Differential Roles of Unsaturated and Saturated Fatty Acids on Autophagy and Apoptosis in Hepatocytes

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Supplemental Materials and Methods

Primary hepatocytes culture. As described previously (Ding et al., 2004), murine hepatocytes were isolated by a retrograde, nonrecirculating perfusion of livers with 0.05% Collagenase Type IV (Sigma). Cells were cultured in William's medium E with 10% fetal bovine serum but no other supplements for 2 hrs for attachment. Cells were then cultured in the same medium without serum overnight before treatment. All cells were maintained in a 37°C incubator with 5% CO_2.

Treatment with unsaturated fatty acid: palmitoleate (PO,16:1)*.* HepG2 cells culture and palmitoleate (PO)/BSA conjugate were prepared as described in the Materials and Methods (main text). Briefly, a 20 mM solution of PO in 0.01 N NaOH was incubated at 70 ºC for 30 min, and fatty acid soaps were then complexed with 5% BSA in PBS at a 7:1 molar ratio of fatty acid to BSA. The PO/BSA conjugate was administered to the cultured cells. BSA was used as a vehicle control.

Measurement of ROS production. Intracellular ROS was measured with the fluoroprobe 2′,7′-dichlorofluorescin diacetate (DCFH-DA) as described previously (Ding et al., 2004). Briefly, HepG2 cells were treated with OA (500 μm) or PA (500 μm) in the presence or absence of NAC (10 mM) for 6 hrs. The cells were further

incubated with 2.5 μM DCFH-DA for 30 min at 37 °C. After DCFH incubation, ROS analysis was carried out with ROS assay kits (Green Fluorescence) (Cell BIOLABS, INC.) in a 96-well plate as modified from the manufacturer's instruction to quantitatively measure cellular ROS level using an Infinite M200 plate reader (Tecan, Durham, NC).

Supplementary Figure Legends

Figure S1. Differential effects of OA and PA on autophagy induction in primary cultured mouse hepatocyte cells. Primary mouse hepatocyte cells were first infected with Ad-GFP-LC3 overnight and then treated with vehicle control (5% BSA), OA (500 μM) or PA (500 μM) in the absence or presence of CQ (20 μM) for 6 hrs followed by fluorescence microscopy. Representative GFP-LC3 images were shown in (**A**). The numbers of GFP-LC3 dots per cell (mean ± SE, n=3) were determined from 3 independent experiments and more than 20 cells were counted in each experiment (**B-C**). (**D**) Total cell lysates were subjected to immunoblot analysis with anti-LC3 and anti-β-Actin antibodies. Densitometry analysis for the expression level of LC3-II was performed using Image J software which was further normalized with its loading control (β-Actin).

Figure S2. PO induces autophagy in HepG2 cells. HepG2 cells were first infected with Ad-GFP-LC3 (100 viral particles per cell) overnight and then treated with vehicle control (5% BSA), PO (500 μM), PO plus CQ (20 μM) or CQ (20 μM) alone for 6 hrs followed by fluorescence microscopy. Representative GFP-LC3 images were shown

in (**A**). The number of GFP-LC3 dots per cell was determined (**B**). Data are presented as mean ± SE from three independent experiments by counting more than 20 cells in each individual experiment. *: $p<0.05$; #: $p<0.01$ (one way ANOVA with Scheffe's post-hoc test). (**C**) HepG2 cells were treated with vehicle control (5% BSA), PO (500 μM), PO plus CQ (20 μM), CQ (20 μM) alone for 6 hrs. Total cell lysates were subjected to immunoblot analysis with anti-LC3 and anti-β-Actin antibodies. Densitometry analysis for the expression level of LC3-II was performed using Image J software which was further normalized with its loading control (β-Actin). Digital data are presented as the ratio of the vehicle control (mean ± SE) from at least three independent experiments.

