UNIVERSITY OF CALIFORNIA, SAN DIEGO

# The Enzymatic Activity of Secondary Organs during Splanchnic Ischemia-Reperfusion

A Thesis submitted in partial satisfaction of the
Requirements for the degree Master of Science

in

Bioengineering

by

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2008

The Thesis of Alexander Humphrey Hu is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2008

# DEDICATION

I would like to dedicate this thesis to my mother, Li-Wen Hu. Without her love, support, humor, and encouragement, this thesis would not exist.

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ABSTRACT OF THE THESIS

**The Enzymatic Activity of Secondary Organs during Splanchnic Ischemia-Reperfusion**

by

Alexander Humphrey Hu

Master of Science in Bioengineering

University of California, San Diego, 2008

Professor Geert W. Schmid-Schönbein, Chair

Shock is one of the major challenges faced by modern medicine. Unless death occurs abruptly, it likely results from multiple organ failure following a shock-induced inflammatory cascade. While the precise events leading to multiple organ failure are still unclear, the intestine has been implicated as one of the key organs in ischemia-reperfusion induced injury. Due to the intestine's unique digestive function, pancreatic enzymes have been implicated to play a major role in the propagation of the inflammatory response.

The objective of this study was to investigate enzymatic activity in selected non-ischemic secondary organs (liver, lung, brain, heart and kidney) following ischemia and reperfusion of the splanchnic circulation by occlusion of the superior mesentery and ciliac arteries. In order to determine the level of lipase and protease activity in these organs, *in situ* zymography was performed. For this technique, frozen sections of tissue were exposed to protease and lipase substrates. In the event of cleavage, the substrate became fluorescent and could be measured and analyzed quantitatively through digital microscopy and imaging techniques.

The results of these measurements show that following splanchnic ischemia-reperfusion, protease activity is only upregulated in the intestine and pancreas, but remains unchanged in the liver, lung, brain, heart and kidney. In contrast, lipase activity was found to be unchanged in all of the organs investigated in this study (brain, heart, intestine, kidney, liver, lung, and pancreas). These results indicate that following splanchnic ischemia-reperfusion, changes in the enzymatic activity of secondary organs may not be critical to their subsequent failure. Rather, exogenous mediators possibly originating from the splanchnic circulation may be instrumental to the eventual failure of these organs in this shock model.

# Chapter 1: Introduction

## Ch 1.1 Shock, Inflammation, and Multiple Organ Failure

Shock, a frequently diagnosed clinical condition, remains poorly understood[1]. There are an estimated 751,000 cases of severe sepsis and septic shock in the United States each year, and these cases are responsible for as many as 215,000 deaths[2]. Further, it is estimated that by the year 2020, there will be over 1,000,000 cases of severe sepsis and septic shock in the United States[2]. Unfortunately, the mortality rate of shock remains exceedingly high—one study reported the mortality rates of septic shock patients exceeding 70%[3].

Shock is currently one of the greatest obstacles to the overall effectiveness of modern medical care—despite advances in critical care management, patients who are now able to survive their initial physiologic insult are later succumbing to the effects of shock[1]. Today, shock and subsequent multiple organ failure are the primary causes of late stage mortality and morbidity in victims of trauma[4].

Shock may be broadly defined as a syndrome that results in inadequate tissue perfusion and cellular oxygenation, either at a systematic or regional level—in fact, all forms of shock have the commonality of inadequate perfusion, or ischemia[1]. Clinically, shock is diagnosed and classified by factors such as blood loss, blood loss percent, pulse rate, blood pressure, pulse pressure, capillary refill, respiratory rate, urine output, and mental status[1] (Table 1.1). Because ischemia is common to shock, readily identifiable changes to the brain, heart, and kidneys may be used to diagnose shock. A decline in cortical function may indicate diminished perfusion of the brain, cardiac dysfunction may

be an early indicator of systemic arterial hypoperfusion, and oliguria may indiciate significant renal hypoperfusion[1].

There are numerous ways to induce shock, including but not limited to hypovolemia, cardiogenic mechanisms, trauma, anesthesia, sepsis, tumors, blood flow obstructions, or ischemia of the intestinal circulation[5]. Hypovolemic shock, in which there is inadequate circulating volume, is the most common form of shock, and nearly all forms of shock include some component of hypovolemia, whether systemic or regional[1].

It is now recognized that shock is the initiator of a complex physiologic response, which may ultimately lead to systemic inflammation and multiple organ failure[1,6,7]. Organ injury following shock is rarely the result of exogenous factors, but is largely a consequence of the host's own endogenously produced inflammatory mediators[6]. It is this inflammatory response that is ultimately responsible for the cascade of events that culminates in multiple organ failure.

