Supplemental Methods

Reagents. Ethanol was purchased from Pharmaco, Inc. (Shelbyville, KY). Other chemicals were from Sigma, Invitrogen, or Calbiochem. Rabbit anti-LC3 antibody was made against an N-terminal peptide of human LC3B. Other antibodies used were anti-β-actin (Sigma, St. Louis, MO), anti-Beclin 1(BD Biosciences, Franklin Lakes, NJ), anti-GFP (Santa Cruz Biotechnology, Santa Cruz, CA), anti-phosphorylated and total p70 S6 kinase and 4E-BP1, and anti-Atg7 antibodies (Cell Signaling Technology, Danvers, MA), anti-LAMP1 (Developmental Studies Hybridoma Bank, University of Iowa), and HRP-labeled secondary antibodies (Jackson ImmunoResearch Laboratories Inc., West Grove, PA).

siRNA information. siRNA specific for human Beclin 1 (5'-GGUCUAAGACGUCCAACA A-3') was obtained from Invitrogen (Carlsbad, CA). Mouse Atg7-speicific siRNA (5' CAGCTCTGAACTCAATAATAA 3') and a negative siRNA (5' AATTCTCCGAACGTGTCACGT 3') were obtained from Qiagen (Valencia, CA).

Preparation of liver lysates and subcellular fractionation. This was performed essentially as previously described (Ref 1). Briefly, murine livers were homogenized with a Dounce homogenizer (pestle B) in Buffer A containing 250 mM mannitol, 70 mM sucrose, 0.5 mM EGTA, and 5 mM HEPES-NaOH, pH 7.2. The homogenates were centrifuged at 1,000 x *g* for 10 min at 4°C to remove intact cells and nuclei. The recovered supernatants were named as the total lysates and were used in most of the biochemical analysis. For some studies, the heavy membrane fraction enriched with the lysosomes was fractionated by further centrifuging the total lysates at 10,000 x *g* at 4 °C for 10 min. The pellets containing the heavy membranes were washed once and resuspended in Buffer A, and subjected to biochemical analysis.

Long-lived protein degradation. Primary hepatocytes (1x 10^5/well in collagen-IV coated 12-well plate, BD BioScience, San Jose, CA), which had been allowed to attached for 3-4 hours in William's medium with 10% serum), and VL-17A (1x10^5/well in a 24-well plate) were labeled with [^{14}C]-valine (0.2 μCi/ml) for 16-22 hours in William's E or DMEM with 10% FCS, respectively. After removal of the [^{14}C]-valine, cells were cultured in the corresponding medium with the addition of 10 mM of valine for 2 hours. The mediums containing the degraded short-lived protein products were then removed and replaced with fresh medium, or starvation medium (EBSS) or with medium containing ethanol for different times. The radio-activity in the TCA soluble fraction of the

supernatant (a) and TCA insoluble fraction of the cells (b) were quantified by liquid scintillation method and the percentage of long-lived protein degradation wad determined using the formulae: a/(a+b) x 100%.

Reference

1. Kim T. H. et al. J. Bio. Chem. 275: 39474, 2000

Table S1. Analysis of autophagy flux in ethanol treatment

Data Source	Experiment system	Assay	Parameter	Synthesis	Degradation	
					CQ only	Ethanol + CQ
Fig. 1D	in vivo-liver-total	WB	LC3-II	0.63	1.3	0.93
Fig. 2E	in vitro-hepatocytes-total	WB	LC3-II	0.58	1.67	1.05
Fig. 1D	in vivo -liver HM fraction	WB	LC3-II	1.2	0.4	1
Fig. 1B	in vivo-liver	FM	GFP-LC3 dots	4.07	2.55	3.69
Fig. 2E	in vitro-hepatocytes	FM	GFP-LC3 dots	25.6	23.16	32.25
Fig. 1F	in vivo-liver	EM	Autophagosomes	4.4	3.1	3.4
Fig. 6E	in vitro-hepatocytes	FM	GFP-LC3 dots with mitochondria	16.4	14.1	21.6
Fig. 6A	in vivo-liver	EM	Autophagosomes with Mitochondria	2.67	0.63	2.16

Autophagy flux was analyzed based on the principles and methods described in Mizushima et al (Ref. 1), Rubinsztein et al (Ref. 2) and Gamerdinger et al (Ref. 3) using data presented in the main figures as indicated in the leftmost column. Original assays were conducted with Western blot (WB), fluorescence microscopy (FM) and electron microscopy (EM) on liver samples from in vivo ethanol-treated mice, or hepatocytes or VL-17A cell lines treated in vitro with ethanol. The synthesis was defined as the difference between the values of ethanol plus CQ and CQ only, whereas degradation was defined as the difference between the values of CQ only and control (CQ only), and between the values of Ethanol plus CQ and ethanol only (ethanol + CQ). Thus the synthesis measured the increase of LC3-II, GFP-LC3 dots or autophagosomes, stimulated by ethanol while in the presence of CQ (which clamps the degradation). A positive value indicates a net increase in these parameters, thus suggesting a promotion of autophagy process. On the other hand, the degradation, assuming at steady status, measures the increase caused by CQ, which otherwise would be degraded in the absence of CQ. If the values of degradation in the ethanol plus CQ group are higher than those in the CQ only group, it may be inferred that ethanol treatment promotes autophagy degradation. See the main text for more discussion.

