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Zinc supplementation attenuates ethanol- and acetaldehyde-induced liver stellate cell activation by inhibiting reactive oxygen species (ROS) production and by influencing intracellular signaling

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Running title: Zinc inhibits activation of liver stellate cells

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## Abstract

*Background/Aims.* Zinc has been reported to prevent and reverse liver fibrosis in vivo; however, the mechanisms of its action are poorly understood. We therefore aimed to determine the antifibrotic potential of zinc.

*Methods.* Assessed was the influence of preincubation of rat HSCs with 30  $\mu\text{M}$   $\text{ZnCl}_2$  on ethanol- (in the presence of 4-methyl pyrazole (4-MP)) or acetaldehyde-induced toxicity; apoptosis; migration, expression of smooth muscle  $\alpha$ -actin ( $\alpha$ -SMA) and procollagen I; release of reactive oxygen species (ROS), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), tumor growth factor- $\beta$ 1 (TGF- $\beta$ 1); metalloproteinase-2 (MMP-2) and tissue inhibitors of metalloproteinases (TIMPs) production. Intracellular signals such as nuclear factor- $\kappa$ B (NF $\kappa$ B), C-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (p38 MAPK) induced by ethanol and its metabolite was also assessed.

*Results.* 30  $\mu\text{M}$  zinc protected HSCs against ethanol and acetaldehyde toxicity and inhibited their apoptosis. Zinc inhibited the production of ROS by HSCs treated with ethanol and acetaldehyde and inhibited their migration. Zinc also inhibited ethanol- and acetaldehyde-induced TGF- $\beta$ 1 and TNF- $\alpha$  production. Zinc down-regulated ethanol- and acetaldehyde-induced production of TIMP-1 and TIMP-2 and decreased the activity of MMP-2. In ethanol- and acetaldehyde-induced HSCs, zinc inhibited the activation of the p38 MAPK as well as the JNK transduction pathways and phosphorylation of I $\kappa$ B and Smad3.

*Conclusion.* The results indicated that zinc supplementation inhibited ethanol- and acetaldehyde-induced activation of HSCs on different levels, acting as an antioxidant and inhibitor of MAPK, TGF- $\beta$  and NF $\kappa$ B/ I $\kappa$ B transduction signaling. The remarkable inhibition of several markers of HCS activation makes zinc a promising agent for antifibrotic combination therapies.

Key words: liver stellate cells, zinc, cytokines, MAPK, NF $\kappa$ B

## 1. Introduction

Liver fibrosis is caused by a variety of etiologic agents, including chronic viral hepatitis, alcohol toxicity, autoimmune disease, and hereditary metabolic disorders. For all of these diseases, there is a common pathologic mechanism that leads to fibrosis: the generation and proliferation of smooth muscle  $\alpha$ -actin ( $\alpha$ -SMA)-positive myofibroblasts of periportal and perisinusoidal origin that arise as a consequence of the activation of hepatic stellate cells (HSCs). HSCs exist in the normal liver as quiescent retinoid-storing cells, which in response to injury activate to become proliferative, profibrogenic cells [1,2]. This event can be recapitulated in a culture model in which isolated HSCs are cultured on plastic in serum-containing media. The activated HSCs are a rich source of type I and III fibrillar collagen and also secrete high levels of the tissue inhibitor of metalloproteinase 1 (TIMP-1) [3]. Many of the morphological and metabolic changes associated with HSC activation during fibrogenesis *in vivo* are also observed in HSCs grown in culture on plastic. These changes include the expression of new receptors, such as platelet-derived growth factor  $\beta$  receptors 1 and 2. Other receptors, such as the receptor for insulin-like growth factor 1 and endothelin are present on both quiescent and activated HSCs [4,5]. Several soluble factors, including growth factors, cytokines, chemokines and oxidative stress products, play a role in the activation of HSCs. Activation of HSCs is associated with the sequential expression of several key cytokines and their surface receptors, including transforming growth factor  $\beta$  (TGF- $\beta$ ) and its receptors [6]. Exogenous expression of TGF- $\beta$  in the liver induces liver fibrosis, and blockade of TGF- $\beta$  signaling by multiple methods prevents progression of liver fibrosis in experimental animals [7]. TGF- $\beta$  downstream signaling is mediated by Smad 2 and Smad 3, which are structurally

similar but functionally distinct. They are differentially activated by TGF- $\beta$  in quiescent and activated HSCs and play different roles in HSC function [8,9].

The development of liver fibrosis in alcoholics has been linked to the oxidation of ethanol to the highly active compound acetaldehyde. At concentrations that have been detected in hepatic venous blood during alcohol consumption, acetaldehyde stimulates type I collagen synthesis and gene transcription in cultured rat and human HSCs through protein kinase C (PKC) activation [10]. Acetaldehyde has also been shown to increase NF $\kappa$ B (p65) and its binding to the  $\alpha_2$ (I) collagen promotor [11] by a mechanism dependent on the accumulation of H<sub>2</sub>O<sub>2</sub> [12,13,14]. CYP2E1 is an important source of reactive oxygen species (ROS) in alcohol-induced injury and fibrosis, generating superoxide (O<sub>2</sub><sup>-</sup>) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). It has been detected that inhibition of CYP2E1 activity by diallylsulfide (DAS) prevents induction of collagen I gene expression in rat stellate cells overexpressing CYP2E1 [15]. Oxidative stress also activates the c-Jun NH<sub>2</sub>-terminal kinase (JNK), a protein which regulates secretion of proinflammatory cytokines by cultured HSCs [16,17].

Matrix metalloproteinases (MMP), a family of zinc metallo-endopeptidases, are promptly expressed by HSCs in response to diverse hepatic toxins. In vitro experiments have demonstrated the role of MMPs in the activation of HSCs. Also proliferation of HSCs was promoted by pericellular collagen I proteolysis acting via  $\alpha_v\beta_3$  integrins [18]. Conversely, MMPs may also contribute to regression of liver fibrosis through cleavage of the fibrillar ECM and promotion of apoptosis among the activated HSCs. Thus MMPs play dual roles in liver fibrosis, depending on the timing [19].

Zinc is an essential nutrient that is necessary for a broad range of biological activities. To date, more than 300 zinc-containing enzymes have been identified. Serum and hepatic zinc concentrations are decreased in chronic liver diseases, and zinc depletion has been suggested as a cause of hepatic fibrosis [20,21]. Zinc supplementation has been shown to have a

favorable effect on fibrosis in vivo and in vitro [22,23]; however, the mechanisms of the anti-fibrotic effect of zinc are not fully understood. In a pilot study in vivo with oral zinc supplementation, down-regulation of TIMP-1 was detected in sera of patients whose zinc levels had been raised [23]. As TIMP-1 has been described [24] to have an antiapoptotic effect on activated HSCs, such a decrease in its production can be beneficial for the resolution of liver fibrosis. It has also been detected that in zinc-deficient HSCs, depletion of intracellular glutathione levels triggers a progression of collagen synthesis [21].

The aim of this study was to investigate the effect of zinc supplementation on the viability, proliferation, apoptosis and activation of rat HSCs cultured in vitro. We also examined whether zinc influenced the production of some cytokines, MMPs, and TIMPs in ethanol- and acetaldehyde-activated HSCs. To assess the mechanisms of zinc influence on HSC activation, we examined whether zinc changed the intracellular signaling involved in HSC activation, such as TGF- $\beta$ -inducing signaling, NF $\kappa$ B, JNK and p38 MAPK activation.

## 2. Materials and Methods

### 2.1. Cell cultures

A moderately activated rat liver stellate cell line CFSC-2G was kindly provided by Dr Marcos Rojkind (Department of Clinical Investigation, Walter Reed Army Medical Center, Washington). CFSC-2G cells were cultured in Eagle's Medium (MEM), supplemented with 5% heat-inactivated fetal calf serum (FCS), 1% nonessential amino acids (NEAA), and 1% Antibiotic-Antimycotic, pH 7.4. The cells were seeded in tissue culture plates (Falcon, Bedford, MA, USA) and incubated at 37<sup>0</sup>C in a humidified atmosphere of 5% CO<sub>2</sub>. CFSC-2G cells were subcultured twice a week by trypsinization in a 0.25% trypsin-EDTA solution after washing with Ca-Mg-free saline. The culture media, antibiotics, 0.25% trypsin-EDTA, FCS 7

and NEAA were obtained from Sigma-Aldrich (Steinheim, Germany). In some experiments, Hanks' Balanced Salt Solution (HBSS) (Sigma-Aldrich) was used.

2.2. The influence of different ethanol and acetaldehyde concentrations on CFSC-2G cell viability after preincubation with  $ZnCl_2$ .

CFSC-2G cells were grown in 96-well plastic plates (Nunc, Roskilde, Denmark),  $2 \times 10^5$  cells/well, in Eagle's Medium (MEM) supplemented with 5% FCS. After 24 h incubation, the medium was replaced with a fresh one with addition of 0.1% FCS and  $30 \mu M ZnCl_2$  (Sigma). After another 24 h of incubation, different ethanol (100-500 mM) or acetaldehyde (0.5-3 mM) concentrations were added. Ethanol and acetaldehyde were purchased from Merck (Darmstadt, Germany) and maintained as 1 M stock solutions. The cells treated with ethanol or acetaldehyde were maintained in a humidified  $CO_2$ -incubator at  $37^{\circ}C$  for 24h. The toxicity of those chemicals was determined by the 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) (Sigma) assay in which the yellow tetrazolium salt was metabolized by viable cells to purple formazan crystals. CFSC-2G cells were incubated for 3 h with the MTT solution (5 mg/ml). Formazan crystals were solubilized overnight in SDS buffer (10 SDS in 0.01 N HCl), and the product was quantified spectrophotometrically by measuring absorbance at 570 nm wavelength using an E-max Microplate Reader (Molecular Devices Corporation, Menlo Park, CA, USA). Data were presented as % of control cell viability. According to the data including in Fig. 1 for further experiments, three ethanol concentrations (5, 10 and 50 mM) and two concentrations of acetaldehyde (75 and  $175 \mu M$ ) were chosen because they did not exhibit any toxicity for CFSC-2G cells.

2.3. Cell proliferation assay.

2.3.1. 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay



CFSC-2G cells were grown in 96-well plastic plates (Nunc, Roskilde, Denmark),  $5 \times 10^3$  cells/well, in Eagle's Medium (MEM) supplemented with 5% FCS for 24 h. Then the medium was replaced with a fresh one with addition of ethanol (0-500 mM), acetaldehyde (0-500  $\mu$ M) or zinc (1-100  $\mu$ M). After 96 h incubation at 37<sup>0</sup>C in a humidified atmosphere of 5% CO<sub>2</sub>, the MTT assay was performed, and the data were presented as % of control cell viability.

### 2.3.2. BrdU assay

CFSC-2G were seeded at a density  $5 \times 10^3$  per well in 96-well plates and cultured in 5% FCS-MEM for 24 h. Then the medium was replaced with a fresh one with addition of ethanol (0-500 mM), acetaldehyde (0-500  $\mu$ M) or zinc (1-100  $\mu$ M). After 96 h incubation at 37<sup>0</sup>C in a humidified atmosphere of 5% CO<sub>2</sub>, the cells were exposed to 10  $\mu$ M BrdU for a 2-h labeling period. The incorporation of BrdU was measured by a colorimetric BrdU cell proliferation ELISA according to the manufacturer's instructions (Roche Diagnostics GmbH, Mannheim, Germany).

## 2.4. Migration assay

### 2.4.1. Cell migration was assessed using an *in vitro* wound closure assay

CFSC-2G cells were plated at  $6 \times 10^5$  on 4-cm culture dishes (Nunc) in 5% FCS-MEM for 24 h. Then one linear wound was scraped in each well with a sterile pipette tip (P300). The wounded monolayers were rinsed twice with culture medium to remove all cellular debris, and the medium was replaced with a fresh one with addition of 0.1% FCS-MEM and, in some cultures, also 30  $\mu$ M ZnCl<sub>2</sub>. After 24 h incubation, 50 mM ethanol or 175  $\mu$ M acetaldehyde was added into wells with or without ZnCl<sub>2</sub>. Control cells were cultured in 0.1% FCS-MEM. The number of cells that had migrated into the wounded area after 24 h was estimated in control and cultures treated with ethanol or acetaldehyde which were pretreated or not with 30  $\mu$ M ZnCl<sub>2</sub>. Plates were stained with the May-Grünwald-Giemsa method. The observation

was performed under an Olympus BX51 System Microscope (Olympus Optical, Tokyo, Japan), and micrographs were prepared using the analySIS software (Soft Imaging System GmbH, Münster, Germany). Cells which had migrated to the wounded areas were counted on micrographs, and the results were expressed as a mean number of cells which had migrated to 100 selected wounded areas taken from three micrographs.

#### 2.4.2. Cell migration through Matrigel.

To mimic the microenvironment of the space of Disse, insert membranes with 8- $\mu$ m pores (6.5 mm Transwell plates, Costar) were covered with Matrigel (Sigma, E1270) (100  $\mu$ g/cm<sup>2</sup>), which was prepared according to the manufacturer's instructions. CFSC-2G cells (2 x 10<sup>4</sup>/well) were added into the upper chamber, and were cultured in MEM supplemented with 0.1% FCS. The bottom wells of the chamber were filled with the same medium with or without 30  $\mu$ M ZnCl<sub>2</sub>. After 24 h incubation at 37<sup>0</sup>C in a humidified atmosphere of 5% CO<sub>2</sub>, acetaldehyde (at a final concentration 175  $\mu$ M) was added into part of the bottom wells. The Transwell plates were incubated for another 24 h to allow possible migration of CFSC-2G cells through the membrane into the lower chamber. The chambers were then immersed in 100% methanol (Sigma) for 1 min for fixation, and all cells were then stained for 5 min with hematoxylin (Sigma). The cells remaining on the top surface of the membrane were completely removed with a cotton swab, and the membrane was removed from the chamber and mounted on a glass slide. The number of infiltrating cells was counted in ten selected regions and determined by the mean count. The experiments were repeated three times.

### 2.5. Apoptosis measurement

#### 2.5.1. Preparation of CFSC-2G cells for caspase 3 activity assay

For the measurement of caspase 3 activity, CFSC-2G were grown in 6-well plastic plates (4 x 10<sup>5</sup> cells/ml, 5 ml/well) in 5% FCS-MEM for 24 h in a humidified CO<sub>2</sub>-incubator at 37<sup>0</sup>C.

Then the medium was replaced with fresh 0.1% FCS-MEM with or without  $\text{ZnCl}_2$  (final concentration 30  $\mu\text{M}$ ). The next day, 50 mM ethanol or 175  $\mu\text{M}$  acetaldehyde were added into the wells with or without  $\text{ZnCl}_2$ , and the wells were incubated for another 18 h at  $37^\circ\text{C}$ . Each sample was prepared in duplicate, with or without the addition of a caspase inhibitor (Z-VAD-FMK, final concentration 20  $\mu\text{M}$ ) (Promega, Madison, WI). Control wells were incubated without the addition of zinc, ethanol or acetaldehyde. After washing with Ca-Mg-free saline, the cells were scraped with a rubber policeman, transferred into Eppendorf tubes with Lysis Buffer (25  $\mu\text{l}$  of cold Lysis Buffer per  $1 \times 10^6$  cells). The cell lysate was incubated on ice for 10 min and then centrifuged at  $10,000 \times g$  for 1 min.

#### 2.5.2. Caspase 3 enzymatic assay

The enzyme activity was determined with a Caspase 3 Colorimetric Assay kit (R&D Systems Inc., Minneapolis, MN). The enzymatic reaction was carried out in 96-well flat bottom microplates. 50  $\mu\text{l}$  of cell lysates (derived from  $2 \times 10^6$  cells) were tested in duplicate experiments by measuring the proteolytic cleavage of DEVD-pNA, a colorimetric substrate specific for caspase 3. Recombinant caspase 3 (R&D Systems Inc., Minneapolis, MN) was used as positive control. After 2 hours of incubation at  $37^\circ\text{C}$ , color development was measured with a microtiter plate reader (E-max, Molecular Devices Co, Menlo Park, CA, USA) using 405 nm wavelength light. Caspase 3 activity in the treated cells was compared to that in the untreated cells, and expressed as a fold change.

