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**Ethanol induced CXC-chemokine synthesis and barrier dysfunction in intestinal epithelial cells.**

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Running title: Effect of ethanol on chemokine synthesis and barrier function

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## ABSTRACTS

**Background:** Ethanol exposure contributes to infectious complications in burn and trauma patients through a process known as “bacterial translocation”. Two major factors, 1) physical disruption of the intestinal mucosal barrier and 2) suppression of immune defense, explain this phenomenon. However, little information is available concerning the immune mechanisms of ethanol-induced bacterial translocation. In this study we investigated the effect of physiological concentrations of ethanol on immune function, especially on CXC-chemokine secretion, neutrophil migration, and barrier function in the small intestine. **Methods :** A rat small intestinal intestinal cell line (IEC-18 cells) was exposed to 50-500 mM ethanol for 24 hr with or without IL-1 $\beta$ . Secretion of CXC chemokines (GRO/CINC-1 and MIP-2) was measured by ELISA assay, and barrier dysfunction was assessed by the apical-to-basolateral flux of HRP-dextran. Neutrophil transmigration was assessed by enzyme histochemistry (AS-D chloroesterase staining).

**Results:** Exposure to ethanol concentrations of 200 mM and over increased GRO/CINC-1 secretion, and MIP-2 secretion increased at 500 mM. Administration of ethanol in combination with IL-1 $\beta$  had no additive effect on the release of GRO/CINC-1 and MIP-2. Exposure of IEC-18 monolayers to ethanol resulted in a dose-dependent increase in permeability but IL-1 $\beta$  had no effect on barrier function. Ethanol had no effect on neutrophil migration in enzyme histochemistry analysis. **Conclusions:** The above observations suggest that ethanol induced physical disruption of the intestine but not neutrophil transmigration is the main cause of the bacterial translocation that leads to bacteremia and endotoxemia in alcoholics. A number of previous studies have shown that

prior ethanol exposure in burn and trauma patients, have a higher incidence of infection than such patients without alcohol exposure. The precise mechanisms of this alcohol-mediated increase in incidence of infection remains unknown. Intestinal epithelial cells (IECs) form the first line of defense against the microorganisms in the intestinal lumen, and actively participate in intestinal immune networks. Under certain conditions, however, bacteria or endotoxins are able to cross the mucosal barrier and spread to the mesenteric lymph nodes, and beyond them to systemic organs. “Bacterial translocation”, is the term used to describe this passage of bacteria or endotoxins from the GI tract to extraintestinal sites, and it has been considered an important cause of infectious complications in alcoholics (Choudhry et al., 2002). Two major factors may contribute to the bacterial translocation in alcoholics: 1) physical disruption of mucosal barrier, and 2) suppression of immune defense. In regard to physical disruption of intestinal mucosal barrier, many *in vivo* and *in vitro* studies have suggested that ethanol impairs both the functional and anatomic integrity of the intestinal mucosa (Banan et al., 1998; Banan et al., 1999; Keshavarzian et al., 1998; Ma et al., 1999). However, extremely high doses of ethanol were used in the *in vitro* studies, and the cells used in the *in vitro* experiments were not suitable as a model for studying barrier function of the small intestine. The present study evaluated the effect of ethanol on intestinal barrier function by using IEC-18 cells, a nontransformed small intestinal cell line, the best model of intestinal epithelium for the study of permeability and paracellular transport. In regard to immune defense, on the other hand, results of previous studies concerning immune function and cytokine synthesis after ethanol exposure have been confusing. Several

investigators have shown that ethanol exposure produces suppression of mitogen-induced gut lymphoid, splenic and peripheral blood T cell proliferation (Choudhry et al., 2002; Jaysinghe et al., 1992). Other previous studies have shown that ethanol suppresses the secretion of many proinflammatory cytokines, including TNF- $\alpha$ , IL-1, IL-6, interferon- $\gamma$ , and IL-8, whereas Boe et al found that ethanol significantly increases the serum IL-8 level (Boe et al., 2001; Pennington et al., 1998; Standiford et al., 2002; Taieb et al., 2002; Zhang et al., 2002). In addition to these systemic immunologic changes, alteration of local immunity in the intestinal mucosa has also been postulated. Among the many local immune functions of the intestine, chemotactic cytokine synthesis and neutrophil accumulation in intestine are considered to be closely related to bacterial translocation. Polymorphonuclear neutrophils (PMNs) play a major role in bacterial destruction through generation of active oxygen metabolites. However, intestinal accumulation of neutrophils which produce excess quantities of free radicals and thereby inflict oxidative tissue damage in the intestine, may also cause bacterial translocation. Fazal found that treatment with antibody to CXC chemokines decreased thermal-injury induced bacterial translocation into mesenteric lymph nodes (Fazal et al., 2000). However, little information is available concerning how ethanol exposure affects host immune defenses, especially epithelial synthesis of CXC chemokines, which are major chemotactic cytokines in the intestine.

Chemokines are a diverse set of low-molecular-weight cytokines that selectively direct the migration and activation of specific populations of leukocytes. The CXC chemokines are defined by the presence of a single amino acid between the first two

NH<sub>2</sub>-proximal cysteines among the four highly conserved cysteine residues that are characteristic of this class of chemokines. MIP-2 and cytokine-induced neutrophil chemoattractant (GRO/CINC-1) are major CXC chemokines in rats, and their secretion by IECs is increased by inflammatory stimuli, such as interleukin-1 $\beta$  (Ohno et al., 1997; Ohtsuka et al., 2001; Yoshida et al., 2001; Zhang et al., 2002). We have shown that GRO/CINC-1 is a potent stimulator that induces not only locomotive but secretagogue activation of neutrophils via a CD18-dependent mechanism in vivo (Suzuki et al., 1994). Production of these cytokines in local tissues is increased during infection and inflammation and generates the chemotactic gradient that required to direct the effective recruitment of PMNs from the systemic circulation.

In the present study, we investigated the mechanisms of bacterial translocation in the small intestine after exposure to ethanol. Our objective was to determine whether acute ethanol intoxication leads to alteration of CXC chemokine synthesis or PMN migration into the small intestine. We also investigated the barrier function of the epithelium after ethanol exposure to identify the factor responsible for increased bacterial translocation in alcoholics.

## **METHODS**

### ***Cell Culture***

IEC-18 cells (derived from fetal rat ileum) were purchased from Dainippon Pharmaceutical Co. (Osaka Japan) and grown at 37 °C in a culture media composed of Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum, 50 U/ml of penicillin, 50 U/ml of streptomycin, and 4 mM of glutamine, under a 5% CO<sub>2</sub> atmosphere.

### ***Cytokine Assays***

Cytokine levels were determined by sandwich enzyme-linked immunosorbent assay (ELISA) by using commercially available kits according to the manufacturer's instructions. GRO/CINC-1 was determined with a rat-specific sandwich immunoassay kit (Immuno-Biological Laboratories Co., Gunma, Japan). MIP-2 concentrations were determined by ELISA with a rat MIP-2 ELISA kit (BioSource International, Camarillo, CA). The chemokine concentrations were calculated by using standard curves for recombinant GRO/CINC-1 and MIP-2. The MIP-2 ELISA was sensitive to 1 pg/ml, and the GRO/CINC-1 was sensitive to 4.7 pg/ml.

