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Ovariectomy Affects Acute Pancreatitis in Mice

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Abstract

Background: Serum estradiol levels in severe acute injury are correlated with in hospital mortality. In acute pancreatitis, serum estradiol levels are strong predictors of disease severity. Studies of whether changes in estradiol levels play a causative role in acute pancreatitis severity are limited. The ovariectomized mouse model has been used to study the effects of estradiol in health and disease.

Aims: We assessed whether the ovariectomized mouse model could be used to assess the effects of estradiol on pancreatitis severity.

Methods: C57BL/6 mice with their ovaries removed were used to simulate low circulating estradiol conditions. Ovariectomized mice were treated with six hourly injections of cerulein to induce mild acute pancreatitis and compared to ovariectomized mice pre-treated with subcutaneous estradiol injections.

Results: Findings suggest ovariectomized model is a problematic preparation to study pancreatitis. At baseline ovariectomy lead to prominent acinar cell ultrastructure changes as well as changes in other select morphologic and biomarkers of pancreatitis. In addition, ovariectomy changed select acute pancreatitis responses that were only partially rescued by estradiol pre-treatment.

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Author Contribution

MW performed data acquisition, data interpretation and statistical analysis. MW and FG performed study concept, design, writing, review, and paper revision.

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Declarations

Consent to publish

No human participants were included in this study, and consent to publish was not required.

Conflicts of interest/competing interests

The authors have no competing interests to report.

Ethics Approval

All animals and procedures within this study were in compliance by the Animal Welfare Act, the Public Health Service Policy, and the Guide for the Care and Use of Laboratory Animals. This research protocol was approved by the Veterans Health Administration Institutional Animal Care and Use Committee (FG009, West Haven, CT, USA).

Data Availability

All data generated or analyzed in this study are included in this published article.

Conclusions: These findings suggest that the ovariectomized mouse as a model of estradiol depletion should be used with caution in pancreatic studies. Future studies should explore whether derangements in other female hormones produced by the ovaries can lead to changes in pancreatic studies.

Keywords

Estrogen; pancreatitis; ovariectomy; zymophagy

Introduction

Circulating estradiol levels have been associated with severe acute injury and higher mortality rates in critical illness.[1, 2] In addition, changes in estradiol levels have been independently associated with mortality among critically ill patients.[2] The effects of estradiol, however, are multifactorial and literature findings are contradictory. For example, while estradiol has been associated with increased risk of death after severe traumatic brain injury, other studies suggest estradiol is neuroprotective.[3, 4] Estradiol has also recently been associated with anti-inflammatory and immunomodulatory properties in the context of immune dysregulation in COVID19 cytokine storm.[5]

Although the effects of estradiol in different disease contexts is variable, increased serum estradiol levels has been associated with worse outcomes in acute pancreatitis. Admission serum estradiol levels is a better predictor of mortality in patients with severe acute pancreatitis than typical severity scores, including the sequential organ failure assessment (SOFA) score, multiple organ dysfunction score, and acute physiology and chronic health care evaluation (APACHE II) score.[6, 7] Further, its analogs have also been associated with the development of hypertriglyceridemic acute pancreatitis in patients with elevated baseline triglyceride levels.[8] In the choline deficient ethionine (CDE)-diet model of acute pancreatitis, young female mice develop acute hemorrhagic pancreatic necrosis; male mice develop this phenotype only after estradiol administration.[9]

Surgical removal of ovaries in animals, ovariectomy (also known as oophorectomy in humans), has been used as a mouse model for various disease states where estradiol levels are low. For example, ovariectomy has been used in studies exploring menopause and aging, osteoarthritis and bone loss, and estrogen replacement therapy in ovariectomized (OVX) mice treated with estrogen.[10–14]

Evidence that ovariectomy affects pancreatitis is limited. To enhance the potential effects of estradiol and eliminate artifact that might be induced by the estrous cycle, we used mice in which the ovaries, the primary site of estradiol production, had been removed. In this model, we examined the effects of ovariectomy (OVX) and estradiol responses on pancreatitis. We found that OVX alone affects baseline pancreatic function and that select parameters of pancreatitis severity were reduced in OVX mice; some of the OVX-associated changes were partially rescued with estradiol.

Methods

Animals

All experiments used C57BL/6J female mice between 20–25 grams at 6–8 weeks old. C57BL/6J OVX mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA). Ovariectomy procedure was completed at Jackson Laboratory and OVX mice were acclimated to laboratory conditions after surgery for one to four weeks prior to pancreatitis induction. All experiments and animal procedures were approved by the Veterans Health Administration Institutional Animal Care and Use Committee (West Haven, CT, USA).

Preparation of Isolated Pancreatic Lobules

Mice were euthanized with CO₂ inhalation after which pancreatic tissue was isolated from the abdominal cavity and cut into fragments of about 5mm diameter. Pancreatic lobules were placed in 24-well Falcon tissue culture plates (BD Biosciences, Franklin Lakes, NJ, USA) in 0.5 mL incubation buffer (10mM Hepes pH 7.4, 95mM NaCl, 4.7 mM KCl, 0.6mM MgCl₂, 1mM NaH₂PO₄, 10mM glucose, 2mM glutamine, 0.1% bovine serum albumin, 1x minimum essential medium amino acids (Gibco, Carlsbad, CA, USA).

Treatment of Isolated Pancreatic Lobules

One hour prior to pancreatitis induction, pancreatic lobules were treated with 1000nM water-soluble estradiol (Sigma Aldrich, St. Louis, MO). Cellular pancreatitis injury was induced using 100nM hyperstimulation cerulein for 30 minutes. For lobule experiments with increasing estradiol concentrations, lobules were pretreated with no estradiol, 10nM estradiol, 100nM estradiol, or 1000nM estradiol and treated with either no cerulein, physiologic cerulein 10⁻¹⁰ M, or hyperstimulation cerulein 10⁻⁷ M. After treatment, 50µl of cell-free medium were removed for amylase secretion. For lobules, the remaining medium containing pancreatic lobules were homogenized manually with 10 strokes in a 2mL glass Teflon homogenizer. Homogenized lobules were then centrifuged at 500g for 10 minutes. Supernatant was used to assay zymogen activation and amylase secretion.

Histology of Isolated Pancreatic Lobules Histology and Immunostaining

Pancreatic tissue were placed into cassettes and fixed in normal buffered formalin for 48 hours before being delivered to Yale Pathology Tissue Services Laboratory in 70% ethanol for paraffin embedding, sectioning, and processing (H&E, TUNEL, Ly6-G). Slides were viewed with a 40x objective (Zeiss Axiophot, Oberkochen, Germany) and histologic markers were scored as described.[15] Images were obtained using digital camera (Spot Imaging, Sterling Heights, MI, USA).