Ischemia can lead to an inflammatory response, both through direct damage to affected tissues, and creation and release of additional inflammatory mediators upon reperfusion, or the restoration of blood flow and oxygenation to the tissue[1,6,7]. Depending on the size and location of the area affected by ischemia-reperfusion, the inflammatory response can become systemic[1,6,7]. Systemic inflammatory responses can also be triggered by pancreatitis, ischemia, multiple trauma and tissue injury, and exogenous administration of inflammatory mediators such as tumor necrosis factors and other cytokines[7].

An excessive and uncontrolled inflammatory response triggers a widespread physiologic response, which results in the activation of neutrophils. In turn, these

neutrophils release reactive oxygen species and lysozomal enzymes, further leading to the release of more inflammatory mediators and eventual organ dysfunction[1,6,8]. It is thought that the failure of one organ system may establish an amplification process that hastens injury to another system, and frequently, multiple organ failure follows the systemic inflammatory response[7].

In multiple organ failure, multiple organ systems fail nearly simultaneously after severe physiologic insult, often resulting in the failure of organs initially uninvolved with the physiologic insult[6,7,9]. Since the ability to salvage patients once multiple organ failure has become established has not improved over the last two decades, it is one of the most common causes of death in the surgical intensive care unit, responsible for 50 – 80 % of all deaths in the unit[3,6,9,10].

One of the major factors limiting the ability to treat patients with multiple organ failure is an incomplete knowledge of the biology and pathophysiology of this syndrome[6,9]. Because organs that fail are not necessarily involved in the primary disease process, and there is a lag phase between initial inciting events and the development of distant organ failure, multiple organ failure is difficult both to diagnose and to treat[6].

## Ch 1.2 The Role of the Intestine in Shock

It has long been recognized that the splanchnic circulation (which provides blood to the stomach, small intestine, colon, pancreas, liver, and spleen) is affected early on by triggers of shock, and that the intestine plays a central role in shock[1,4,5,11-13]. In fact, shock is associated with particularly deleterious functional and structural changes to the gastrointestinal tract, and it is hypothesized that in the majority of shock cases, the

intestine is the primary source of inflammatory mediators leading to systemic inflammation[4,10,14,15]. Though shock may cause injury to the gut in a variety of ways[16], one theory states that as a result of trauma and blood loss, blood flow is redistributed to the vital organs such as the brain and heart at the expense of the gastrointestinal tract, resulting in intestinal ischemia[1,17].

Though the exact source of the damage caused to the intestine as a result of ischemia is difficult to pinpoint, the end result of ischemia is a weakening of the intestinal mucosa and elevated endothelial permeability[4,11,12,15,18,19]. It has been suggested that the intestinal barrier is particularly susceptible to ischemic damage due to the microcirculation of the villi, and that oxygen radicals may play a large role in the injury of the intestine during ischemia[19,20]. Furthermore, during ischemia, cytotoxic mediators begin to accumulate in the intestine, possibly further contributing to its damage[4]. Regardless, gut mucosal deficits are instrumental to the initiation and propagation of multiple organ failure[21].

When blood flow is restored to the intestine, a more pronounced damage, the so-called reperfusion injury, occurs[17]. This injury results in the release of soluble inflammatory factors from the intestine, which could include pancreatic enzymes, lipid derivatives, endotoxins, cytokines, reactive oxygen species, and pancreatic enzyme-derived mediators[12,14,22]. It is thought that these activating factors, which eventually induce systemic inflammation and multiple organ failure, are generated from a variety of sources.

Oxygen free radicals may be primed for production during ischemia; as a result of ischemia, hypoxanthine is accumulated and xanthine dehydrogenase is converted to

xanthine oxidase[19]. Upon reperfusion, xanthine oxidase converts hypoxanthine to xanthine while simultaneously reducing oxygen to superoxide, an oxygen radical which is converted to a number of free radicals which injure cell membranes, and attract and activate neutrophils[23]. The accumulation of activated neutrophils further inflicts injury upon the intestine by releasing additional oxygen free radicals, cytotoxic granules, and proteases, amplifying the inflammatory response[18,19,24-26].

While the above-mentioned injury mechanism occurs in any tissue experiencing ischemia-reperfusion, its development in the intestine is more dangerous and more likely to initiate a systemic inflammatory response. As a result of the weakened intestinal barrier, content from the lumen of the intestine is able to enter the intestinal wall, and upon reperfusion, something in the luminal content appears to result in greatly amplified systemic inflammation and damage. The three major candidates in the lumen which may infiltrate the intestinal wall are digestive enzymes, digested food, and bacteria and their products[4].