### ***Determination of Cell Viability by MTT Assay***

Cells were cultured in 96-well multiwell plates in DMEM containing 10% FCS. After the cells had attached to their substrate, the culture medium was switched to DMEM containing 0.1% FCS for 24 hr. Cell numbers were determined 24 hr after exposure to ethanol alone or to ethanol plus IL-1 $\beta$  (0.1 ng/ml and 1.0 ng/ml). At the termination of the incubation, reduction of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salts) to formazan was assayed by using a CellTiter 96 AQ non-radioactive cell proliferation assay kit (Promega Co. Madison, WI, USA) according to the manufacturer's instructions. The absorbance of the formazan at 490 nm was measured with an MTP-120 Microplate Reader (Corona Electric Co. Ibaragi, Japan).

### ***Assessment of Barrier Integrity***

Barrier integrity was assessed by measuring the apical-to-basolateral flux of horseradish peroxidase-dextran (HRP-dextran, molecular weight, 44 kDa), as described previously (Wang

et al., 2002). Polycarbonate filters (8.0  $\mu\text{m}$  pore size) and a 24-well cluster plates were used in these experiments. HRP-dextran (20  $\mu\text{g}/\text{ml}$ ) was added to the upper layer. After 60 minutes of incubation at 37  $^{\circ}\text{C}$ , 100  $\mu\text{l}$  of the medium in the lower compartment was collected and kept on ice; and 30  $\mu\text{l}$  of the culture medium was collected from the lower compartment and added to 860  $\mu\text{l}$  of a reaction buffer containing 50 mmol/l of monobasic sodium phosphate and 5 mmol/l of guaiacol (Sigma Chemical Co. St. Louis, MO). This experiment was performed in duplicate. The reaction was started by addition of 100  $\mu\text{l}$  of hydrogen peroxide (0.6 mmol/l), and the medium was incubated for 30 minutes at room temperature. The samples were read using MTP-120 Microplate Reader set at an absorbance of 450 nm. Data were calculated as follows:

$$\text{OD}_{450} = \text{Sample OD}_{450} - \text{blank OD}_{450}.$$

The data were expressed as the  $\text{OD}_{450}$  for the permeation of HRP across a Transwell filter.

### ***Animals***

Male C57BL/J mice weighing 15-20 g were used as the experimental animals and maintained on a standard diet and water (Charles River Japan, Inc. Kanagawa, Japan). They were divided into two different groups, each consisting of three mice, and fasted 24 hr. Group 1 was gavaged with 0.5 ml of distilled water. Group 2 was gavaged with 3.0 g/kg body weight of ethanol in a 40% solution. The mice were sacrificed 24 hr later and samples of intestine were collected.

### ***Histology***

A 1cm segment of small intestine was immersed in 10% neutral buffered formalin and embedded in paraffin, and then sections were cut at a thickness of 5- $\mu\text{l}$ . Neutrophils were stained by a naphthol AS-D chloroesterase staining method, which identifies specific leukocyte esterases according to the method described in a previous report (Li et al.,

1973). The stained neutrophils were identified on the basis of their nuclear morphology and red small granules scattered within the cytoplasm.

### ***Statistical analysis***

Cytokine levels are expressed as the means  $\pm$  SEM of results obtained from at least three individual experiments performed in duplicate. Differences among groups were evaluated by one-way ANOVA and Fisher's posthoc test. Statistical significance of difference was set at  $p < 0.05$ .

## **RESULTS**

### ***Effect of Ethanol and Ethanol Plus IL-1 $\beta$ on Cell Viability***

Exposure of IEC-18 cells to a range of ethanol concentrations (50 mM-500 mM) for 24 hr did not affect cell viability as determined by the MTT assay (Table 1). We then investigated whether stimulation of IEC-18 cells with both IL-1 $\beta$  (0.1 ng/ml and 1 ng/ml) and ethanol (50 mM-500 mM) would decrease cell viability. A low concentration of IL-1 $\beta$  (0.1 ng/ml) plus ethanol (50 mM~500 mM) did not affect cell viability. A high concentration of IL-1 $\beta$  (1.0 ng/ml) plus ethanol (50 mM~100 mM) also did not affect cell viability; however, IL-1 $\beta$  (1.0 ng/ml) plus ethanol at concentrations of 200 mM and over significantly decreased cell viability.

### ***Effect of Ethanol on GRO/CINC-1 and MIP-2 Secretion by Rat IECs***

We assessed secretion of the CXC chemokines GRO/CINC-1 and MIP-2 by IEC-18 cells stimulated with ethanol at a concentration of 50 mM, 100 mM, 200 mM, or 500 mM for 24 hr. As shown in Fig. 1A, IEC-18 cells constitutively secreted low levels of GRO/CINC-1, but conditioned medium from IEC-18 cells exposed to ethanol at

concentrations of 200 mM and 500 mM contained greater amounts of GRO/CINC-1 than control cells. Similarly, conditioned medium from unstimulated IEC-18 contained low concentrations of MIP-2, while ethanol administration at a concentration of 500 mM resulted in an increase in MIP-2 concentration, as shown in Fig. 1B. The synthesis of these CXC chemokine is not attributable to TNF- $\alpha$  release by intestinal epithelial cells, because TNF- $\alpha$  secretion does not increase by IECs after ethanol exposure (data not shown).

#### ***Combined Effect of Ethanol and IL-1 $\beta$ on GRO/CINC-1 and MIP-2 Release by Rat IECs***

Administration of IL-1 $\beta$  (0.1 ng/ml and 1 ng/ml) for 24 hr by itself markedly increased GRO/CINC-1 and MIP-2 secretion by IECs. Administration of ethanol in combination with IL-1 $\beta$  (0.1 ng/ml and 1 ng/ml) had no significant additive effect on GRO/CINC-1 release compared with administration of IL-1 $\beta$  alone (Fig. 2A), however, addition of ethanol at a concentration of 500 mM had a suppressive effect on MIP-2 release induced by IL-1 $\beta$  (1 ng/ml). (Fig. 2B).

To investigate chemokine production after exposure to a low level of IL-1 $\beta$  (0.1 ng/ml) and ethanol, MIP-2 and GRO/CINC-1 protein concentrations were measured after 24 hr of exposure. Both GRO/CINC-1 and MIP-2 protein production was suppressed after administration of 0.1 ng/ml of IL-1 $\beta$  and ethanol concentration of 500 mM (Fig. 2C, 2D).

#### ***Neutrophil Transmigration in the Intestine after Ethanol Exposure***

Histological examination showed no significant change in neutrophil accumulation in the intestine in ethanol-exposed mice (Fig 3B) compared to control mice (Fig 3A). Histologic

investigation after ethanol exposure (3 g/kg) for 4 hr also showed no significant change in neutrophil infiltration (data not shown). There were no significant differences in MPO activity after ethanol exposure. (data not shown).