#### 2.6. Treatment of CFSC-2G cells with ethanol in the presence of cytochrome P4502E1 or ADH inhibitors

CFSC-2G cells were grown in 96-well plastic plates (Nunc, Roskilde, Denmark),  $2 \times 10^4$  cells/well with nontoxic concentrations of ethanol metabolism inhibitors: cytochrome P4502E1 – 2 mM DAS (Sigma) or ADH – 2 mM 4-MP (Sigma). After 24 h of incubation, an

oxygen burst was induced by ethanol at a concentration of 50 mM, and the production of superoxide anion was measured by the cytochrome c reduction assay [25].

## 2.7. Treatment of CFSC-2G cells with ethanol or acetaldehyde after preincubation with 30 $\mu$ M ZnCl<sub>2</sub>

CFSC-2G cells were grown in 6-well plastic plates ( $4 \times 10^5$  cells/ml, 5 ml/well) in 5% FCS-MEM for 24 h in a humidified CO<sub>2</sub>-incubator at 37°C. Then the medium was replaced with fresh 0.1% FCS-MEM with or without 2 mM 4-MP and with or without 30  $\mu$ M ZnCl<sub>2</sub> for another 24 h at 37°C. Then the inductors, ethanol at different concentrations (final concentrations 5, 10 or 50 mM) or acetaldehyde (final concentration 175  $\mu$ M), were added into wells. The wells, to which ethanol was added had been supplemented with 2 mM 4-MP. Plates were prepared in duplicate; a) after 20 min of incubation and washing twice with PBS, the cells were collected for Western blot analysis of phospho- and total NF $\kappa$ B, phospho- and total I $\kappa$ B, phospho- and total JNK, and phospho- and total p38 b) after 24 h of incubation, the cultures were washed twice with PBS and the cells were harvested for Western blot analysis of  $\alpha$ -SMA, procollagen I, TIMP-1, TIMP-2, MMP-13, and phospho- and total Smad 3.

## 2.8. Treatment of CFSC-2G cells with ethanol or acetaldehyde in the presence of the protein kinase C inhibitor and with or without preincubation with 30 $\mu$ M ZnCl<sub>2</sub>

CFSC-2G cells were grown in 6-well plastic plates ( $4 \times 10^5$  cells/ml, 5 ml/well) in 5% FCS-MEM for 24 h in a humidified CO<sub>2</sub>-incubator at 37°C. Then the medium was replaced with fresh 0.1% FCS-MEM with 2 mM 4-MP and with or without 1  $\mu$ M calphostin C (a protein kinase C inhibitor) (Sigma) or without 30  $\mu$ M ZnCl<sub>2</sub> for another 24 h at 37°C. Then the inductors, ethanol at different concentrations (final concentrations 5, 10 or 50 mM in wells with 4-MP) or acetaldehyde (final concentration 175  $\mu$ M), were added into wells. After 24 h

of incubation, the cultures were washed twice with PBS and the cells were harvested for Western blot analysis of  $\alpha$ -SMA.

#### 2.9. Measurement of superoxide anion ( $O_2^-$ ) production by cytochrome c reduction assay [25]

CFSC-2G cells were grown in 96-well plastic plates ( $2 \times 10^4$  cells/well) for 24 h at  $37^\circ\text{C}$  in a humidified atmosphere of 5%  $\text{CO}_2$ . Then, the cultures were washed twice with HBSS and the culture medium was replaced with fresh 0.1% FCS-MEM with or without  $30 \mu\text{M}$   $\text{ZnCl}_2$ . The next day, an assay for superoxide anion was performed. Briefly, HBSS ( $207.5 \mu\text{l}$ ),  $12.5 \mu\text{l}$  of cytochrome c solution in HBSS (final concentration  $75 \mu\text{M}$ ),  $5 \mu\text{l}$  of either SOD solution (final concentration  $60 \text{ U/ml}$ ) or  $5 \mu\text{l}$  HBSS, and  $25 \mu\text{l}$  ethanol solution in HBSS (final concentration of 5, 10 or  $50 \text{ mM}$ ) or  $25 \mu\text{l}$  acetaldehyde solution in HBSS (final concentration of 75 or  $175 \mu\text{M}$ ) were added into each well on a 96-well plate. Also control wells were used, where cells were incubated without ethanol or acetaldehyde. The microplate was incubated at  $37^\circ\text{C}$  for 60 min and transferred to the microplate reader. The absorbance values at 550 nm (the differences in OD between samples with and without SOD) were converted to nanomoles of  $O_2^-$  based on the extinction coefficient of cytochrome c:  $\Delta E_{550} = 21 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$ . The results were expressed as nanomoles of  $O_2^-$  per  $1 \times 10^6$  cells per 60 min. All chemicals were purchased from Sigma –Aldrich.

#### 2.10. Measurement of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) production [26]

HepG2 cells were grown in 96-well plastic plates ( $4 \times 10^4$  cells/well) with or without  $30 \mu\text{M}$   $\text{ZnCl}_2$ . After 24 h incubation at  $37^\circ\text{C}$ , the cultures were washed twice with HBSS, and HBSS ( $100 \mu\text{l/well}$ ) with an addition of ethanol (final concentration of  $50 \text{ mM}$ ) or acetaldehyde (final concentration of  $175 \mu\text{M}$ ) for 60 min at  $37^\circ\text{C}$  into wells was added. In control wells, cells were incubated without ethanol or acetaldehyde. Those wells were washed

twice with HBSS and a measurement of intracellular hydrogen peroxide was performed. The assay was based on horseradish-dependent peroxidation (HRPO) of phenol red by  $H_2O_2$  leading to the formation of a compound that exhibited absorbance at 600 nm. Briefly, the cells were covered with 100  $\mu$ l/well of the assay solution, which was prepared on the day of the experiment and consisted of HBSS, phenol red (Sigma, final concentration of 0.56 mM), HRPO (Serva, Heidelberg, Germany, final concentration of 20 U/ml). Additionally, 10  $\mu$ l/well of 1N NaOH was added. After 3 min of incubation, the plate was read at 600 nm in the microplate reader. The results were expressed as nanomoles  $H_2O_2$  per  $10^6$  cells per 60 min based on the phenol red extinction coefficient ( $\Delta E_{600} = 19.8 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ ).

#### 2.11. TNF- $\alpha$ , TGF- $\beta$ , MMP-2 and TIMP-1 assay

CFSC-2G cells were cultured, in duplicate, in 24-well plastic plates (Nunc) at a density of  $2 \times 10^5$  cells/ml/well in 5% FCS-MEM with or without 2 mM 4-MP for 24 h in a humidified  $CO_2$ -incubator at  $37^\circ C$ . Then the culture medium was replaced with 0.1% FCS-MEM containing or not 30  $\mu$ M  $ZnCl_2$  and 2 mM 4-MP. After 24 h, a selected concentration of ethanol (10 or 50 mM) or acetaldehyde (175  $\mu$ M) was added. After incubation for another 24 h at  $37^\circ C$ , 5%  $CO_2$ , cell culture supernatants were collected, centrifuged and frozen immediately at  $-80^\circ C$  for a further TNF- $\alpha$ , TGF- $\beta$ , MMP-2 and TIMP-1 assay. Culture supernatants were stored for no longer than 3 weeks. Rat TNF- $\alpha$ , TGF- $\beta$ , MMP-2 and TIMP-1 present in the supernatants from CFSC-2G cells were measured by the ELISA method using kits from Bender MedSystems Diagnostics (TNF- $\alpha$ ) and R&D Systems (TGF- $\beta$ , MMP-2, TIMP-1). The kits contained a specific monoclonal antibody immobilized on a 96-well microtiter plate that bound TNF- $\alpha$ , TGF- $\beta$ , MMP-2 or TIMP-1 in the aliquot and a second enzyme-conjugated specific polyclonal antibody. Following several washings to remove unbound substances and antibodies, a substrate solution was added to the wells. Color

development was stopped by sulfuric acid and the intensity of color was measured using a microtiter plate reader (E-max, Molecular Devices Co, Menlo Park, CA) at 450 nm (correction at 550 nm or 620 nm). The detection limits were TNF- $\alpha$ >11.2 pg/ml, TGF- $\beta$ >4.61 pg/ml, MMP-2>0.16 ng/ml and TIMP-1>3.5 pg/ml. Intra-assay variations were less than 10%.

## 2.12. Western blot analysis

CFSC-2G cells were harvested and lysed in RIPA buffer (50 mM Tris/HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1% sodium deoxycholate, 0.1% SDS, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 10 mM NaF, and a protease inhibitor cocktail) and then centrifuged 10,000 rpm/5 min at 4<sup>0</sup>C. Proteins were assayed using a BCA Protein Assay Kit (Pierce, Rockford, Ill, USA). For Western blot analysis, supernatants of RIPA cell lysates were solubilized in 5xSDS sample buffer (100 mM Tris/HCl pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue, 3%  $\beta$ -mercaptoethanol) and then boiled for 5 min at 100<sup>0</sup>C. Equal amounts of the total cellular protein extract were separated on 10% SDS-PAGE at 200 V for 1 h under reducing conditions and electrotransferred in a semi-dry way to polyvinylidene difluoride membranes (PVDF, Millipore, Whatman) at 15 V for 15 min in a transfer buffer (47.8 mM Tris/HCl, 0.293% glycine, 20% methanol), pH 8.1 . After blocking for 15 min at room temperature with 10% dried nonfat milk/TBS/0.1% Tween 20, the membranes were probed overnight at +4<sup>0</sup>C with the primary antibody (diluted in 1% BSA/TBS/0.1% Tween 20) as follows: rabbit polyclonal anti-procollagen type I (1:250, Santa Cruz Biotechnology), mouse monoclonal anti- $\alpha$ -SMA (1:1000, Sigma), mouse monoclonal anti- $\beta$ -actin (1:4000, Sigma), mouse monoclonal anti-TIMP-1 (1:500, R&D Systems), rabbit monoclonal anti-TIMP-2 (1:1000, Sigma), rabbit polyclonal anti-MMP-13 (1:200, USBiological), rabbit monoclonal anti-total Smad 3 (1:2000, Epitomics), rabbit monoclonal anti-phospho Smad 3 (1:1000, Epitomics), rabbit polyclonal anti-total NF $\kappa$ B p65 (1:2000, Chemicon), rabbit

polyclonal anti-phospho NF $\kappa$ B p65 (1:500, Rockland), rabbit polyclonal anti-total I $\kappa$ B- $\alpha$  (1:2000, Sigma), mouse monoclonal anti-phospho I $\kappa$ B- $\alpha$  (1:1000, USBiological), rabbit polyclonal anti-total JNK (1:2000, Sigma), rabbit polyclonal anti-phospho JNK 1/2 (1:1000, Sigma), rabbit polyclonal anti-total p38 (1: 10 000, Sigma), and rabbit polyclonal anti-phospho p38 (1:1000, Sigma). After repeated washing (TBS/0.1% Tween 20), the membranes were incubated with the horseradish peroxidase-conjugated secondary (anti-rabbit or anti-mouse) antibody (1:4000, in 1% BSA/TBS/0.1% Tween 20, Amersham Biosciences, Buckinghamshire, UK), and visualized using enhanced chemiluminescence reaction (ECL Western Blotting System, Amersham Biosciences, Buckinghamshire, UK). Protein bands were scanned, and the band intensities quantified using ImageJ densitometry software. All chemicals were purchased from Sigma-Aldrich.

### 3. Statistical analysis

Values are expressed as mean  $\pm$  S.D. The significance of differences was determined with the use of an analysis of variance (Statistica computer package). A number of statistical tests were used; these included the two-way ANOVA test with post-hoc Tukey's test and Wilcoxon's paired test for comparisons inside groups. P values  $<0.05$  were considered to be significant.

### 4. Results

#### 4.1. The influence of preincubation of HSCs with 30 $\mu$ M ZnCl<sub>2</sub> on their viability, proliferation and apoptosis after activation in vitro with ethanol and acetaldehyde

HSCs cultivated on plastic plates and silenced by a low (0.1%) content of FCS in the medium were very resistant to ethanol and acetaldehyde toxicity. To demonstrate the effect of zinc supplementation to cell viability, the high concentrations of both ethanol and



acetaldehyde were used. Ethanol at a 50 mM concentration and acetaldehyde at an 0.5 mM concentration exhibited no toxicity for HSCs in vitro. As can be seen from Fig 1., preincubation of HSCs with 30  $\mu$ M (non-toxic)  $ZnCl_2$  significantly inhibited the toxicity of ethanol and acetaldehyde. Moreover, the 100 mM ethanol and 175  $\mu$ M acetaldehyde concentrations did not influence HSC growth as measured by the MTT method and the BrdU incorporation method (data not presented). However, 100 mM ethanol caused 2-fold increase in level of cell apoptosis, as measured by caspase 3 activation, and zinc supplementation totally inhibited the apoptosis of HSCs nearly to the level of control (Table 1).

#### 4.2. HSCs metabolize ethanol via the ADH and the C4502E1 pathways, and zinc supplementation inhibits ROS production

HSCs were incubated with ethanol and with inhibitors of ethanol metabolism, diallylsulfide (DAS) as an inhibitor of C4502E1 or 4-methylpyrazole (4-MP) as an inhibitor of alcohol dehydrogenase (ADH), used separately or in combination. Inhibition of ADH also caused an inhibition of superoxide anion production as did the use of the P4502E1 inhibitor. Both inhibitors used together inhibited the production of superoxide anion nearly totally (Fig. 2A). These results indicate that, in accordance with earlier publications, HSCs contain the enzymes of oxidative ethanol metabolism, including the ADH and C4502E1 proteins [27,28]. When HSCs were preincubated with 30  $\mu$ M  $ZnCl_2$  and thereafter with ethanol or acetaldehyde, a significant inhibition of superoxide anion production was observed (Fig 2B). When the level of hydrogen peroxide released from the cells was measured,  $ZnCl_2$  significantly inhibited its production induced by ethanol or acetaldehyde (Fig. 2C).

#### 4.3. The influence of $ZnCl_2$ supplementation on ethanol- and acetaldehyde-induced $\alpha$ -SMA and procollagen I production. Zinc induces HSC quiescence

HSCs were starved by incubation of the cells in a medium with 0.1% FCS and subsequently incubated with ethanol in the presence of 4-MP as an inhibitor of ADH or with acetaldehyde. The intracellular levels of  $\alpha$ -SMA and procollagen type I were estimated by Western blot. Ethanol and acetaldehyde induced the production of both  $\alpha$ -SMA and procollagen type I in a concentration-dependent manner (Fig. 3A and B). Preincubation with  $ZnCl_2$  significantly inhibited the expression of markers of HSC activation.

#### 4.4. The influence of $ZnCl_2$ supplementation on HSC motility.

HSC migration was assessed using the wound assay method. A cell monolayer was wounded with a pipette tip and the number of cells which had migrated into the wounded area after 24 h of incubation with ethanol in the presence of 4-MP or with acetaldehyde was estimated in control and in cultures preincubated with 30  $\mu$ M  $ZnCl_2$ . Zinc supplementation strongly inhibited the migratory activity of HSCs (Fig 4A). In another experiment, we examined the behavior of HSCs seeded on the top of Matrigel-coated filters to mimic the motility of HSCs in the liver. The lower compartment contained a medium with an addition of 175  $\mu$ M acetaldehyde. Zinc supplementation significantly inhibited acetaldehyde-induced migration of HSCs through the membrane coated with Matrigel (Fig. 4B).

#### 4.5. Zinc supplementation down-regulates TGF- $\beta$ 1 and TNF- $\alpha$ production in ethanol- or acetaldehyde-activated HSCs

Preincubation of HSCs with 30  $\mu$ M  $ZnCl_2$  for 24 h caused a significant decrease in ethanol-induced TGF- $\beta$ 1 and TNF- $\alpha$  production in the presence of 4-MP (Fig 5 A and B). Under similar experiment conditions, zinc supplementation also inhibited acetaldehyde-induced TGF- $\beta$ 1 and TNF- $\alpha$  production (Fig 5 C and D).

