

The Metabolism and Liver Toxicity of N,N-dimethylformamide in the Isolated Perfused Rat Liver

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N,N-dimethylformamide (DMF) is metabolized by the microsomal cytochrome p-450 into mainly N-hydroxymethyl-N-methylformamide (HMMF), which further breaks down to N-methylformamide (NMF).

However, the detailed mechanism of its toxicity remains unclear.

We investigated the metabolism and the toxicity of DMF using the isolated perfused liver model. DMF was added to the recirculating perfusate of the isolated perfused rat liver at concentrations of 0, 10 and 25 mM. Samples were collected from the inferior vena cava at 0, 30, 45, 60, 75, and 90 minutes following addition of the DMF. The metabolites of DMF were analyzed using Gas-chromatography (GC). The changes in the rate of oxygen consumption by the DMF were monitored during perfusion. The enzyme activities (aspartic aminotransferase:AST, alanine aminotransferase:ALT, and lactic dehydrogenase:LDH) in the perfusate were monitored to see if DMF caused hepatotoxicity.

As the perfusion progressed, the DMF concentration in the perfusate decreased, but the level of NMF increased to a maximum of 1.16 mM. The rate of oxygen consumption increased at DMF concentrations of 10 mM and 25 mM. However, when a known inhibitor of cytochrome p-450, SKF 525A (300 μ M), was used to pretreat the perfusate prior to the addition of the DMF, the rate of oxygen consumption was significantly inhibited, indicating the cytochrome p-450 system was responsible for the conversion of DMF to NMF. On addition of the DMF, the activities of the enzymes AST, ALT and LDH

were significantly increased a time and dose dependent manner. However, following pretreatment with SKF 525A, their releases were inhibited.

Key Words: NN dimethylformamide, N methylformamide, isolated perfused rat liver, cytochrome p-450, Hepatotoxicity

INTRODUCTION

N,N-dimethylformamide (DMF) is a colorless, or slightly yellow solvent, with the molecular formula $(\text{CH}_3)_2\text{NCHO}$, and a relative molecular mass of 73.09. It is widely used as an industrial solvent due to its solubility in both aqueous and lipid media, and is especially useful in manufacturing synthetic leather. As DMF is readily absorbed by inhalation and dermal contact,¹ with the primary effects of respiratory failure and an atonic muscle state, occurring at high dosages.^{2,3} Occupational exposure to DMF causes hepatic damage, pancreatic disorders, and intolerance to alcohol.⁴⁻⁸ Ducatmann et al.⁹ reported the detection of carcinomas of the testes in several workers exposed to DMF. Therefore, American Conference of Governmental Industrial Hygienists (ACGIH) and Occupational Safety and Health Administration (OSHA) have recommended that the threshold limited value of DMF should be 10ppm (atmospheric threshold), 30 mg/m³ (skin). A biological exposure indices (BEI) limit for the concentration of N-methylformamide (NMF) in urine of 20 mg/l.¹⁰

A growing body of research has focused on the metabolism of DMF. For example, Scailteur et al.¹¹ demonstrated that N-hydroxymethyl- N-methyl-

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formamide (HMMF) was the main metabolite of DMF (50% of absorbed dose), which broke down to NMF (4% of absorbed dose) from gas chromatographic (GC) analysis. Barnes and Ranta¹² reported that the isolation and identification of N-methylformamide as a metabolite in rat urine following the addition of DMF. Brindley et al.¹³ also identified the urinary metabolite as HMMF, which on the basis of GC analysis was previously identified as NMF. The confusion arose because HMMF is stable in aqueous solution, but is thermally degraded to NMF on the gas chromatography column. Mraz et al.⁶ compared the three DMF metabolites, HMMF, NMF, and N-acetyl-(N-methylcarbonyl) cysteine in humans and rodents. N-acetyl-(N-methylcarbonyl) cysteine (AMCC) is a major metabolite of DMF in humans, but it is only a minor rodent metabolite, with the metabolic mechanism of DMF remaining unclear. A few researchers have reported that DMF is N-methylated by microsomal enzymes in the liver, and a cytochrome P450 mono-oxygenase has been shown to play a pivotal role in the biotransformation of DMF.¹⁴⁻¹⁶