Bacteria and their products have traditionally been thought to be the main source of inflammatory mediators in shock[1]. Bacterial translocation is based on the idea that intestinal bacteria and/or bacterial products like endotoxin pass through the intestinal barrier and into the circulation during ischemia, causing infection, inflammation, and sepsis[5]. Despite this, many studies have been unable to determine a decisive role for bacterial translocation as the main mechanism for cell activation or multiple organ failure[24,27-29]. In the absence of endotoxin, it is possible to induce cell activation and death, and in fact, it has been shown that measurements of plasma concentrations of endotoxin are not predictive of the development of the inflammatory response after

trauma[24,27]. Hence, bacterial sepsis is probably not the essential cause of multiple organ failure[28].

Intestinal food has been explored as a source of cytotoxic mediators[4]. It was found that homogenized food became highly cytotoxic only following digestion with luminal fluid containing pancreatic enzymes, suggesting that the root of inflammatory and activating factors may in fact be enzymatic in nature[4].

## Ch 1.3 The Role of Enzymatic Activity in Shock

The theory that enzymatic activity may play a critical role in the inflammatory response to shock started when the pancreas was discovered as a major source of inflammatory mediators[15,30,31]. Homogenates of the ischemic pancreas were found to induce inflammation and cause result of injury to the mucosal barrier in ischemia, infiltration of active proteases can cytotoxicity when applied to fresh neutrophils[31]. Furthermore, these homogenates contained activators that stimulated inflammatory cell functions in the microvasculature and caused cell death in the mesentery parenchyma[31].

It was hypothesized that pancreatic enzymatic activity could be a source of the generation of inflammatory mediators, and that pancreatic enzymes, synthesized in an inactive form in the pancreas, were being inappropriately activated in shock[31]. It was also hypothesized that enzymes synthesized in the pancreas and secreted into the lumen of the intestine were infiltrating the intestinal wall following ischemia-reperfusion injury[32]. Under normal physiologic conditions, the intestinal mucosal barrier protects the intestinal wall from auto-digestion, but as a result of ischemia, the intestinal mucosal barrier's protective function is compromised. These enzymes, once in the wall, could

initiate auto-digestion of the intestinal wall and generate a variety of inflammatory mediators[4].

Indeed, while it has been shown that neither homogenates of nonischemic intestines nor pure pancreatic enzymes were cytotoxic[4,15], homogenates of nonischemic intestines digested with pancreatic enzymes became cytotoxic and induced blebs in neutrophils [4,12,15]. Of the four general classes of pancreatic enzymes (proteases, amylases, lipases, nucleases), significant cytotoxicity only resulted from digestion with serine proteases (trypsin, chymotrypsin, elastase) and lipases, but not nucleases or amylases[8,24,30,33].

Further evidence for the role of enzymatic activity as a critical source of inflammatory mediators was provided through experiments inhibiting enzymatic activity in the lumen of the intestine[34-36]. Inhibitors of pancreatic enzymes injected into the intestinal lumen served to attenuate the inflammatory response following ischemia-reperfusion injury[15,33-36]. Certain inhibitors were able to reduce gut and lung injury, as well as the neutrophil activating ability of intestinal lymph[33]. Protease inhibition was found to reduce intestinal injury, decrease the level of cell activation in the microcirculation, and help restore blood pressure after splanchnic arterial occlusion induced shock[35]. Application of an inhibitor of serine proteases, lipases, and phospholipases served to completely inhibit the activation of circulating leukocytes during ischemia and reperfusion, and prevented the appearance of activators in the portal venous and systemic arterial plasma[15]. Early inflammation and initial symptoms of multiple organ injury in shock were attenuated as well[15].

It was then hypothesized that specific tissues or organs other than the intestine or pancreas might also serve as sources of activators in the inflammatory response[24,31]. It was found that homogenates of these organs were not cytotoxic or neutrophil stimulating on their own, but upon incubation with pancreatic proteases and/or lipases, many of them activated cells and/or became cytotoxic[24]. Hence, many organs were found to have the potential to induce inflammation, containing bioactive factors or precursors of bioactive factors that could activate cells, but only upon digestion with pancreatic enzymes were these factors released.

These important pieces of evidence all support the hypothesis that enzymatic activity plays a critical role in the systemic inflammatory response.

## Ch 1.4 Inflammatory Mediators and Cell Death

### Ch 1.4.1 The Nature of Inflammatory Mediators Involved in Shock

In order to better understand the progression of the inflammatory cascade, it is important to determine the nature of the inflammatory mediators which lead to systemic inflammation and multiple organ failure.