### ***Effect of Ethanol and IL-1 $\beta$ on Permeability***

Exposure of IEC-18 monolayers to a range of ethanol concentrations (50-500 mM) for 24 hr resulted in dose-dependent increases in permeability (Fig. 4A). To determine whether IL-1 $\beta$  affects ethanol-induced increases in IEC-18 cell permeability, rat intestinal epithelial monolayers were exposed to ethanol (50-500 mM) and IL-1 $\beta$  (1 ng/ml) for 24 hr, and barrier integrity was assessed. IL-1 $\beta$  did not appear to affect the disruption in epithelial barrier function caused by ethanol exposure (Fig. 4B).

## **DISCUSSION**

It has recently been reported that alcoholics with chronic liver disease exhibit increased intestinal permeability that allows endotoxins to escape into the portal circulation and initiate a hepatic necroinflammation cascade when they arrive at the liver (Choudhry et al., 2002; Keshavarzian et al., 1998; Ma et al., 1999). Tamai et al. found that both long-term ethanol feeding and acute ethanol ingestion facilitate the endotoxin absorption from the rat small intestine as a result of an increase in intestinal permeability (Tamai et al., 2000; Tamai et al., 2002), and several other investigators found that ethanol exposure causes intestinal barrier dysfunction *in vitro* (Banan A et al., 1999; Ma et al., 1999). These *in vitro* studies were used Caco-2 monolayers, which were originally derived from a human colon carcinoma cell line. Although Caco-2 cells form tight junctions and exhibit the morphological and functional characteristics of small intestinal

enterocytes, the net electrical resistance of Caco-2 cells is much higher than that of normal small intestinal tissue. The resistance of normal small intestinal tissue, without the muscularis and serosal layers is 20-45  $\Omega \cdot \text{cm}^2$ , whereas Caco-2 cells have an epithelial resistance of 160-240  $\Omega \cdot \text{cm}^2$ . IEC-18 cells, a nontransformed small intestinal cell line, on the other hand, have a resistance of 28  $\Omega \cdot \text{cm}^2$  (Ma et al., 1992). We used IEC-18 cells, the best model for studying the permeability of the epithelium of the small intestine, and the results showed that abnormal permeability was induced by ethanol at concentrations >200 mM without affecting cell viability.

Chronic ethanol exposure alters cytokine gene expression in the small intestine under experimental conditions in which no direct effect of ethanol on the liver is observed. Fleming et al. recently reported that ethanol exposure upregulates basal gene expression of IL-1 $\beta$  in the ileum in a chronic alcohol gavage mouse model (Fleming et al., 2001). We therefore focused on the effect of the interaction between ethanol and IL-1 on cytokine synthesis and barrier function in IEC-18 cells. Interestingly in our own study, IL-1 $\beta$ , which is known to induce secretion of enormous amounts of several cytokines in the intestinal mucosa, did not affect intestinal cell permeability. Mascarenhas also found that the cell layer integrity of IEC-6 cells is unaffected by IL-1 $\beta$  or TNF- $\alpha$  stimulation, and our findings are consistent with their results (Mascarenhas et al., 1996).

The ethanol concentrations used in this study seem to be higher than under physiological conditions, but there are several points that are relevant to this particular concern. Levitt observed that 89% of the total dose of ethanol entered the duodenum and that 10% was absorbed across the gastric mucosa (Levitt et al., 1997). This means that the

major fraction of a sizable ethanol dose reaches the proximal jejunum, even though several studies have shown that the stomach contains sufficient alcohol dehydrogenase activity to metabolize a significant amount of ethanol (Lim et al., 1993). In a human study, Halsted et al. found that the ethanol concentration in the proximal jejunum reached 5000 mg/100 ml (about 1000 mM) 45 min after oral administration of ethanol (25% solution), and that the concentration remained at 400 mg/100 ml (about 100 mM) for more than 60 min (Halsted et al., 1973). There is some recent evidence that the pattern of drinking may be important to determining the health effects of alcohol. "Binge drinking" is defined as the consumption of 160 g/day or more of pure ethanol usually lasting for a week and over (Malyutina et al., 2002), and we speculate that during such binge drinking, high ethanol concentrations (>200 mM) may be sustained for 24 hr even in the small intestine.

Although many investigators have found that ethanol induces barrier disruption in the intestine, few reports have referred to the intestinal immune response, especially chemokine synthesis and neutrophil transmigration. The study by Tabata is the only study that referred to an association between ethanol and neutrophil migration. They found that acute ethanol intoxication plus sham laparotomy caused a three-fold increase in PMN accumulation in the wall of the small intestine compared to sham laparotomy group (Tabata et al., 1995). Their experiment, however, did not rule out the effect of laparotomy and manipulation of the intestine, suggesting that effect of ethanol alone has not been investigated accurately. PMN-induced oxidative mucosal damage increases intestinal permeability, and high-density PMN transmigration itself produces epithelial wounds and markedly diminishes epithelial barrier function (Nusrat et al., 1997). Therefore, our study

was performed to test hypothesis that both physical disruption of barrier integrity and excess PMN migration could affect bacterial translocation after ethanol exposure. However, our data showed that IECs synthesize and release biologically active proinflammatory chemokines, but that neutrophil migration is unaffected by acute ethanol exposure. The results of our study show that ethanol has little effect on IL-1 $\beta$ -induced chemokine synthesis. The histologic study also show that ethanol has little effect on IL-1 $\beta$ -induced PMN migration (data not shown). These findings support the notion that a direct effect of ethanol on the barrier function of the intestinal mucosa rather than neutrophil-associated damage to the intestinal mucosa is responsible for bacterial translocation after ethanol exposure. The discrepancy between the chemokine synthesis and neutrophil migration can be explained by the pathophysiology of PMN recruitment. Neutrophil infiltration occurs in four stages: selectin-mediated “rolling”, chemoattractant-mediated “activation”, integrin-mediated “adhesion”, and immunoglobulin-superfamily-molecules mediated “transmigration”, suggesting that chemokine are one of the factors that affect neutrophil migration (Stadnyk et al., 2002).

In conclusion, the results of the present study indicated that abnormal intestinal permeability is induced by ethanol even at physiological concentrations. We performed this study on IEC-18 cells, the best model for studying the permeability of the epithelium in the small intestine. Our experiments also demonstrated that the physiological concentration of ethanol has little effect on PMN transmigration in the intestine, even though biologically active CXC-chemokine synthesis increased after ethanol exposure. These observations suggest that the ethanol modulation of intestinal epithelial tight

junction barrier is a main causes of the ethanol-induced gastrointestinal epithelial damage that leads to endotoxemia and bacteremia in alcoholism.

#### **ACKNOWLEDGEMENTS**

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#### **FIGURE LEGEND**

Table 1 Cell viability after exposure to ethanol and ethanol plus IL-1 $\beta$

Ethanol alone or ethanol plus IL-1 $\beta$  was added to IEC monolayers for 24 hr, and cell viability was assessed using MTT assay. The means  $\pm$  SEM of 3 experiments is shown. #  $p < 0.05$  vs controls.

Fig 1

Effect of graded concentrations of ethanol (50 mM~500 mM) on GRO/CINC-1 release (Fig 1A) and MIP-2 release (Fig 1B) by intestinal epithelial cells. The means  $\pm$  SEM of data from three experiments are shown. #  $p < 0.05$  vs controls.

