

## Protective Effect of Gabexate Mesilate (FOY) against Pancreatic Injuries Induced by Ethanol in Rats

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### Abstract

Four-hour intravenous ethanol infusion at two doses of 0.5 and 1.0 g/kg · hr caused mild, but significant, rises in serum amylase and pancreatic water content as well as pancreatic histological changes such as interstitial edema in rats. These doses of ethanol also caused an impaired pancreatic adenylate energy charge levels and increased pancreatic mitochondrial fragility. The dose of 0.2 g/kg · hr caused only marginal changes in these parameters. Moreover, gabexate mesilate (FOY) at the dose of 20 mg/kg · hr inhibited almost completely all these pancreatic injuries induced by ethanol, exerting significant protective effects. These results suggest that impaired pancreatic energy metabolism and increased mitochondrial fragility seem to play an important role in the pathogenesis of ethanol-induced pancreatic injuries, and that some unknown protease activity, which can be inhibited by FOY, also seems to play an important role. Finally, FOY seems to be useful in protecting the exocrine pancreas in the alcoholic patients.

Excessive intake of ethanol often precedes the development of both acute and chronic pancreatitis, and pancreatitis occurs more commonly in alcoholics than in the general population<sup>1</sup>. Thus, alcohol has been reported to be one etiological factor in the pathogenesis of human pancreatitis<sup>2,3</sup>. However, little is known about the mechanism whereby alcohol induces pancreatic acinar cell injuries. Moreover, there have been few reports regarding the effect of ethanol on pancreatic adenylate energy metabolism.

Recently, we have reported the important role of subcellular organellar fragility in the triggering of pancreatic injuries in other models of pancreatitis such as secretagogue-induced<sup>4,5</sup> and pancreatic duct obstruction<sup>6</sup>.

In this study, we evaluated the effect of ethanol administration at various doses on the exocrine pancreas from several parameters including pancreatic adenylate energy charge levels and subcellular organellar fragility as well as the protective effect of a synthetic protease inhibitor, gabexate mesilate (FOY) [ethyl-4-(6-guanidino hexanyloxy benzoate) methanesulfonate; M.W. 417 daltons].

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Key words: Ethanol, Pancreatic energy metabolism, Mitochondria, Malate dehydrogenase.

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## Materials and Methods

Thirty one male Wistar rats weighing about 350 g were obtained from Shizuoka Experimental Animals, Shizuoka, Japan. They were kept in light-dark cycle regulated (light; 5 : 00-17 : 00) and air-conditioned ( $23 \pm 3^\circ\text{C}$ ) animal quarters in our institute before the experiments, and were given free access to tap water and laboratory food (Oriental Rodent Chow, Tokyo, Japan). The animals were maintained throughout the study in accordance with the guidelines of the Committee on Animal Care of Kyoto University, and this study was approved by the committee.

### Experimental Designs

After a 16-hour fast, under general anesthesia with intraperitoneal injection of sodium pentobarbital (Nembutal<sup>®</sup>, Abbott Co., North Chicago, IL, USA) (30 mg/kg), a PE-50 catheter (Clay Adams, Parsippany, NJ, USA) was placed in the superior vena cava through the right external jugular vein. At this point, all the rat were divided into the following five groups:

(a) Alcohol (ALC<sub>1</sub>, ALC<sub>2</sub> and ALC<sub>3</sub>) groups (n=6, in each group). Ethanol (Kanto Chemical Co., Tokyo, Japan) was infused at three doses of 0.2 (ALC<sub>1</sub>), 0.5 (ALC<sub>2</sub>) and 1.0 g/kg · hr (ALC<sub>3</sub>) for 4 hours in heparinized saline (20 U/ml) at a rate of 1.58 ml/hr by an infusion pump (Truth Type B-6, Nakagawaseikodo, Tokyo, Japan).

(b) Control (CONT) group (n=5). Only heparinized saline was infused for 4 hours.

In all the rats, anesthesia was maintained for 4 hours, and the rats were put on a heating pad at 40°C with overhead lamps to maintain their core temperature.

### Serum Ethanol Amylase and Malate Dehydrogenase (MDH) Levels

In each group, at 2 and 4 hours after the induction of ethanol infusion or saline infusion, 0.8 ml of blood was withdrawn from the venous catheter for the determination of serum ethanol, amylase and MDH levels. After blood samplings, the same volume of saline was infused through venous catheter for the blood loss.