#### 4.6. Effect of zinc supplementation on metalloproteinase and TIMP production in ethanol- and acetaldehyde-activated HSCs

The influence of preincubation of HSCs with 30  $\mu\text{M}$   $\text{ZnCl}_2$  on the production of MMP-2 and MMP-13 was examined. When total production of MMP-2 was examined by the ELISA method, zinc significantly decreased ethanol-induced production of this metalloproteinase (Fig 6A and B). Zinc supplementation did not influence the activity of MMP-13 in rat HSCs incubated with ethanol or acetaldehyde (Fig 7 A and B). In Western blot analysis, two forms of MMP-13, proMMP-13 and its activated form, were detected. Ethanol, independently of the concentration used, did not influence the production of proMMP-13 or its activated form. Acetaldehyde, in turn, significantly inhibited the amounts of activated MMP-13. Zinc did not influence its activation. Production of TIMP-1 and TIMP-2 by HSCs incubated with ethanol in the presence of 4-MP or with acetaldehyde was examined by ELISA and Western blot. As can be seen from Fig 7A and B, ethanol and acetaldehyde significantly increased TIMP-1 and TIMP-2 production. Zinc supplementation was a strong inhibitor of TIMP-1 production, as detected by more sensitive ELISA method, but in Western blot analysis inhibition was observed only when high 50 mM concentration of ethanol was used. TIMP-2 production estimated using only Western blot analysis was 85% inhibited by zinc supplementation in HSCs treated with 50 mM ethanol or 29% inhibited when induced by 175  $\mu\text{M}$  acetaldehyde (Fig 7a and B).

#### 4.7. Ethanol and acetaldehyde-induced NF $\kappa$ B activation is antagonized by zinc

NF $\kappa$ B is a ubiquitous transcription factor involved in the regulation of cytokine production and action and in the regulation of cell apoptosis. A number of studies have demonstrated that zinc influences the status of NF $\kappa$ B in various cell types [29]. Activation of NF $\kappa$ B is linked to phosphorylation and proteolytic degradation of I $\kappa$ B $\alpha$  [30]. Therefore, we

examined the influence of zinc supplementation on the levels of ethanol or acetaldehyde-induced NF $\kappa$ B in HSCs and also on its phosphorylation. Moreover, we examined the total level of I $\kappa$ B $\alpha$  and its phosphorylation. The experiment revealed that both 50 mM ethanol and 175  $\mu$ M acetaldehyde increased total levels of NF $\kappa$ B and significantly increased phosphorylation of its inhibitor I $\kappa$ B $\alpha$ , while decreasing the total level of the latter. Preincubation of HSCs with 30  $\mu$ M ZnCl<sub>2</sub> decreased the total level of ethanol- and acetaldehyde-induced NF $\kappa$ B, increased the total level of its inhibitor I $\kappa$ B $\alpha$ , while significantly inhibiting the latter's phosphorylation (Fig. 8A and B).

#### 4.8. Zinc supplementation influences MAPK activation in HSCs

Because cell growth and the expression of genes involved in cell growth and cytokine production are widely regulated through MAPK signal cascades, we assessed the effect of zinc supplementation on MAPK activity, including JNK and p38. Treatment of HSCs with both 50 mM ethanol in the presence of 4-MP and with 175  $\mu$ M acetaldehyde significantly enhanced the phosphorylation of JNK and only slightly increased the phosphorylation of p38 MAPK (Fig 8A and B). Preincubation of HSCs with 30  $\mu$ M ZnCl<sub>2</sub> inhibited ethanol-induced phosphorylation of JNK and p38 MAPK by nearly 31% and 26%, respectively, and acetaldehyde-induced phosphorylation of both JNK and p38 MAPK (Fig 8A and B) by nearly 50% and 30%, respectively.

#### 4.9. Effect of zinc supplementation on the TGF- $\beta$ -signaling pathway

Because TGF- $\beta$  signal cascades through Smad 2 and Smad 3 strongly regulate the expression of type I collagen genes [9], we evaluated the effect of zinc supplementation on ethanol- and acetaldehyde-induced phosphorylation of Smad 3. Treatment with zinc

suppressed ethanol- and acetaldehyde-induced phosphorylation of Smad 3 by nearly 75% (Fig 9A and B).

#### 4.10. Zinc supplementation inhibits other than ethanol- or acetaldehyde-induced PKC signaling

Since it was detected that acetaldehyde-elicited overproduction of  $\alpha$ -SMA as a marker of HSC activation is, at least in part, connected with PKC activation, we examined whether preincubation of HSCs with 30  $\mu$ M ZnCl<sub>2</sub> might further inhibit  $\alpha$ -SMA production in the presence of calphostin, a well-known inhibitor of PKC. Calphostin significantly inhibited ethanol- and acetaldehyde-induced  $\alpha$ -SMA, but concomitant zinc supplementation inhibited  $\alpha$ -SMA expression even more efficiently (Fig. 10A and B). It seems likely that zinc may inhibit other signaling pathways induced by ethanol or its metabolite than the PKC pathway.

## 5. Discussion

Our study explored the multiple effects of zinc supplementation on ethanol- or acetaldehyde-activated rat stellate cells (HSCs). Pretreatment of ethanol or acetaldehyde-activated HSCs with 30  $\mu$ M ZnCl<sub>2</sub> influenced several symptoms of HSC activation, caused a decrease in  $\alpha$ -SMA and procollagen type I production, and inhibited their motility, but had no effect on HSC proliferation activity. To assess the mechanisms by which zinc inhibited HSC activation, we examined its antioxidative properties. It is known that zinc supplementation attenuates ethanol-induced hepatic zinc depletion and suppresses ethanol-elevated C4502E1 activity, but increases the activity of ADH in the liver. In consequence zinc suppresses alcohol-induced oxidative stress [31]. In our experiments, zinc supplementation inhibited ethanol-induced (in the presence of the ADH inhibitor 4-methylpyrazole) or acetaldehyde-induced production of superoxide anion and hydrogen peroxide in HSCs. These results are in

agreement with the results of other authors who detected that in HSCs zinc deficiency causes a reduction in intracellular glutathione levels and activation of the cells. Zinc supplementation reverses this process [21]. Of particular interest is the interaction between TGF- $\beta$  and ROS formation. In cultured HSCs, TGF- $\beta$  increases the production of hydrogen peroxide [32], which in turn induces the expression of  $\alpha 1(I)$  procollagen mRNA [33,34]. The direct profibrogenic effect of hydrogen peroxide has also been observed in co-cultures of HSCs with HepG2 cells overexpressing CYP2E1 [35]. It should be stressed that in our study zinc supplementation inhibited both ROS generation and TGF- $\beta$  production.

In addition to its essential role in various immunological functions [36], zinc has a dual effect on the secretion of pro-inflammatory cytokines. It has been reported to trigger the release of these cytokines by monocytes [37], but suppression of cytokine production has also been found [38,39]. This is explained by a concentration-dependent process, in which low zinc doses potentiate cytokine production, while its high concentrations inhibit cytokine secretion [40]. In our experiment, we chose a rather low 30  $\mu$ M concentration of zinc, which did not exhibit any toxicity for HSCs in vitro, and which represented a maximal level of zinc attainable for humans after oral supplementation [41,42]. Zinc at this concentration significantly inhibited ethanol- and acetaldehyde-induced TGF- $\beta 1$  and TNF- $\alpha$  production in HSCs. In the experiments done by other authors in a mouse model of non-alcoholic steatosis, palaprezinc (a zinc-carnosine chelate compound) slightly reduced lipid peroxidation and suppressed HSC activation as well as mRNA expression of TGF- $\beta 1$  and TNF- $\alpha$  [43].

A number of reports show that zinc modulates several signaling pathways, among others NF $\kappa$ B activation and the MAPK pathway [44]. It is also known that the NF $\kappa$ B and MAPK pathways, including p38 and JNK, participate in HSC activation [45,46,47]. Therefore, we examined the influence of 30  $\mu$ M zinc supplementation on NF $\kappa$ B, JNK and p38 MAPK activation and demonstrated an inhibitory effect of zinc on ethanol- or acetaldehyde-induced

activation of the signaling pathways. It has been demonstrated that NF $\kappa$ B binding activity to  $\kappa$ B binding sites in several genes increases in liver macrophages and hepatocytes after CCl<sub>4</sub> treatment of rats, and ongoing production of proinflammatory cytokines regulated by NF $\kappa$ B is believed to play a major role in CCl<sub>4</sub>-induced liver fibrosis [48,49,50]. The key feature of liver fibrosis is the increase in collagen type I synthesis. It has been reported that Col1A2 promoter contains at least 2 putative NF $\kappa$ B binding sites [51]. Oxidative stress is the major factor inducing the phosphorylation of I $\kappa$ B, which releases NF $\kappa$ B, translocating it than to the nucleus to activate the transcription of target genes [52]. Moreover, acetaldehyde itself is able to induce an early activation of NF $\kappa$ B and AP-1 in HepG2 cells (via a PKC-dependent pathway) and in rat HSCs [53,54,55]. Additionally, acetaldehyde-elicited  $\alpha$ 2(I) collagen gene expression in human HSCs is inhibited by calphostin, an inhibitor of PKC [53,54]. In our study, we also detected that, at least in part, overproduction of  $\alpha$ -SMA as a marker of HSC activation involved the PKC pathway, as calphostin inhibited ethanol- and acetaldehyde-induced  $\alpha$ -SMA expression.

A number of studies have implicated zinc in the control of NF $\kappa$ B and AP-1 activation [56,57]. However, the results of these studies regarding the outcome of zinc treatment with regard to the status of NF $\kappa$ B and AP-1 activities are inconsistent. Some reports suggest that zinc supplementation reduces the DNA binding activities of NF $\kappa$ B and AP-1 [56], while zinc chelation increases NF $\kappa$ B and AP-1 DNA binding [57]. Conversely, other studies demonstrate that the addition of zinc exerts an opposite effect on these transcription factors increasing the DNA binding activity of NF $\kappa$ B and AP-1 [58]. In yet another paper, zinc was shown to inhibit NF $\kappa$ B activity, but promote AP-1 activation in cancer cells [59]. Together these data indicated that if zinc inhibits NF $\kappa$ B activity, such inhibition occurs via blocking of I $\kappa$ B kinase (I $\kappa$ K) activity. Indeed, recent studies have demonstrated that thiol-reactive metal compounds inhibit

NF $\kappa$ B activation by blocking I $\kappa$ K [60]. In our study, we also detected that zinc supplementation significantly inhibited phosphorylation of I $\kappa$ B while increasing its total level.

The mechanisms by which ethanol and its metabolite regulate extracellular matrix (ECM) gene expression as markers of HSC activation have not been completely elucidated. Several centers have reported that the MAPK and PI-3K pathways are involved [47,61]. Anania et al. [47] noted that in rat HSCs, phospho-JNK was elevated following exposure to acetaldehyde. It seems likely that JNK is the principal mediator of acetaldehyde-induced  $\alpha$ 1(I) collagen gene up-regulation in rat HSCs. These findings are consistent with those previously reported by Anania et al. [53]. Moreover, in rat pancreatic stellate cells (PSCs) ethanol and acetaldehyde at clinically relevant concentrations (50 mM and 200  $\mu$ M, respectively) activated JNK and p38 MAPK. As ethanol- and acetaldehyde-induced activation of MAPK was blocked by the antioxidant N-acetyl-cysteine, the role of oxidative stress in the signal transduction was suggested [62]. The JNK pathway may be involved in the migration of HSCs within the Disse space to the sites of tissue damage because the JNK inhibitor SP 600125 inhibits HSC migration induced by a TGF- $\beta$  signal [47,61]. The results of our study confirm the involvement of the p38 and JNK pathways in ethanol- and acetaldehyde-induced HSC activation. Moreover, JNK activation occurs concomitantly with the enhanced HSC migratory activity. Zinc inhibited both JNK phosphorylation and HSC migration. It has also been detected that the inhibiting of either p38 MAPK or Smad signaling reduces  $\alpha$ 1(I) collagen gene expression in untreated HSCs, and when both signaling pathways are simultaneously inhibited,  $\alpha$ 1(I) collagen gene expression is essentially blocked [63]. These data indicate that not only MAPK pathways but also TGF- $\beta$ -induced signaling is important in the activation of HSCs. In our study, zinc supplementation also significantly inhibited phosphorylation of Smad 3, suggesting that zinc can inhibit cellular processes upstream of



both MAPK and TGF- $\beta$ -induced signaling (probably oxidative stress), which, as described earlier, are responsible for the activation of MAPK and TGF- $\beta$  production in HSCs.

Our study has shown that HSCs can be a rich source of several MMPs, among others MMP-2 and MMP-13. Both ethanol, in the presence of 4-MP as an inhibitor of ADH, and acetaldehyde significantly modulated MMP activity. Moreover, our study demonstrated the expression of TIMP-1 and TIMP-2 proteins, especially after incubation of HSCs with ethanol and acetaldehyde. This is in accordance with the studies of Knittel et al. [64], who demonstrated that HSCs are a major source of MMP-2 and MMP-9 in the liver, and that MMP-2 is largely secreted in a latent form. Recent studies have also demonstrated that rat HSCs express mRNA for MMP-13. Both MMP-2 and MMP-9 are known to degrade basement membrane collagen while MMP-13 is known to degrade fibrillar collagen. Studies with HSCs have established that when activated, HSCs synthesize increased ECM proteins, particularly fibrillar collagen, but shut-down the expression of proteases such as MMP-13, which degrade fibrillar collagen [65,66,67]. Together these changes may facilitate the profibrogenic action of activated HSCs. Increased MMP-2 production at the early stages of cell activation may also be profibrogenic because it increases degradation of normal basement membrane collagen. Moreover, products of collagen degradation may exert a proliferative effect on HSCs [67] leading to an increase in the number of activated HSCs. Proliferation and invasiveness of HSCs could be prevented by specific MMP-2 inhibitors and antioxidants. On the other hand the later overexpression of MMP-2 may be important in the remodeling of matrix during tissue repair processes [68,69]. The results of our study indicating enhanced MMP-2 activity after ethanol- or acetaldehyde-induced activation, suggest a profibrogenic action of ethanol and its metabolite. Zinc, which was detected to inhibit MMP-2 production seems to be antifibrotic in its action. However, the data concerning protective effect of zinc

supplementation are contradictory because in some experiments zinc administration in already cirrhotic animals reduced lipid peroxidation but did not affect liver collagen content [70].

Regulation of ECM synthesis and its degradation by MMPs and their inhibitors (TIMPs) is a complex process. In general, TIMPs inhibit MMP activity by binding to active sites of MMPs. Our study has shown that TIMP-1 and TIMP-2 secretion was induced in HSCs by ethanol and acetaldehyde. This finding is similar to previously reported results demonstrating a significant TIMP expression after HSC activation [71]. In our study, zinc supplementation significantly inhibited the production of both TIMP-1 and TIMP-2. These results seem to confirm data from in vivo experiments with oral zinc supplementation, in which an increased level of zinc in patients down-regulated the level of TIMP-1 in their sera [23]. As TIMP-1 was described [72] to have an antiapoptotic effect on activated HSCs, such a decrease in its production can be beneficial for liver fibrosis resolution.

Summing up, the results of our experiments revealed that zinc can exert an antifibrotic activity by silencing ethanol- or acetaldehyde activated HSCs. The possible mechanisms of antifibrotic effect of  $ZnCl_2$  are presented on Fig. 11. Zinc supplementation inhibited ethanol- and acetaldehyde-induced ROS production, which seems to be a key mechanism in its inhibitory action on  $\alpha$ -SMA and procollagen I expression. Zinc was also shown to inhibit TGF- $\beta$  and TNF- $\alpha$  production, probably by its inhibitory action on the NF- $\kappa$ B pathway. Moreover, zinc also attenuated HSC activation via down-regulation of p38 MAPK, JNK and TGF- $\beta$  signaling activation induced by ethanol or acetaldehyde. Inhibition by zinc of MMP-2, TIMP-1 and TIMP-2 production by HSCs seems to be an additional mechanism of its antifibrotic activity.

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#### Figure legends

##### Fig. 1

The influence of zinc supplementation on ethanol- (A) and acetaldehyde- (B) induced toxicity in CFSC-2G cells.

CFSC-2G cells were preincubated with 30  $\mu$ M ZnCl<sub>2</sub> for 24 h. Thereafter, ethanol or acetaldehyde at indicated concentrations were added. After 24 h of incubation, the toxicity was determined by MTT method. Values are means  $\pm$  SD of results from five experiments.

\* Statistically significant at  $p \leq 0.05$  in comparison to cells incubated with ethanol or acetaldehyde alone (Wilcoxon test).

