Tamoxifen-Induced Gastric Injury: Effects of Dose and Method of Administration

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Materials and Methods

Mice

Lgr5-eGFP-IRES-CreERT2[1] (JAX 008875), *ROSA-CAG-LSL-tdTomato-WPRE*[2] (JAX 007914), gastrin-deficient[3] (JAX 031681) and C57BL/6 mice were in-house bred and housed in ventilated and automated-watering cages under specific pathogen-free conditions. Mice of both sexes aged 2-3 months were treated with tamoxifen (TX). Gastric tissue was collected at various times post TX after an overnight fast with free access to water. Mouse use was approved by The University of Michigan Committee on Use and Care of Animals.

TX Administration

TX (Sigma) was either dissolved in 100% ethanol (EtOH) and then added to corn oil (Sigma) (5 or 10% final EtOH concentration), or TX was dissolved directly in corn oil without EtOH, to a final concentration of 10 mg/ml or 20 mg/ml. TX was administered via intraperitoneal (IP) injection, subcutaneous injection or oral gavage. Corn oil with EtOH (5% final concentration), or without EtOH was used as the vehicle control. EtOH and corn oil were heated to 65°C for all preparations until TX was completely dissolved. TX solutions were stored at 4°C for up to 1 month.

Measurement of mRNA Abundance

Total RNA was isolated using Trizol extraction (Invitrogen). RNA was purified and treated with DNase1 using RNeasy columns (Qiagen). Reverse-transcriptase (RT) reactions with 1μg of RNA were performed with an iScript kit (Bio-Rad). Triplicates from each cDNA sample were amplified by quantitative PCR (qPCR) to measure specific mRNA abundance using an ICycler (Bio-Rad) with SYBR Green dye (Molecular Probes), as previously described[4]. Melt curve analysis was used to assess product purity. Expression levels were normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*). Primer sequences for *Atp4a* and *Gapdh* were as previously described[4]. Primer sequences for *Cd44* were as follows: Forward: 5'- CAC ATA TTG CTT CAA TGC CTC AG-3' and Reverse: 5'-CCA TCA CGG TTG ACA ATA GTT ATG-3' to amplify a 111bp product.

Histological Analysis

For paraffin-embedded tissue sections, stomachs were removed and fixed overnight in 4% paraformaldehyde in phosphate buffered saline (PBS) before tissue blocks were prepared, as described[5]. Paraffin sections (4 μm) were stained with hematoxylin and eosin (H&E) for evaluation of general histology. For cryo-sections, stomachs were removed and fixed in 4% paraformaldehyde in PBS for 1 hour then placed in 30% sucrose in PBS overnight before cryo-blocks were prepared by embedding the tissue in O.C.T. (Tissue Tek) and freezing on dry ice.

H/K-ATPase-α staining was performed as previously described[4]. For proliferation analysis, mice were injected with the thymidine analog 5-ethynyl-2´-deoxyuridine (EdU) (Invitrogen) (25 mg/kg) 1.5 hours before tissue collection. Paraffin or cryo-sections (4 μm) were treated with the EdU Click-it reaction according to the manufacturer's instructions (Invitrogen). After staining, coverslips were mounted on slides with Prolong Gold plus 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Life Technologies) for nuclear staining. Histological images were captured on a Nikon E800 microscope with Olympus DP controller software.

Lineage Tracing

Lgr5-eGFP-IRES-CreERT2;ROSA-CAG-LSL-tdTomato-WPRE mice were treated IP with vehicle, 25mg/kg, 50mg/kg, 100mg/kg, or 200mg/kg TX and examined 24 hours later for tdTomato activation in antral glands.