In vivo Pancreatitis Induction and Estradiol Treatment

To induce pancreatitis, mice were treated with six hourly intraperitoneal injections of 40µg/kg cerulein in saline. One hour prior to pancreatitis induction, mice were treated with 100µg/kg subcutaneous water-soluble estradiol injection. Six hours after initial cerulein injection at time of collection, mice were anesthetized with 0.1mL/10kg of a combination of 100mg/kg ketamine and 10mg/kg xylazine and euthanized with cardiac puncture and

exsanguination (West Haven VA Pharmacy, CT, USA). Pancreatic tissue was isolated and plasma was collected by removing the supernatant of blood centrifuged at 2000g for fifteen minutes (BD Biosciences, Franklin Lakes, NJ).

Amylase Assay

Plasma samples were diluted 1:10 in deionized water and amylase levels were assessed using a commercial phaebedas kit read at 620nm in duplicate (Magle Life Sciences, Lund, Sweden).

Zymogen Activation Assay

Pancreatic tissue samples were thawed and manually homogenized with 2mL glass Teflon homogenizer in zymogen assay buffer (50mM Tris buffer, 150mM NaCl, 1mM CaCl₂, pH 8.1). 20µl of supernatant and zymogen assay buffer were incubated at room temperature in a 24-well Falcon tissue culture plate (BD Biosciences, Franklin Lakes, NJ) with 50µl zymogen enzyme substrate (40mM final; Chymotrypsin from Calbiochem Division, EMD chemicals, Gibbstown, NJ, Trypsin from Peptides International, Louisville, KY). Plate was read using a fluorometric plate reader Flx800 (Biotek Instruments, Winnski, VT) at excitation wavelength 380nm and emission wavelength 440nm. Lobules were normalized total amylase content and pancreatic homogenates were homogenized to total protein concentration obtained using Pierce 660nm protein assay kit (ThermoFisher Scientific, Waltham, MA).

Edema Measurements

Tissue edema was determined by immediately weighing pancreatic tissue after removal from mice and re-weighed after drying at 60°C for 48 hours. Percent wet weight was calculated using the formula: $\text{wet weight} - \text{dry weight} / \text{wet weight} \times 100$. Histological edema was measured by scoring on a zero to three scale as assessed by the amount of space between lobules and acinar cells on histology and averaging across 20 observations per slide at 400x.

Immunofluorescence

Pancreatic tissue were isolated from animals, manually cut into 10mm cubes, incubated in 2% paraformaldehyde in PBS for 30 minutes on ice, and place din 15% sucrose in PBS overnight at 4°C. Tissue pieces were then snap frozen on dry ice in OCT Compound in cryomolds (Sakura Finetek, Torrance, CA), and placed on slides after being cut into thin sections at -20°C. Tissue sections were permeabilized using TBS and 0.05% Saponin (TBS-S) and quenched with TBS-S with 50mM NH₄Cl and 5% goat serum. Tissues were incubated with primary antibody (Amylase: 1:200 (Sigma Aldrich), Trypsin 1:1000 (previously made in laboratory), Lamp-2: 1:100 (Sigma Aldrich) diluted in TBS-S overnight at 4°C and incubated with secondary antibody with Alexa Fluor 488 at 1:500 in TBS-S. Slides were mounted using Vectashield hard set mounting medium with DAPI (Vector Labs, Burlingame, CA), examined using fluorescence microscope (Zeiss Axiophot, Oberkochen, Germany), and imaged using images digital camera (Spot Imaging, Sterling Heights, MI).

Electron Microscopy Preparation

Fresh pancreatic tissues of 1mm x 1mm were placed in buffer provided by the Yale Center for Cellular and Molecular Imaging Electron Microscopy facility and processed for imaging at the facility.

Pancreatic Homogenate Preparation

Fresh pancreatic samples were suspended in homogenization buffer (0.3mM sucrose, 10mM Tris, pH 6.4, protease and phosphatase inhibitor (Complete Protease Inhibitor mini, EDTA-free cocktail, 1 tablet per 15 mL stock solution, Roche, Mannheim, Germany), 10mM benzamidine, 0.1mg/ml soybean trypsin inhibitor, and phosStop tablet (1 tablet per 10ml stock solution, Roche, Mannheim, Germany) and manually homogenized in 2mL glass Teflon homogenizer. Homogenates were centrifuged at 500g for 10 minutes. Supernatant was removed and stored at -80°C .

Western blot

Pancreatic homogenate samples were diluted to 20–50 μg and loaded onto electrophoresis gel (Biorad, Hercules, CA, USA) prior to being transferred to Immobilon-P membranes (Millipore, Billerica, MA, USA). Membranes were blocked with blotto (5% milk powder in TBS-Tween: 1x TBS, 0.05% Tween 20) at room temperature before probing with primary antibody in 5% bovine serum albumin in TBS-Tween (LC3B: 1:750 (Cell Signaling Technology, Danvers, MA, USA), p62: 1:750 (BD Biosciences, Franklin Lakes, NJ, USA) at 4°C overnight. Membranes were incubated with secondary antibody (Sigma-Aldrich, St. Louis, MO, USA) and labeled bands were detected using Supersignal West Pico Chemiluminescence ECL reagent (Pierce, Thermo Fisher Scientific, Waltham, MA, USA) and imaged with ChemiDoc Touch Imaging System (Biorad, Hercules, CA, USA). *Stain-Free* imaging technology was used to measure total lane protein and to normalize signals in each lane by using a proprietary trihalo compound that provides an in-gel fluorescence signal that is linear with respect to the level of each protein band (Biorad, Hercules, CA, USA). For normalization of loads, the protein content per gel band was integrated to give the total protein per lane.

Statistical Analysis

Data were analyzed using Mann-Whitney U test to compare between samples and represented as means using Prism Version 8 (Graphpad, San Diego, CA, USA). All statistically analyzed samples included at least 4 separate samples. Data were considered statistically significant if p-value was ≤ 0.05 .

Results

Ovariectomy may lead to apparent nonselective changes in ultrastructure of unclear significance at baseline

When examining female and OVX mouse pancreatic acinar cells under electron microscopy, OVX acinar cells demonstrated endoplasmic reticular (ER) dilation compared to female acinar cells (Figure 1A–B). This finding was present in OVX acinar cells examined either

one week or one month after ovariectomy. Several studies have associated pancreatic acinar cell injury and downstream ER stress responses.[16] ER dilation is phenotypically associated with ER stress in acinar cells. Our observation suggests that the process of ovariectomy itself and its long-term changes may be associated with baseline changes in pancreatic acinar cell function. The findings on electron microscopy of differences between female and OVX pancreatic acinar cells suggest that this intervention could affect baseline acinar cell biology.