##### Fig. 2

CFSC-2G cells metabolize ethanol via the ADH and the P4502E1 pathways. (A) The cells were incubated with 2 mM DAS or 2 mM 4-MP or with a mixture of the inhibitors. After 24 h of incubation, oxygen burst was induced by 50 mM ethanol. Superoxide anion production was

measured by cytochrome c reduction assay. Preincubation of CFSC-2G cells for 24 h with 30  $\mu\text{M}$   $\text{ZnCl}_2$  inhibits ethanol and acetaldehyde induced superoxide anion (B) or hydrogen peroxide (C) production. Results are expressed as mean  $\pm$  SD of four independent experiments. \*Significantly different from respective control (cells incubated without inhibitors) (A), cells not treated or treated only with zinc (B,C),  $p \leq 0.05$ . #Statistically significant at  $p \leq 0.05$  in comparison to cells treated with ethanol or acetaldehyde alone. Zinc significantly changed the ethanol/acetaldehyde effect,  $p \leq 0.001$  (two-way ANOVA).

Fig. 3

Preincubation of CFSC-2G cells for 24 h with 30  $\mu\text{M}$   $\text{ZnCl}_2$  induces quiescence of cells activated by 24 h incubation with ethanol in the presence of 4-MP (A) or with acetaldehyde (B). The markers of HSCs activation such as  $\alpha$ -SMA and procollagen I were measured by Western blot.  $\beta$ -actin expression served as loading control. On the right, the arrows indicate the position of the molecular weight markers used in the experiments. Representative blots are shown. Experiments were done in triplicates and the bar represents the mean  $\pm$  SD. \*Significantly different from respective control (cells incubated with 4-MP) (A), cells not treated or treated only with zinc (A,B); \* $p \leq 0.05$ , \*\* $p \leq 0.01$ . #Statistically significant at  $p \leq 0.05$  in comparison to cells treated with ethanol or acetaldehyde alone. Zinc significantly changed the ethanol/acetaldehyde effect,  $p \leq 0.001$  (two-way ANOVA).

Fig. 4

Preincubation of CFSC-2G cells with 30  $\mu\text{M}$   $\text{ZnCl}_2$  inhibits cell migration. (A) A wound healing assay was performed on CFSC-2G cells grown to a confluent cell layer in which a wound was scraped to remove a linear area of cells, and cultures were treated with 30  $\mu\text{M}$   $\text{ZnCl}_2$  for 24 h. Then ethanol or acetaldehyde at indicated concentrations were added and the

cells were allowed 24 h to migrate. Representative images of different conditions are shown. A chemotaxis assay (B) was performed in serum starved cells placed in Boyden chambers, in which the bottom chamber contained 30  $\mu\text{M}$   $\text{ZnCl}_2$  enriched medium. After 24 h of incubation, 175  $\mu\text{M}$  acetaldehyde was added into the bottom chamber. The number of cells migrating through membranes coated with Matrigel was counted under a microscope and shown as mean  $\pm$  SD of three independent experiments. \*Statistically significant at  $p \leq 0.05$  in comparison to respective control (cells not treated or treated only with zinc). #Statistically significant at  $p \leq 0.05$  in comparison to cells treated with ethanol or acetaldehyde alone (Wilcoxon test).

Fig. 5

Preincubation of CFSC-2G cells with 30  $\mu\text{M}$   $\text{ZnCl}_2$  inhibits ethanol (A,B) or acetaldehyde (C,D) induced production of TGF- $\beta$  and TNF- $\alpha$ . The cells were preincubated with 30  $\mu\text{M}$   $\text{ZnCl}_2$  for 24 h and thereafter induced to produce cytokines by addition of ethanol or acetaldehyde at indicated concentrations. The level of cytokines was measured by ELISA and shown as mean  $\pm$  SD of three independent experiments. \*Significantly different from respective control (cells not treated or treated only with zinc),  $p \leq 0.01$ . #Statistically significant at  $p \leq 0.05$  in comparison to cells treated with ethanol or acetaldehyde alone. Zinc significantly changed the ethanol/acetaldehyde effect,  $p \leq 0.01$  (two-way ANOVA).

Fig. 6

Preincubation of CFSC-2G cells with 30  $\mu\text{M}$   $\text{ZnCl}_2$  decreases production of MMP-2 induced by ethanol (A) or acetaldehyde (B). The MMP-2 level was estimated by ELISA. \*Significantly different from respective control (cells not treated or treated only with zinc),  $p \leq 0.01$ . #Statistically significant at  $p \leq 0.05$  in comparison to cells treated with ethanol or

acetaldehyde alone. Zinc significantly changed the ethanol/acetaldehyde effect,  $p \leq 0.01$  (two-way ANOVA).

Fig. 7

The effect of preincubation of CFSC-2G cells with 30  $\mu\text{M}$   $\text{ZnCl}_2$  on parameters related to extracellular matrix remodeling induced by ethanol (A) or acetaldehyde (B). Western blot analysis for TIMP-1, TIMP-2 and MMP-13 was performed on cell lysates derived from cells preincubated for 24 h with 30  $\mu\text{M}$   $\text{ZnCl}_2$  and thereafter for 24 h with the indicated ethanol or acetaldehyde concentrations. The upper panel shows representative blots, the middle - a densitometry analysis of bands, and the lower - TIMP-1 ELISA assay. \*Significantly different from respective control (cells not treated or treated only with zinc),  $p \leq 0.01$ . #Statistically significant at  $p \leq 0.05$  in comparison to cells treated with ethanol or acetaldehyde alone. Zinc significantly changed the ethanol/acetaldehyde effect,  $p \leq 0.01$  (two-way ANOVA).

Fig. 8

The effect of preincubation of CFSC-2G cells with 30  $\mu\text{M}$   $\text{ZnCl}_2$  on phosphorylation of  $\text{NF}\kappa\text{B}$ ,  $\text{I}\kappa\text{B}$ , JNK and p38 MAPK. The cells were preincubated with 30  $\mu\text{M}$   $\text{ZnCl}_2$  for 24 h and thereafter not exposed or exposed to 50 mM ethanol (A) or 175  $\mu\text{M}$  acetaldehyde (B) for 20 min. The relative densitometry readings (mean  $\pm$  SD) from three independent experiments are shown. The upper panel shows representative blots of total and phosphorylated forms of  $\text{NF}\kappa\text{B}$ ,  $\text{I}\kappa\text{B}$ , JNK and p38 MAPK. \*Significantly different from respective control (cells incubated with 4-MP (A), cells not treated or treated only with zinc (A,B);  $*p \leq 0.05$ ,  $**p \leq 0.001$ . #Statistically significant at  $p \leq 0.05$  in comparison to cells treated with ethanol or acetaldehyde alone (Wilcoxon test).

Fig. 9

The effect of preincubation of CFSC-2G cells with 30  $\mu\text{M}$   $\text{ZnCl}_2$  on phosphorylation of Smad 3. The cells were preincubated with 30  $\mu\text{M}$   $\text{ZnCl}_2$  for 24 h and then induced by 50 mM ethanol (A) or 175  $\mu\text{M}$  acetaldehyde (B) for 24 h. The amounts of phosphorylated and total Smad 3 (used as loading control) were measured by Western blotting. The upper panel shows representative blots. Each figure is representative of three independent experiments. Band intensities were measured, and the ratio of phosphorylated Smad 3 in the absence of  $\text{ZnCl}_2$  and ethanol or acetaldehyde was used as a control (100%). The values shown are the mean  $\pm$  SD of triplicate determination. \*Significantly different from respective control (cells incubated with 4-MP (A), cells not treated or treated only with zinc (A,B), \* $p \leq 0.05$ . #Statistically significant at  $p \leq 0.05$  in comparison to cells treated with ethanol or acetaldehyde alone (Wilcoxon test).

Fig. 10

Zinc supplementation inhibits pathways other than the PKC pathway. CFSC-2G cells were preincubated with 30  $\mu\text{M}$   $\text{ZnCl}_2$  for 24 h in the presence of 4-MP and with the addition or in the absence of 1  $\mu\text{M}$  calphostin C (a PKC inhibitor), and then ethanol at different concentrations (A) or 175  $\mu\text{M}$  acetaldehyde (B) were added. The cells were incubated for another 24 h and harvested for Western blot analysis.  $\alpha$ -SMA as a marker of cell activation was estimated. The upper panel shows representative blots. The lower panel shows a densitometry analysis of bands. \*Significantly different from respective control (cells not treated or treated with 4-MP, calphostin C or zinc alone),  $p \leq 0.01$ . #Statistically significant at  $p \leq 0.05$  in comparison to cells treated with ethanol or acetaldehyde alone. Zinc significantly changed the ethanol/acetaldehyde effect,  $p \leq 0.01$  (two-way ANOVA).

Fig. 11

Possible mechanisms of  $ZnCl_2$  antifibrotic effects in hepatic stellate cells treated with ethanol or acetaldehyde. Zinc attenuates oxidative stress, phosphorylation of kinases p38 and JNK as well as Smad 3. In the effect, it down-regulates collagen type I gene expression.

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Table 1.

The influence of preincubation of CFSC-2G cells with 30  $\mu$ M zinc for 24h on apoptosis induced by ethanol or acetaldehyde. Caspase 3 activity was determined by Colorimetric Assay Kit.

	Caspase 3 (fold increase)	
	-	Zn 30 $\mu$ M
Ethanol 100 mM	1,96 $\pm$ 0,3*	1,21 $\pm$ 0,2#
Acetaldehyde 175 $\mu$ M	1,3 $\pm$ 0,24	1,14 $\pm$ 0,17
-	1	1,1 $\pm$ 0,15

\* statistically significant in comparison to control (cells incubated without inductors),  $p \leq 0.05$

# statistically significant in comparison to cells not treated with ZnCl<sub>2</sub>,  $p \leq 0.05$  (two-way ANOVA)

Fig. 1.

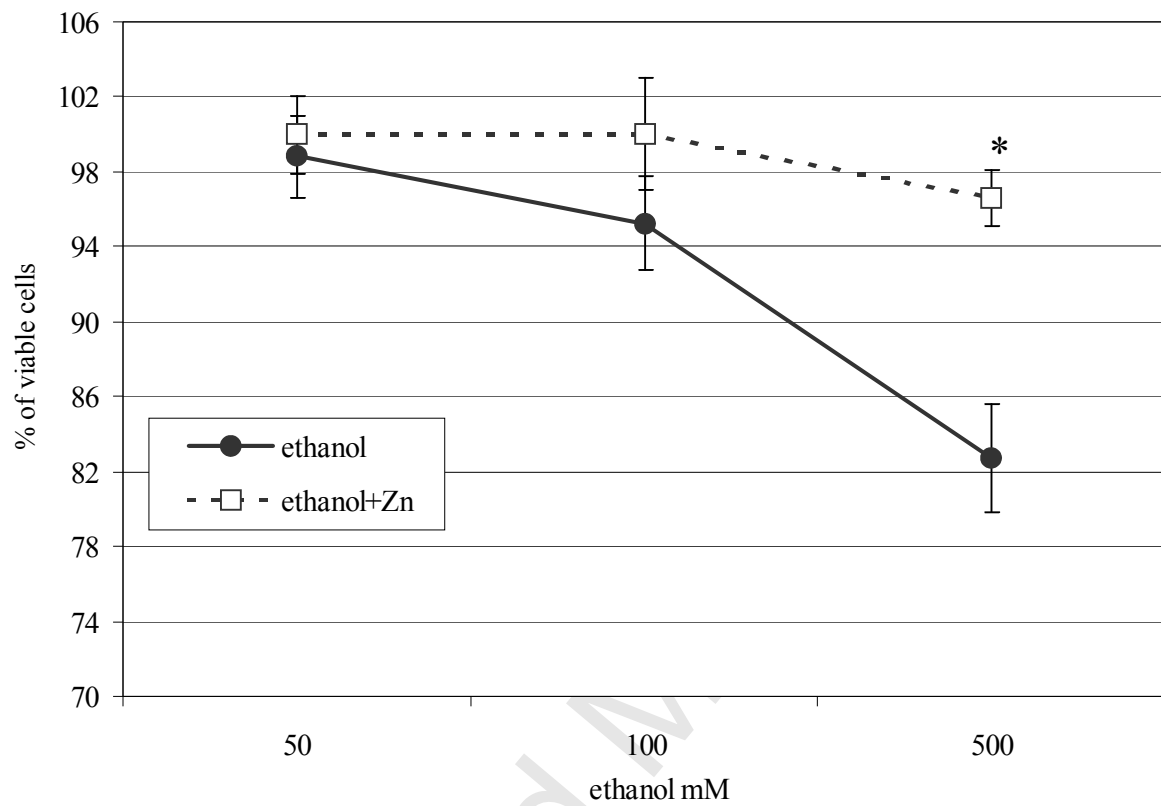
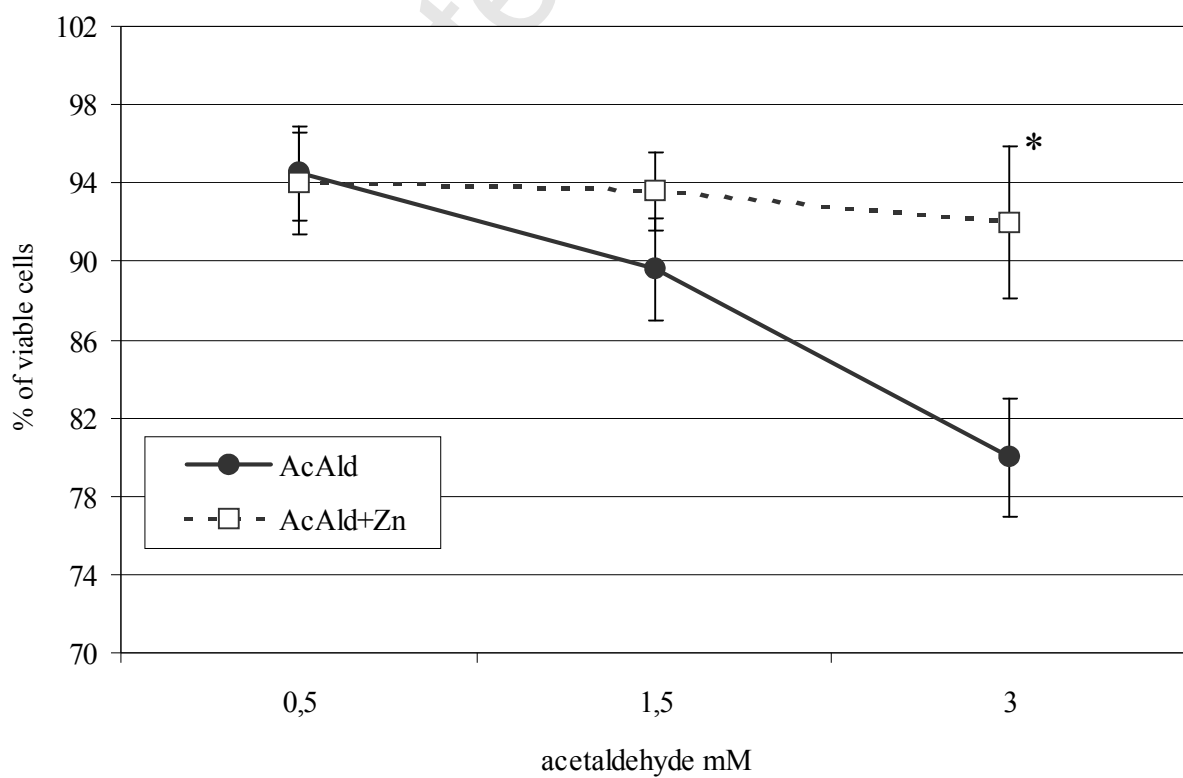
**A****B**