### Pancreatic Water Content and Pancreatic Histological Changes

At the selected times after the 4-hour experiments, rats were killed painlessly by a large dose of intravenous pentobarbital (80 mg/kg). The pancreas was removed quickly. One small portions of the pancreas from the splenic portion were fixed overnight by immersion in phosphate-buffered (pH 7.4) 10% neutral formalin, and pancreatic histological changes such as interstitial edema, acinar cell vacuolization and inflammatory cell infiltration were examined by a blinded observer on a O (no changes) to 4+ (maximum changes) scale.

About 300 mg of the pancreas was used for the quantification of pancreatic water content by comparing the weight immediately after removal (wet weight) with that of the same sample after incubation at 150°C for 48 hours (dry weight) in a dessicator (Sanyo Drying Over<sup>®</sup>, Sanyo, Tokyo, Japan).

### Mitochondrial Fragility in *In-Vitro* Incubation

About 400 mg of the pancreas was used for the subcellular fractionation. The excised and trimmed pancreas was homogenized in 6 ml of cold (4°C) 5 mM MOPS (3-(N-morpholino) propanesulfonic acid) (Sigma Chemical Co., St. Louis, MO, USA) buffer (pH 6.5) containing 1 mM MgSO<sub>4</sub> and 250 mM sucrose, and divided into its various subcellular fractions by differential centrifugation as described previously<sup>7,8,9,10</sup>. Briefly, the homogenate was centrifuged (150 × g at 4°C for 15 min) to remove unbroken cells and debris, and the resulting supernatant was centrifuged (1300 × g at 4°C for 15 min) to obtain a zymogen-rich pellet and a supernatant. This supernatant

was centrifuged ( $12,000 \times g$  at  $4^\circ\text{C}$  for 12 min) to pellet a mitochondria-rich pellet. This fraction, arbitrarily considered to contain 100% of mitochondrial enzyme activity, was resuspended in 2 ml of 5 mM MOPS buffer and incubated for varying intervals (60 and 120 min) at  $25^\circ\text{C}$  in a shaking water bath under room air as reported previously<sup>11,12</sup>. The samples then centrifuged ( $12,000 \times g$  at  $4^\circ\text{C}$  for 12 min) to separate the particulate from the soluble mitochondrial enzyme activity, each of which was individually measured after separation. As a mitochondrial enzyme, MDH activity was measured both in the pelleted and the soluble fractions. Centrifugation and subsequent measurement of particulate and soluble mitochondrial enzyme activity identified the rate and extent of in-vitro rupture of mitochondrial enzyme containing organelles. Soluble MDH activity was expressed as a percentage of the total activity to give an index of mitochondrial fragility.

#### Pancreatic Adenylate Concentrations and Energy Charge Levels

About 500 mg of the pancreas was frozen in liquid nitrogen. The frozen tissue was powdered with a mortar and pestle in a liquid nitrogen bath and homogenized in 4 times volume of chilled 6% (W/V) perchloric acid containing 1 mM edetic acid. The extract was centrifuged at  $1,000 \times g$  at  $0^\circ\text{C}$  for 15 min. The Supernatant was adjusted to pH 6.0 with cold 69% (W/V)  $\text{K}_2\text{CO}_3$  and recentrifuged at  $10,000 \times g$  at  $0^\circ\text{C}$  for 5 min. The final supernatant was used to determine the concentrations of adenine nucleotides (ATP, ADP and AMP) in the pancreatic tissue. The energy charge (E.C.) was calculated by the formula proposed by Atkinson<sup>13</sup>;  $\text{E.C.} = (\text{ATP} + 1/2 \text{ADP}) / (\text{ATP} + \text{ADP} + \text{AMP})$ .

#### Protective Effect of Gabexate Mesilate (FOY)

In another new group of rats (FOY group) ( $n=8$ ), ethanol was infused as in the ALC<sub>2</sub> group (0.5 g/Kg · hr for 4 hours), but FOY (Ono Pharmaceutical Co., Osaka, Japan) was infused throughout the 4-hour experiment at a dose of 20 mg/Kg · hr from the venous catheter (PE-50) placed in the inferior vena cava via the left femoral vein in heparinized saline (0.58 ml/hr). After this 4-hour experiment, all the protocols as in the other groups were performed to evaluate the protective effect of FOY in this model.