References:

1. Mizushima, N. et al, Cell: 140: 313-326, 2010
2. Rubinsztein, D.C. et al, Autophagy, 5:5, 585-589, 2009
3. Gamerdinger M. et al, EMBO J. 28: 889-901, 2009

Supplemental Figure Legend

Figure S1. Ethanol treatment of hepatocytes in vitro increases the number and the size of autophagosomes.
Primary hepatocytes were treated with ethanol at different doses and then processed for EM analysis. The number of autophagosomes per given area (A) and the diameter of autophagosomes (B) was quantified (mean+SD). *: $p<0.01$ and #: $p<0.05$, one way ANOVA. The results indicate a dose-dependent increase in autophagosome number and size in response to ethanol treatment.

Figures S2. Ethanol induced mainly apoptosis in VL-17 cells that could be enhanced by autophagy inhibition.
VL-17A cells were treated with ethanol at the indicated dose with or without 3-MA (10 mM) for 6 hours. Cells were harvested and stained with Annexin-V-FITC and PI, following by flow cytometry. The dot plots were shown in panel A while the percentages (mean+SD) of cells positive only for Annexin V staining (indicative of apoptosis) or only for PI staining (indicative of necrosis) were shown in panel B and panel C, respectively. The results suggest that ethanol mainly induced apoptosis in VL-17 cells, which was affected by autophagy, and that ethanol did not consistent affected necrosis, although autophagy might have an independent role in that aspect.

Figure S3. Inhibition of autophagy enhanced ethanol-induced hepatocyte death in vivo.
(A). Wild type mice were treated as indicated for 16 hours. Livers were harvested and paraffin-embedded. Liver sections were subjected to Hoechst 33342 staining for the nucleus (blue) and TUNEL for cells with fragmented DNA (green). (B). TUNEL positive cells per field were quantified from more than 10 field per sample (mean+SEM), *: $p<0.01$, one way ANOVA. The results indicate that ethanol-induced hepatocyte death could be enhanced by suppression of autophagy in vivo.

Figure S4. Pharmacological modulation of autophagy alters hepatic steatosis in mice treated with ethanol.
Wild type mice (at least three per group) were given the following treatment: saline (a, g), ethanol (b, h), ethanol plus CQ (c, i), ethanol plus rapamycin (d, j), CQ only (e, k) or rapamycin only (f, l). Livers were harvested, paraffin-embedded and H-E stained. Images were acquired with a 20x (a-f) or a 40x (g-l) objective lens. Acute ethanol treatment induced microvesicular steatosis, which were enhanced by the co-treatment with chloroquine, but reduced by the co-treatment with rapamycin. Changes in the degree of steatosis were confirmed by Bodipy staining (Fig. 7C), which was quantified (Fig. 7D). Gross parenchymal alternations and cell death were not obvious in these treatments. Note that rapamycin treatment caused a hypochromic staining of the cytosol, likely due to the inhibition of mTOR and protein synthesis by this chemical.

Figure S5. Genetic modulation of autophagy alters hepatic steatosis in mice treated with ethanol.
Wild type mice (three per group) were given negative siRNA (a, b) or Atg7-specific siRNA for 48 hours and then given ethanol treatment for 16 hours. Livers were harvested, paraffin-embedded and H-E stained. Images were acquired with a 20 x (a, c) or 40 x (b, d) objective lens. Microvesicular steatosis induced by acute ethanol treatment was further enhanced by Atg7-specific siRNA, which was confirmed and quantified in Fig. 7F. Other gross parenchymal changes were not obvious.

Figure S6. Lipid staining with BODIPY 493/503 and BODIPY 581/591-C11 resulted in the same pattern.
Cryosection of liver tissues from ethanol-treated mice were stained with BODIPY 493/503 (0.1 μm) and BODIPY 581/591 (1 μm) for 15 minutes followed by confocal microscopy. Note the staining patterns of the two dyes are almost completely overlapped, suggesting that the both dyes could be used for detecting lipids with comparable results.

Figure S7. Ethanol did not promote or inhibit long-lived protein degradation at basal status or under starvation, in VL-17A

VL-17A cells were cultured in DMEM with 10% FCS or in starvation medium (EBSS) without or with ethanol for 15 hours. Long lived proteins degradation was then performed as described in the supplemental method. *: $p<0.001$. Note that ethanol did not inhibit long lived protein degradation at the basal status or under starvation, nor did it promote long-lived protein degradation.