Fig. 2

A) Effect of graded concentrations of ethanol (50 mM~500 mM) plus IL-1 $\beta$  (1.0 ng/ml) on GRO/CINC-1; B) MIP-2 release; C) ethanol (50 mM~500 mM) plus IL-1 $\beta$  (0.1 ng/ml) on GRO/CINC-1; and D) MIP-2 release, in IEC-18 monolayers. The means  $\pm$  SEM of three experiments are shown. #  $p < 0.05$  vs IL-1 $\beta$  alone.

Fig. 3

Histopathological appearance of naphthol AS-D chloroesterase stained sections of small intestine. A) 24 hr after oral administration of distilled water. B) 24 hr after oral administration of ethanol.

Fig. 4

Effect of ethanol (Fig 4A) and ethanol plus IL-1 $\beta$  (Fig 4B) on transepithelial monolayer permeability measured by the passage of horseradish peroxidase (HRP). The means  $\pm$  SEM of data from three experiments are shown. #  $p < 0.05$  vs controls.

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Fig 1A

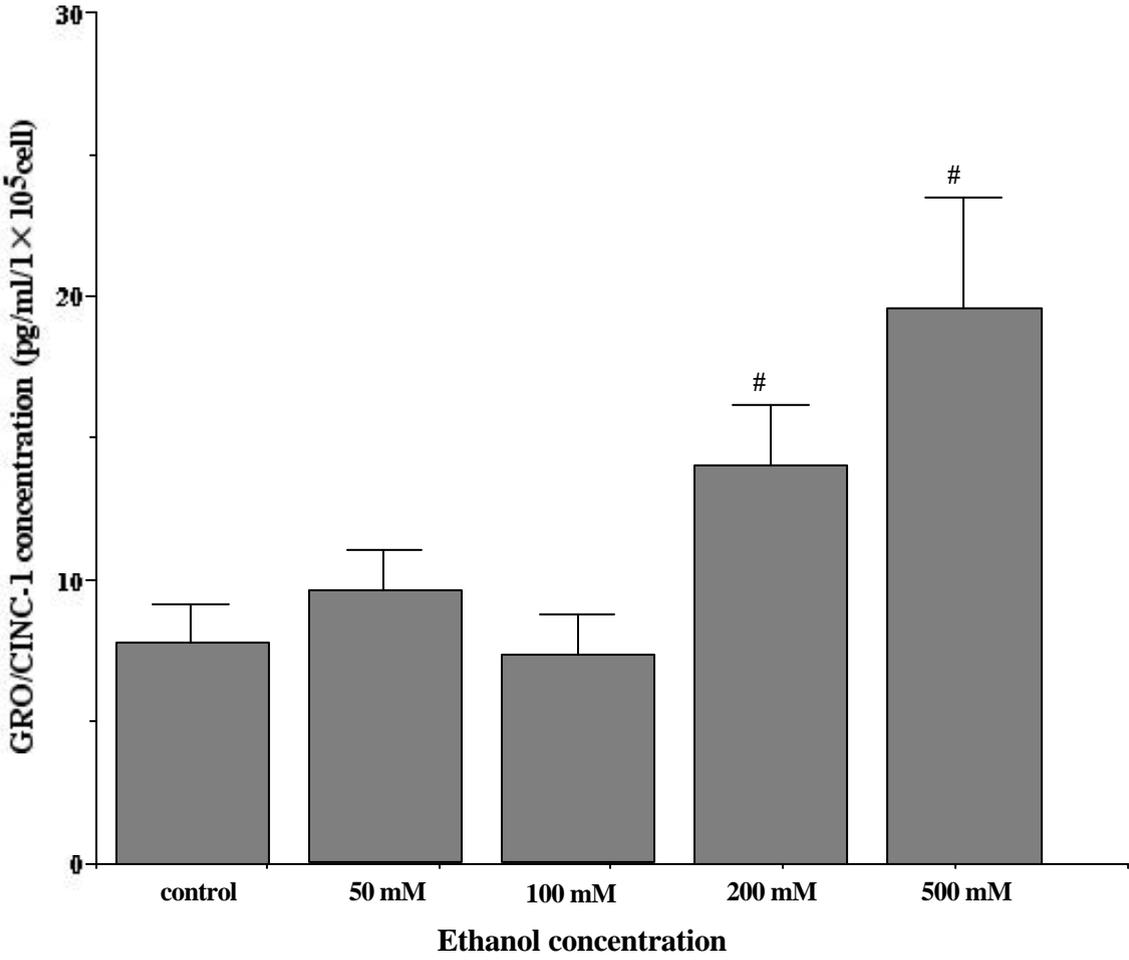


Fig 1B