#### Assays

Serum concentrations of ethanol were determined by the alcohol dehydrogenase method by the method of BONNICHSEN<sup>14</sup>. Amylase activity was measured with blue starch (Shionogi Amylase A-Test, Shionogi Co., Osaka, Japan) as the substrate by the method of IRIE and co-workers<sup>15</sup>. MDH activity was measured spectrometrically by the method of BERGMAYER<sup>16</sup>. ATP and ADP were measured enzymatically by the method of LAMPRECHT and TRAUTSCHOLD<sup>17</sup>, and AMP was measured by the method of JAWOREK and co-workers<sup>18</sup>.

#### Data Presentation

The results reported in this study represent the means  $\pm$  SEM for each value ( $n$ ) obtained using a different animal. The significance of chances was evaluated by analysis of variance (ANOVA). If ANOVA indicated significant differences, the data were analyzed by using the TUKEYS method as a post hoc test for differences between groups. For the histological changes, WILCOXON'S rank sum test was used.  $p < 0.05$  was considered to be significant.

## Results

#### Serum ethanol, Amylase and MDH Levels

Four-hour intravenous ethanol infusion caused marked high levels of serum ethanol (0.2 g/Kg

· hr; 2 hours:  $46 \pm 12$  mg/dl, 4 hours:  $61 \pm 15$  mg/dl, 0.5 g/Kg · hr; 2 hours:  $79 \pm 18$  mg/dl, 4 hours:  $92 \pm 21$  mg/dl, 1.0 g/Kg · hr; 2 hours:  $105 \pm 24$  mg/dl, 4 hours:  $119 \pm 28$  mg/dl) (Fig. 1).

4-hour intravenous ethanol infusion at the doses of 0.5 g/Kg · hr and 1.0 g/Kg · hr caused

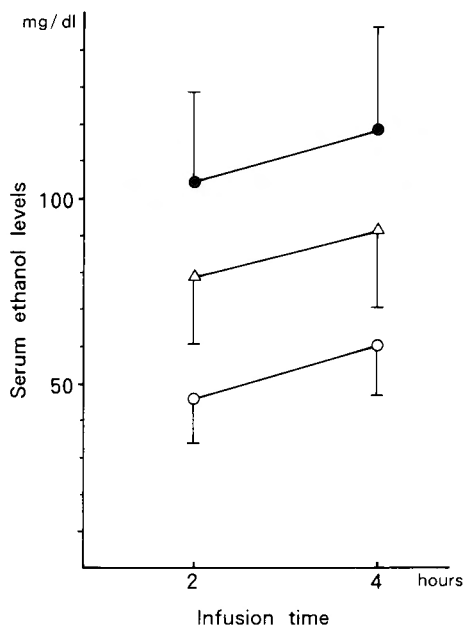


Fig. 1 Effect of intravenous ethanol infusion on serum ethanol levels in rats.

Ethanol was infused at three doses of 0.2 (○), 0.5 (△) and 1.0 g/kg · hr (▲) Each group had 6 animals.

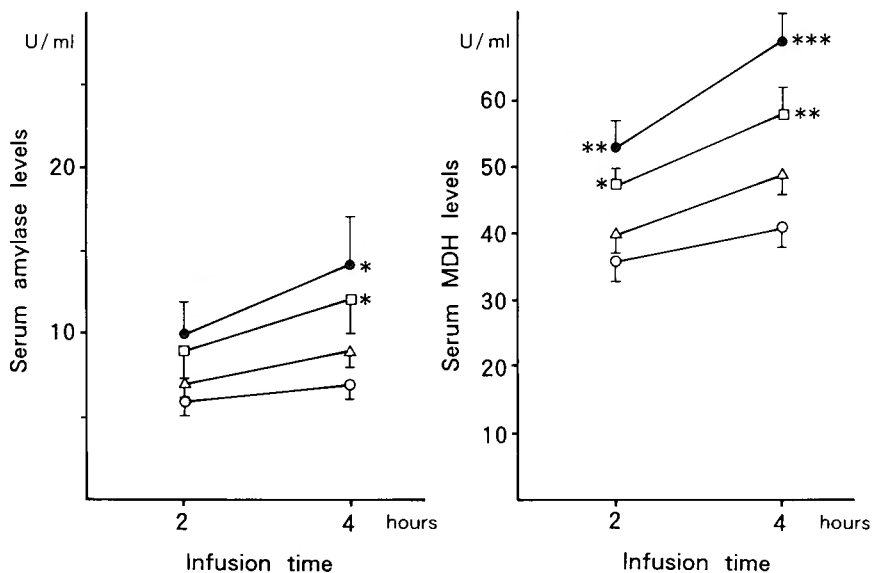


Fig. 2 Effect of intravenous ethanol infusion on serum amylase and MDH levels in rats.