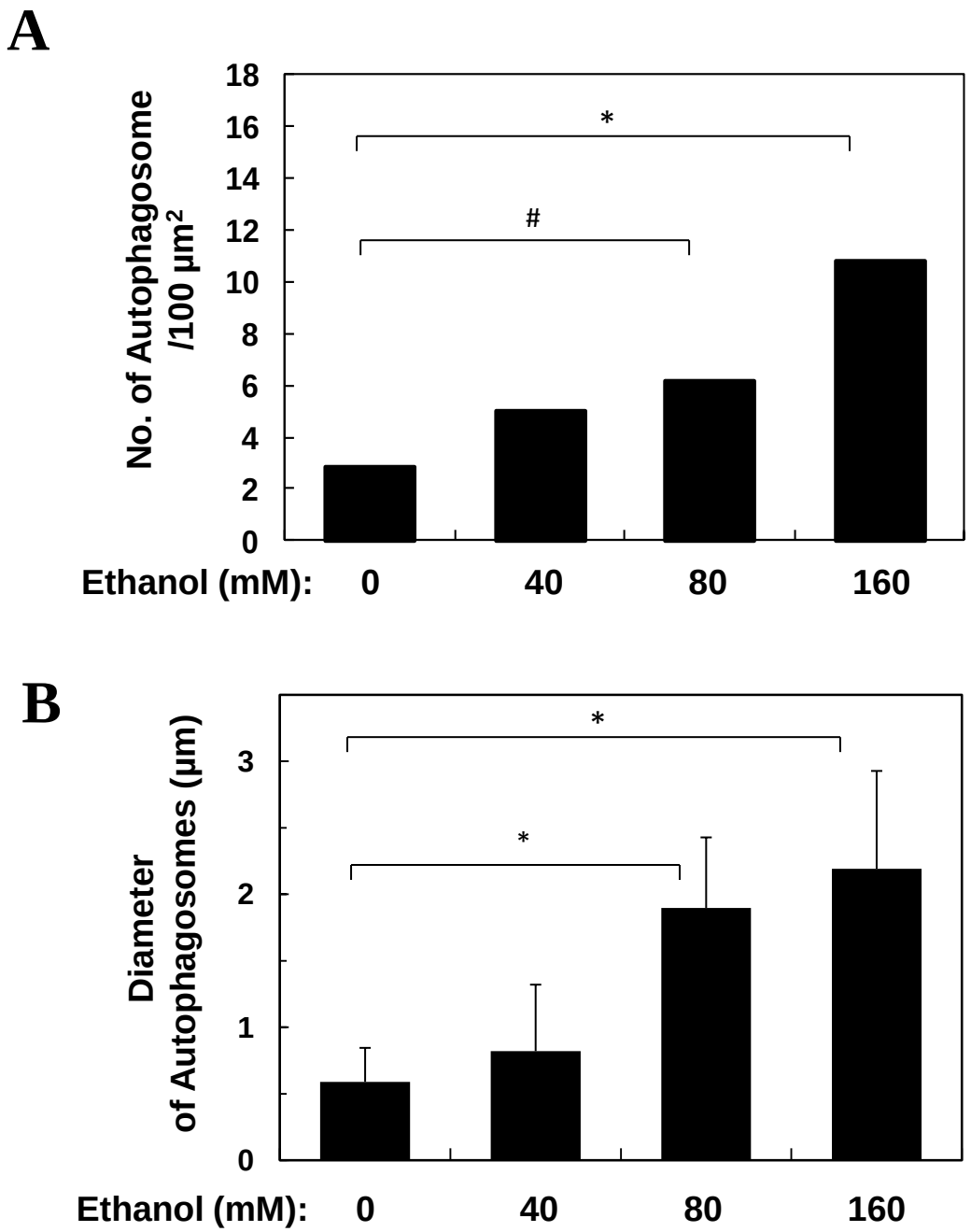
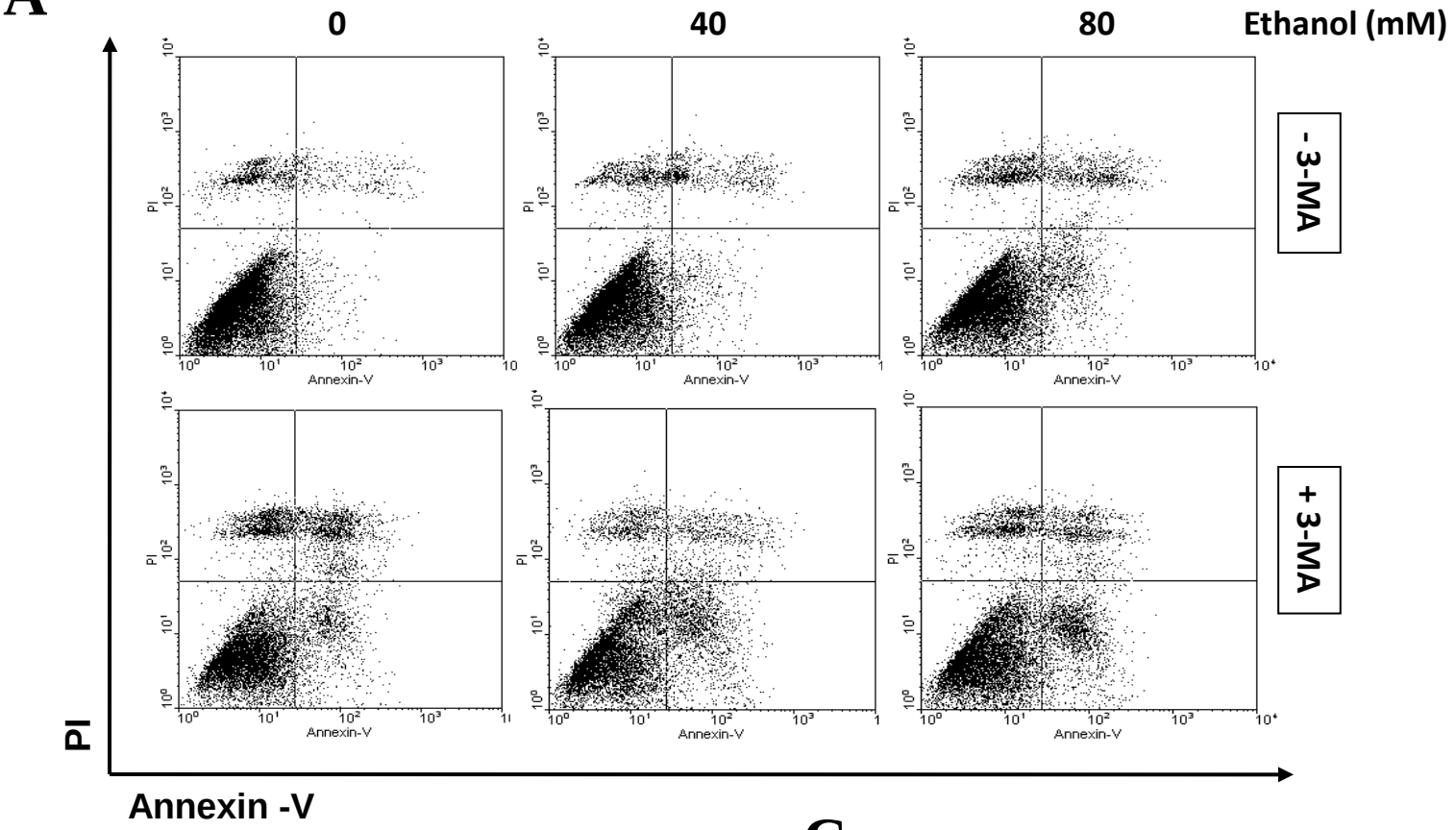
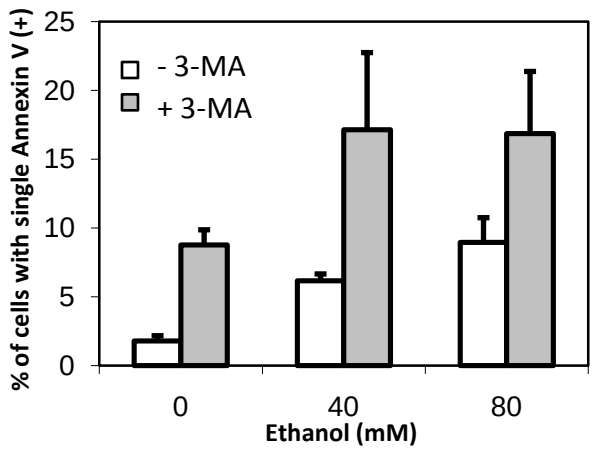