Morphometric Analysis

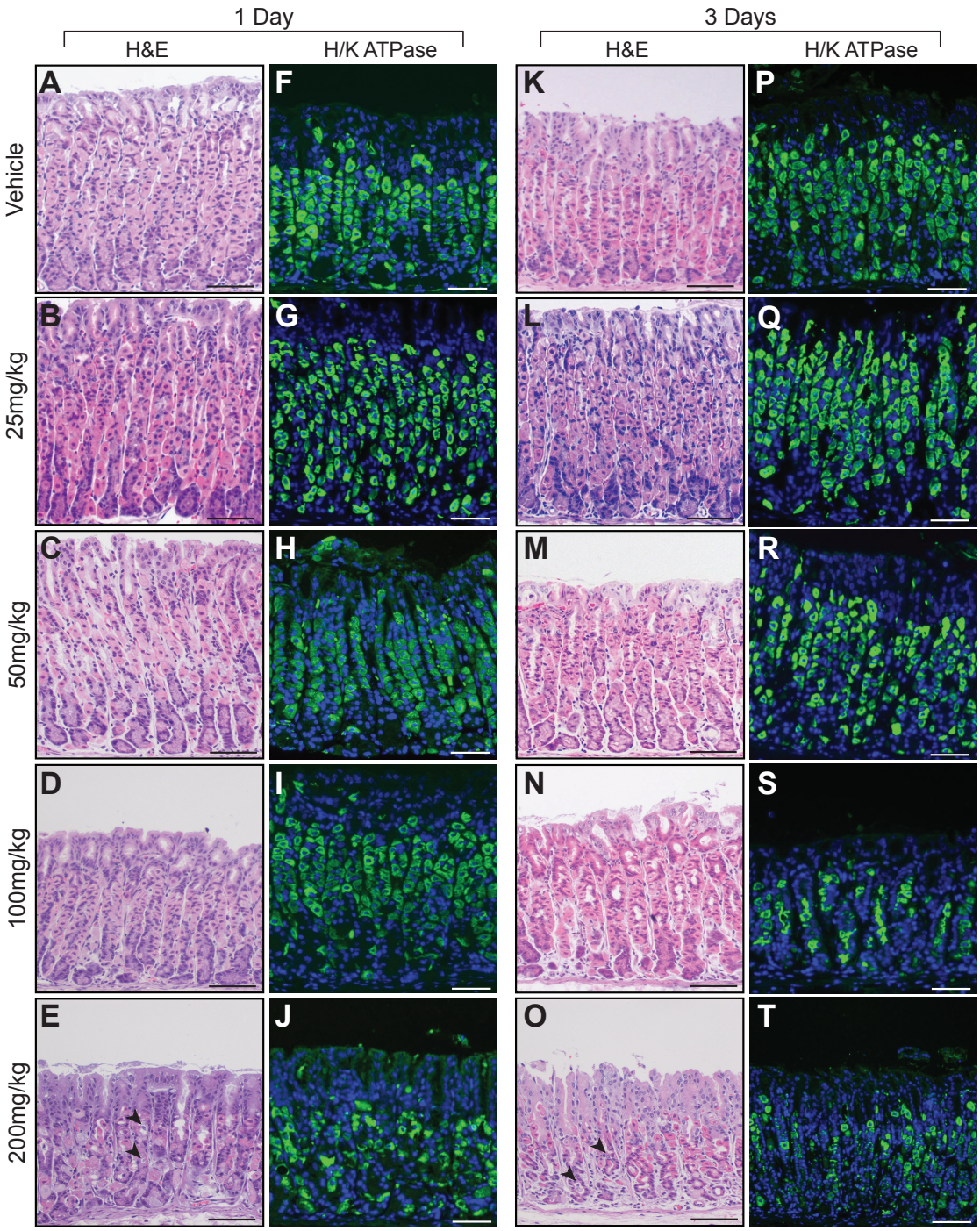
Image J [version 1.34u, Wayne Rasband, National Institutes of Health (http://rsb.info.nig.gov/ij/)] was used to calculate length of the tissue from 5-9 field views per animal (n = 3-8 animals per group). The number of EdU-positive cells was counted, and the data were expressed as number of positive cells per length of epithelium in μm. For lineage activation, tdTomato-labeled glands were counted along a length of antral tissue, divided by the total number of antral glands present.