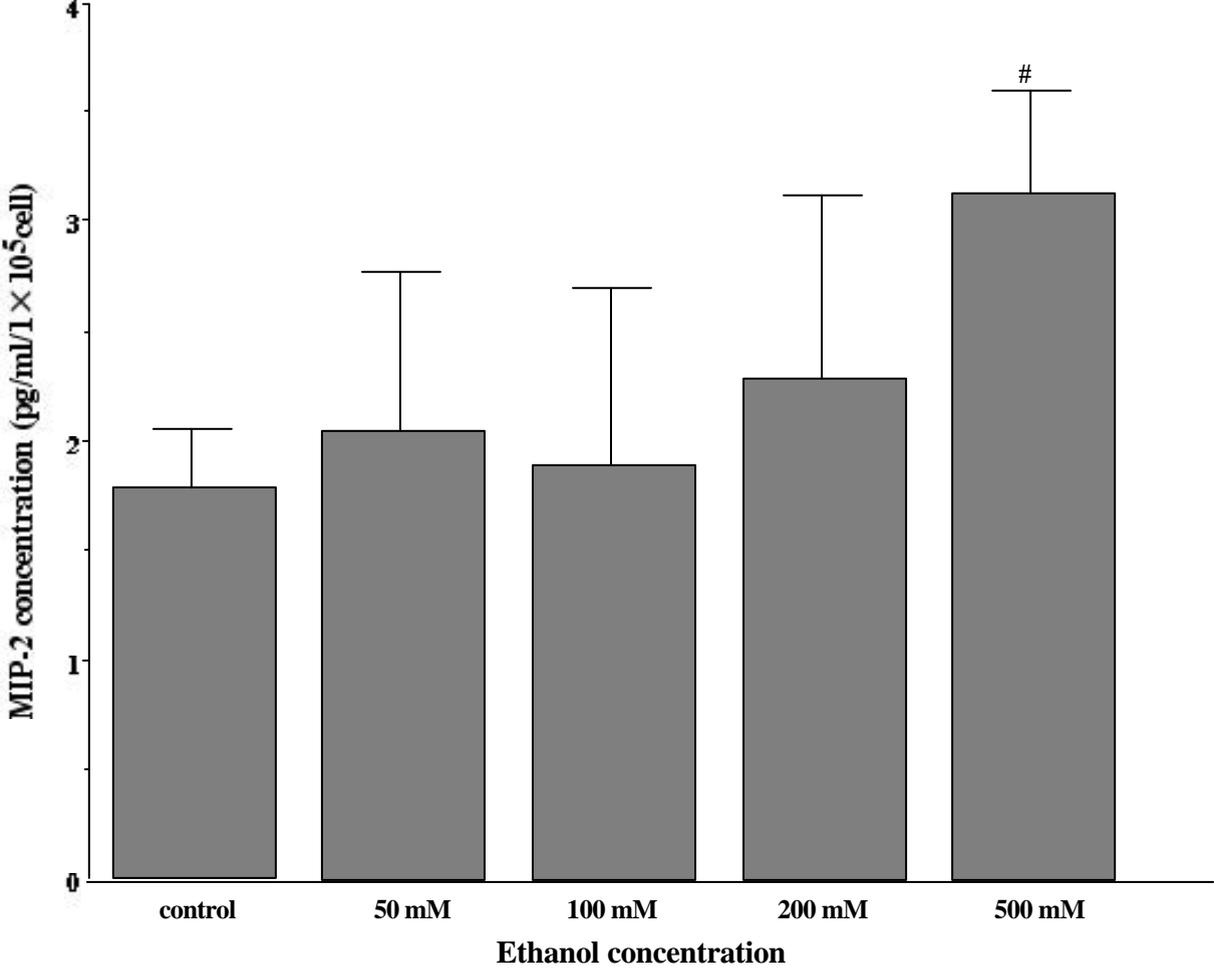


Fig 2A

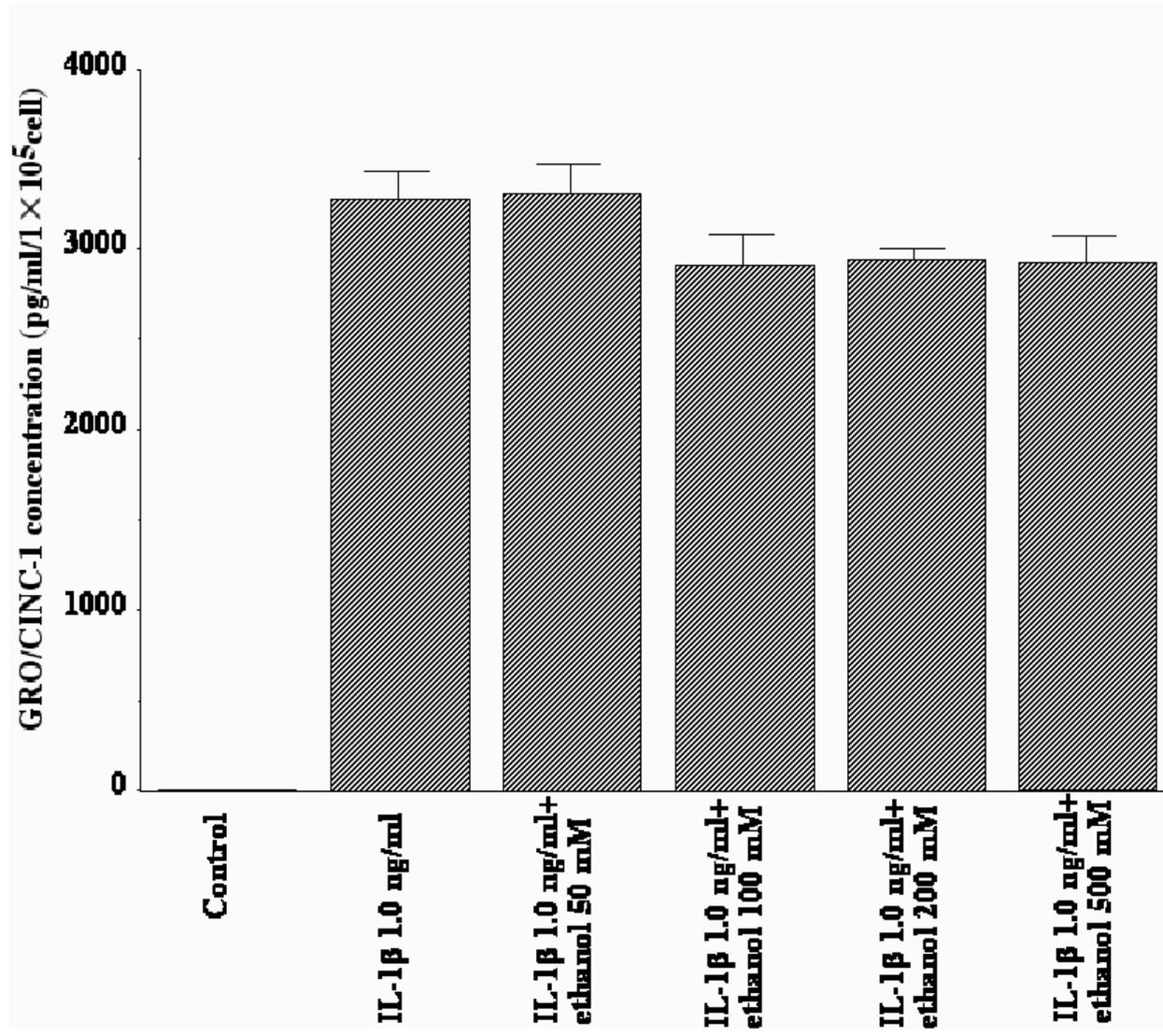


Fig 2B

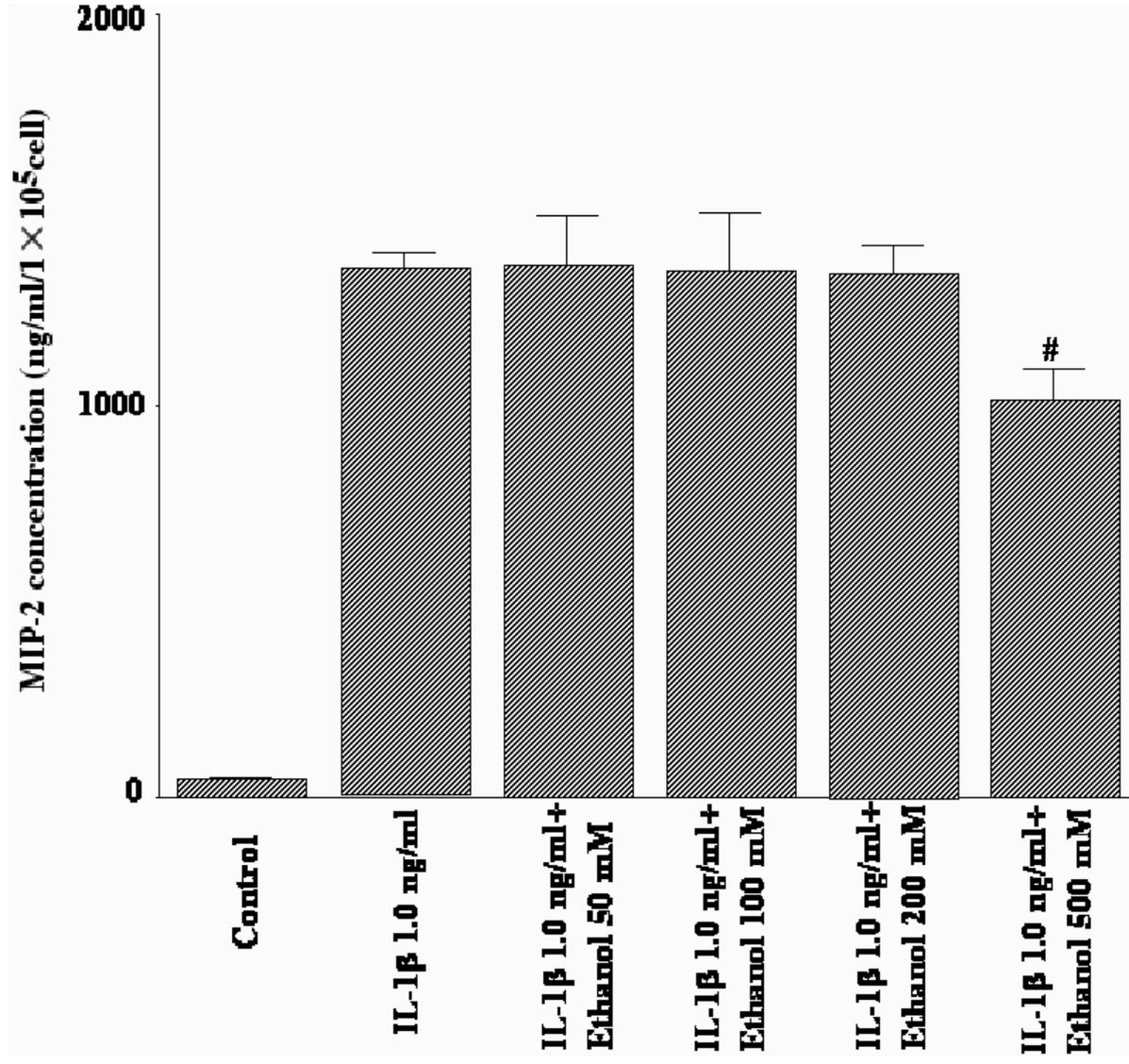


Fig 2C

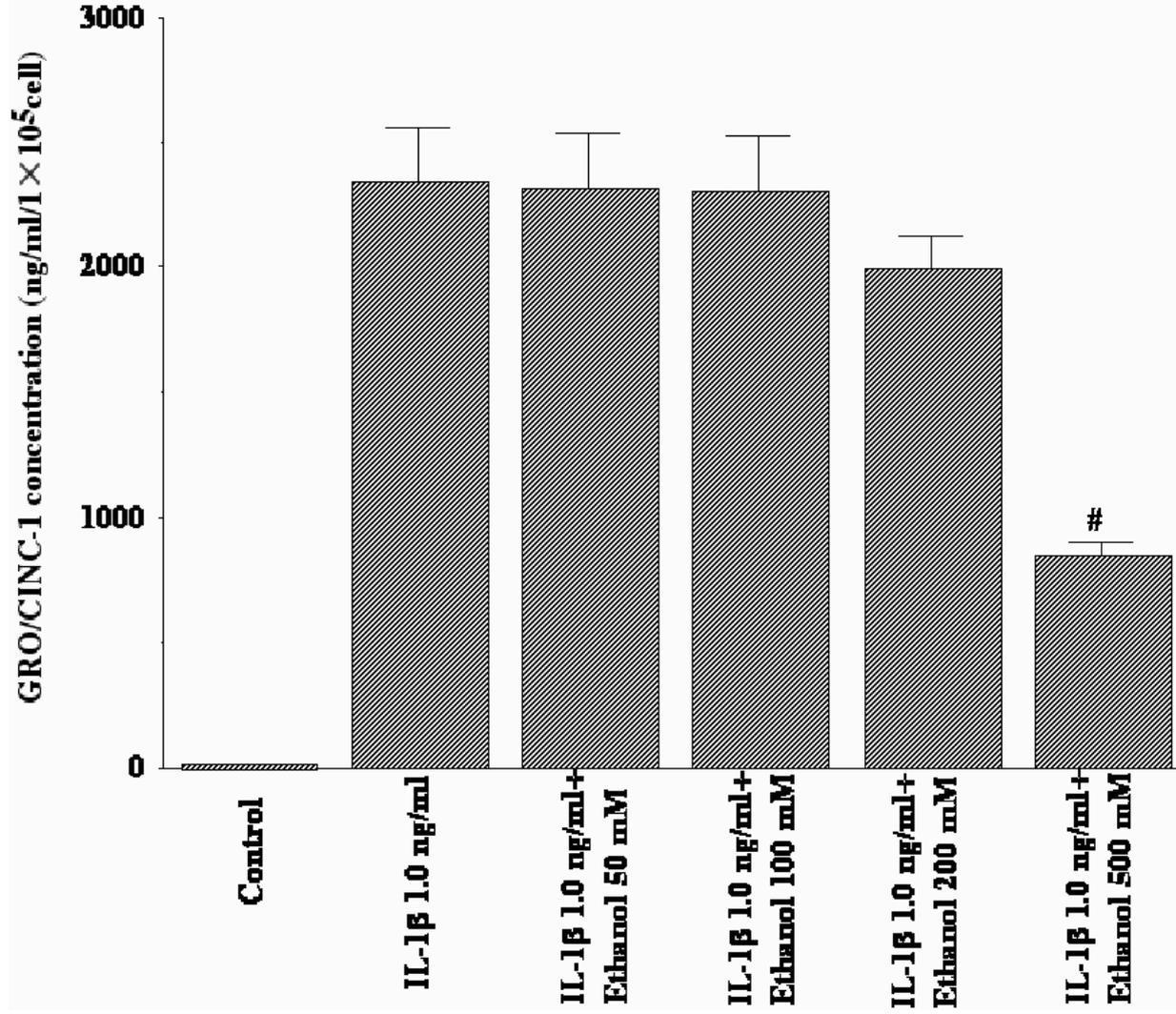


Fig 2D

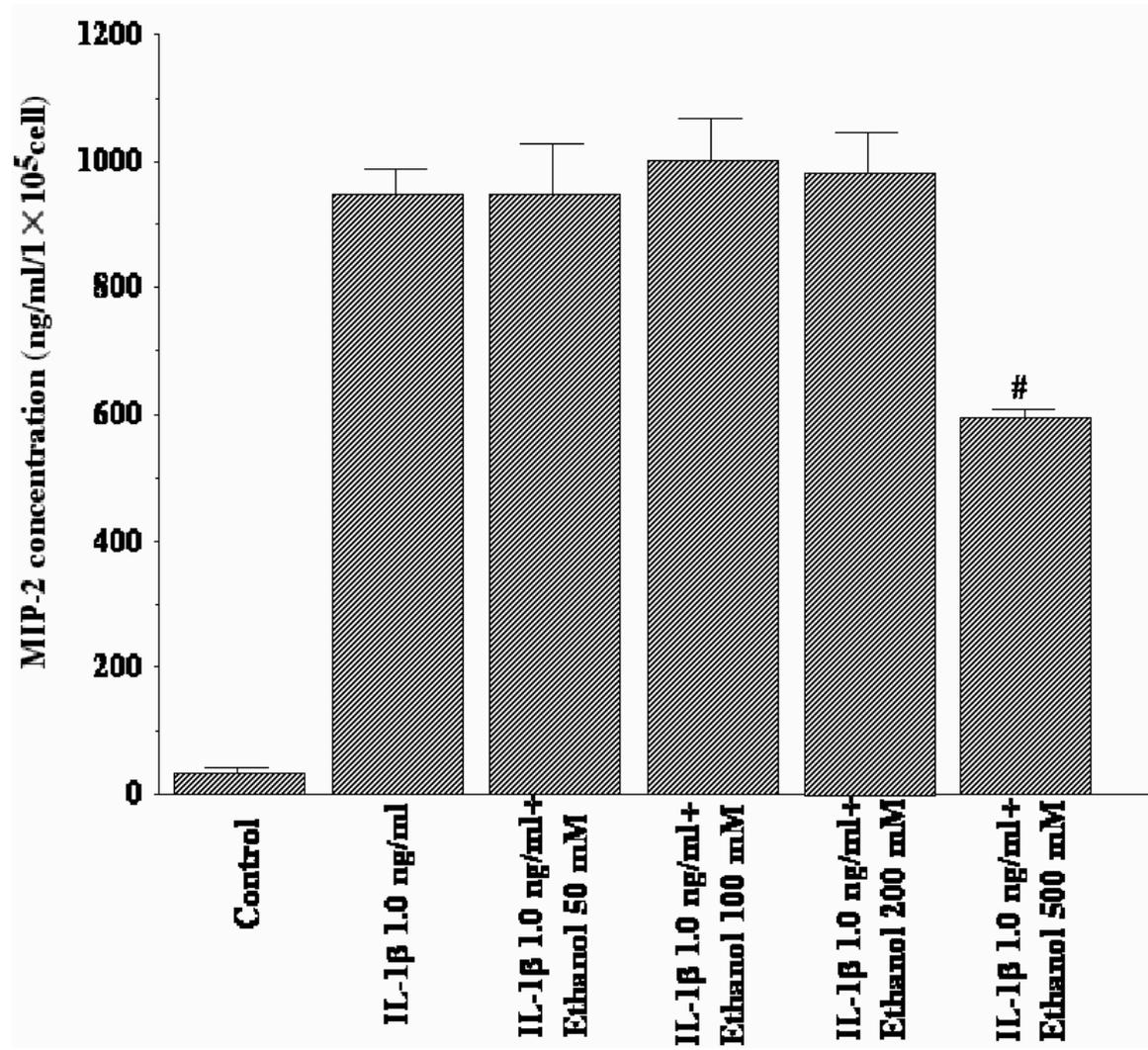


Fig 3

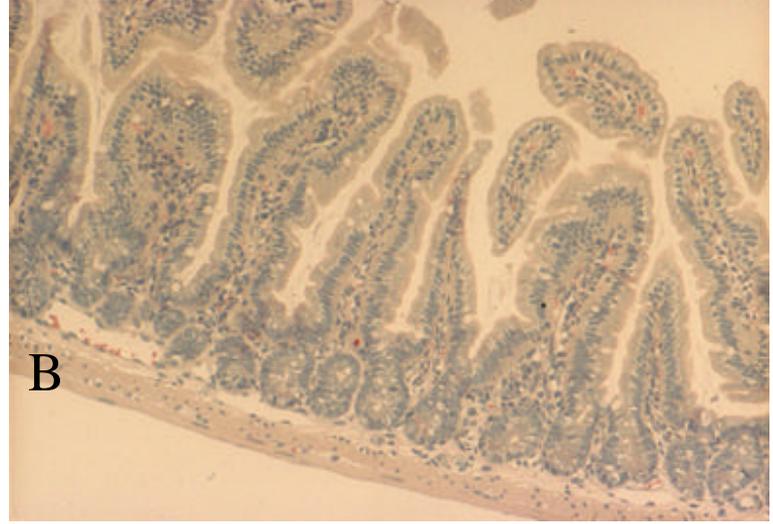
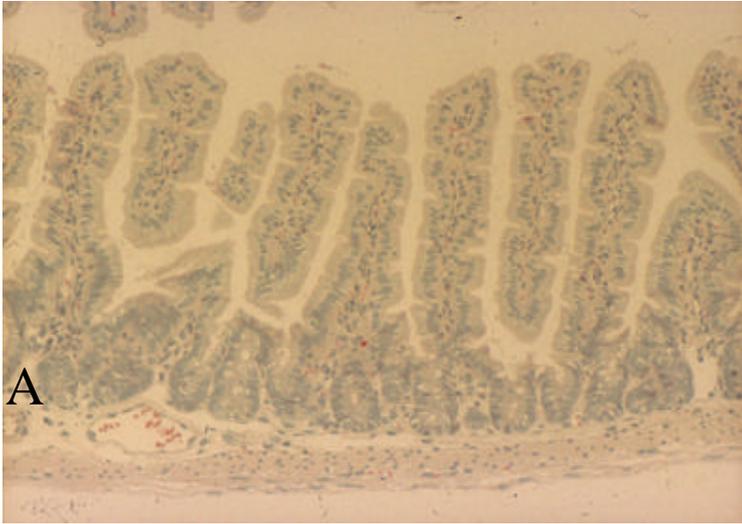