A
No. of Autophagosome /100 μm²
18
16
14
12
10
8
6
4
2
0
*
#
Ethanol (mM): 0 40 80 160
B
Diameter of Autophagosomes (μm)
3
2
1
0
*
*
Ethanol (mM): 0 40 80 160

Figure S2


A
0
40
80
Ethanol (mM)
- 3-MA
+ 3-MA
PI
Annexin-V
PI
Annexin -V


B
% of cells with single Annexin V (+)
- 3-MA
+ 3-MA
0
5
10
15
20
25
0
40
80
Ethanol (mM)

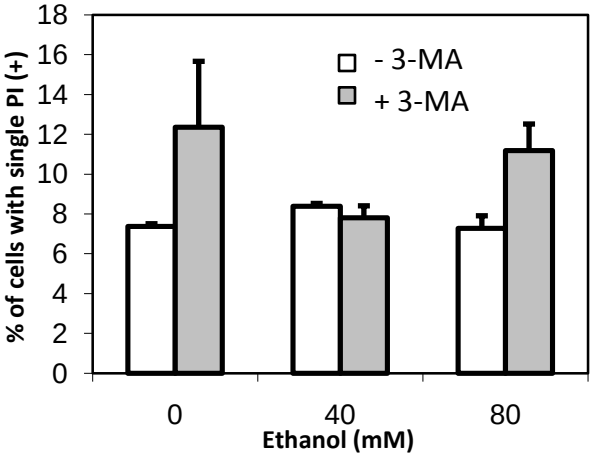
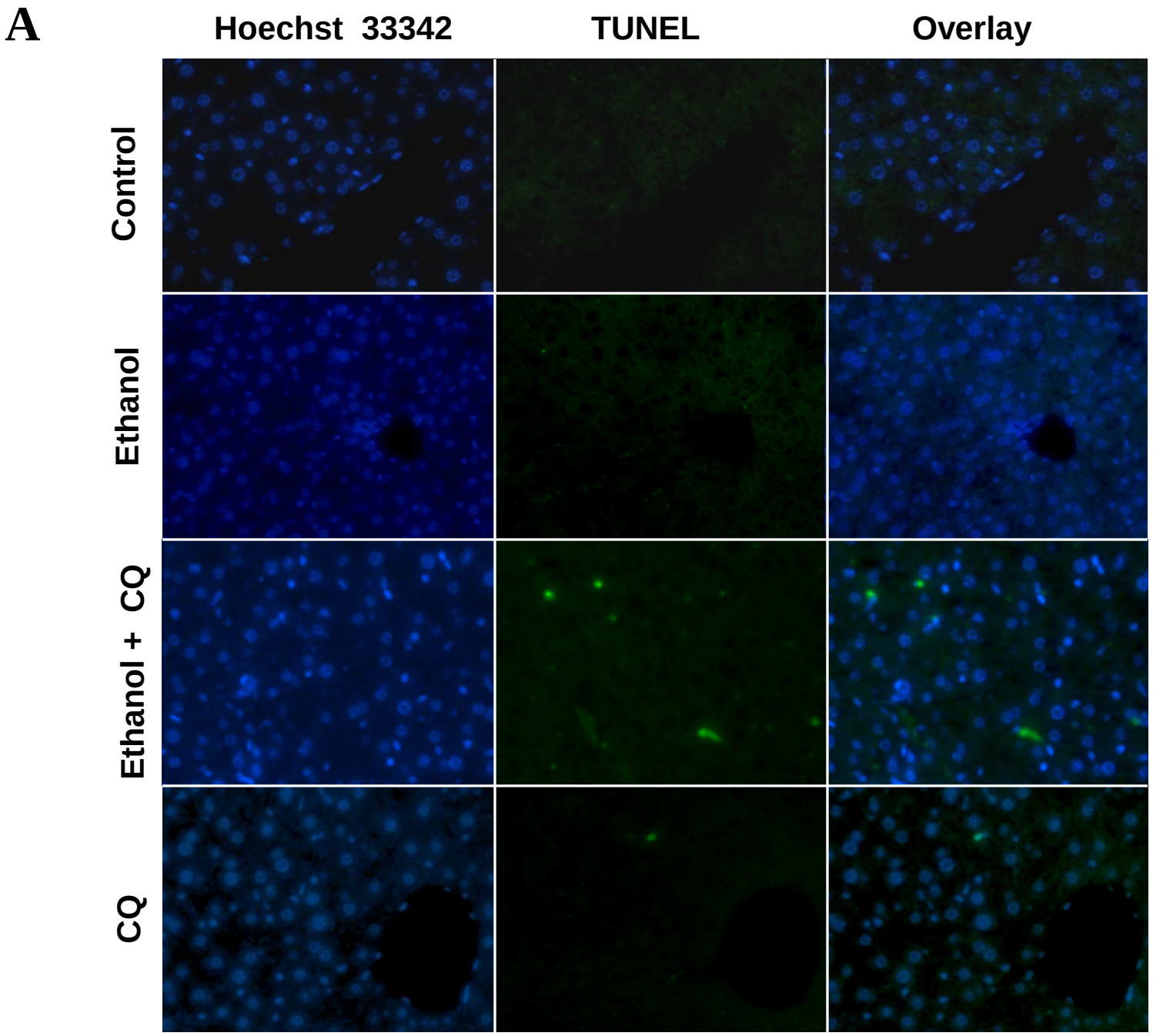

C
% of cells with single PI (+)
- 3-MA
+ 3-MA
0
2
4
6
8
10
12
14
16
18
0
40
80
Ethanol (mM)