Statistics

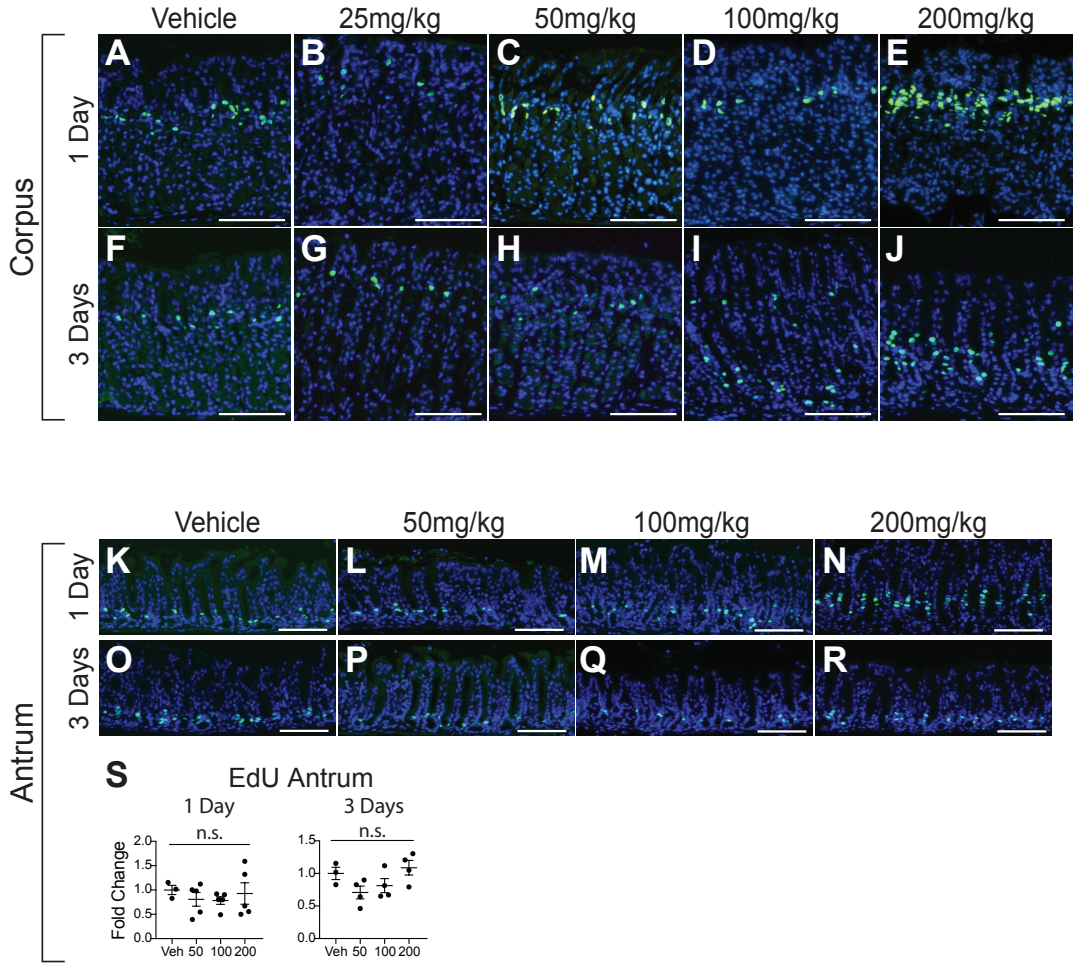
GraphPad Prism software was used for statistical analysis and preparation of graphs. Quantitative data are presented as mean ± SEM and analyzed by Student's t-test, 1-way ANOVA with Dunnett's post-test, or 2-way ANOVA with a Tukey post hoc test, as noted in figure legends. $P < 0.05$ was considered significant.

Supplementary References

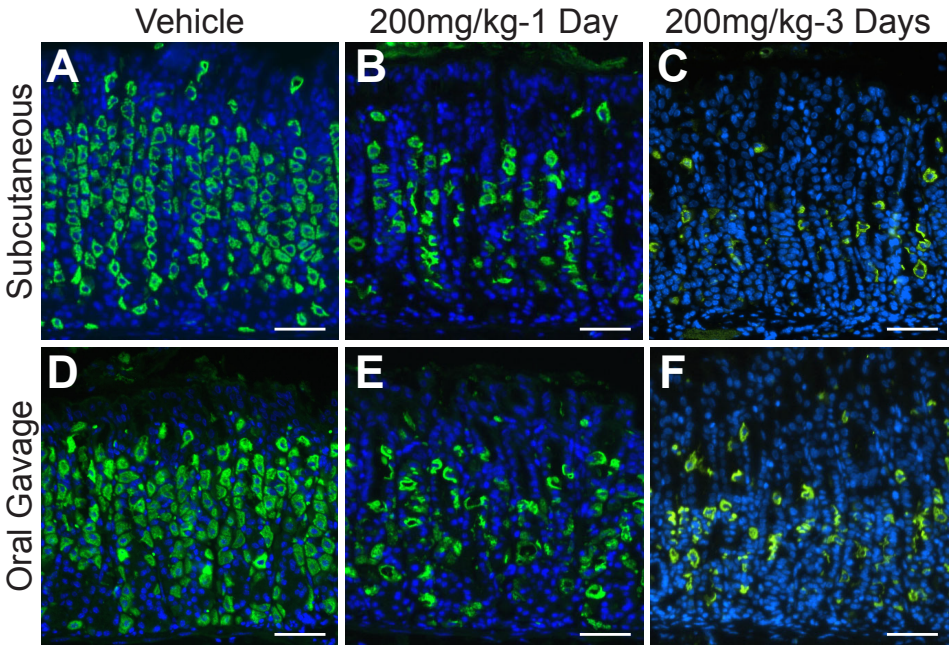
1. Barker N, et al. Nature 2007;449:1003-7.
2. Madisen L, et al. Nat Neurosci 2010;13:133-40.
3. Friis-Hansen L, et al. Am J Physiol Gastrointest Liver Physiol 1998;274:G561-8.
4. Keeley TM, Samuelson LC. Am J Physiol Gastrointest Liver Physiol 2010;299:G1241-51.
5. Lopez-Diaz L, et al. Am J Physiol Gastrointest Liver Physiol 2006;290:G970-9.