Figure S3. Fatty acid-treatment increases lipid droplet in primary mouse hepatocytes. (**A**) Primary mouse hepatocytes were treated with BSA vehicle control OA (500 μM), PA (500 μM) and PO (500 μM) for 6 hrs and fixed with 4% paraformaldehyde. The cells were further stained with Bodipy 493/503 (0.1 μM) for lipid droplets and Hoechst 33342 (0.5 μg/mL) for the nuclei followed by fluorescence microscopy. (**B**) The number of lipid droplets per cell was quantified and data are presented as mean ± SE from at least three independent experiments. #: $p<0.01$ (one way ANOVA with Scheffe's post-hoc test).

Figure S4. Effects of NAC on fatty-acid-induced ROS production in HepG2 cells. HepG2 cells were treated with vehicle control (5% BSA), OA (500 μM), PA (500 μM) in the presence or absence of NAC (5 mM) for 6 hrs. The cells were further incubated with 2.5 μM DCFH-DA for 30 min at 37 °C. After DCFH incubation, ROS

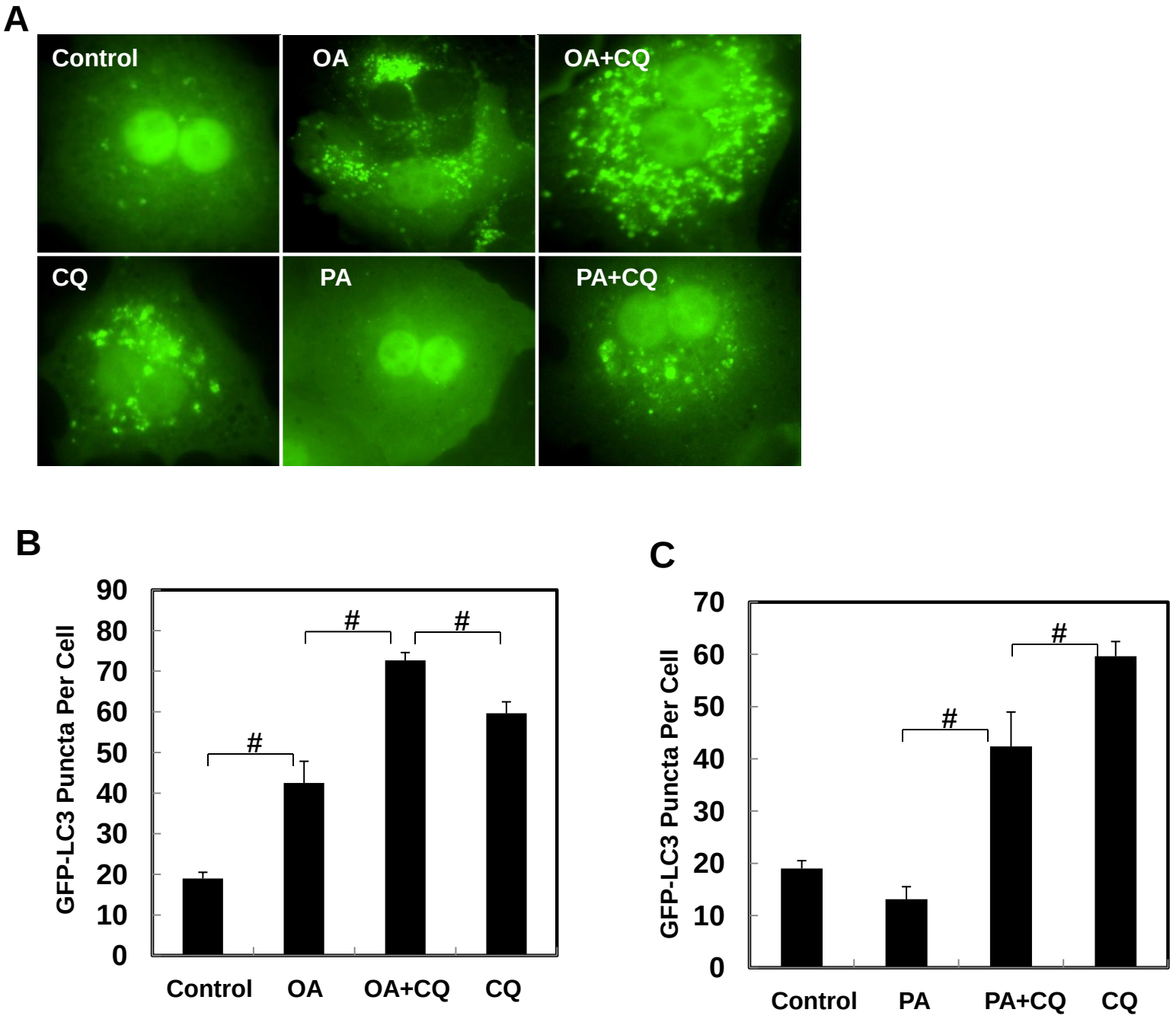
analysis was carried out with the ROS assay kits (Green Fluorescence) (Cell BIOLABS, INC.) in a 96-well plate as modified from the manufacturer's instruction.