Figure S3


A
Hoechst 33342
TUNEL
Overlay
Control
Ethanol
Ethanol + CQ
CQ

B

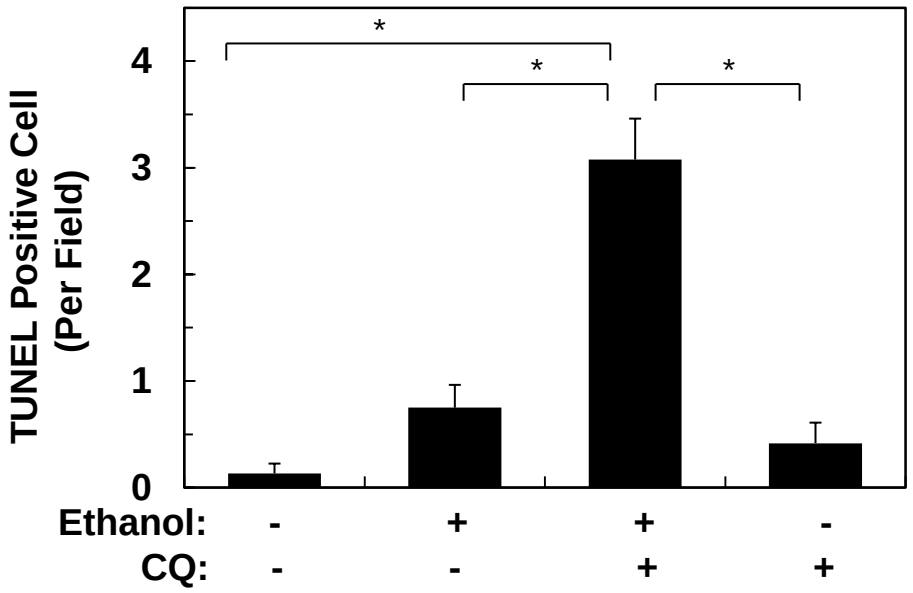
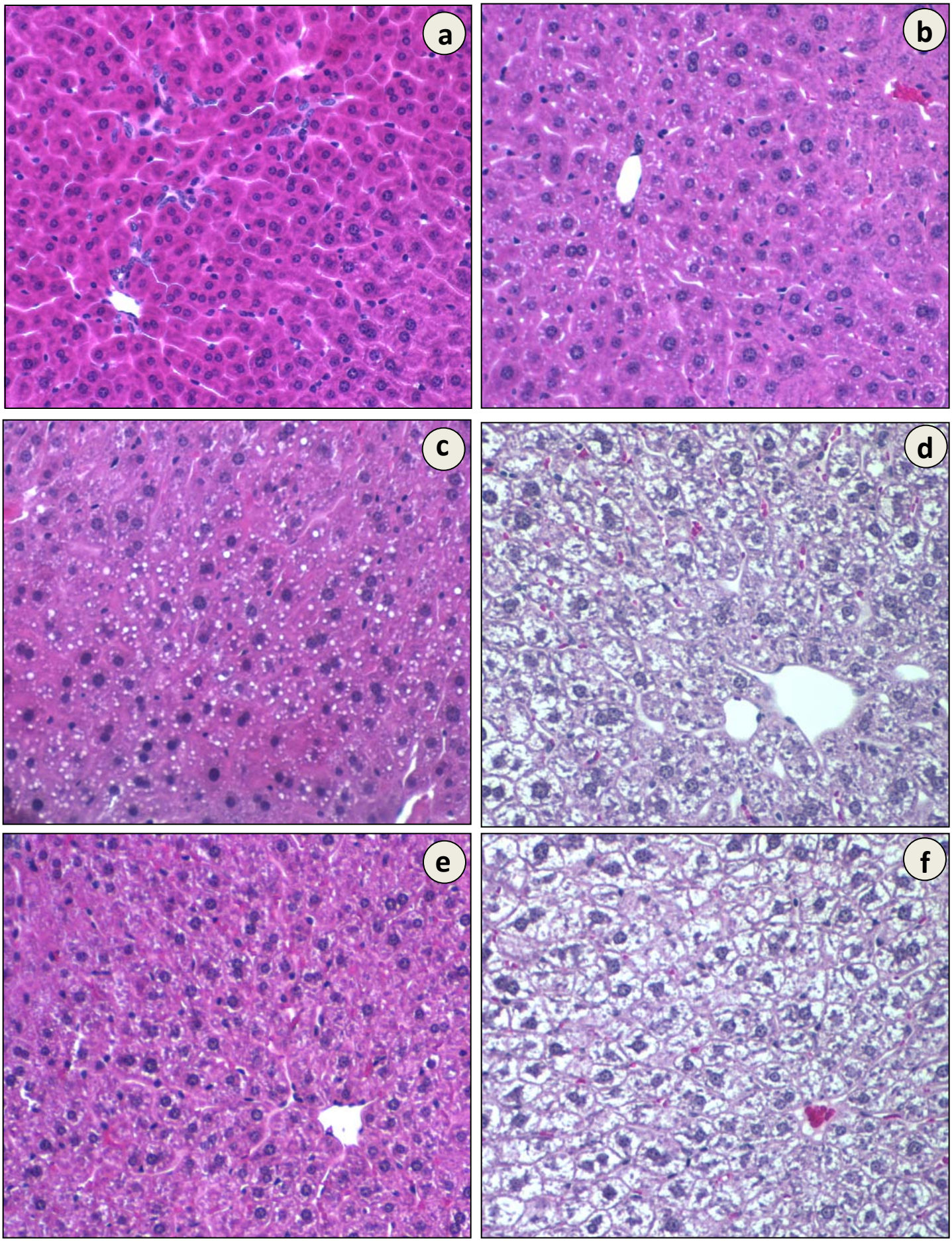