Ethanol was infused at three doses of 0.2 (△, n=6), 0.5 (□, n=6) and 1.0 g/kg · hr (●, n=6). There were 5 rats in the control group (○). \* $p < 0.05$ , \*\* $p < 0.02$ , and \*\*\* $p < 0.01$  vs CONT group.

significant rises in serum amylase levels (0.5 g/Kg · hr;  $12 \pm 2$  U/ml, 2 hours:  $9 \pm 2$  U/ml, 1.0 g/Kg · hr;  $14 \pm 3$  U/ml, 2 hours:  $10 \pm 2$  U/ml) compared with the CONT group ( $7 \pm 1$  U/ml, 2 hours:  $6 \pm 1$  U/ml). However, ethanol at the dose of 0.2 g/Kg · hr caused no significant changes ( $9 \pm 1$  U/ml, 2 hours:  $7 \pm 1$  U/ml) (Fig. 2a).

4-hour intravenous ethanol infusion at the doses of 0.5 g/Kg · hr and 1.0 g/Kg · hr caused significant rises in serum MDH levels (0.5 g/Kg · hr; 2 hours:  $47 \pm 3$  U/ml, 4 hours;  $58 \pm 4$  U/ml, 1.0 g/Kg · hr; 2 hours:  $53 \pm 4$  U/ml, 4 hours:  $69 \pm 4$  U/ml) compared with the CONT group (2 hours;  $36 \pm 3$  U/ml, 4 hours;  $41 \pm 3$  U/ml). However, ethanol at the dose of 0.2 g/Kg · hr caused no significant changes (2 hours;  $40 \pm 3$  U/ml, 4 hours;  $49 \pm 3$  U/ml) (Fig. 2b).

#### Pancreatic Water Content and Histological Changes

Four-hour intravenous ethanol infusion at the doses of 0.5 and 1.0 g/Kg · hr caused significant

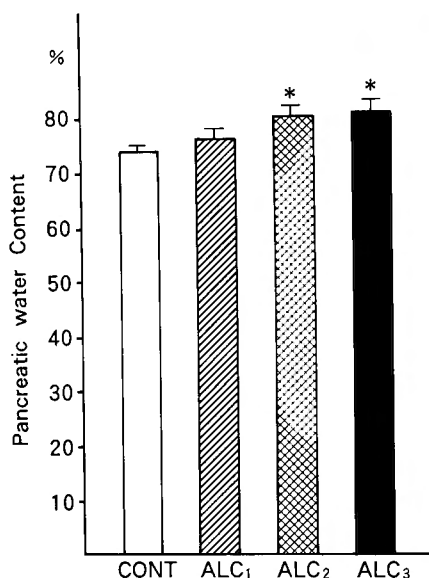


Fig. 3 Effect of intravenous ethanol infusion on pancreatic water content in rats.

Ethanol was infused at three doses of 0.2 (●, n=6), 0.5 (●, n=6) and 1.0 g/kg · hr (■, n=6) for 4 hours. There were 5 rats in the control group (□). \*p<0.05 vs. CONT group.