OVX changes acute pancreatitis measurements compared to female mice

Compared to female mice under the same conditions, OVX mice treated with cerulein-induced acute pancreatitis demonstrated reduced trypsin activity (13.11 RFU/sec/mg protein vs. 6.05 RFU/sec/mg protein, $p = 0.029$, Figure 2A) and reduced chymotrypsin activity (3.07 RFU/sec/mg protein vs. 0.00 RFU/sec/mg protein, $p = 0.029$, Figure 2B). There was no statistically significant difference in serum amylase or pancreatic edema between female and OVX mice treated with cerulein (Figure 2C–D). Although there was no statistically significant difference in histology scores for edema, vacuoles, pyknotic nuclei, or necrosis, OVX mice trended to show increased TUNEL staining (6.48 cells/400x field vs. 12.95 cells/400x field, $p = 0.057$, Figure 3A) and a decrease in neutrophil infiltration (2.48 cells/400x field vs. 1.88 cells/400x field, $p = 0.057$, Figure 3B) at 6 hours. Taken together, these findings suggest that OVX mice differentially affect acute pancreatitis at 6 hours compared to intact female mice.

Estradiol administration in OVX mice does not fully reverse acute pancreatitis changes

To determine whether the changes in pancreatitis responses in OVX mice were due to estradiol, OVX mice were treated with a subcutaneous injection of 200 μ g/kg of estradiol one hour prior to acute pancreatitis induction. Compared to OVX mice, OVX mice treated with estradiol demonstrated a decreased in serum amylase (6.42 U/ml vs. 4.64 U/ml, $p = 0.029$, Figure 2D) but increased pancreatic trypsin (6.05 RFU/sec/mg protein vs. 15.60 RFU/sec/mg protein, $p = 0.029$, Figure 2A) and chymotrypsin (0.00 RFU/sec/mg protein vs. 3.13 RFU/sec/mg protein, $p = 0.029$, Figure 2B) activities. OVX mice treated with estradiol trended to have decreased tissue edema (75.73% vs. 70.21%, $p = 0.126$, Figure 4A & Figure 2C), histologic edema score (1.90 vs. 0.49, $p = 0.057$, Figure 4A & B), histologic apoptosis/necrosis score (1.60 vs. 0.26, $p = 0.057$, Figure 4A & E), and neutrophil infiltration (5.93 cells/400x field vs. 2.48 cells/400x field, $p = 0.057$, Figure 3B) compared to OVX mice without estradiol treatment. There was no significant difference in vacuole formation, pyknotic nuclei, or TUNEL staining (12.95 cells/400x field vs. 3.50 cells/400x field, $p = 0.114$, Figure 3A & 4C–D). These findings suggest that estradiol administration does not fully reverse many of the effects of OVX on acute pancreatitis in female mice. However, estradiol did reverse the effect of OVX to increase trypsin and chymotrypsin levels or apoptosis. Estradiol also tended to decrease in edema and neutrophil infiltration in OVX mice (not significant). Therefore, the administration of exogenous estradiol may also have effects independent of OVX on several aspects of pancreatitis.

Estradiol administration in OVX mice increased zymophagy compared to OVX mice

Acinar cells in OVX mice demonstrated a baseline increase in large, vacuoles filled with eosinophilic content; though infrequent, some reached the size of the nucleus and observed in both in vivo and in vitro preparations. In experiments with pancreatic lobules exposed to estradiol, the number of large, eosinophilic structures also increased as concentration of estradiol increased (Figure 5A–E & Figure 6A–B). Similar structures were not observed in ovariectomized or female controls. Immunofluorescence of intact pancreatic tissues demonstrated large discrete vacuolar structure containing both amylase and trypsin immunoreactivity which are consistent with zymophagic vacuoles (Figure 5B–D). These structures co-distributed with LAMP-2 antibody labeling, a marker for the autophagic-lysosomal pathway (Figure 5E). Figure 5E demonstrates both areas of co-distribution as well as separate LAMP-2 and amylase distribution. Electron microscopy also showed that OVX mice estradiol treatment increased the numbers of membrane bound vacuoles containing round-electron dense structures consistent with zymogen granules and zymophagy (Figure 5F). These findings suggest that there is an increase in zymophagy in OVX mice compared to controls and that this increases further in mice treated with estradiol treatment.

To further assess the overall activity of autophagic pathways, OVX mice pancreatic samples were homogenized to determine levels of the autophagic markers LC3–2 and p62. Compared to female mice treated with cerulein, OVX mice treated with and without estradiol showed increased LC3–2 and p62 levels (Figure 7A–D).

Discussion

Studies have demonstrated a correlation between increased serum estradiol levels and worse injury, including more severe acute pancreatitis.[1, 2, 6] We hypothesized that removal of the primary source of estradiol production by ovariectomy would reduce pancreatitis severity. We found that OVX itself had distinct but substantial effects on features of acute pancreatitis that were partially rescued with estradiol treatment.

Additionally, we found on electron microscopy that at baseline, OVX mice had apparent nonselective changes in ultrastructure of unclear significance compared to female control mice. Our finding is also consistent with other studies that suggest ovariectomy may lead to baseline pancreatic changes in pancreatic biology. Garcia et al identified ultrastructural changes on electron microscopy in rat pancreatic islet cells in rats with short-term ovariectomy, defined as 14 days, including dilation of the ER.[17] Although some of the baseline changes were reversed with estrogen administration, ER dilation persisted.[17] These findings suggest OVX can change diverse baseline pancreatic responses and may have limited utility for studying the effects of female hormones on this organ.

In our study, we demonstrate that compared to female mice, OVX differentially affected commonly used pancreatic parameters. OVX mice demonstrated a reduction in select pancreatitis parameters including trypsinogen and chymotrypsinogen activation. We also found that OVX mice trended to have a decrease in neutrophil infiltration and an increase in apoptosis. In studies exploring the effect of apoptosis / necrosis in the development of acute pancreatitis, evidence suggests that cell death by apoptosis, as opposed to necrosis, favors

a less severe disease response.[18] Whether the enhanced apoptosis with OVX we observed was protective in remains unclear.

The pancreatitis effects of OVX were partially rescued with estradiol treatment. Although OVX mice treated with estradiol had an increase in zymogen activation and a decrease in apoptosis, other changes, including a decrease in serum amylase and neutrophil infiltration, suggest that exogenous estradiol treatment may also lead to effects on pancreatitis independent of ovariectomy.