DMF hepatotoxicity has been reported in experimental animals in laboratory studies.¹⁷ Sakai et al.¹⁸ demonstrated that the workers exposed to DMF showed abnormal liver function values. A study by Massman¹⁹ found the LD₅₀ of DMF to be 3500 mg/kg. Recent studies have focused more on the toxicity and metabolism of DMF, HMMF, and NMF in order to gain a better understanding of the underlying mechanism.

In our experiments, we evaluated both the metabolism and hepatotoxicity of DMF using isolated perfused rat liver. Since Bernard first reported the use of the isolated perfused rat liver, the model has been a valuable and commonly used tool for exploring the physiology and pathophysiology of the liver.²⁰ Without a doubt, the isolated perfused rat liver model is an important experimental model despite the availability of more modern techniques (e.g., liver slice, isolated and cultured cells, and isolated organelles) for the evaluation of hepatic function. Its popularity is due to, in contrast to *in vivo* models such as the bile fistula rat, the fact it allows repeated sampling of the perfusate, easy exposure of the liver to different concentrations of test substances, and is

amenable to alterations in temperature that would not be tolerated *in vivo*. Furthermore, the experiments can be performed independently without the influence of other organ systems, plasma constituents, and neural-hormonal effects.²¹ In contrast to other *in vitro* models, such as isolated and cultured hepatocytes, the isolated organelles, hepatic architecture, cell polarity and bile flow are preserved.

The purpose of this study is to elucidate the change in concentration of NMF, a metabolite of DMF, and the hepatotoxicity of DMF in the isolated perfused rat liver

MATERIALS AND METHODS

Materials

Chemicals

N, N-dimethylformamide (Merck, New Jersey, USA) and N-methylformamide (Fluka, Buchs, Switzerland), both 99% pure, were used in this experiment. The methanol used to mix the sample, and for the GC analyses on the Gas Chromatograph (GC)(Varian, Walnut Creek, CA, USA) was 99% pure.

Animals

Adult male Sprague-Dawley rats, weighing between 350g and 350g, were acclimatized for 10 weeks before starting experiment. The animals were allowed free access to food (commercial rat chow) and water.

Method

Analytical method

Samples (perfusate) were collected from the inferior vena cava at 0, 30, 45, 60, 75, and 90 minutes following the addition of DMF. After 1 ml of the standard solution and sample were added to 1ml of methanol, the mixture was centrifuged at 1000 × g. GC analysis was performed with 1 µl of the supernatant. The GC was equipped with FID and a Quadrex Carbowax 20 M column (15 m long, 0.53 mm ID, 3 µm film thickness). The oven temperature was programmed from 60°C to 220°C at 30°C/min. The injector and detector tempera-

ture were kept at 230°C. The helium carrier gas was used with a flow rate of 10 ml/min. The GC conditions are detailed in Table 1.

Calibration graph and calculation

Calibration graphs were constructed from the area under the curve obtained from standard solutions of DMF and NMF versus the concentration, after applying least-squared analyses.

Isolated perfused rat liver model

Isolation of liver

The rat was anesthetized with pentobarbital sodium (40 mg/kg) via the intraperitoneum. The abdomen was opened through midline and midtransverse incisions, and the inferior vena cava and portal vein exposed. After ligating the abdominal vena cava above the renal vein, a PE-260 polyethylene catheter was inserted into the portal vein and tied in place. The thorax was then opened by a transverse incision just above, and along the line of the diaphragm and by a longitudinal cephalad incision. A polyethylene catheter was inserted and secured in the thoracic vena cava via penetration of the right atrium. The liver was rapidly excised, transferred onto the liver platform, and placed in the perfusion chamber (Fig. 1).