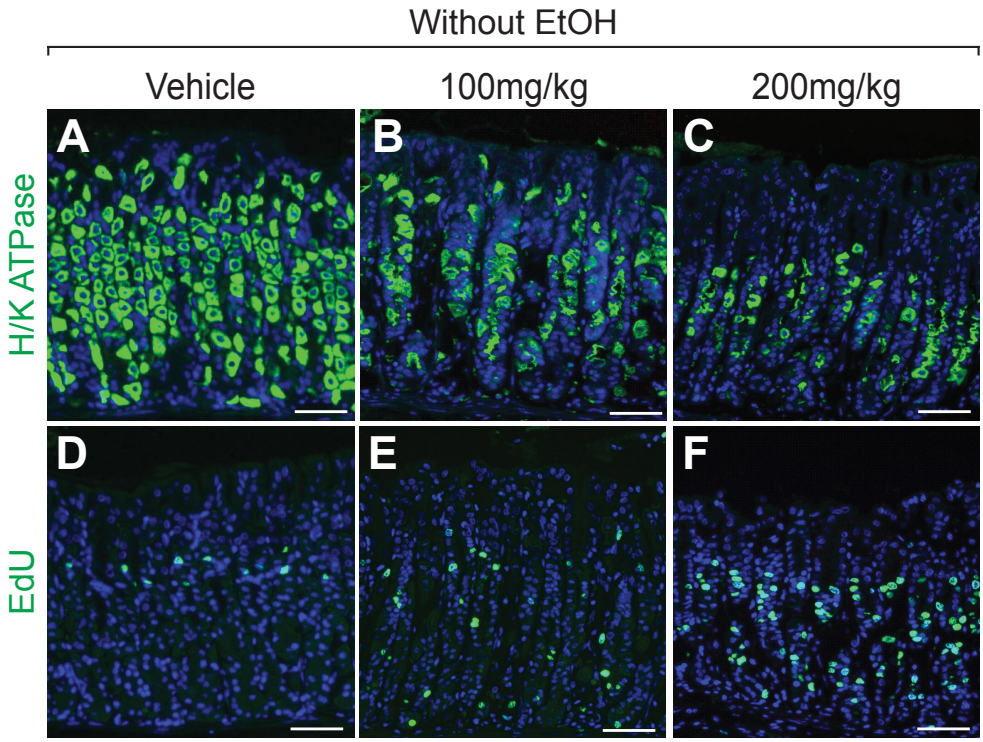
Supplementary Figure 1: Tamoxifen (TX) treatment induces parietal cell loss. Adult C57BL/6 mice were treated once with vehicle, 25mg/kg, 50mg/kg, 100mg/kg, or 200mg/kg TX injected IP and examined 1 day (A-J) or 3 days (K-T) later. Paraffin sections were H&E stained to examine tissue morphology (A-E and K-O), or immunostained for H/K-ATPase-α (green) to examine parietal cells, with DAPI (blue) nuclear stain (F-J and P-T). Arrowheads indicate delaminated cells. Scale=100μm.



Supplementary Figure 2: Gastric epithelial cell proliferation after TX treatment. Mice were injected IP with vehicle (A,F,K,O), 25mg/kg (B,G), 50mg/kg (C,H,L,P), 100mg/kg (D,I,M,Q), or 200mg/kg (E,J,N,R) TX, and examined 1 day (A-E and K-N) or 3 days (F-J and O-R) later. Paraffin sections from the gastric corpus region were stained for EdU (green) incorporation with DAPI (blue) nuclear stain (A-J). Paraffin sections from the gastric antral region were stained for EdU (green) incorporation with DAPI (blue) nuclear stain (K-R). Scale=100μm. (S) Morphometric counting of EdU^{+} cells in the antral region 1 and 3 days after TX. Quantitative data are presented as mean ± SEM (*n* = 3-5 mice/treatment; n.s.= not significant by 1-way ANOVA).



Supplementary Figure 3: Route of TX administration does not affect the gastric damage response. Mice were treated by subcutaneous injection (A-C) or oral gavage (D-F) with vehicle, or 200mg/kg TX. Paraffin sections were immunostained for H/K-ATPase-α (green) with DAPI (blue) nuclear stain to examine parietal cells at 1 day (B,E) and 3 days (C,F) post TX treatment. Scale=100μm.



Supplementary Figure 4: TX-induced gastric injury is not ethanol (EtOH)-dependent. (A-C) Mice were injected IP with vehicle (A,D), 100mg/kg (B,E), or 200mg/kg (C,F) TX without EtOH, and examined 3 days later. (A-C) H/K-ATPase-α (green) immunostaining with DAPI (blue) nuclear stain. (D-F) EdU (green) staining with DAPI (blue) nuclear stain. Scale=100μm.