Figure S5. Effects of ZVAD on PA-induced GFP-LC3 puncta formation in HepG2 cells. (**A**) HepG2 cells were first infected with Ad-GFP-LC3 (100 viral particles per cell) overnight and then treated with vehicle control (5% BSA), PA (500 μM), PA plus ZVAD (50 μM) or ZVAD (50 μM) alone for 6 hrs followed by fluorescence microscopy. (**B**) The number of GFP-LC3 dots per cell (mean ± SE) was quantified from three independent experiments and more than 20 cells were counted in each individual experiment. *: $p<0.05$; #: $p<0.01$ (one way ANOVA with Scheffe's post-hoc test).

Figure S6. OA protects against PA-induced apoptosis. HepG2 cells were treated with OA (500 μM), PA (500 μM) or OA (500 μM) plus PA (500 μM) for 24 hrs. Apoptotic cell death was analyzed by nuclear staining with Hoechst 33342 (mean ± SE, n=3).

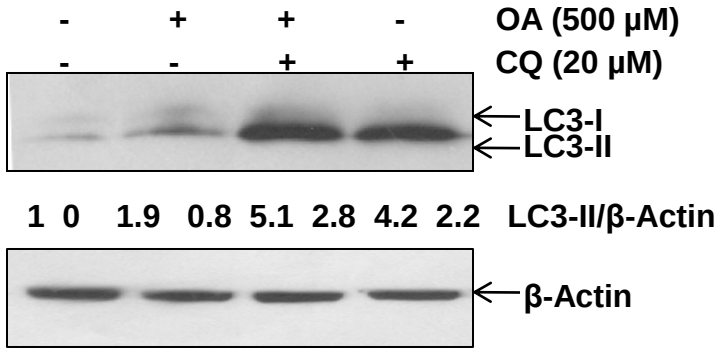
Figure S7. Effects of autophagy modulation on fatty acid-induced lipid accumulation. (**A**) HepG2 cells were treated with vehicle control (5% BSA), OA (500 μM), OA plus 3MA (10 mM) or 3MA (10 mM) for 6 hrs. Lipid droplets were analyzed by staining with Bodipy 493/503(0.1 μM) and the number of lipid droplets per cell was quantified (mean ± SE) from three independent experiments. (**B**) HepG2 cells were treated with vehicle control (5% BSA), OA (500 μM) in the presence or absence of rapamycin (Rap, 10 μM) for 6 hrs. The cellular TG contents were quantified (mean ± SE) from three independent experiments.