Perfusate

We used Krebs-Ringer bicarbonate buffer (KRB) as the perfusion medium. The solution contained (in mM) NaCl 117, KCl 4.7, CaCl₂ 1.91, KH₂PO₄ 1.19, MgSO₄ 1.44 and NaHCO₃ 24.8. The KRB was saturated with an O₂/CO₂ (95:5 V/V) gas mixture, and the pH adjusted to 7.4 at 36°C by NaOH and HCl (Table 2).

Measurement of perfusion flow

The changes in the flow in the portal vein under

the constant perfusion pressure (28cmH₂O) were measured. After a 30-min stabilization period, the initial perfusion flow was 50.5 ± 2.45 ml/min. The perfusion rate was measured by a calibrated spherical float flowmeter (Gilmont flowmeter; barnant, Barrington, Illinois, USA). A Gilmont

Table 2. Components of Krebs-Ringer Bicarbonate Buffer Solutions

Component	Concentration (mM)
NaCl	117
KCl	4.7
CaCl ₂	1.9
KH ₂ PO ₄	1.2
NaHCO ₃	24.8
MgSO ₄	1.4
Glucose	5.5

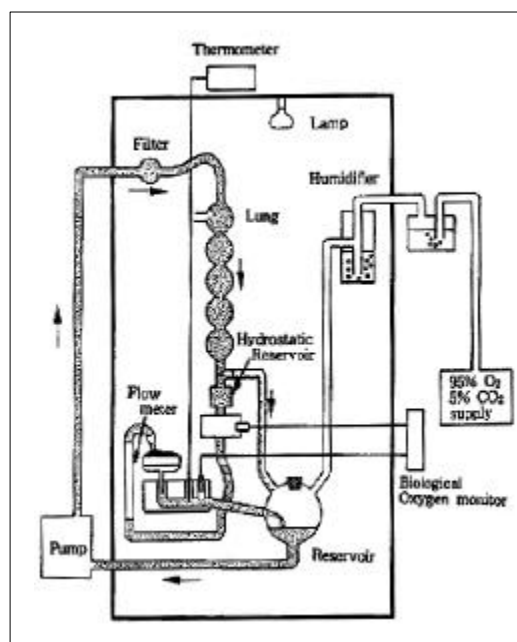


Fig. 1. Isolated perfused rat liver model.

Table 1. Gas Chromatography Conditions for the Detection of Dimethylformamide Metabolite

Description	Conditions
Column	Ø 0.53 mm × L 15 m, Quardrex Carbowax 20 M
Injector	split/splitless (Initial 0.5 min; splitless, and then split)
Gas	He 10 ml/min (40°C)
Temperature	detector: 230°C, injector: 230°C column: 60°C(1 min) 30°C/min, 120°C(1 min) 20°C/min, 220°C(1 min)

Flowmeter F-1300 (accuracy, $\pm 2\%$) was used for the measurement of the portal vein perfusion flow. The real perfusion flow rate (ml/min) was calculated from the scale of the flowmeter via a calibration chart.

Measurement of oxygen consumption

The oxygen tension was measured using a Biologic Oxygen Monitor (YSI, Yellow Springs, Ohio, USA). The oxygen probe was a Clark type polarographic electrode. Two oxygen probes were established- one at the site entering into the liver, the other at the site leaving the liver. O_{input} and O_{output} represented the oxygen tension differences between the two probes, which indicated the oxygen uptake by the liver, and was measured at intervals of 30 seconds. Oxygen consumption rates were calculated from the oxygen uptake and the perfusion flow as follows:

$$\begin{aligned} \text{Amount of oxygen in the solution: } SO_2 (\mu\text{l/ml}) \\ &= \alpha \times PO_2 (\alpha: 0.024 \text{ ml } O_2/\text{ml of water}/ \\ &\quad 760 \text{ mmHg at } 37^\circ\text{C}) \\ &= 0.024 \text{ ml } O_2/\text{ml of solution}/760 \text{ mmHg} \\ &\quad \times (760 \times 95/100) \text{ mmHg} \\ &= 0.00228 \text{ ml } O_2/\text{ml of solution} (=22.8 \mu\text{l} \\ &\quad O_2/\text{ml of solution}) \end{aligned}$$