Figure S4



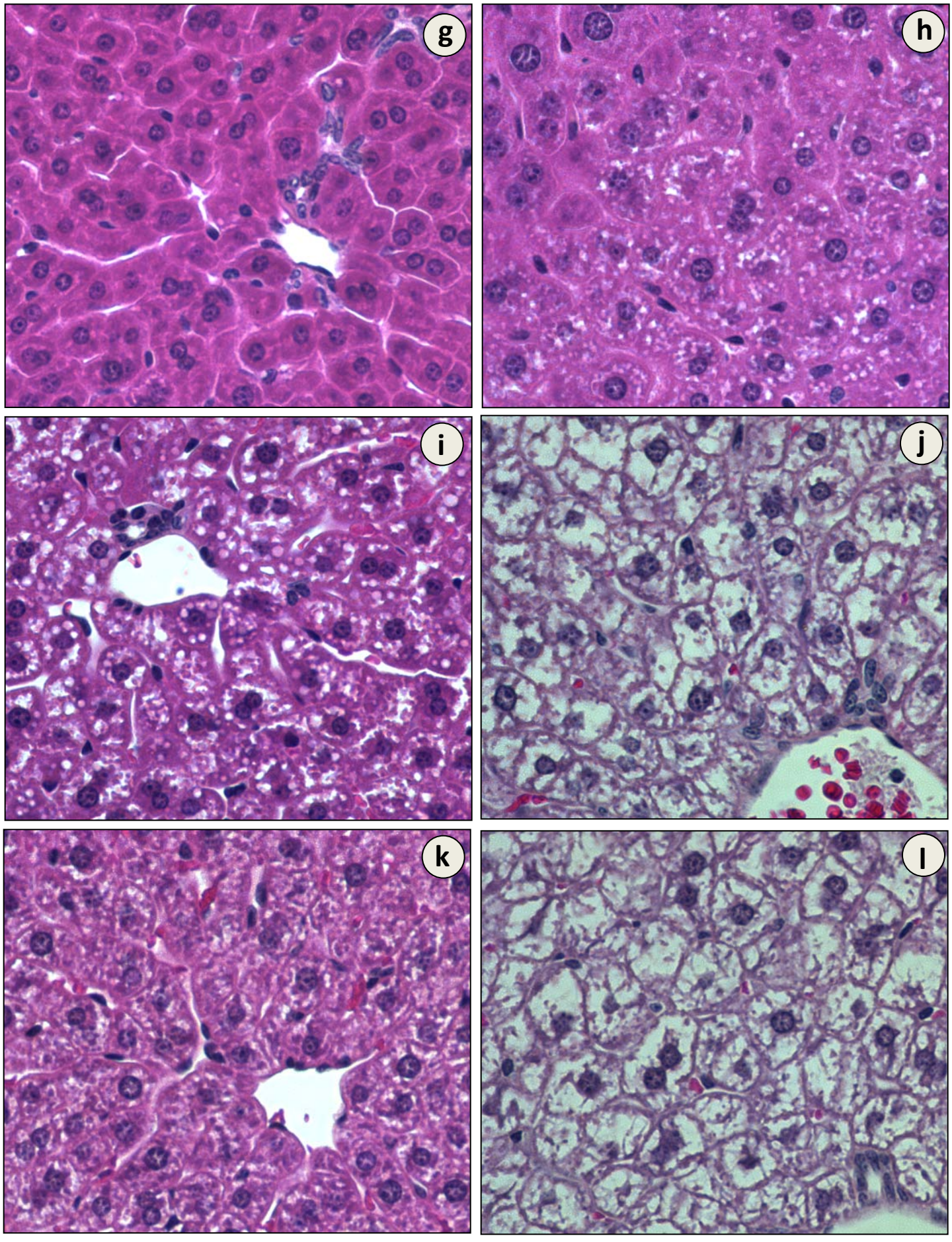

g
h
i
j
k
l

Figure S5

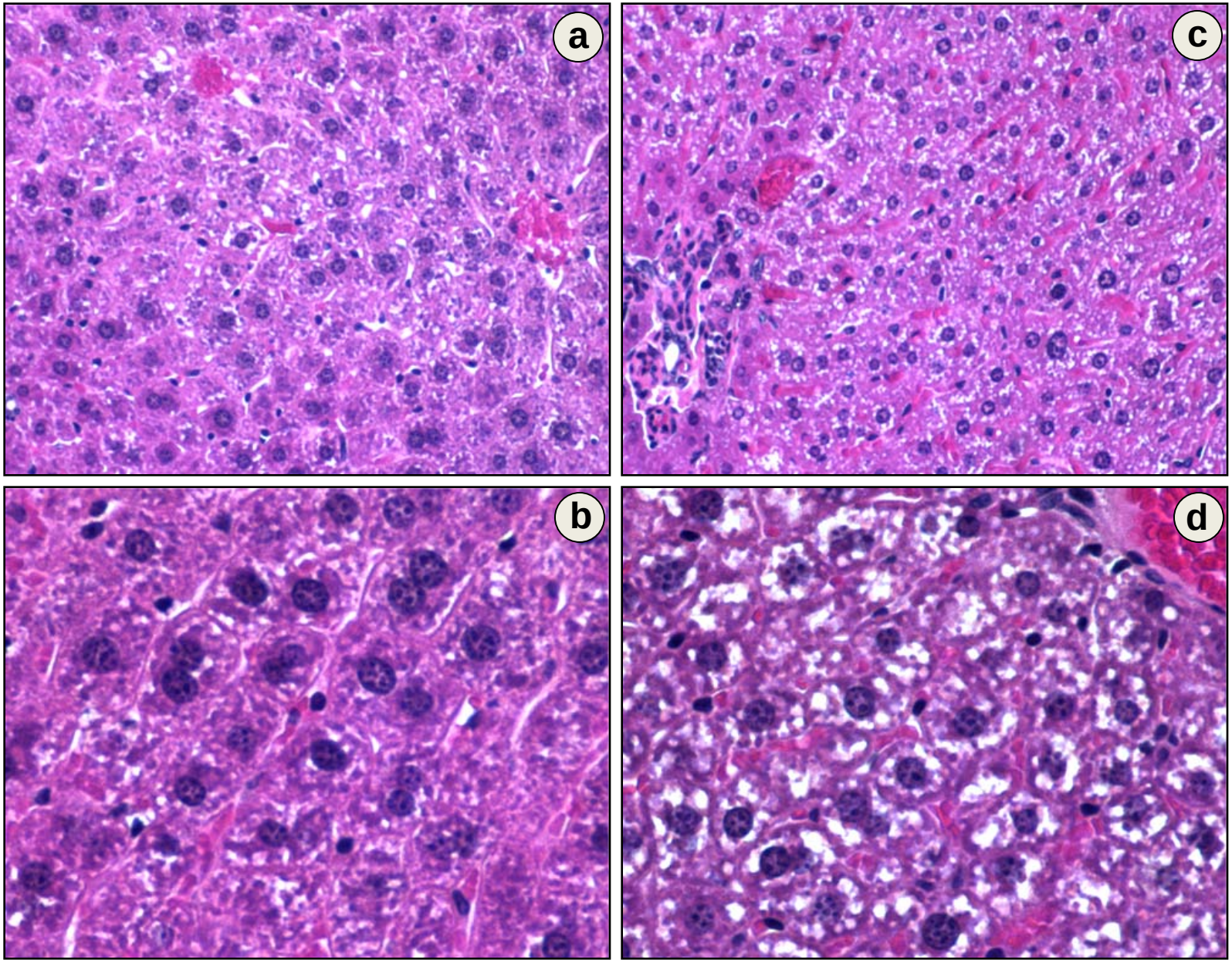


Figure S6

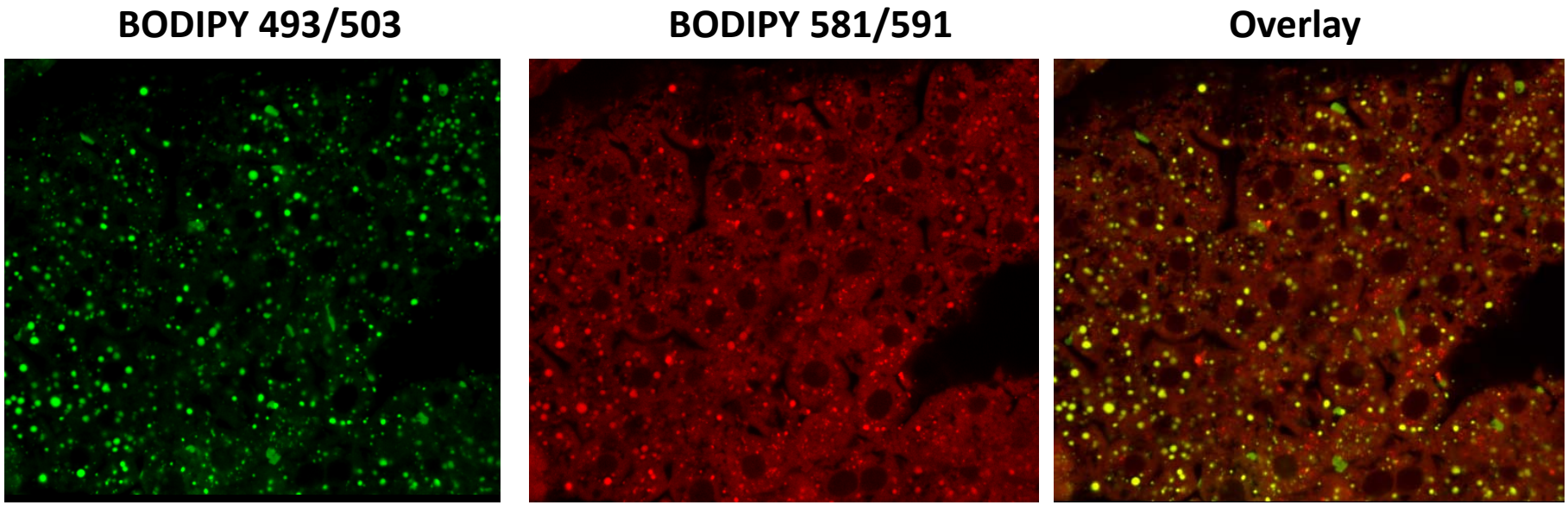