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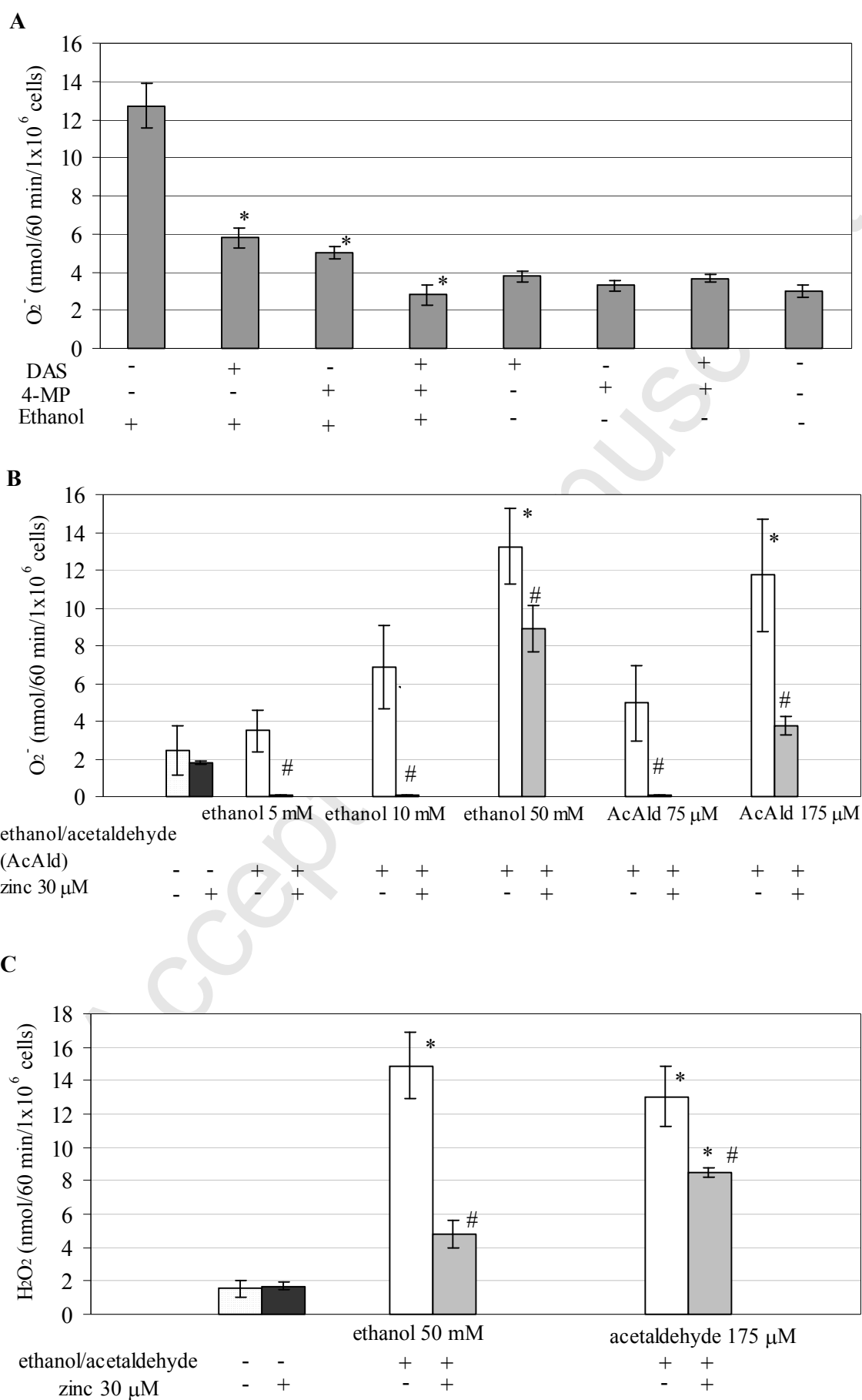


Fig. 3 A

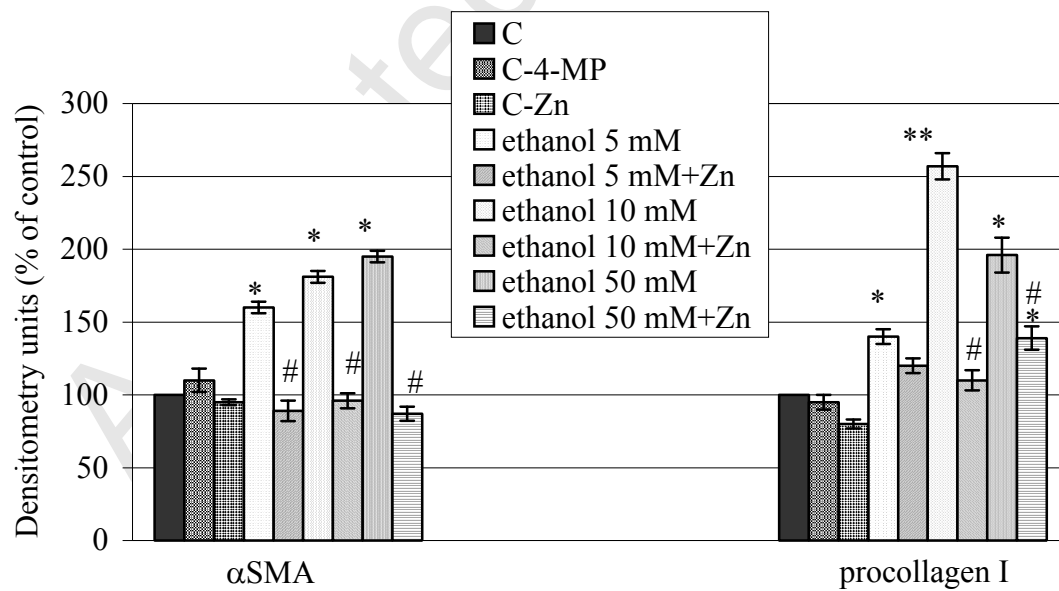
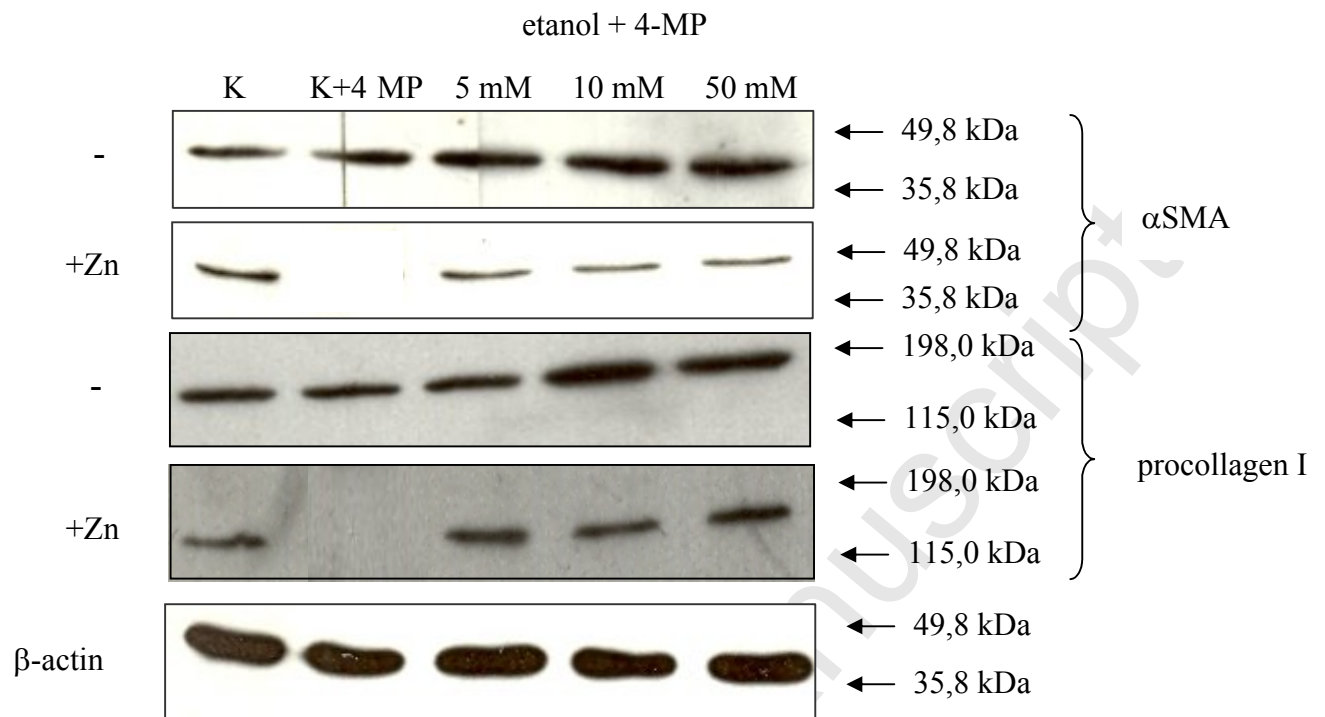


Fig. 3 B

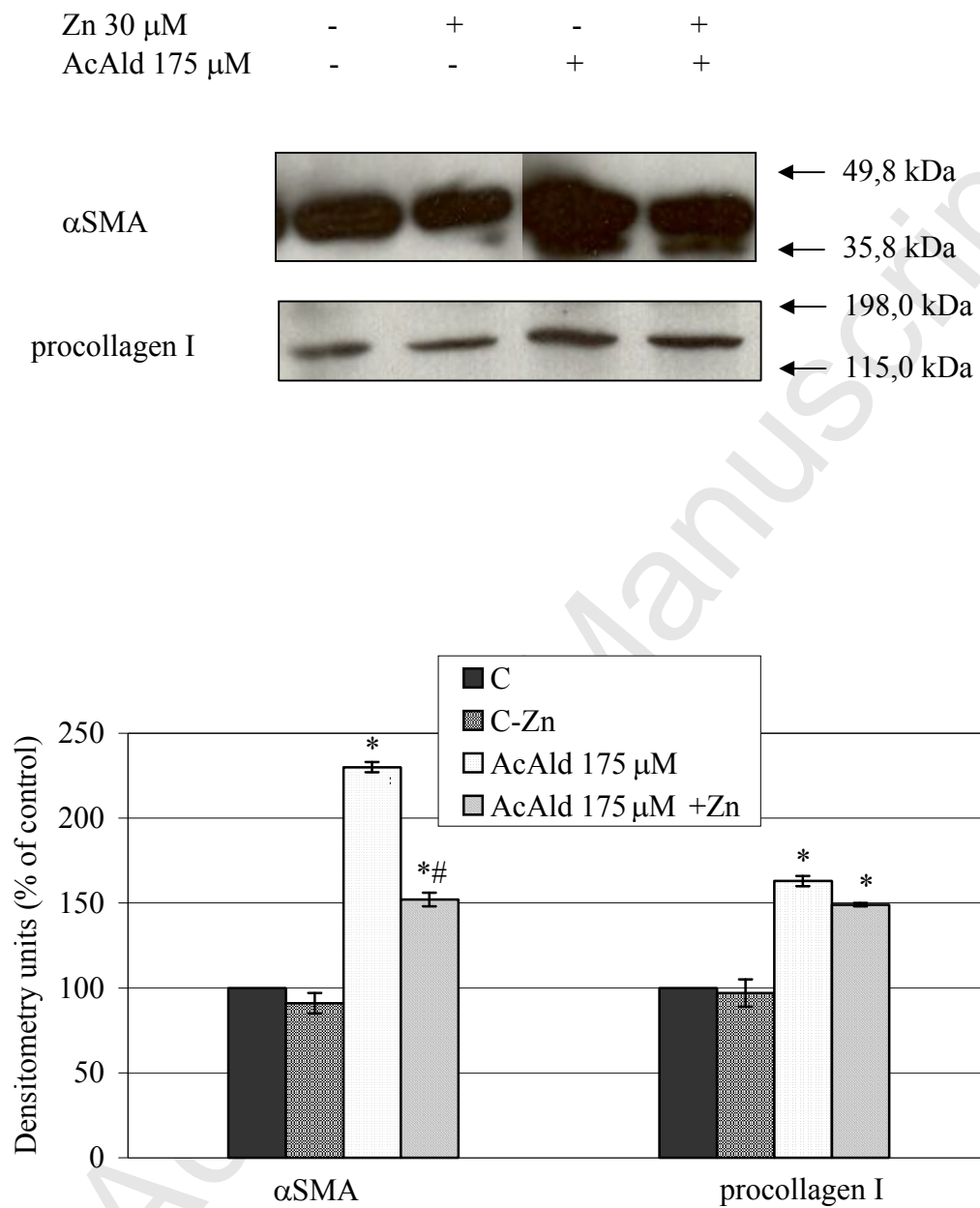


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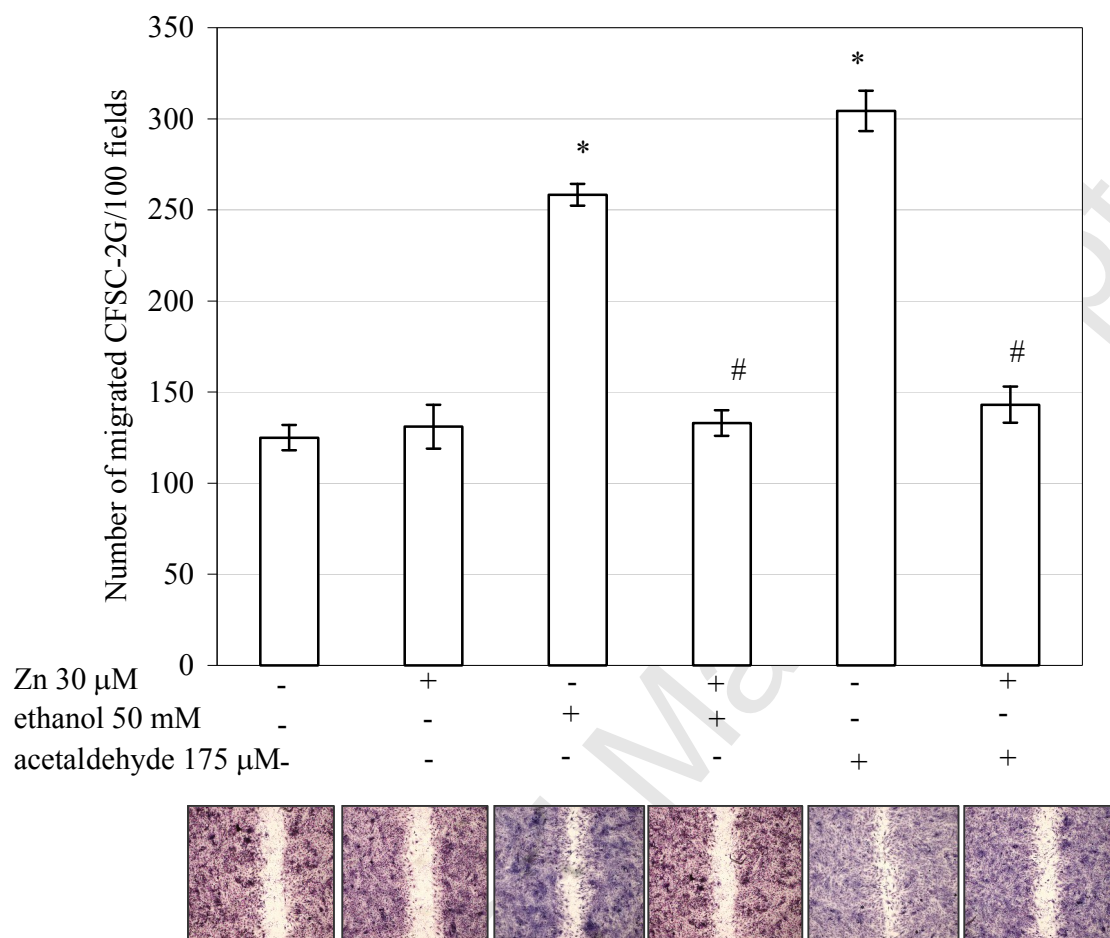