$$\begin{aligned} \text{Oxygen Uptake by the liver; } (\mu\text{l/min}) \\ &= \text{Oxygen amount in solution} \times \text{Flow} \\ &\quad \text{rate} \times \text{Difference in saturation } \%/100 \\ \text{Oxygen consumption rate: } (\mu\text{l } O_2/\text{min/gm}) \\ &= \text{Oxygen uptake by the liver} \times (1/\text{Liver} \\ &\quad \text{weight}) (\text{gm}) \end{aligned}$$

Enzyme assay

The enzyme activities (Alanine aminotransferase, Aspartic aminotransferase, Lactic dehydrogenase) in the perfusate were measured to find if the DMF caused hepatotoxicity. The enzyme assays were performed in the perfusate with a BM/Hitachi 747 at 37°C .^{22,23}

Statistical analysis

The results are presented as the mean \pm standard deviation. The Kruskal-Wallis one-way ANOVA was used to analyze the effect of DMF toxicity.

RESULTS

Concentrations of metabolite

Controls (i. e. 0), 10, and 25 mM of DMF were added to the recirculating perfusate of the isolated perfused rat liver. Samples were collected from the inferior vena cava at 0, 30, 45, 60, 75, and 90 minutes following the addition of the DMF. The metabolites were analyzed by the GC (Fig. 2). It was found that the DMF concentration decreased with time, and a level of NMF of 1.16 mM was detected (Table 3).

Effect of DMF on perfusion flow

The effect of DMF on the changes in flow in the portal vein, under the constant perfusion pressure (28 cmH₂O), was measured after a 30 min stabilization period. As shown in Fig. 3, the DMF decreased the flow in proportion to its concentration, but this difference was not statistically significant.

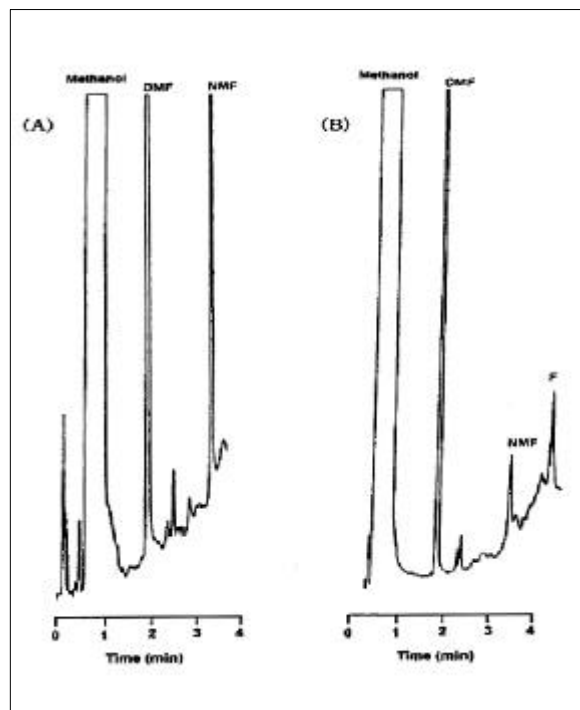
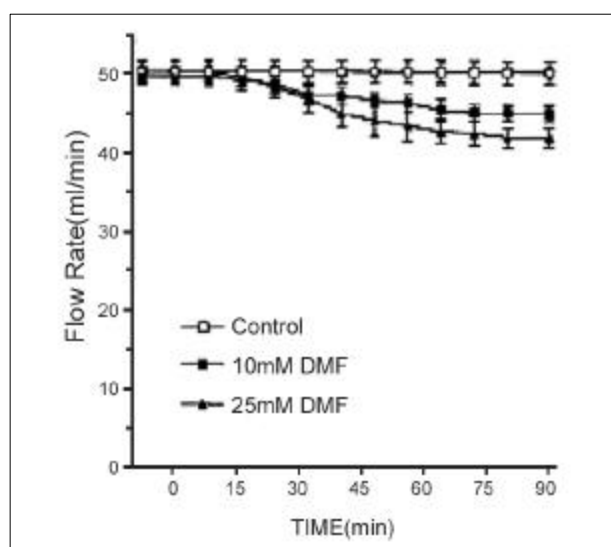
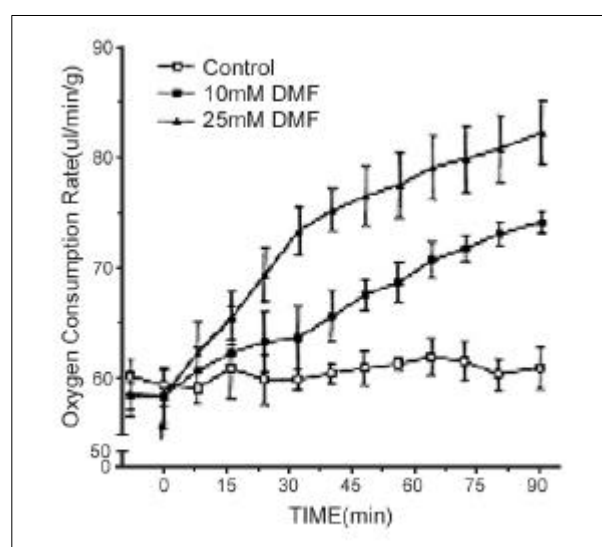