Finally, we found that the addition of estradiol in OVX mice led to an increase in zymophagy identified through histology and autophagy protein production. Zymophagy, a specialized form of autophagy in which there is selective engulfment of zymogen granules, has been identified within the cerulein model of pancreatitis.[19] An increase in zymophagy is associated with reduced severity of pancreatic injury in pancreatitis.[20] We found an increase in autophagy protein production and an increase in zymophagy organelles on histology and electron microscopy in OVX mice and with additional estradiol treatment. This finding could be due to stimulation of zymophagy or reduction of autophagic-lysosomal flux from this pathway. Our studies do not differentiate between these mechanisms, though increases in the general autophagic marker p62 would favor a reduction in autophagic-lysosomal flux. Grossman et al found that ovariectomy led to a depletion of zymogen granule in acinar cells that were restored upon estradiol treatment, which could be related to zymophagy and sequestration in light of our findings.[21] The effects of autophagy in other disease models suggests that estradiol's relationship with autophagy is quite complex.[22] Yang et al found that estradiol decreased apoptosis and increased autophagy biomarkers in osteoblasts, similar to our findings in acinar cells.[23] However, while many studies suggest that estradiol promotes autophagy, some studies have shown that estradiol can modulate autophagy, particularly when it is abnormally stimulated.[22] For example, in osteoblasts in OVX mice, an increase in oxidative stress leads to an increase in autophagy that is decreased with estradiol treatment.[24] OVX also induces uterine autophagy, which is decreased with estradiol treatment.[25] Our study therefore contributes to the complexity of the relationship between estradiol and autophagy, suggesting that in OVX mice, estradiol treatment might reduce autophagic flux leading to more severe pancreatitis. Future studies, however, are necessary to determine whether OVX and estradiol can change autophagic flux.

We demonstrate that OVX itself may induce underlying changes in the pancreas. Ovariectomy has been traditionally used to create an estradiol-depleted model in experimental designs.[10, 13] Our studies, however, suggest that the OVX model should be used with caution in pancreatitis studies. First, removal of ovaries not only reduces estradiol production, but also affects production of other hormones such as progesterone, luteinizing hormone, and follicular-stimulating hormone. Whether effects seen in OVX model are due to estradiol depletion or changes in levels of other hormones is unclear.[26] Additionally, in addition to ovaries, steroidogenic tissues including adrenals and brain and non-steroidogenic tissues including liver, kidney, adipose tissue, breast, and heart express steroidogenic enzymes capable of increasing circulatory levels of steroid hormones such as estradiol. Therefore, the removal of ovaries may not be sufficient as a truly estradiol

depleted model.[27] Second, OVX led to basal effects on pancreatic tissue of unclear clinical significance. These effects need to be better understood before the OVX model can be effectively used in experiments with pancreatic tissue. Finally, estradiol treatment only partially rescued responses seen in the OVX model, suggesting that OVX itself can lead to changes independent of estradiol, perhaps through other hormonal changes as described.

In summary, our study provides evidence of persistent estradiol dependent and independent pancreatic effects. Though we used only a single dosing estradiol dosing regimen and one pancreatitis model, we believe the findings document the challenges with use of this model. Thus, ovariectomy might result in pancreatic changes that make it a suboptimal model for the study of estradiol in pancreatitis. Use of other models such as females with coordinated estrous cycles or male mice may provide a better baseline for studies of estradiol effects on pancreatitis. Future studies also should further explore the complex relationship between estradiol's effects on injury and recovery in pancreatitis to ultimately identify not only potential predictive values in serum estradiol levels but also potential therapeutic options particularly in patients with high levels of serum estradiol.

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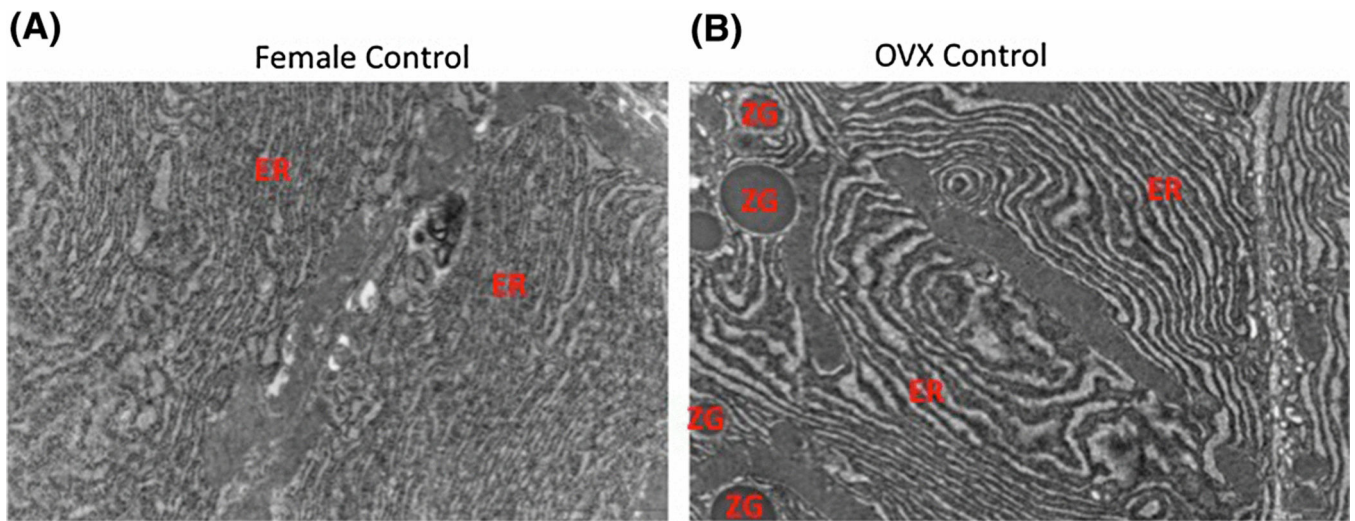


Figure 1. Ovariectomy leads to changes in acinar cell ultrastructure.