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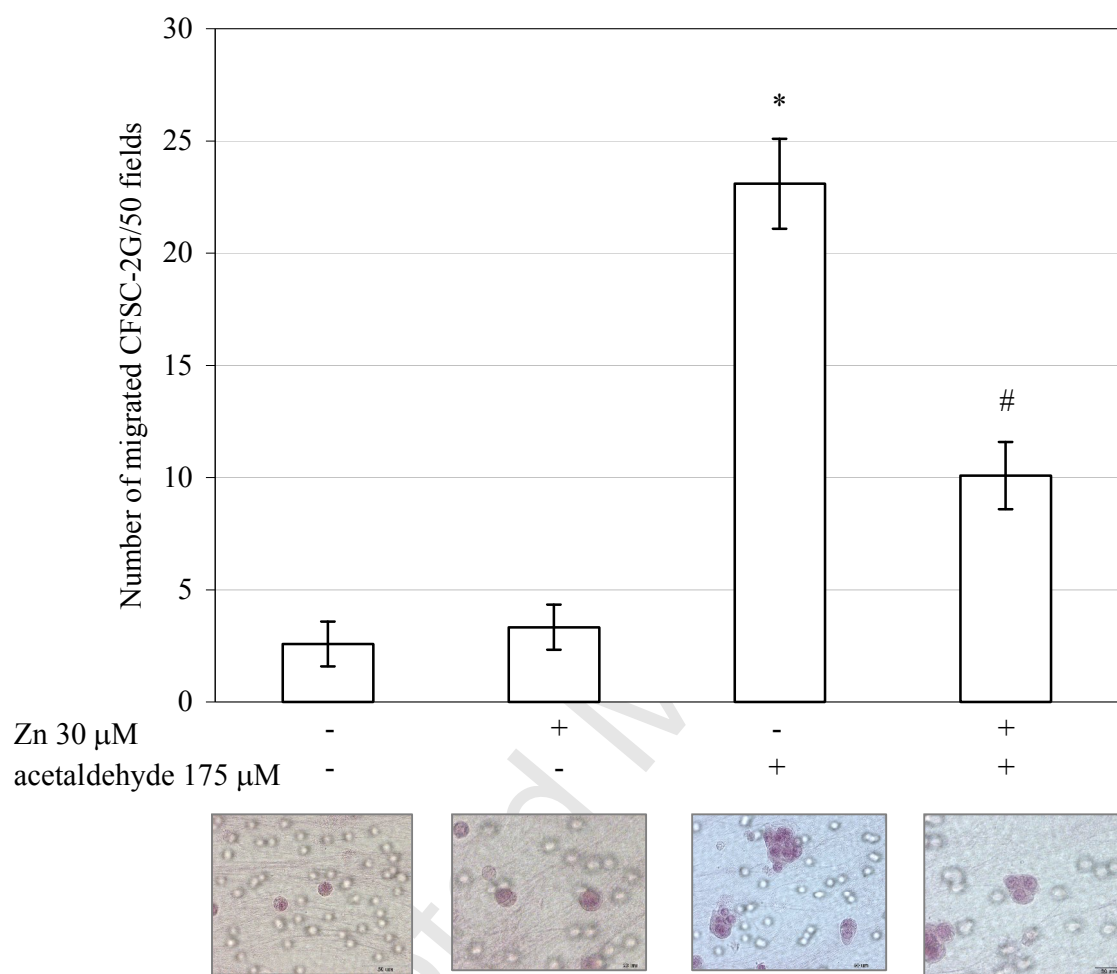


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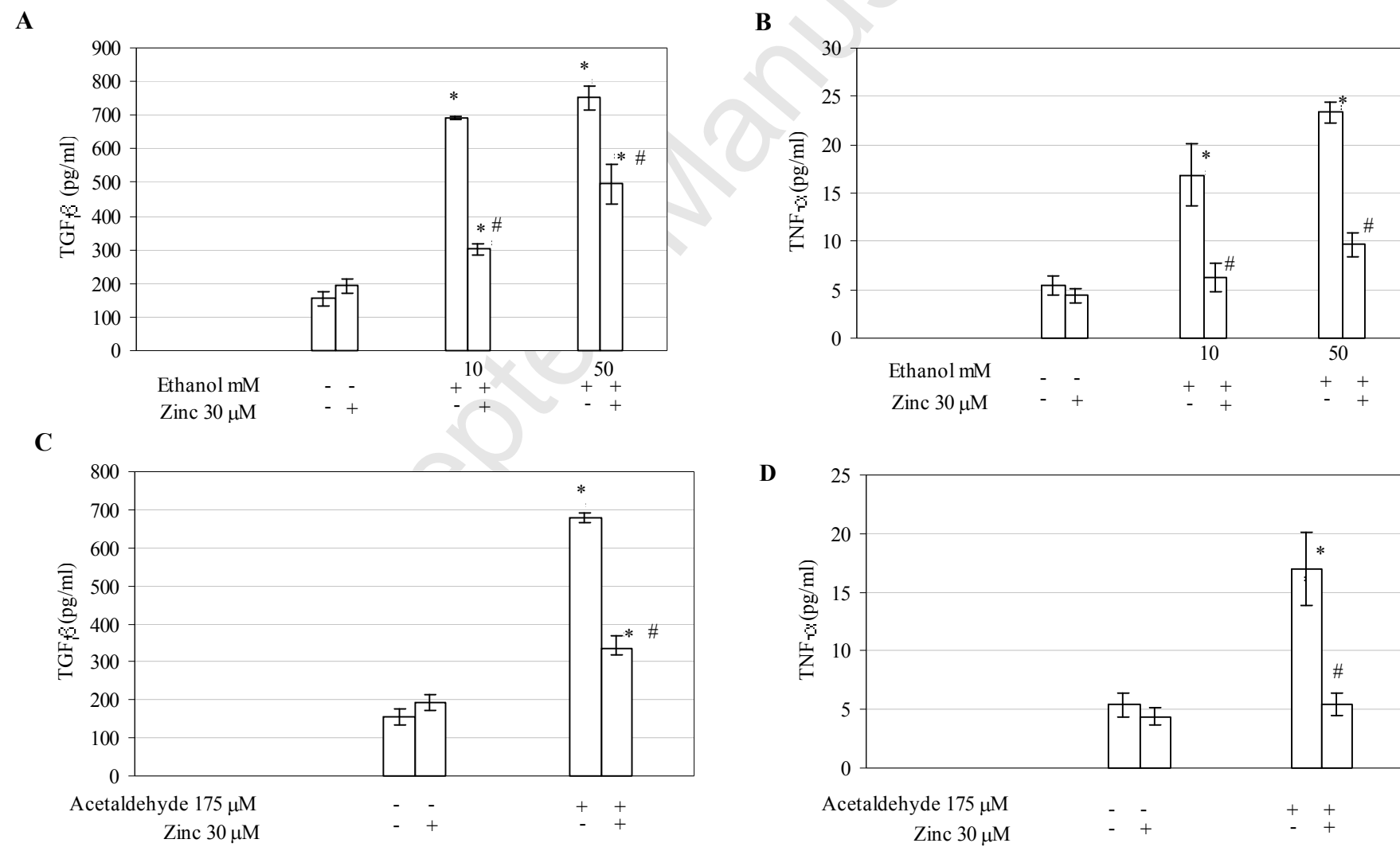




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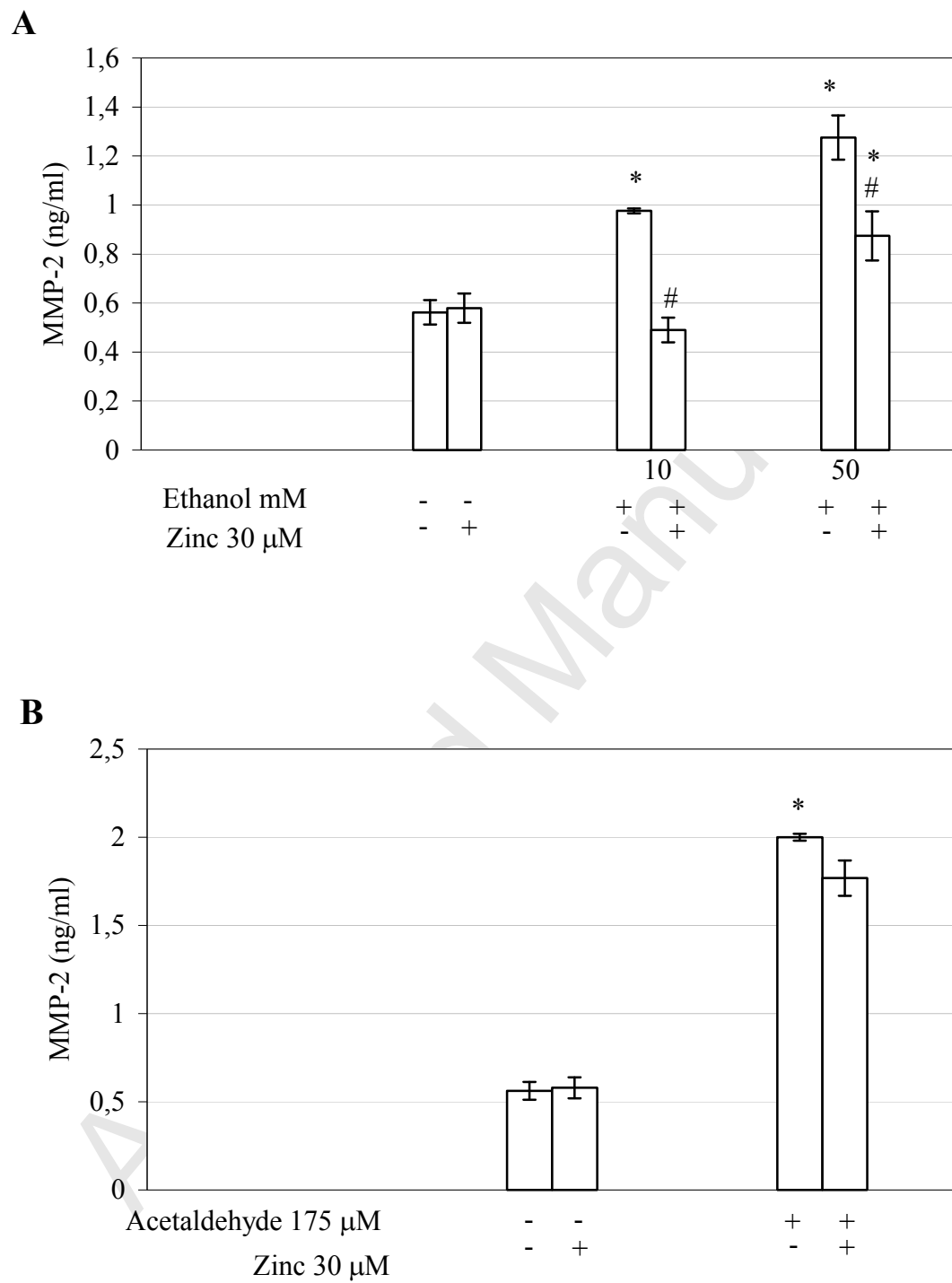


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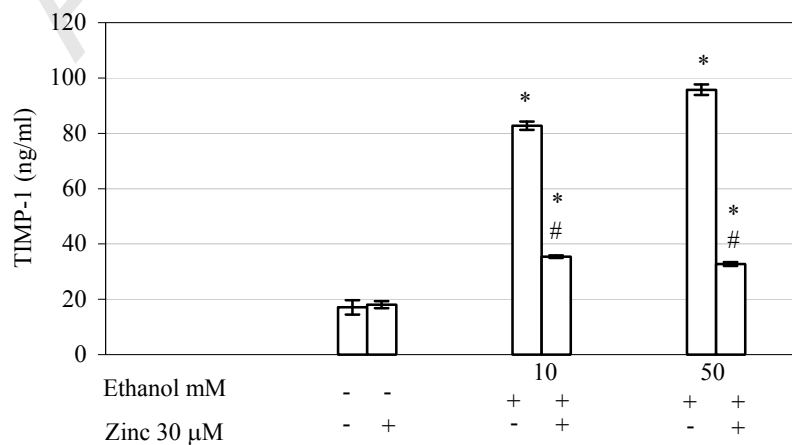
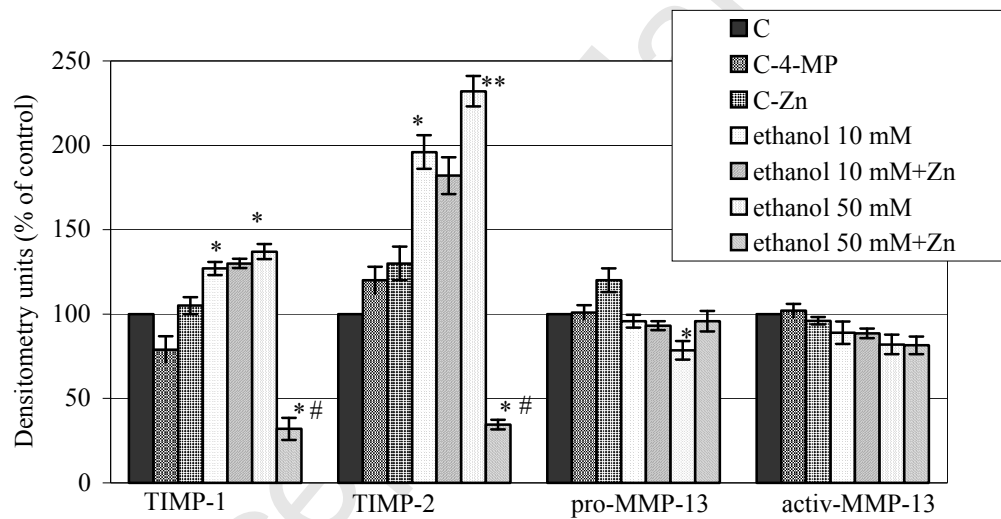
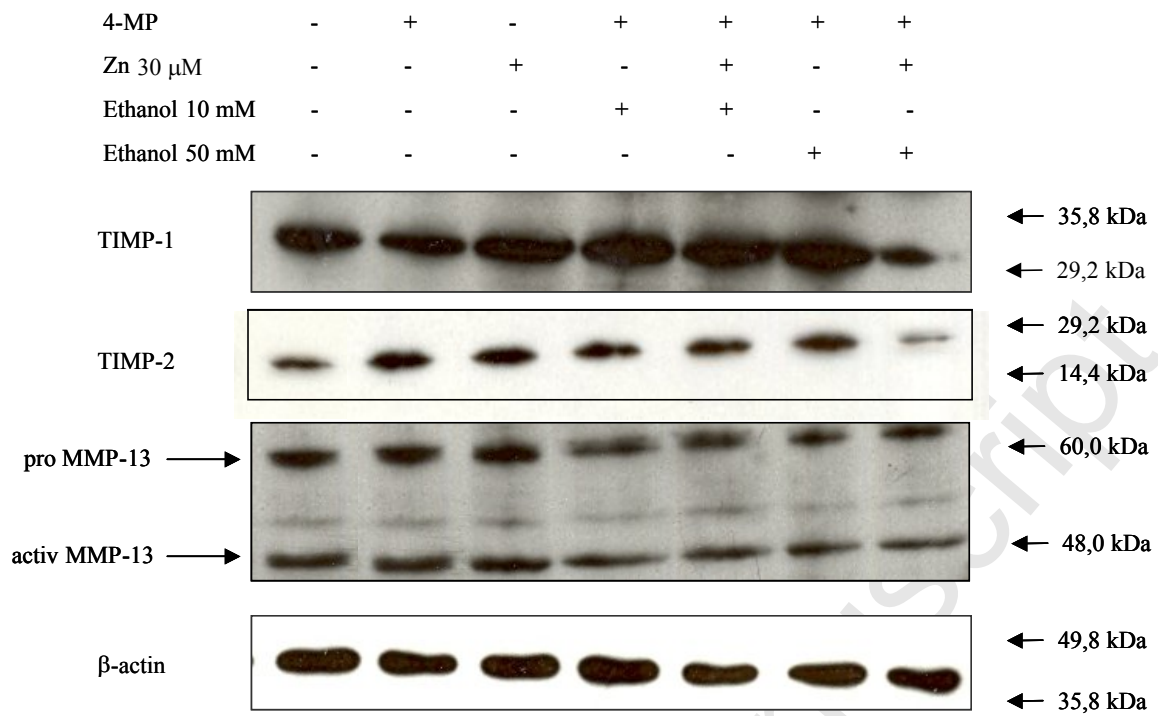