Fig. 2. Gas chromatograms of DMF standard solution and DMF in perfusate. (A) Gas chromatogram of DMF standard solution. (B) Gas chromatogram of DMF in perfusate.

Table 3. Concentration of DMF and NMF in Perfusate with Time

Time (min)	DMF	NMF
0	24.98 ± 0.04	-
30	16.09 ± 0.02	0.13 ± 0.01
45	13.41 ± 0.16	0.26 ± 0.02
60	11.63 ± 0.03	0.39 ± 0.05
75	10.36 ± 0.12	0.80 ± 0.04
90	9.34 ± 0.09	1.16 ± 0.11

**Fig. 3.** Effect of DMF on perfusion flow in the portal vein.**Fig. 4.** Effect of DMF on oxygen consumption rate with time.

Effect of DMF on oxygen consumption in the perfused liver

The oxygen consumption of the perfused liver was calculated from the perfusion flow and oxygen extraction measurements. Fig. 4 represents the rate of oxygen consumption with various dosages of DMF. The rate of oxygen consumption in the control group did not change due to the constant perfusion flow and oxygen extraction during the perfusion period. Although the DMF decreased the perfusion flow, the rise of oxygen extraction following the addition of the DMF was greater than the reduction in the perfusion flow, such that the rate of oxygen consumption was increased in a dose dependent manner ($p < 0.05$). However, when a known inhibitor of cytochrome p-450, SKF 525A (300 μ M), was used to pretreat the perfusate

prior to the addition of the DMF, the rate of oxygen consumption was significantly inhibited (Fig. 5).

Hepatotoxicity of DMF

The activities of AST, ALT and LDH in the perfusate were measured to compare the hepatotoxic effect of the DMF at concentrations of 10, and 25 mM with that in the controls. In the controls, only small amount of changes were found in the perfusate. With the addition of the DMF, the activities of AST, ALT and LDH significantly increased in a time and dosage dependent manner (Fig. 6-8). However, the pretreatment of the perfused liver with SKF 525A resulted in the gradual inhibition of AST, ALT and LDH release (Fig. 9-11).