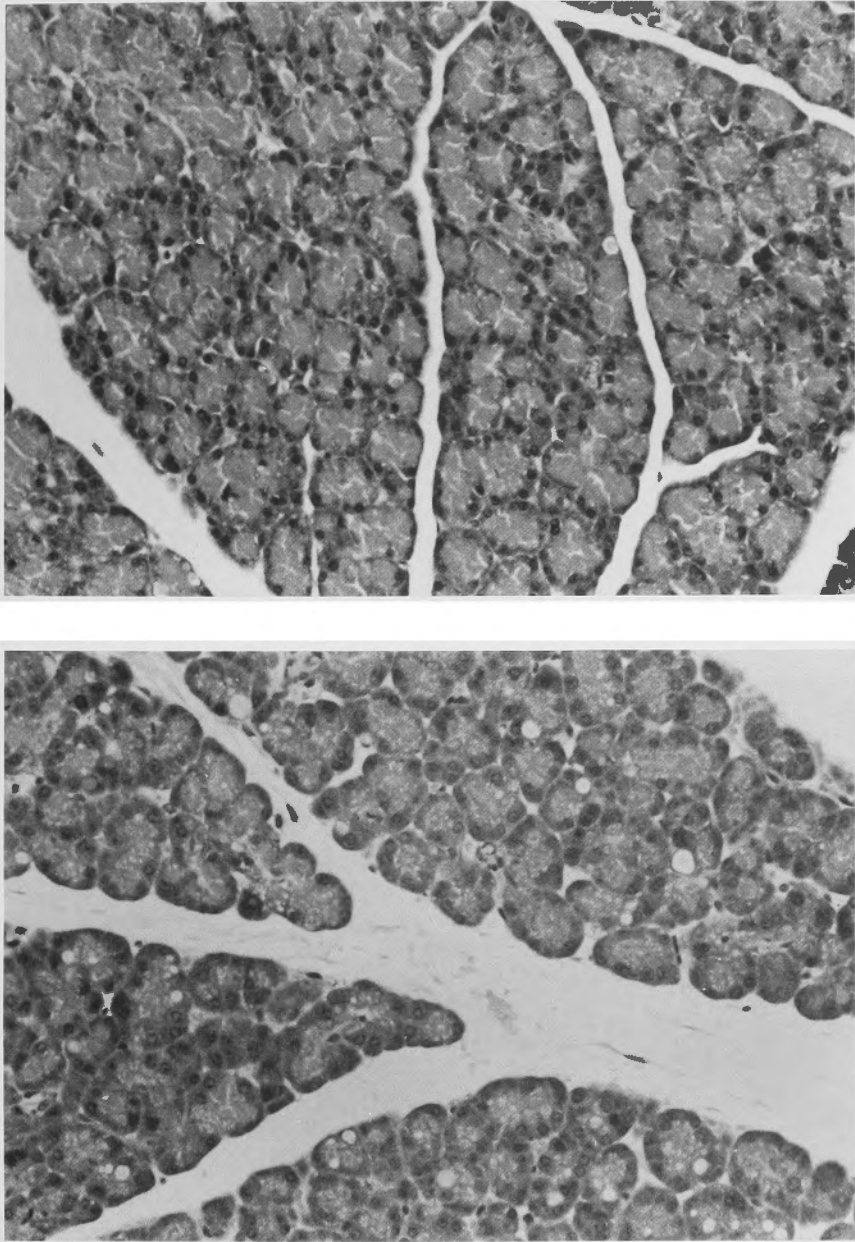
Table 1 Effect of 4-hour intravenous ethanol infusion at three doses on pancreatic histological changes in rats

Group	n	Pancreatic histological changes		
		Interstitial edema	Acinar cell vacuolization	Inflammatory cell infiltration
ALC <sub>1</sub>	6	0 (0-1) [0.3±0.2]	0 (0-1) [0.2±0.2]	0 (0) [0]
ALC <sub>2</sub>	6	1+* (1-2) [1.2±0.2]	0 (0-1) [0.3±0.2]	0 (0-1) [0.2±0.2]
ALC <sub>3</sub>	6	1+* (1-2) [1.3±0.2]	1+ (0-1) [0.8±0.2]	0 (0-1) [0.2±0.2]
CONT	5	0 (0) [0]	0 (0) [0]	0 (0) [0]

[ ]; means±SEM, ( ); ranges of the scores, ALC<sub>1</sub>; 0.2 g/kg. hr of ethanol infusion, ALC<sub>2</sub>; 0.5 g/kg. hr of ethanol infusion, ALC<sub>3</sub>; 1.0 g/kg. hr of ethanol infusion, CONT; saline infusion, \*p<0.05 vs. CONT group.

rises in pancreatic water content ( $0.5 \text{ g/Kg} \cdot \text{hr}$ ;  $80 \pm 2\%$ ,  $1.0 \text{ g/Kg} \cdot \text{hr}$ ;  $81 \pm 2\%$ ) compared with the CONT group ( $74 \pm 1\%$ ). However, ethanol at the dose of  $0.2 \text{ g/Kg} \cdot \text{hr}$  caused no significant changes ( $76 \pm 2\%$ ) (Fig. 3).