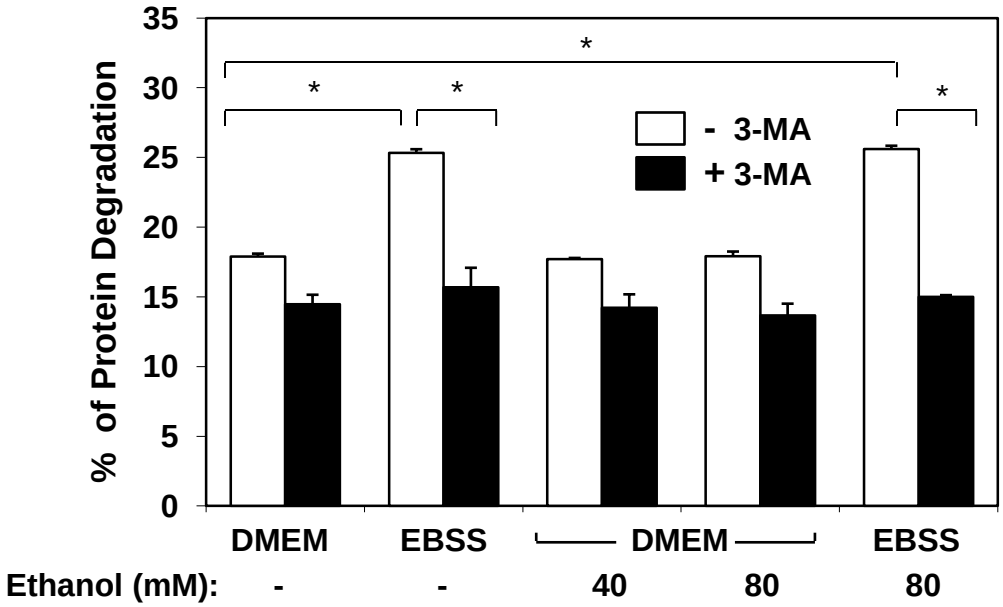


Figure S7


% of Protein Degradation
35
30
25
20
15
10
5
0
*
*
*
*
- 3-MA
+ 3-MA
DMEM
EBSS
DMEM
EBSS
Ethanol (mM):
-
-
40
80
80