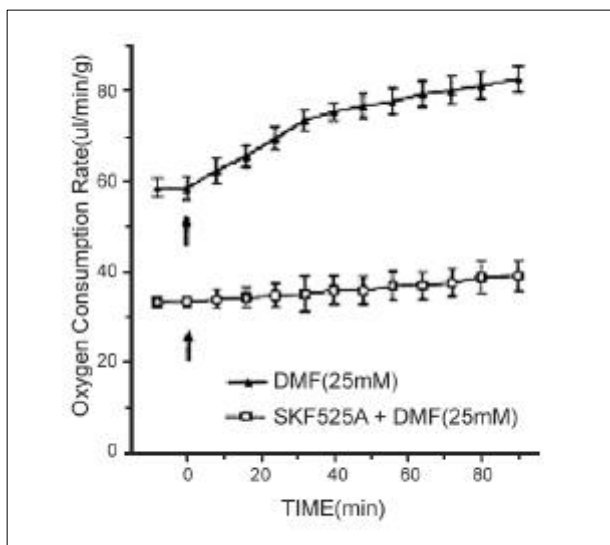


Fig. 5. Effect of DMF on oxygen consumption rate after pretreatment with SKF 525A (300 μ M).

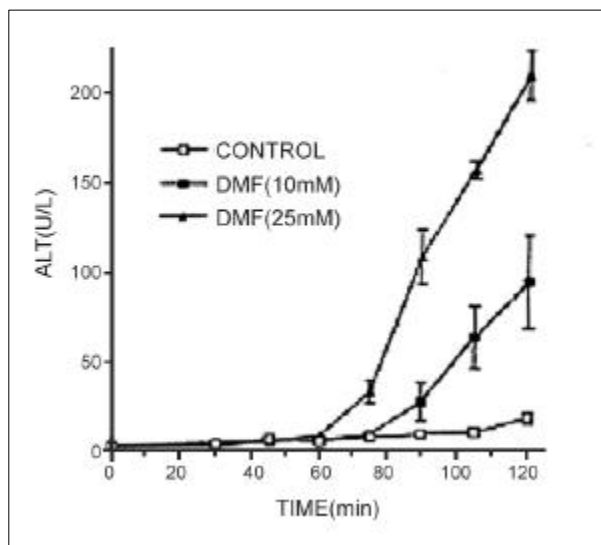


Fig. 7. Perfusate ALT concentration with time after administration DMF.

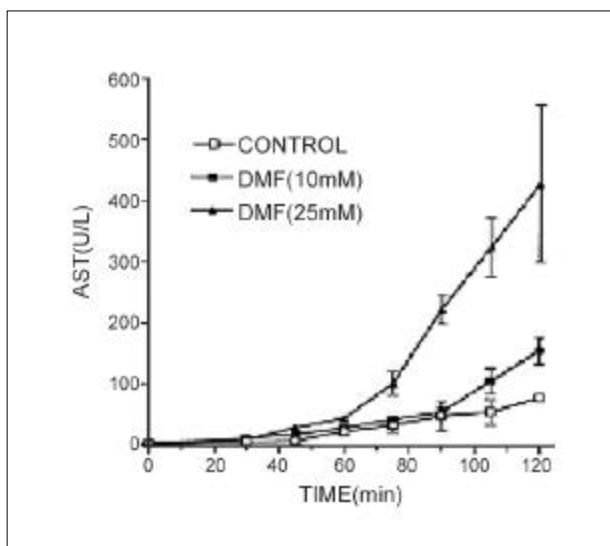


Fig. 6. Perfusate AST concentration with time after administration DMF.

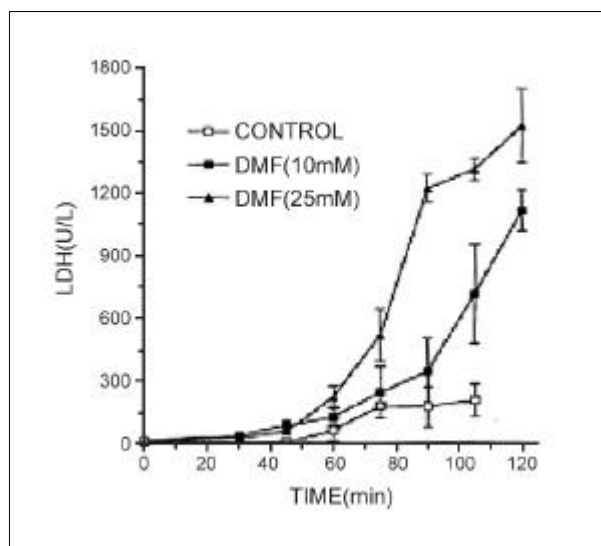


Fig. 8. Perfusate LDH concentration with time after administration DMF.