4-hour intravenous ethanol infusion at the doses of  $0.5$  and  $1.0 \text{ g/Kg} \cdot \text{hr}$  caused a mild, but



**Fig. 4** Effect of intravenous ethanol infusion at three doses of  $0.2$  (a),  $0.5$  (b) and  $1.0 \text{ g/kg} \cdot \text{hr}$  (c) on pancreatic histological changes in rats. (d); control rats  $\times 200$ .

significant interstitial edema histologically. Ethanol at the dose of 1.0 g/Kg · hr also caused a slight and not significant acinar cell vacuolization. However, ethanol at the dose of 0.2 g/Kg · hr caused only marginal changes (Table 1 and Fig. 4).

Mitochondrial Fragility in *In-Vitro* Incubation

Four-hour intravenous ethanol infusion at the doses of 0.5 and 1.0 g/Kg · hr caused significant

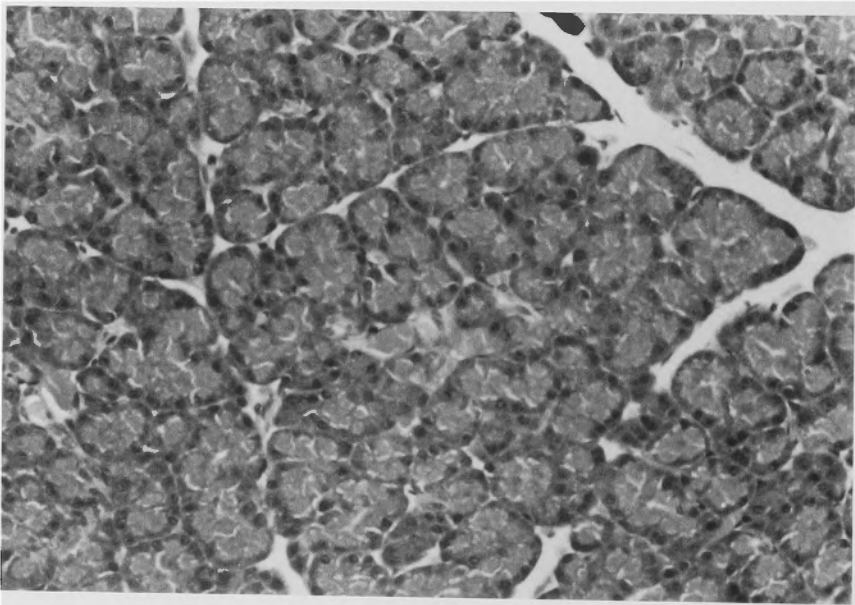
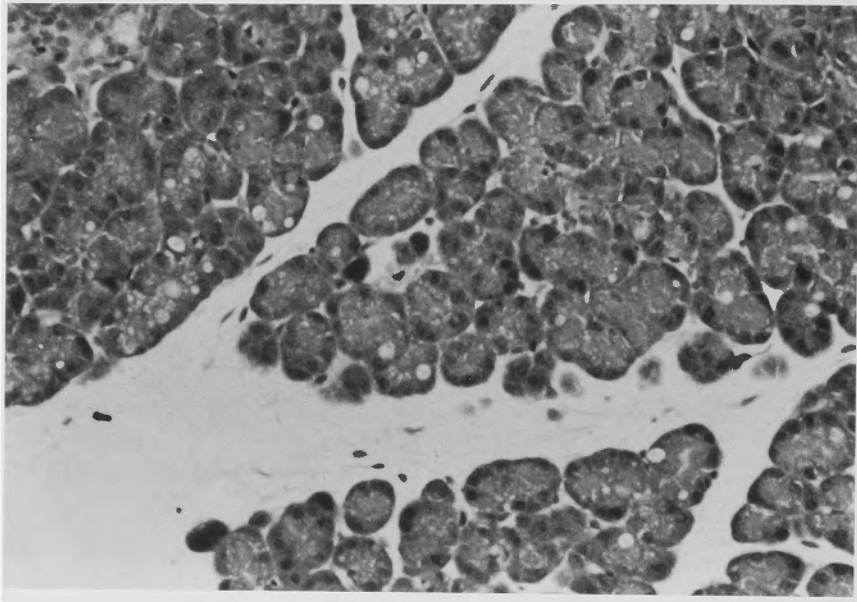