(A) Representative electron microscopy image of age-matched female control mouse without cerulein treatment with several zymogen granules, and endoplasmic reticulum. (B) Representative electron microscopy image of ovariectomized control mouse with nucleus and disorganized endoplasmic reticulum suggestive of endoplasmic reticular stress. ER = endoplasmic reticulum; ZG = zymogen granule; OVX = ovariectomized

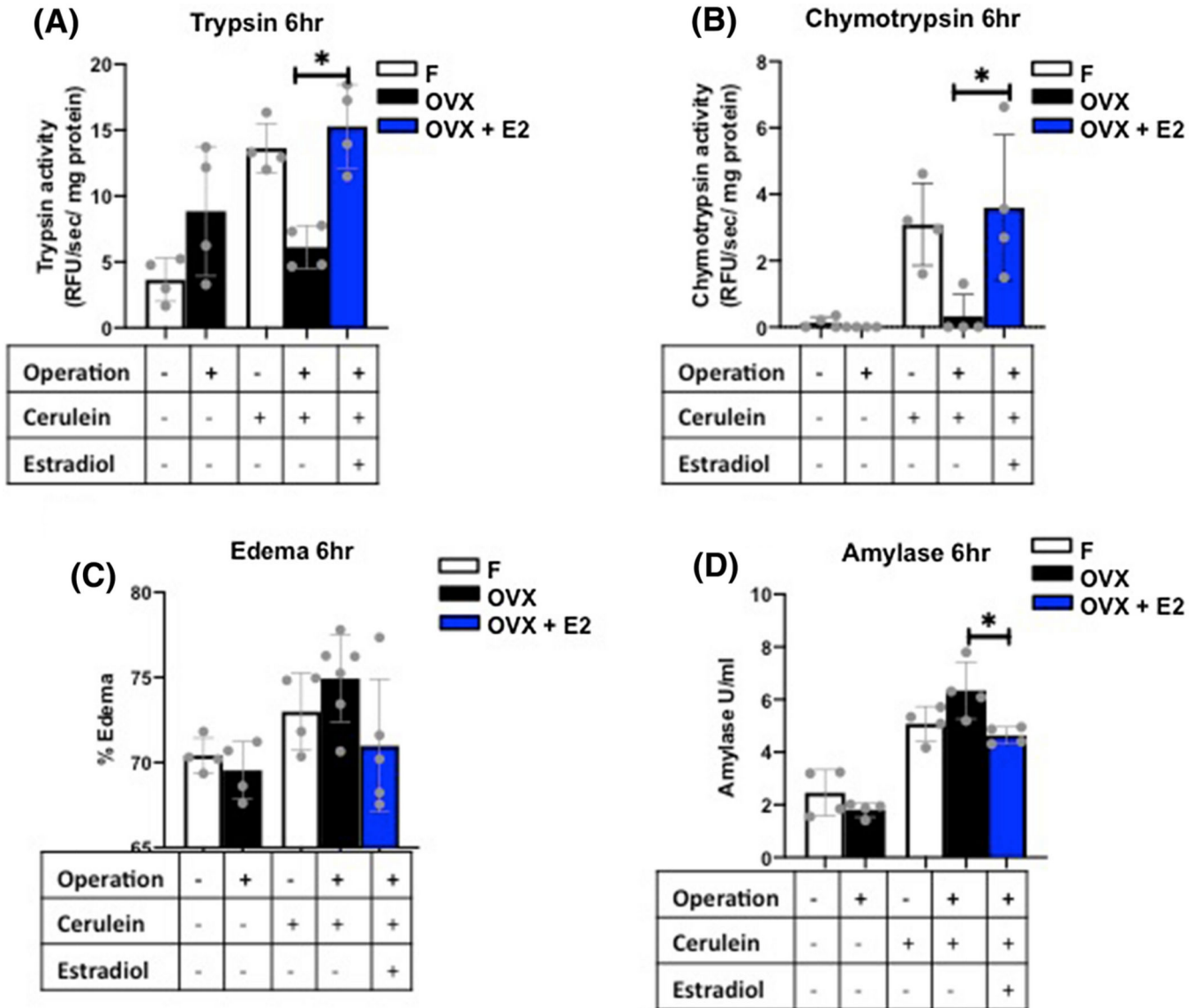


Figure 2. Ovariectomized mice treated with cerulein demonstrated select changes pancreatitis parameters compared to female mice. (A) pancreatic trypsin activity (B) pancreatic chymotrypsin activity (C) pancreatic edema by wet/dry weight (D) serum amylase. Degree of statistical significance is denoted by number of asterisks (One asterisk denotes $p < 0.05$, Two asterisks denotes $p < 0.01$, No asterisks between bars denotes non-significance); Operation (+) = received ovariectomy, Operation (-) = female without ovariectomy, F = female, OVX = ovariectomy; Ctrl = control, E2 = estradiol

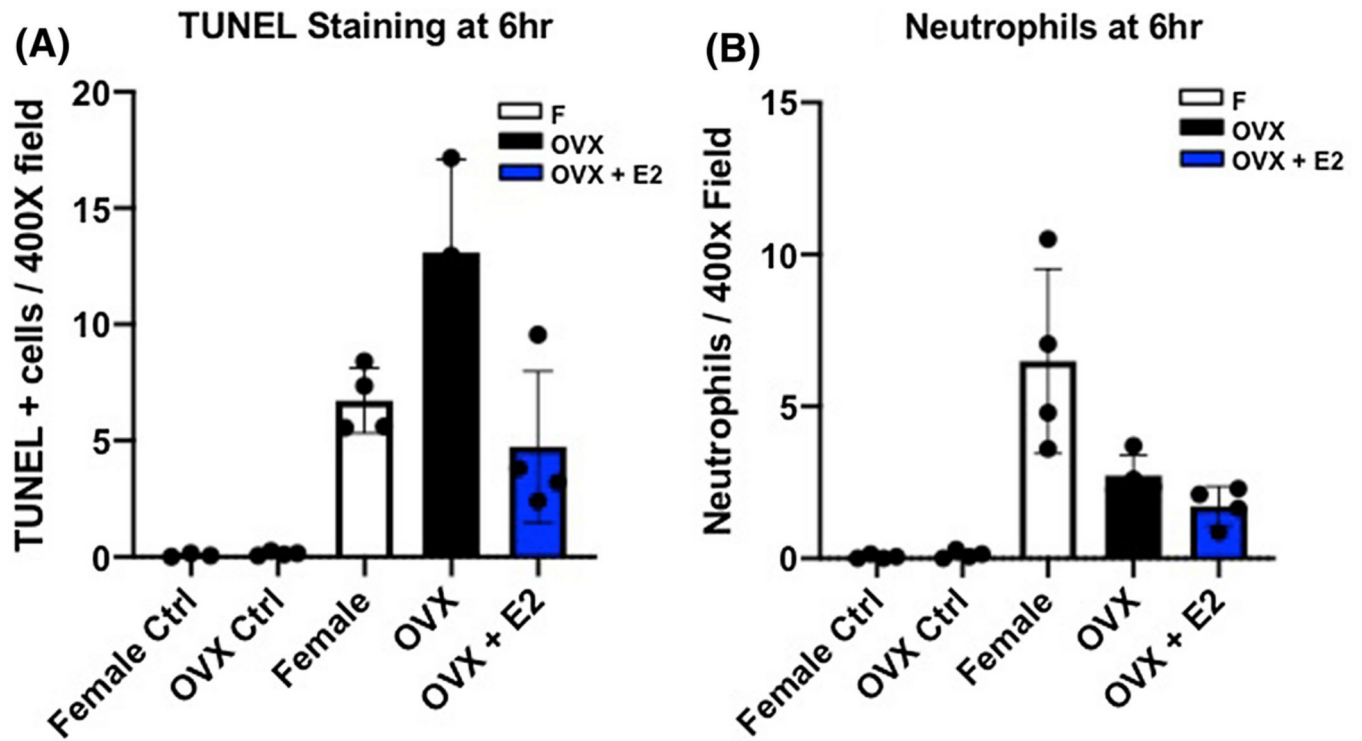


Figure 3. Ovariectomized mice treated with cerulein demonstrated increased apoptosis and decreased neutrophil infiltration compared to female mice.