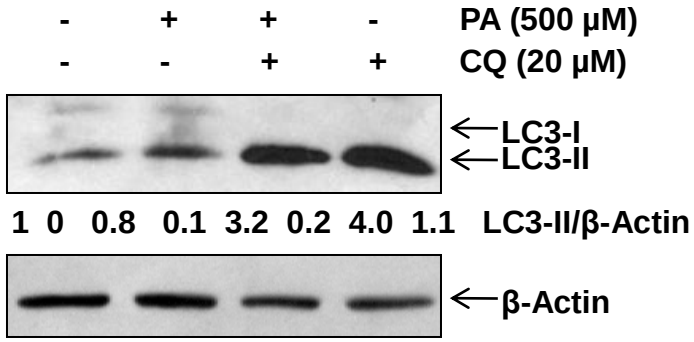
Supplemental Figure 1



Supplemental Figure 1

D





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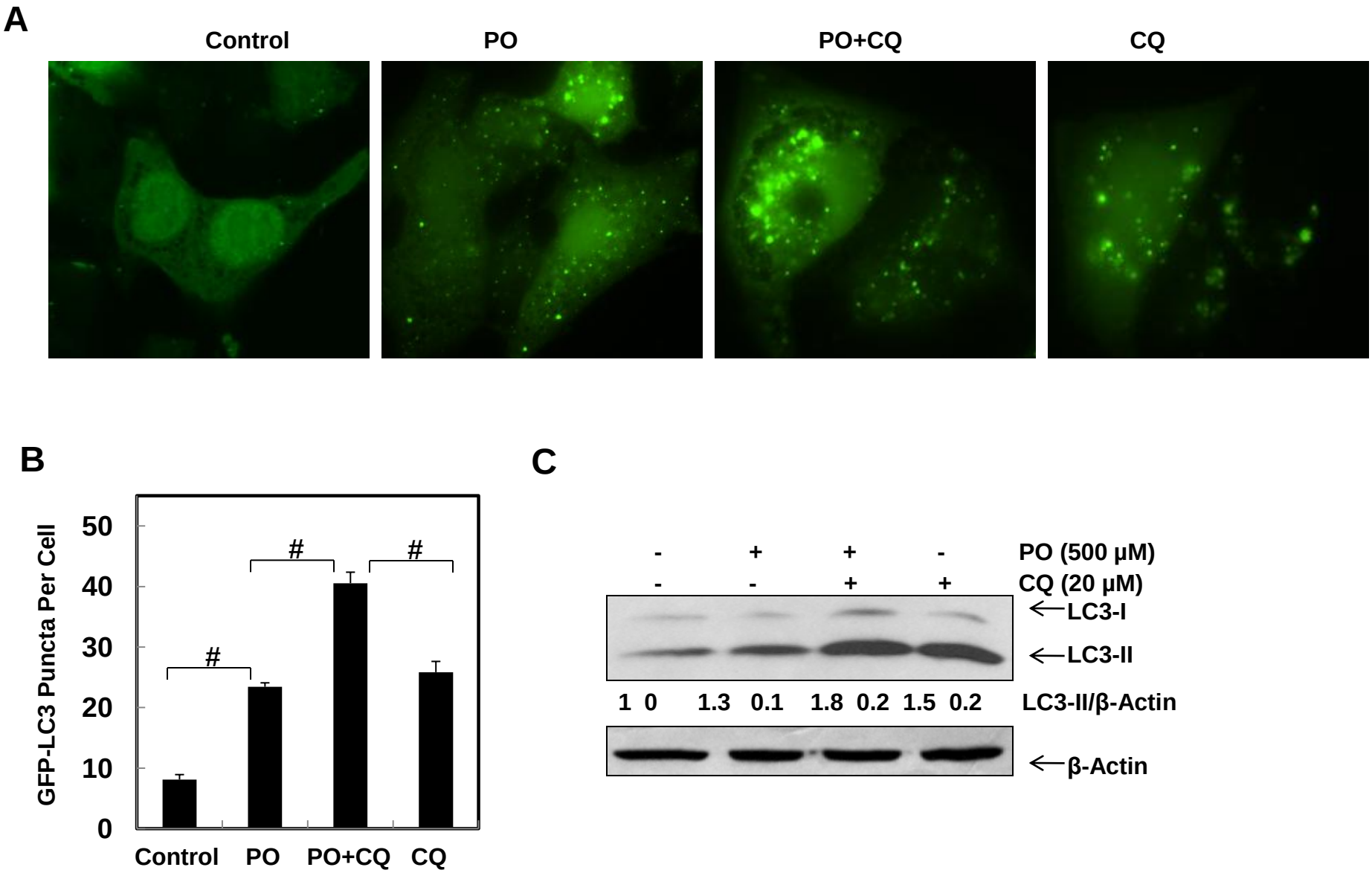

