is dissolved in an organic vehicle, and the control group, in which only the vehicle is used. It would be good to include, at least in the first experiment, one more control group, in which only pure saline or distilled water is used, to better understand the effect of the vehicle. The dose of solvent or detergent added to the vehicle should be as small as possible and explicitly noted. It is probably better to use vehicles that interfere less with APAP toxicity. This means that Tween 20 and ETOH should be considered first, and then PG. The usage of DMSO or DMF should be limited to occasions when it is inevitable. Alternatively, some water-insoluble substances become soluble if the pH of the saline solution is changed. If some general hepatoprotective mechanism is investigated, it would be helpful to see whether it also protects against the GalN plus LPS model of hepatotoxicity, since this model appears to be more resistant to vehicle usage.

Finally, in the effort to avoid a possible experimental artifact with water-insoluble drugs, it is necessary to reduce the amount of injected biologically active organic solvent (or reduce its concentration in in vitro experiments) to the minimum dose possible, use an organic solvent with a smaller biological effect, or try to dissolve the substance in aqueous solvent with changed pH. Nonetheless, it is very important to include control groups in which the effect of the drug vehicle alone is tested.

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