Fig. 7 B

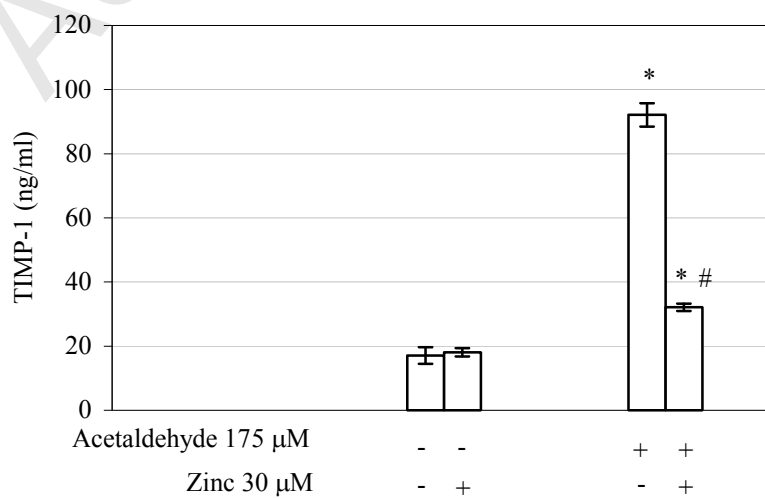
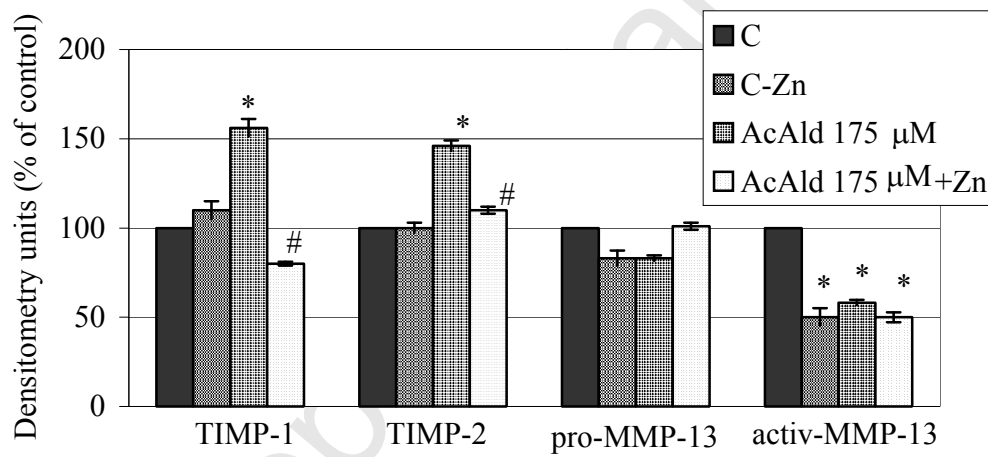
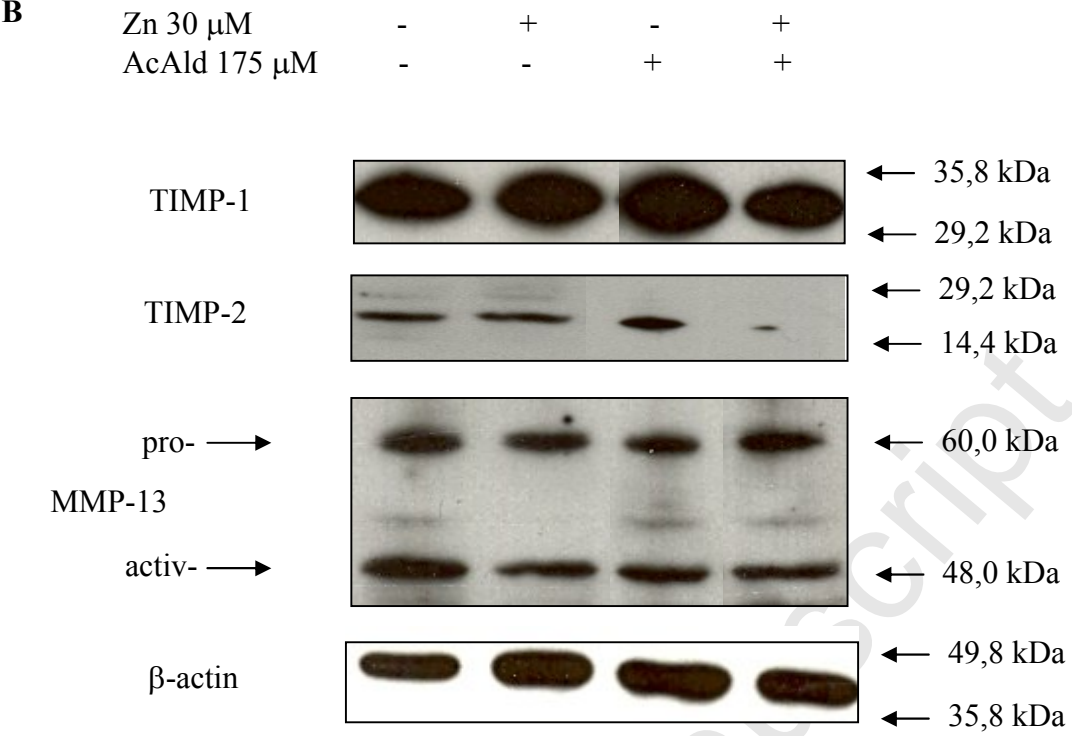


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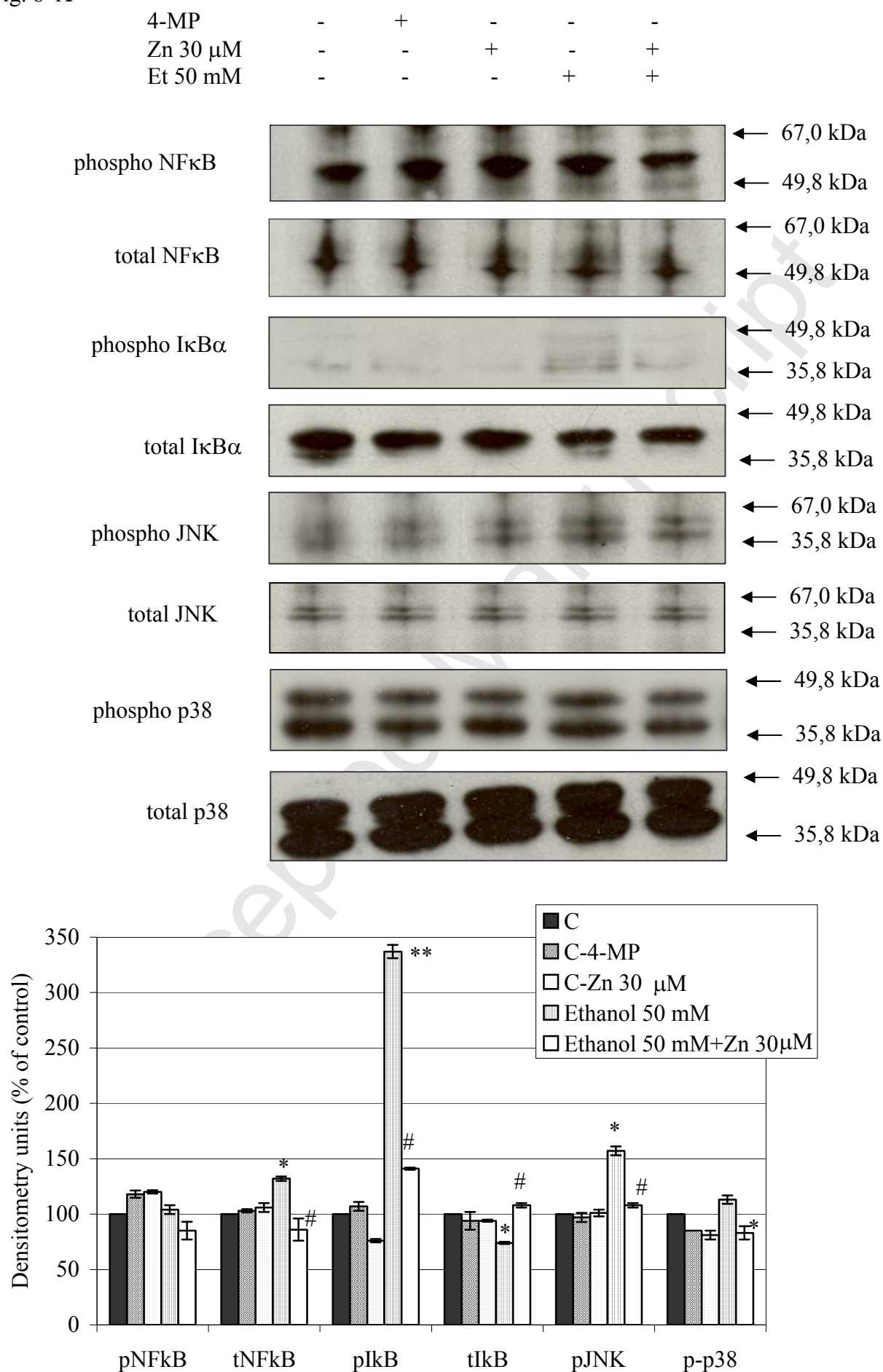


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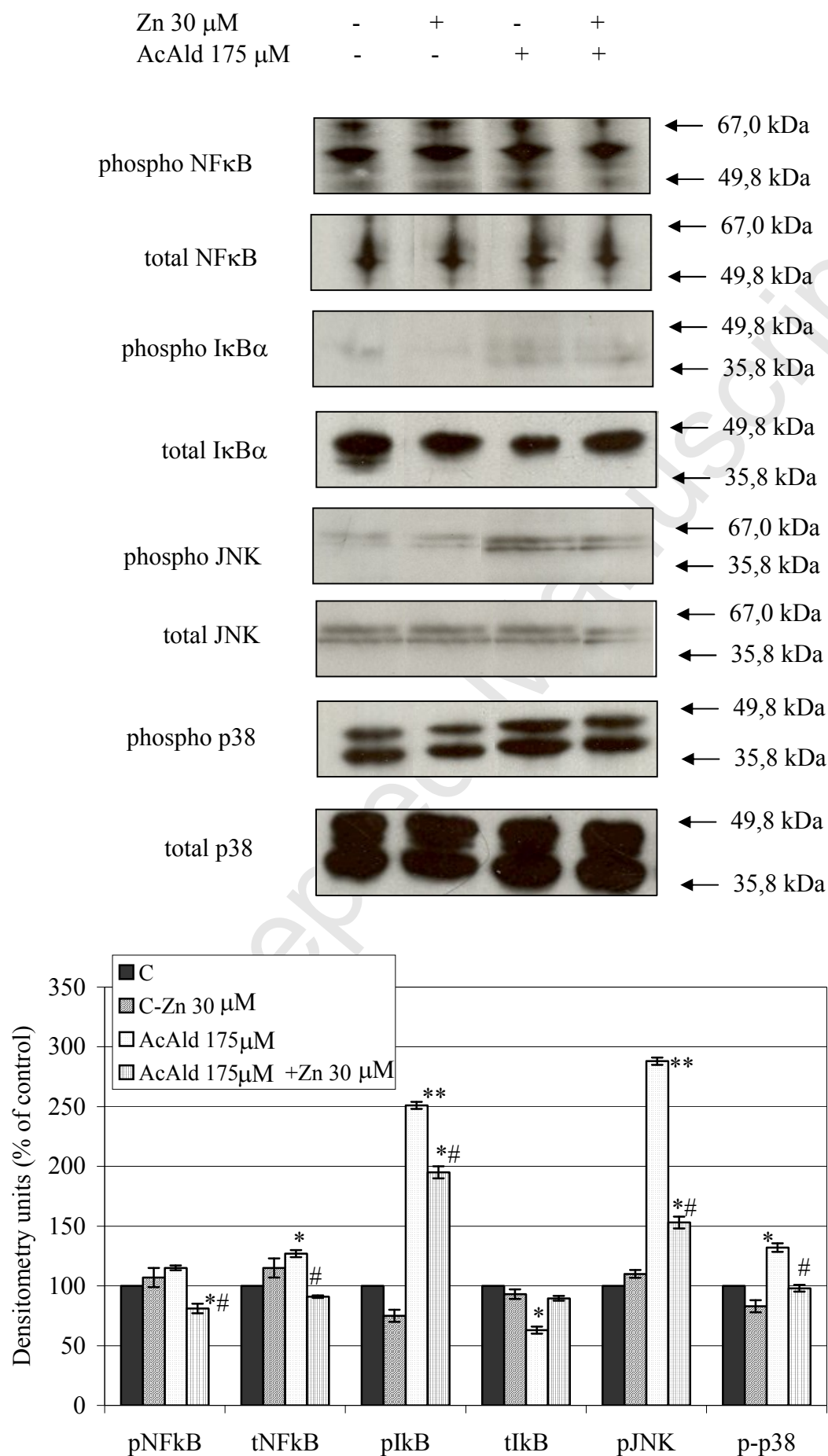


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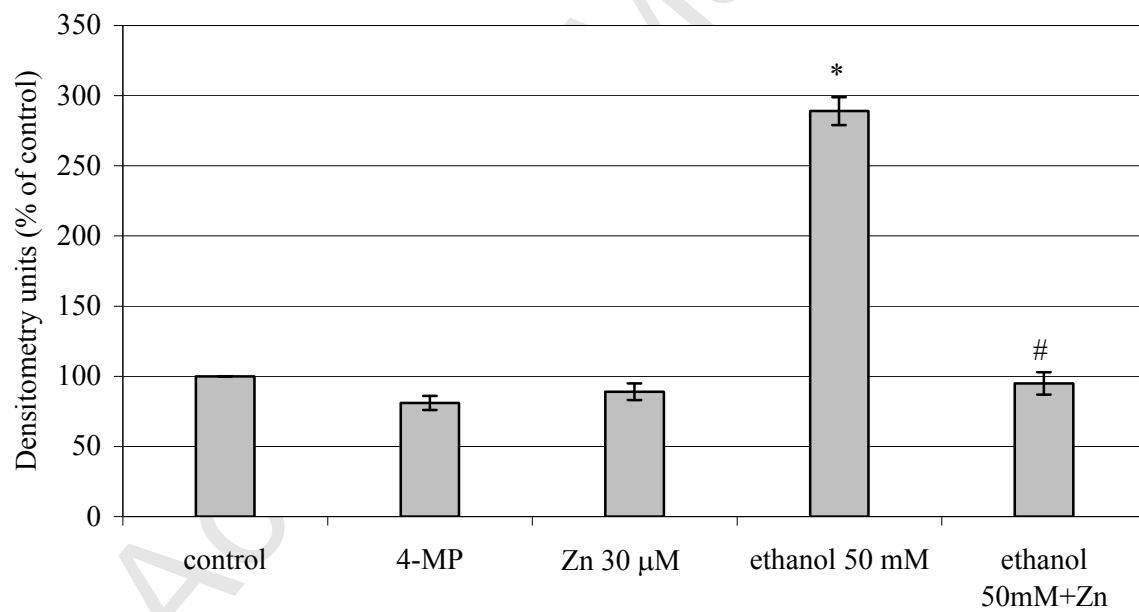
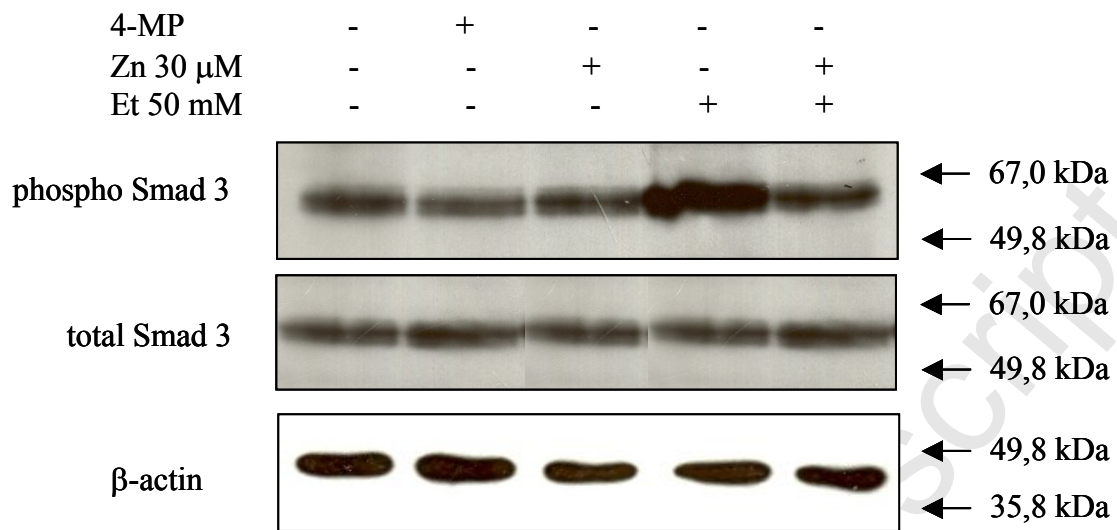