Fig. 4 c. d

accelerated leakage of MDH from mitochondria in *in-vitro* incubation (0.5 g/Kg · hr; 60 min:  $17 \pm 2\%$ , 120 min:  $38 \pm 3\%$ , 1.0 g/Kg · hr; 60 min:  $19 \pm 2\%$ , 120 min:  $42 \pm 3\%$ ) compared with the CONT group (60 min:  $11 \pm 1\%$ , 120 min;  $23 \pm 2\%$ ). However, ethanol at the dose of 0.2 g/Kg · hr caused no significant changes (60 min;  $14 \pm 2\%$ , 120 min;  $29 \pm 2\%$ ) (Fig. 4).

#### Pancreatic Adenylate Energy Charge Levels

Four-hour intravenous ethanol infusion at all the doses of 0.2, 0.5 and 1.0 g/Kg · hr caused significant decreases of pancreatic ATP concentration and significant increases of AMP concentrations compared with the CONT group. Reflecting these changes, 4-hour intravenous ethanol infusion caused significant decreases in pancreatic adenylate energy charge levels, particularly at the doses of 0.5 and 1.0 g/Kg · hr.

#### Protective Effect of Gabexate Mesilate (FOY)

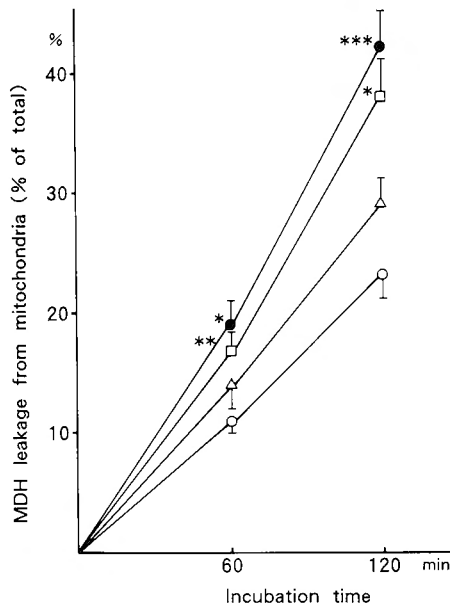


Fig. 5 Effect of intravenous ethanol infusion on MDH leakage from mitochondria in rats.

Ethanol was infused at three doses of 0.2 (△, n=6), 0.5 (□, n=6) and 1.0 g/kg · hr (●, n=6) for 4 hours. There were 5 rats in the control group (○). \*p<0.02, \*\*p<0.05, and \*\*\*p<0.01 vs. CONT group.

Table 2 Effect of 4-hour intravenous ethanol infusion at three doses on pancreatic adenine nucleotides concentrations in rats

Group	n	Pancreatic adenine nucleotides concentrations (micromoles/g. wet pancreas)			Pancreatic energy charge levels
		ATP	ADP	AMP	
ALC <sub>1</sub>	6	2.094 ± 0.117*	0.226 ± 0.042	0.194 ± 0.016***	0.88 ± 0.02
ALC <sub>2</sub>	6	1.705 ± 0.119**	0.219 ± 0.038	0.224 ± 0.031*	0.85 ± 0.02***
ALC <sub>3</sub>	6	1.628 ± 0.124**	0.207 ± 0.049	0.248 ± 0.035*	0.83 ± 0.03***
CONT	5	2.873 ± 0.082	0.245 ± 0.037	0.125 ± 0.010	0.92 ± 0.02

Symbols in the groups have the same meanings as in Table 1.

\*p<0.01, \*\*p<0.001 and \*\*\*p<0.05 vs. CONT.



FOY at the dose of 20 mg/Kg · hr in the ALC<sub>2</sub> group had significant protective effects against serum amylase levels (2 hours;  $7 \pm 2$  U/ml, 4 hours;  $8 \pm 1$  ( $p < 0.05$ )) and MDH levels (2 hours;  $39 \pm 2$  U/ml ( $p < 0.05$ ), 4 hours;  $44 \pm 3$  U/ml ( $P < 0.02$ )) compared with the ALC<sub>2</sub> group. FOY also exerted a significant protective effect against pancreatic edema ( $75 \pm 2\%$ ,  $p < 0.05$ ). FOY also inhibited the histological changes (interstitial edema; 0 (0~1) [ $0.3 \pm 0.2$ ], ( $p < 0.05$ )) significantly.