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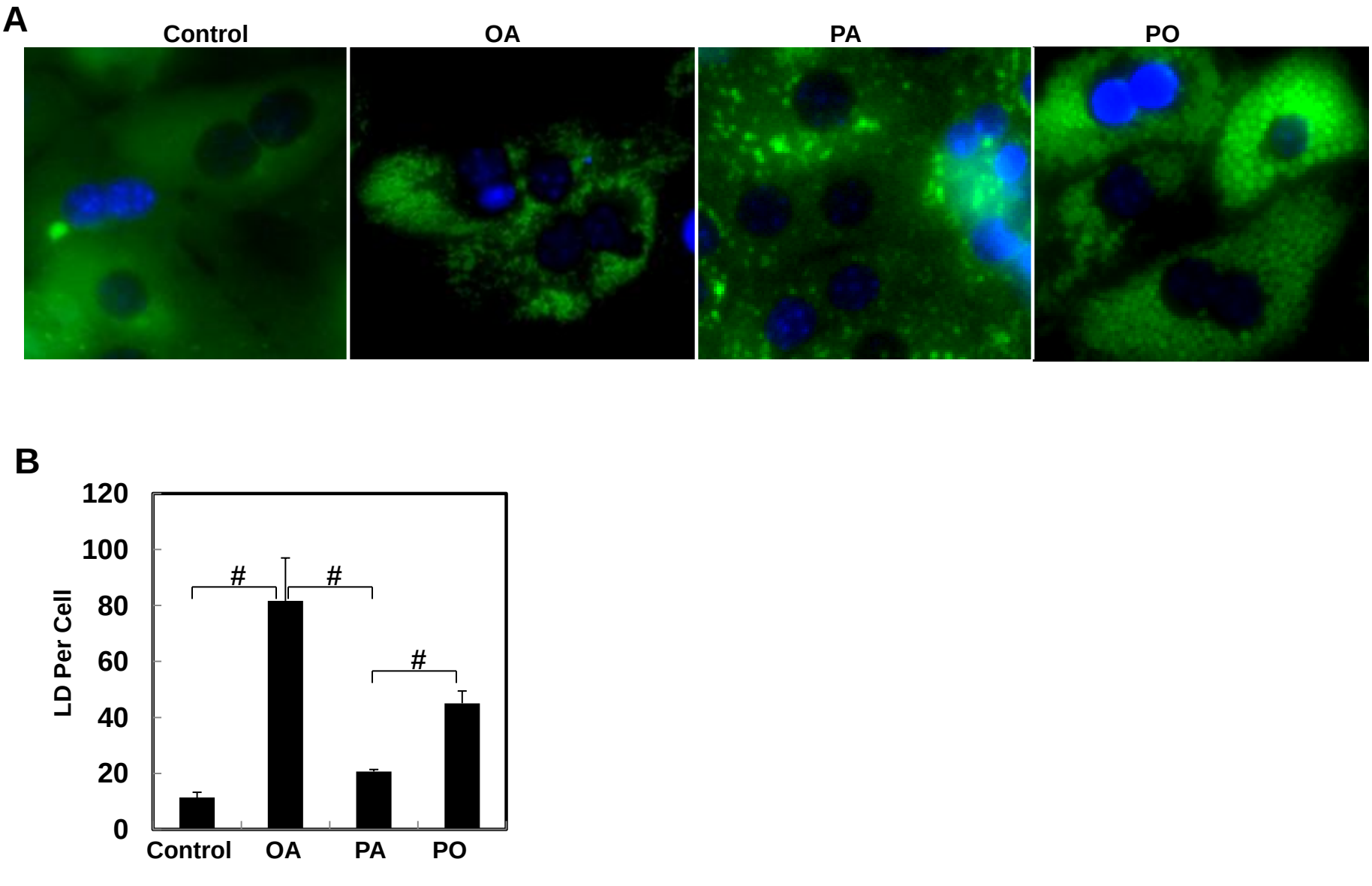