(A) pancreatic TUNEL staining score for apoptosis and (B) pancreatic Ly6b staining score for neutrophil infiltration. Degree of statistical significance is denoted by number of asterisks (One asterisk denotes $p < 0.05$, Two asterisks denotes $p < 0.01$, No asterisks between bars denotes non-significance); F = female, OVX = ovariectomy; E2 = estradiol

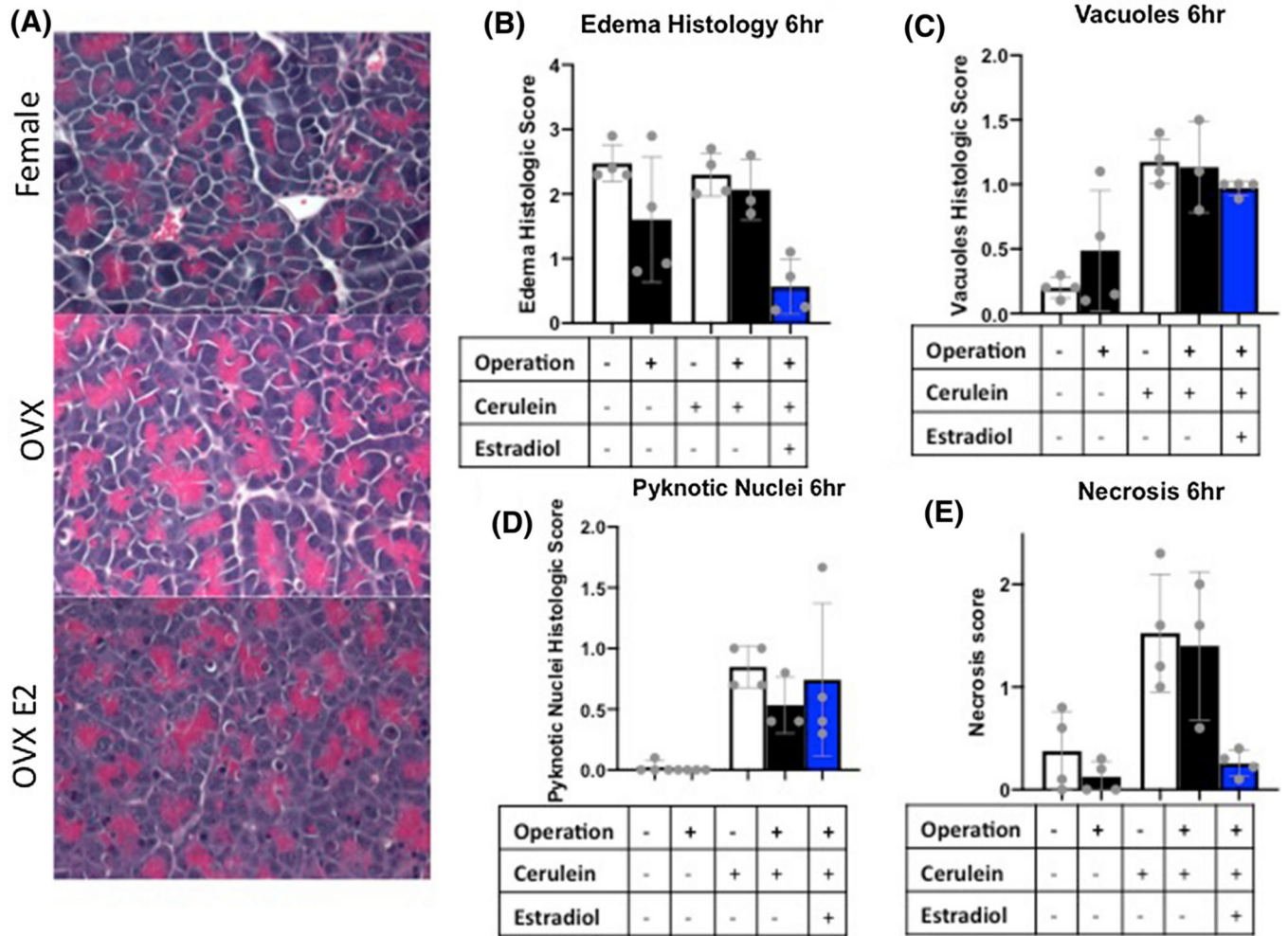


Figure 4. Ovariectomized mice treated with cerulein and estradiol demonstrated minimal histological changes compared to female mice.

(A) Representative histology at 40x of pancreatic acinar cells in female mice, ovariectomized (OVX) mice, and ovariectomized mice pre-treated with estradiol (OVX E2) (B) pancreatic edema by histology (C) vacuole formation (D) pyknotic nuclei and (E) necrosis (One asterisk denotes $p < 0.05$, Two asterisks denote $p < 0.01$, No asterisks between bars denotes non-significance); Operation (+) = received ovariectomy, Operation (-) = female without ovariectomy, F = female, OVX = ovariectomy; Ctrl = control, E2 = estradiol