Fig 4A

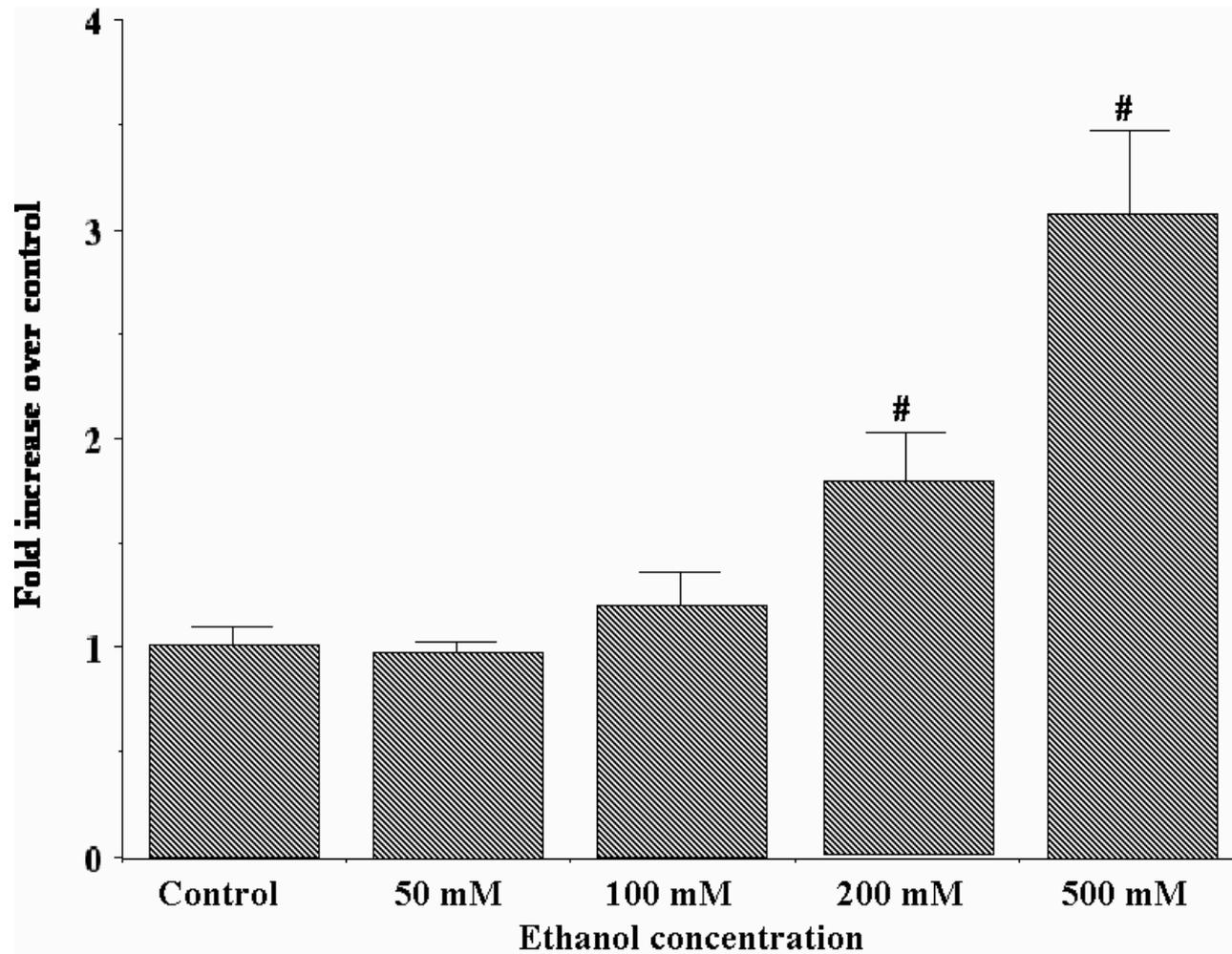


Fig 4B

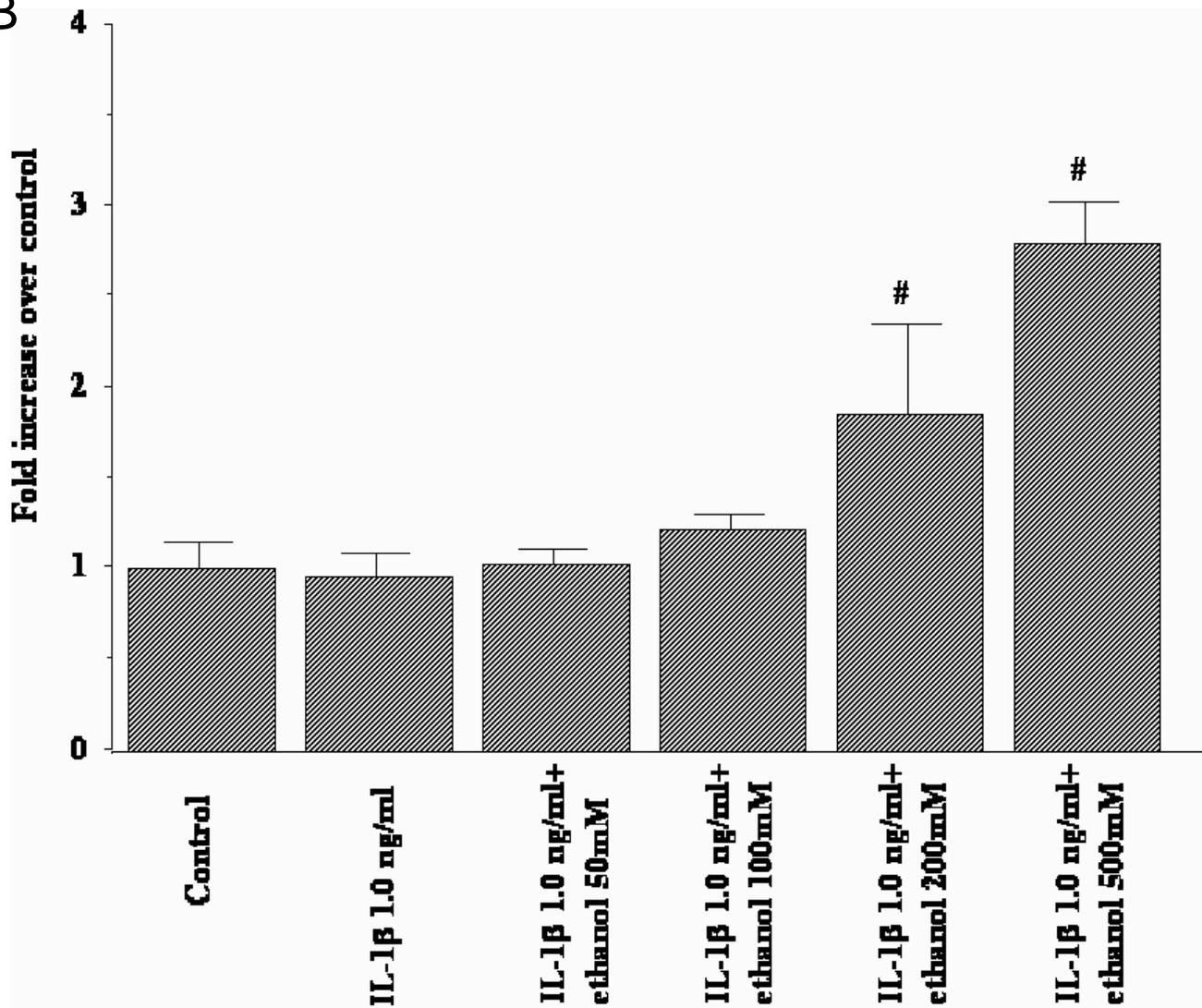


Table 1

Treatment	Fold increase over control
control	1.0
ethanol 50 mM	1.0±0.05
ethanol 100 mM	0.9±0.13
ethanol 500 mM	1.0±0.07
IL-1 $\beta$ 0.1 ng/ml	1.0±0.02
IL-1 $\beta$ 0.1 ng/ml+ethanol 50 mM	0.9±0.04
IL-1 $\beta$ 0.1 ng/ml+ethanol 100 mM	1.0±0.04
IL-1 $\beta$ 0.1 ng/ml+ethanol 200 mM	1.0±0.07
IL-1 $\beta$ 0.1 ng/ml+ethanol 500 mM	1.0±0.07
IL-1 $\beta$ 1.0 ng/ml	1.0±0.01
IL-1 $\beta$ 1.0 ng/ml+ethanol 50 mM	1.0±0.01
IL-1 $\beta$ 1.0 ng/ml+ethanol 100 mM	0.9±0.09
IL-1 $\beta$ 1.0 ng/ml+ethanol 200 mM	0.8±0.06#
IL-1 $\beta$ 1.0 ng/ml+ethanol 500 mM	0.7±0.10#