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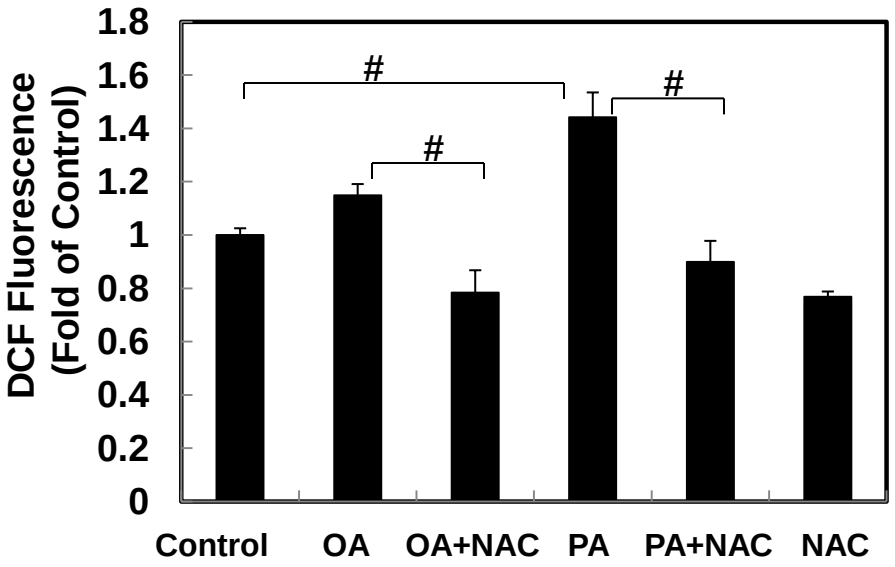

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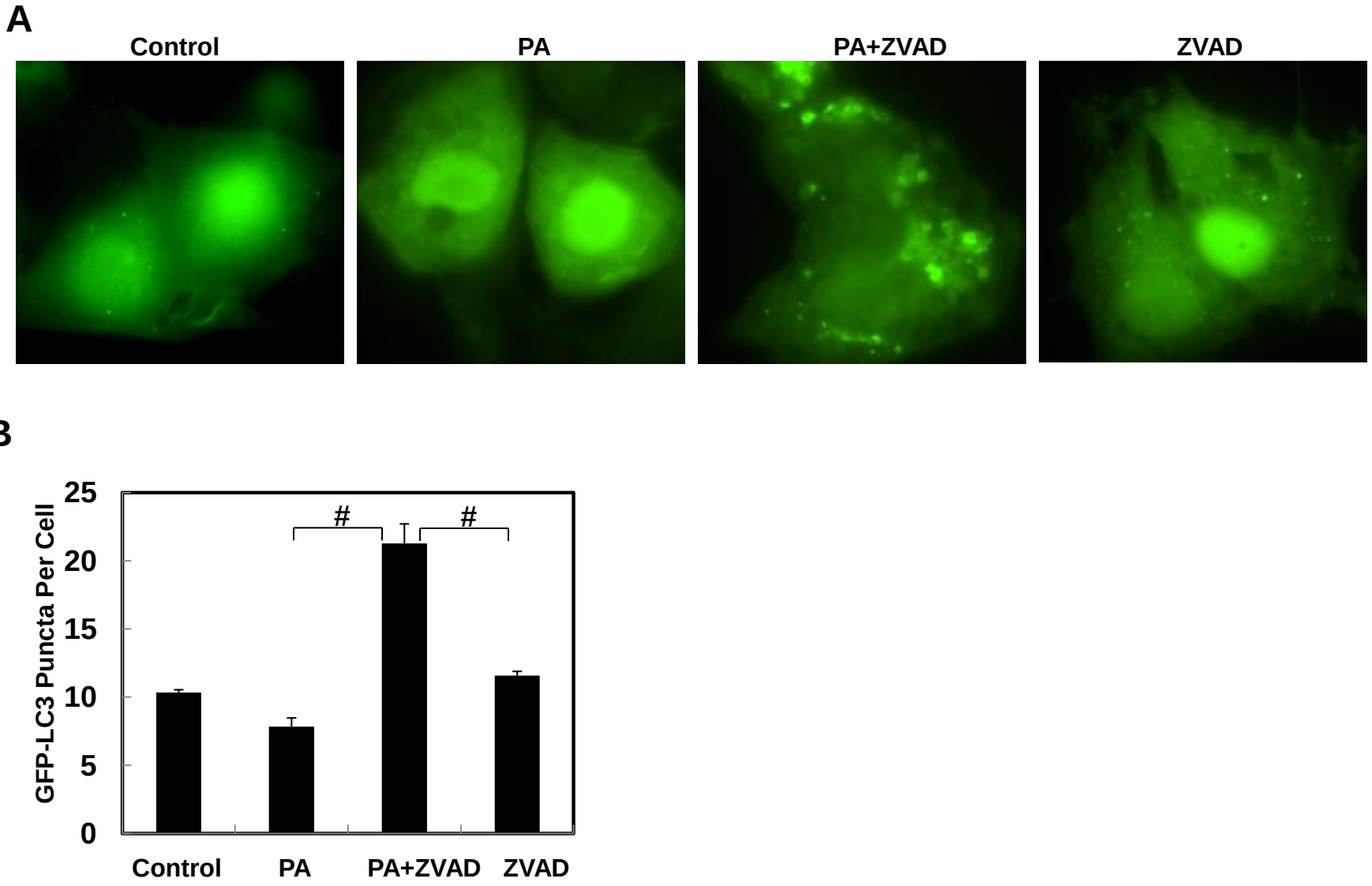