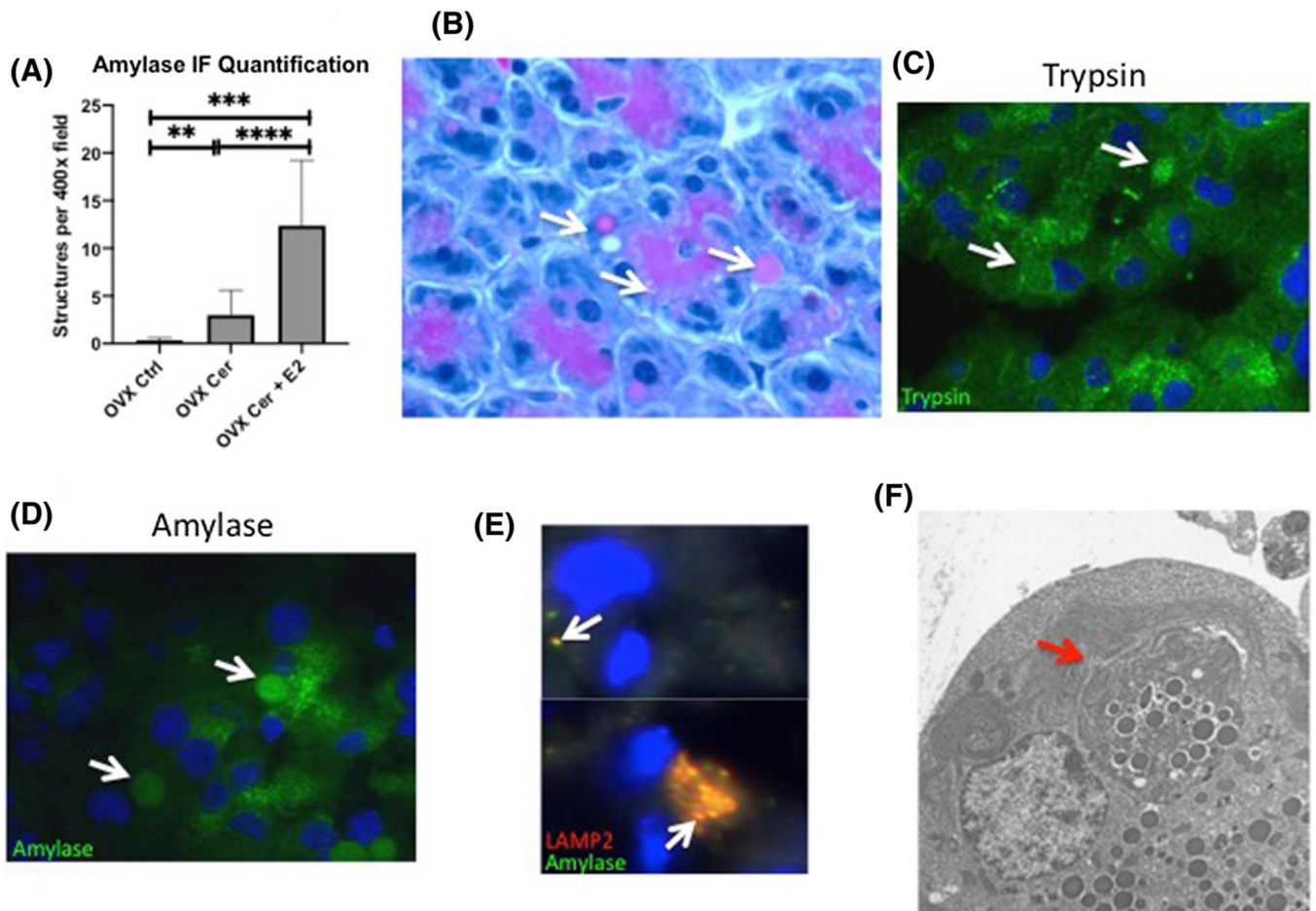


Figure 5. Ovariectomized mice treated with cerulein and estradiol demonstrated increased zymophagy compared to female mice.

(A) Quantification of zymophagic granules on histology at 400x in ovariectomized mice; Degree of statistical significance is denoted by number of asterisks (One asterisk denotes $p < 0.05$, Two asterisks denotes $p < 0.01$. Three asterisks denotes $p < 0.001$, Four asterisks denotes $p < 0.0001$, No asterisks between bars denotes not significant) (B) Representative histology of pancreatic acinar with white arrows pointing to eosinophilic structures consistent with zymophagy (C) Representative immunofluorescence figures of pancreatic acinar cells with white arrow pointing to circular staining at 400x with Green = Trypsin and Blue = DAPI (nucleus) (D) Representative immunohistochemistry figures of pancreatic acinar cells with white arrow pointing to circular staining at 400x with Green = Amylase and Blue = DAPI (nucleus) and (E) Representative immunofluorescence figures of pancreatic acinar cells with white arrows pointing to examples of co-distribution of LAMP-2 and Amylase at 40x with Green = Amylase, Blue = DAPI (nucleus), Red = LAMP-2, and Yellow = Amylase and Lamp-2 co-distribution. (F) Representative electron microscopy with red arrow pointing to zymophagic granule next to nucleus; OVX = ovariectomized, E2 = estradiol