DISCUSSION

DMF is metabolized in the liver and excreted in the urine. The main metabolite used for its biological monitoring is urinary NMF. With the recent unequivocally identification of N-acetyl-(N-methylcarbonyl) cysteine (AMCC) in human urine following exposure to DMF, several studies have suggested AMCC to be a suitable biomarker for

assessing DMF,^{1,18,24} but the metabolic pathway of DMF is still unclear. It has been reported in some investigations that DMF appears to metabolize to NMF.^{12,25} This transformation was thought to be responsible for the liver-damaging properties of DMF, as NMF is a powerful hepatotoxicant in rodents.^{6,26} However, the urinary metabolite of DMF, which was previously identified as NMF on the basis of the GC analysis, but has recently been

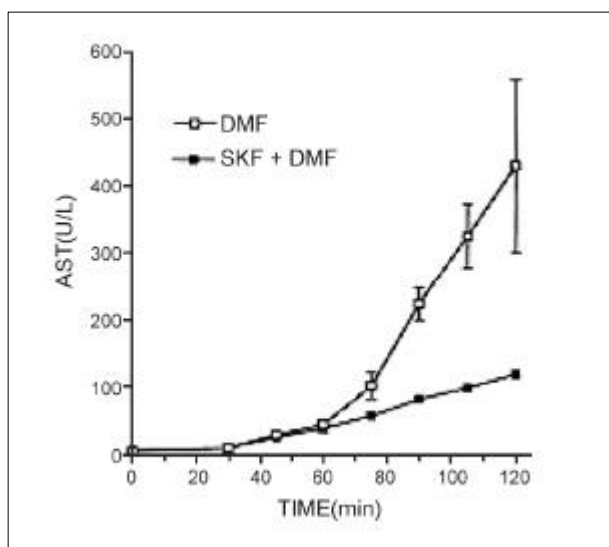


Fig. 9. Perfusate AST concentration with time after pretreatment with SKF 525A (300 μ M).

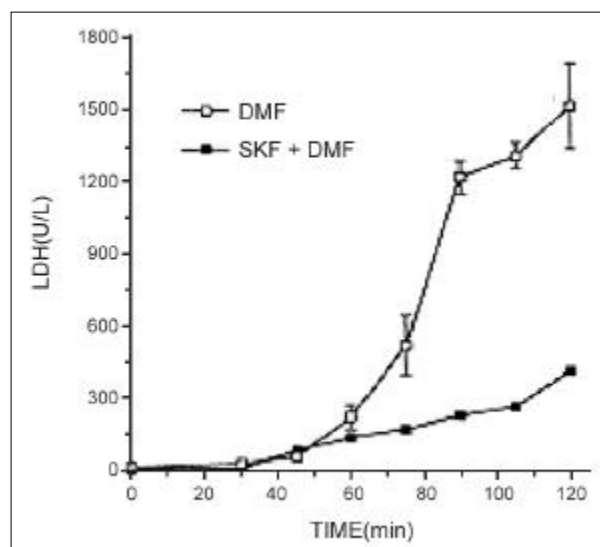


Fig. 11. Perfusate LDH concentration with time after pretreatment with SKF 525A (300 μ M).