Fig. 9 B

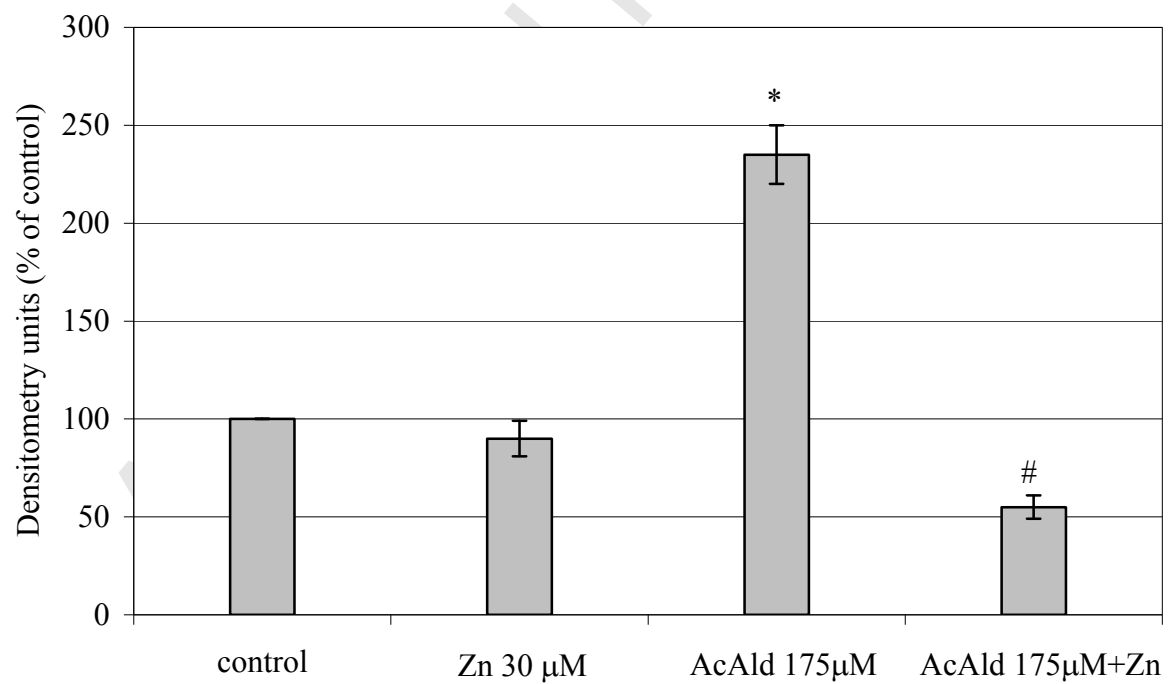
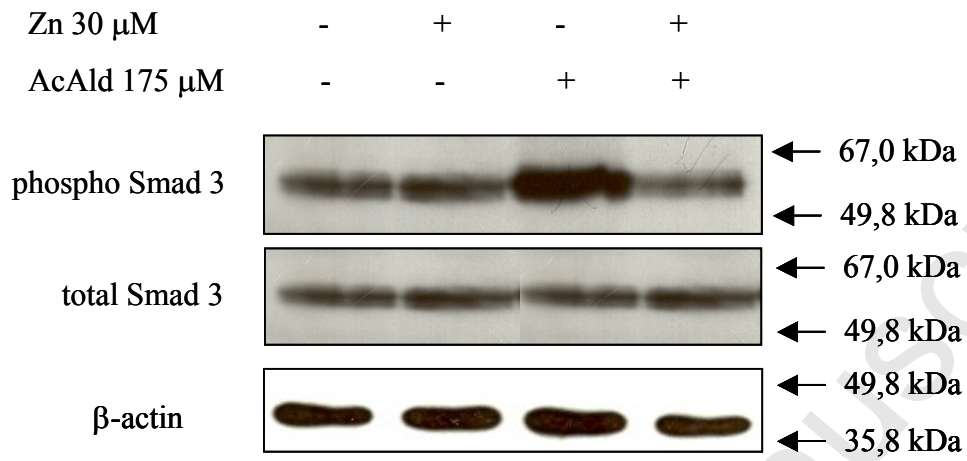


Fig 10 A

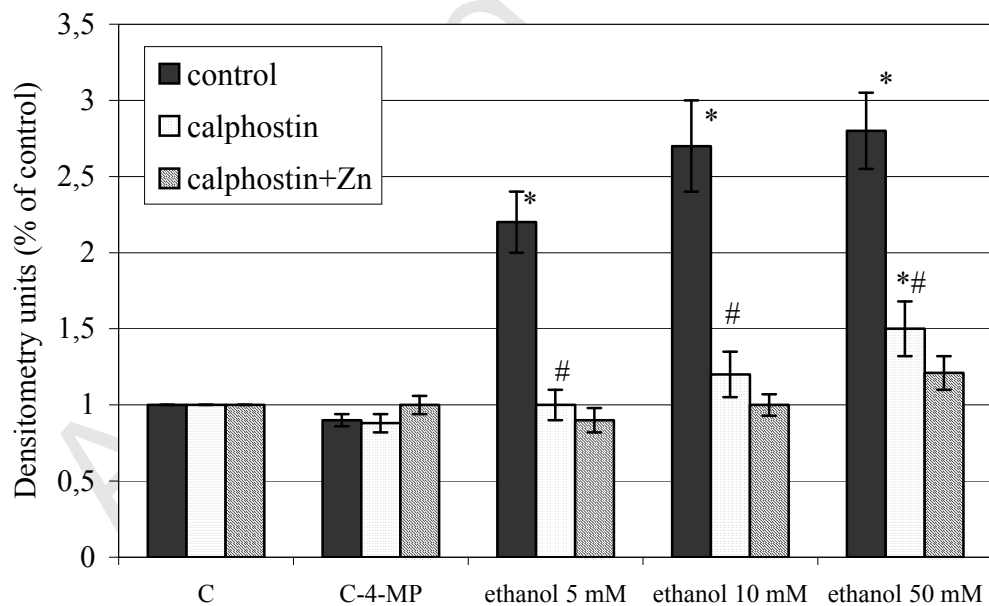
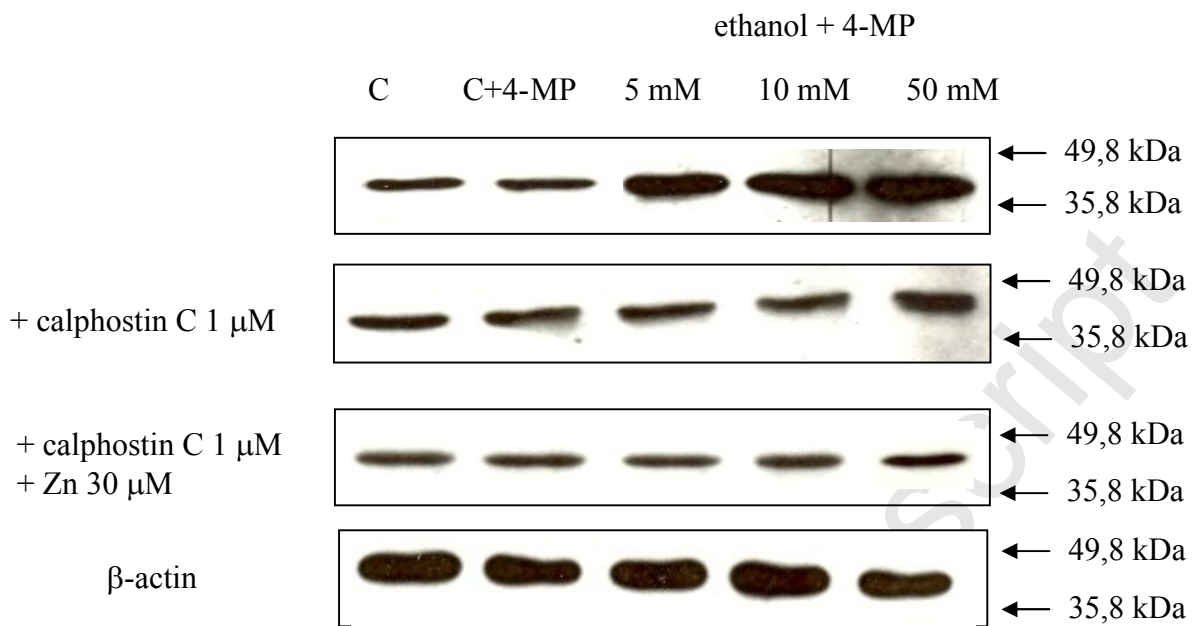




Fig. 10 B

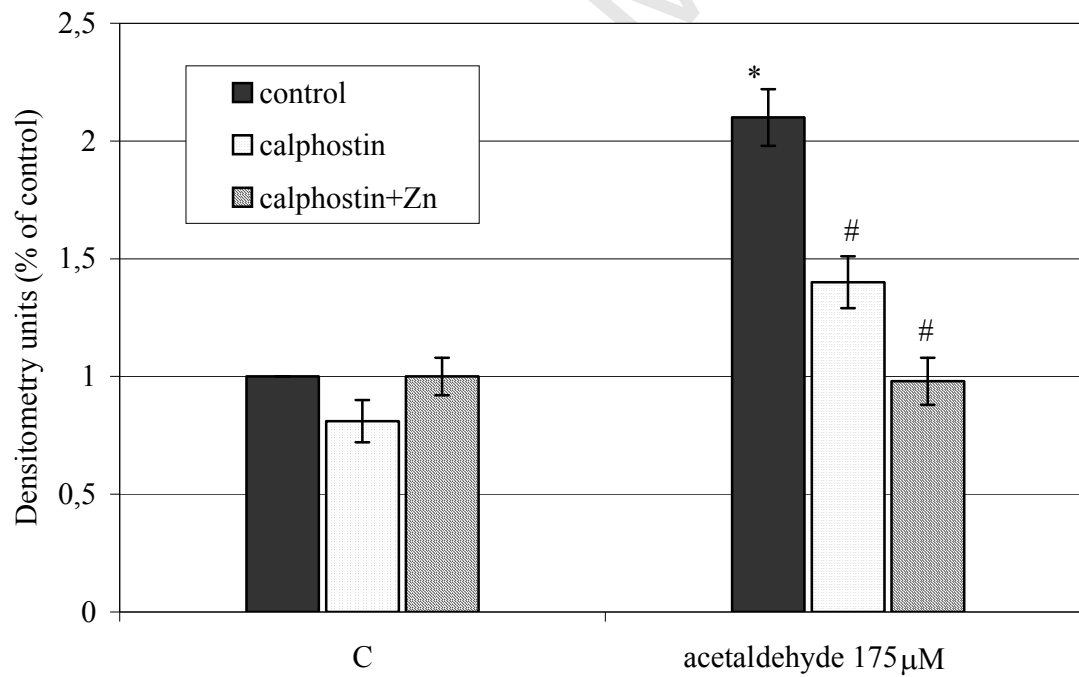
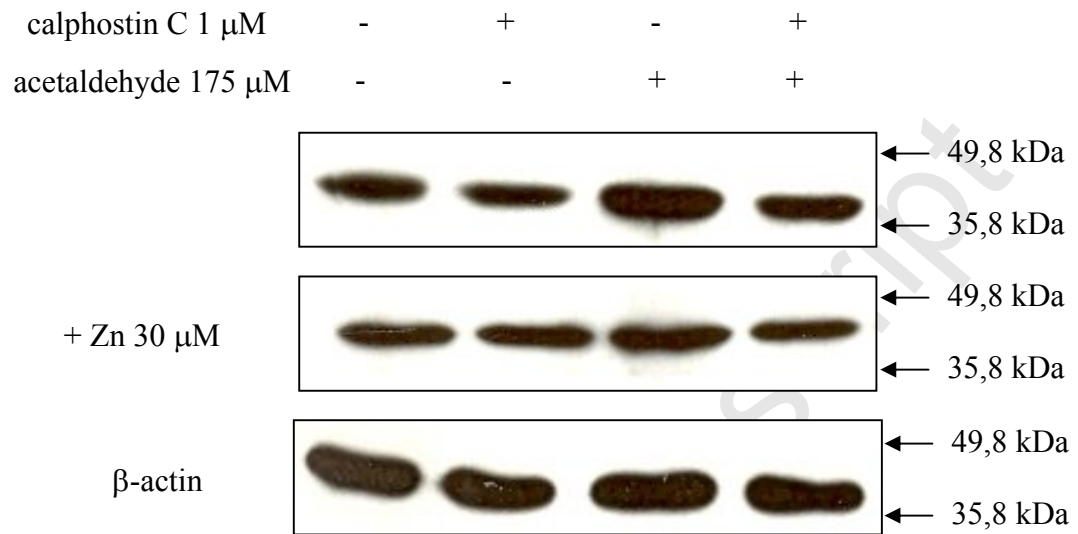
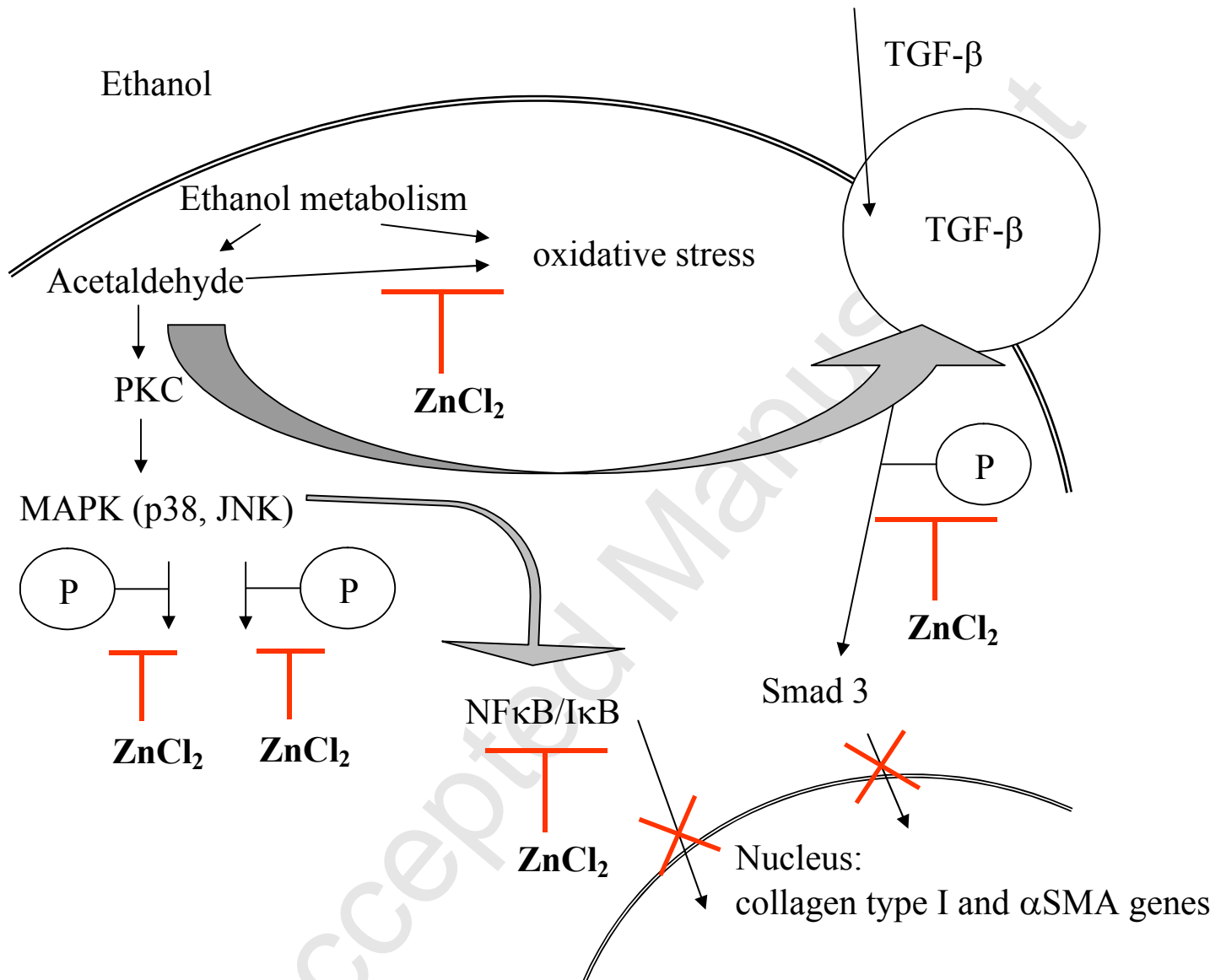


Fig. 11

Possible mechanisms for the antifibrotic effects of ZnCl<sub>2</sub> on hepatic stellate cells.



Possible mechanisms for the antifibrotic effects of zinc chloride on hepatic stellate cells.