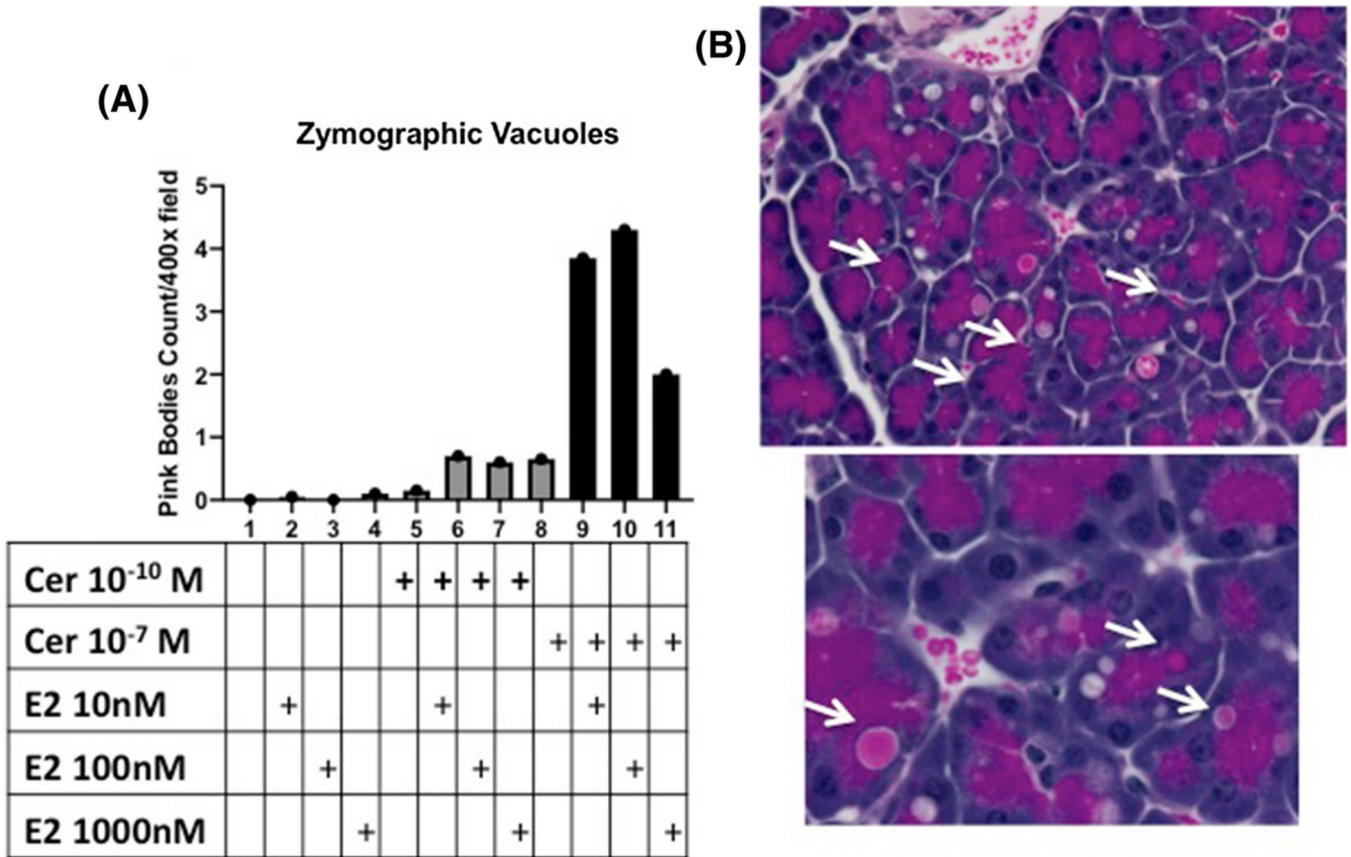


Figure 6. Estradiol treatment in vivo increases zymophagy in OVX mice.

(A) Number of eosinophilic / pink bodies per 400x field on histology scoring in lobules treated with increasing concentration of estradiol pre-treatment and cerulein treatment; Degree of statistical significance is denoted by number of asterisks (No asterisks between bars denotes non- significance) (B) Representative histological figures at 400x and 600x of pancreatic acinar cells in lobules pre-treated with 100nM estradiol and 10^{-7} M cerulein with white arrows pointing to eosinophilic structures; Cer = cerulein; E2 = estradiol; OVX = ovariectomized

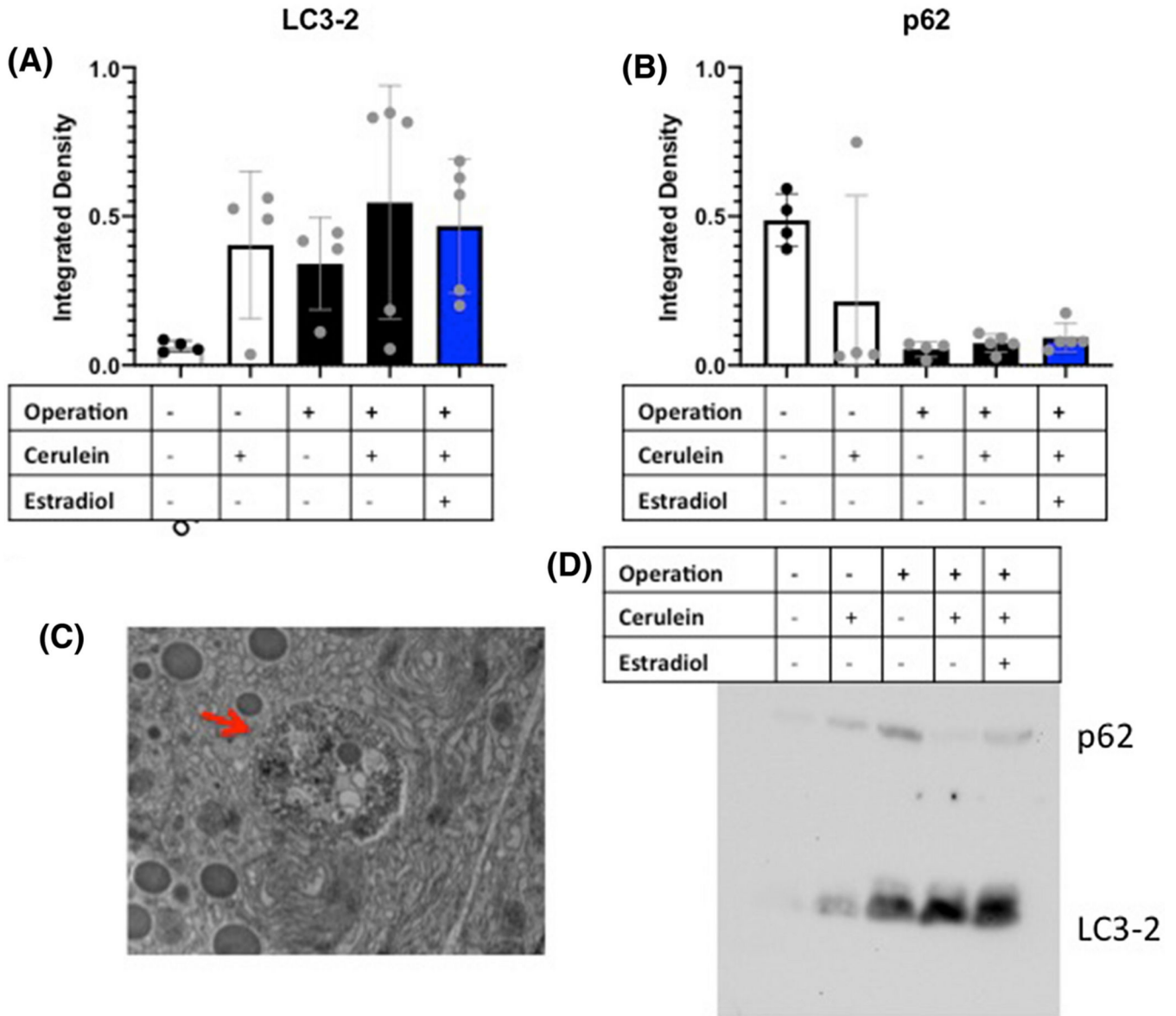


Figure 7. OVX mice treated with estradiol have increased autophagic protein production and reduced autophagic flux.

(A) Integrated density of LC3-2 bands detected on immunoblots of pancreatic homogenates

(B) Integrated density of p62 on bands detected on immunoblots of pancreatic homogenates

(C) Representative electron microscopy of zymophagy indicated by a red arrow, and (D)

Representative immunoblot of pancreatic homogenate samples; (One asterisk denotes p < 0.05, Two asterisks denotes p < 0.01, (No asterisks between bars denotes non-significance);

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