FOY had a significant protective effect against the accelerated leakage of MDH from mitochondria (60 min;  $12 \pm 2\%$  ( $p < 0.05$ ), 120 min;  $25 \pm 2\%$  ( $p < 0.02$ )). FOY significantly inhibited all of the decrease of ATP concentration ( $2.49 \pm 0.095$ ,  $p < 0.001$ ), increase of AMP concentration ( $0.148 \pm 0.012$ ,  $p < 0.01$ ) and decrease of pancreatic energy charge levels ( $0.90 \pm 0.02$ ,  $p < 0.05$ ).

### Discussion

Four-hour infusion of ethanol caused a mild, but significant, hyperamylasemia and pancreatic edema, and slight, but also significant, histological changes such as interstitial edema. Moreover, subcellular fractionation and *in-vitro* incubation study showed that ethanol causes an impaired pancreatic energy metabolism. Thus our present data suggest a possible role for alcohol in the development of pancreatitis. Ethanol-induced reduction in the cellular energy supply might well disrupt homeostatic mechanism sufficiently to produce cell injuries in pancreatic acinar cells.

On the other hand, 4-hour infusion of ethanol caused a significantly high levels of serum MDH and accelerated leakage of MDH from mitochondria, indicating the increased mitochondrial fragility. If mitochondrial fragility is increased, such an impaired energy metabolism will be expected, since mitochondria is a main organelle for cellular energy metabolism.

Synthetic protease inhibitor, gabexate mesilate (FOY) is one of guanidino acid esters, which has been reported to inhibit several key enzymes such as trypsin, phospholipase A<sub>2</sub> and elastase<sup>19</sup>, and also reported to be effective in another model of acute pancreatitis; secretagogue-induced pancreatitis<sup>4,11</sup>). Such an effectiveness encouraged us to evaluate its protective effects against the ethanol-induced pancreatic injuries in this study. We used the dose of 20 mg/Kg · hr for FOY, since this dose was effective against secretagogue-induced pancreatitis<sup>4,11,20</sup>).

The results in this study indicate that sustained high blood ethanol levels will induce pancreatic injuries including hyperamylasemia and pancreatic edema, and also suggest one possible mechanism for alcoholic pancreatitis probably via increased mitochondrial fragility and impaired pancreatic adenylate energy metabolism.

Our present study seems to provide a direct toxic effect of ethanol on the exocrine pancreas. However, ethanol is metabolized to acetaldehyde by the enzyme alcohol dehydrogenase, primarily in the liver. Since we have not examined the effect of acetaldehyde on the exocrine pancreas in this study, there still seems to be a possible role of acetaldehyde in the pathogenesis of pancreatic injuries, and this issue will be needed to be studied in the future study. Moreover, some unknown protease activity, which can be inhibited by FOY, seems to be closely involved in the pathogenesis of pancreatic injuries induced by ethanol infusion, and synthetic protease inhibitor such as FOY will also be useful in protecting the exocrine pancreas in the alcoholic patients.

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## 和文抄録

ラットにおけるエタノールの静脈内投与が膵エネルギー代謝と  
膵ミトコンドリアの脆弱性に及ぼす影響と  
FOY の保護効果について

京都大学医学部 第一外科学教室

平野 鉄也

4時間のエタノール静脈内投与 (0.5 g/Kg・hr と 1.0 g/Kg・hr)により, ラットにおいて高 amylase 血症と膵浮腫が出現した. これらの用量にてさらに膵エネルギーチャージレベルの障害とともに膵ミトコンドリアの脆弱性の亢進も観察された. エタノールの 0.2 g/Kg・hr の用量では有意な変化は観察されなかった. さらに, 20 mg/Kg・hr の FOY を投与すると, こ

れ ら

の変化は有意に抑制された. 今回の結果はアルコール性膵障害の発生過程にて, 膵エネルギー代謝の障害と膵臓ミトコンドリアの脆弱性の亢進が重要な役割を果していることを示唆するとともに, アルコール多飲者における膵保護における FOY の有用性をも示唆させるものであった.