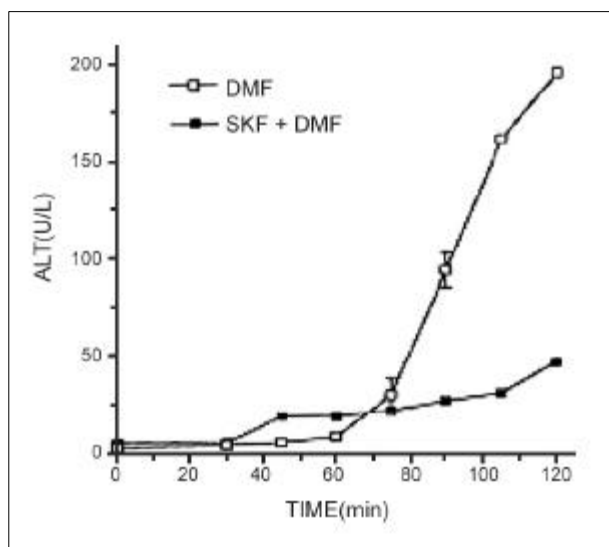


Fig. 10. Perfusate ALT concentration with time after pretreatment with SKF 525A (300 μ M).

shown to be HMMF, which is stable in aqueous solution, but thermally degrades to NMF on the GC column. Although the biological properties and metabolic fate of HMMF are unknown, some studies on its analog N-(hydroxymethyl) formamide (HMF) suggest it is not hepatotoxic.²⁷

In addition, another metabolic pathway has been documented in humans. The metabolism of DMF and NMF to AMCC proceeds via a previ-

ously unidentified reactive metabolite, perhaps methylisocyanate (MIC), which in turn reacts with glutathione (GSH). The GSH conjugate is biotransformed further to the mercaptopuric acid AMCC.^{1,28,29} There is a difference between the metabolisms of DMF in humans compared to that in rodents. In humans, 14.5% of a dose absorbed by the inhalation of DMF was found as AMCC, whereas the amount measured in mice, or rats following ip administration was only one third, or less of this percentage.⁶ These findings suggest the biotransformation of DMF to AMCC is an important pathway in humans but not in rodents, so we restricted our study to the metabolism of DMF to NMF an animal population was used.

To maintain a stabilized condition for the GC detection, we kept the injection port temperature at 230°C, this being higher than the evaporation temperature of NMF before the analysis. In our results, the NMF was detected at 30 min following the addition of 25 mM of DMF, NMF concentration in the perfusate increased with time, reaching a maximum of 1.16 mM, this being 4.6% of the DMF concentration at 90 min. However, Mraz et al.⁶, and Bulcke et al.³⁰ demonstrated variations in the NMF concentration between 8.4% and 47.3% of the DMF dose. This discrepancy in the result might be due to the use of the isolated perfused liver model, with a life-span of approximately 2

hours under usual condition, or to the use of different GC conditions used in its detection, as suggested in the work of Lareo et al.²⁴

The metabolite of DMF is produced via its enzymatic oxidation in the liver, in which cytochrome P-450 has been shown to play a pivotal role. Imazu et al.¹⁴ investigated the effects of repeated exposure to DMF on the hepatic microsomal monooxygenase system. Mraz et al.¹⁵ reported that monospecific antibody against rat liver P450 inhibited the metabolism of DMF. Thruman and Scholz³¹ measured the redox change of cytochrome p-450 in perfused rat liver by an increased oxygen uptake following the addition of aminophyrin.

In our experiment, the rate of oxygen consumption was monitored in a perfused liver from control and DMF treated groups in order to measure the change in cytochrome p-450. Our results indicate the rate of oxygen consumption was unchanged in the control group, but at 10 and 25 mM DMF was increased because the rate of oxygen extraction was higher than the decrease in the perfusion flow. With the addition of DMF following pretreatment with SKF 525A (300 μ M), the rate of oxygen consumption was inhibited. Our findings suggest that DMF may be metabolized to NMF in the liver, and cytochrome P-450 monooxygenase plays a role in the biotransformation of NMF, which is consistent with other studies.³

Therefore, the time course of DMF toxicity, in relation to NMF formation, is compatible with the hypothesis that the hepatotoxicity of DMF is mediated by NMF. Further studies with *in vivo* experiments are still required, and should be performed through a toxicological approach.

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