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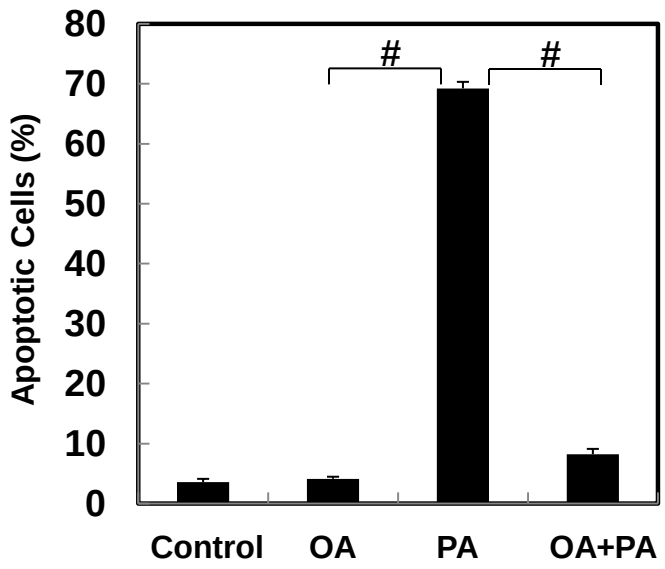
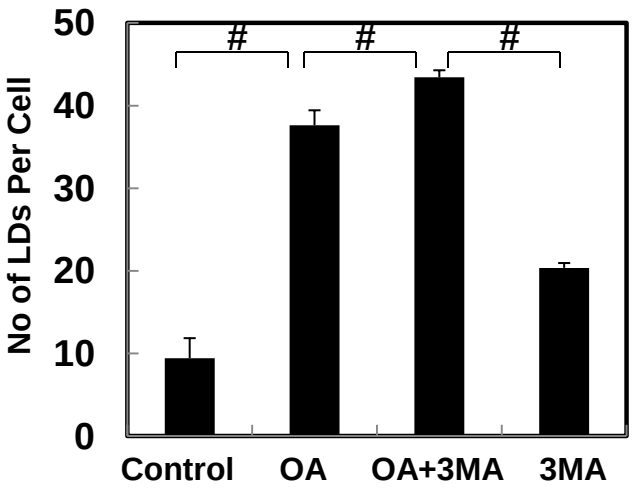


Supplemental Figure 7

A



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