At the site of organ injury, it has been suggested that protease digestion of the extracellular matrix may yield hidden bioactive epitopes with inflammatory activity[12,37]. Biologically active cryptic sites within matrix molecules, or matricryptic sites, may be exposed after injury, attracting neutrophils[37]. In healthy tissue, this serves as a repair mechanism, but in shock, this may cause the further generation of inflammatory mediators and cell death.

As for soluble inflammatory mediators, pancreatic homogenates were separated into lipid and protein fractions, and it was found that lipids in the hydrophobic fractions induced both neutrophil activation and instant lethality when injected intravenously[38].

When small intestine homogenates of healthy rats were separated into lipid fractions and aqueous soluble and non-soluble (sedimented) protein fractions, only the lipid fraction was found to be cytotoxic[39]. Recombining the lipid fraction with protein fractions prevented cell death, but digestion of the protein fractions with proteases eliminated that particular protection[39]. Addition of albumin, a lipid binding protein, was also able to prevent cell death[39]. Further, inhibiting lipase activity helped prevent cytotoxicity in protease digested intestinal homogenates[39]. These findings suggest that cytotoxic free (non-esterified) fatty acids generated by lipases and normally bound to fatty-acid binding proteins such as albumin are released as a result of protease activity.

**Ch 1.4.2 Cell Death**

The observed *in vitro* cell death caused by these cytotoxic lipid mediators mimics that observed in ischemia-reperfusion injury. Cell death is considered one of the most important mechanisms for the pathogenesis of inflammation, and it was observed that following ischemia-reperfusion injury, morphological features of cell death in the heart and kidney were consistent with oncosis[21]. Oncosis is a type of programmed cell death characterized by swelling, membrane dysfunction, and cell detachment, distinctly different from apoptosis and necrosis[21]. Though the cell death caused by application of cytotoxic lipid fractions was not explicitly characterized as oncosis, observations of the dead cells are consistent with oncosis—membrane disruption and swelling of the cells[39]. Importantly, cells dying through oncosis are at a considerable risk for membrane rupture,

with the release of proinflammatory mediators[21]. This finding suggests that cell death may also play a significant role in tissue damage during systemic inflammation. As lipases and proteases are responsible for the creation of free fatty acids and their release from binding proteins, respectively, it becomes important to determine if they might be introducing cytotoxic lipids in other organs besides the intestine or pancreas.

## Ch 1.5 Research Goals

### Ch 1.5.1 Objectives and Experimental Design

It is clear that enzymatic activity plays an important role in the progression of the inflammatory cascade and multiple organ failure following shock. In the intestine and pancreas, aberrant enzymatic activity is known to follow ischemia-reperfusion injury, which may result in cell death. Additionally, previously non-cytotoxic organ homogenates, once digested with pancreatic enzymes, become cytotoxic. Hence, it is reasonable to hypothesize that the enzymatic activity of some secondary organs may be changed as a result of ischemia-reperfusion injury. If enzymatic activity were altered in secondary organs, cytotoxic mediators could be directly generated at the sites of these organs, leading to cell death, local inflammation, and the progression of multiple organ failure.

In this study, our objective is to determine if the enzymatic activity of secondary organs is affected by splanchnic ischemia-reperfusion injury either through endogenous enzymatic activity or enzymes traveling from the intestine or pancreas. The enzymatic activities of interest are serine proteases and lipases, both of which are known to generate cytotoxic and inflammatory mediators *in vitro*. Using *in situ* zymography, it will be

possible to detect changes in the enzymatic activity of tissue sections through the use of fluorescent enzymatic substrates. While it will be impossible to determine whether pancreatic enzymes escape through a permeable intestinal barrier and reach a variety of organs during splanchnic ischemia-reperfusion or inflammatory mediators from the intestine stimulate the release of enzymes from these organs, the information gained in this study will allow us to determine if the enzymes themselves, as opposed to their products, contribute to remote site injury in multiple organ failure.

**Ch 1.5.2 Hypothesis**

In this study, we hypothesize that there may be a change in the enzymatic activity of some secondary organs following splanchnic ischemia-reperfusion injury. An increase in protease or lipase activity in non-ischemic secondary organs could result in the generation of cytotoxic and inflammatory mediators directly at the site of these organs, resulting in leukocyte activation, cell death, and necrosis directly at these tissues.

**Ch 1.5.3 Specific Aim**

Utilize *in situ* zymography to quantitatively determine serine protease and lipase enzymatic activity in the brain, heart, intestine, kidney, liver, lung, and pancreas following splanchnic ischemia-reperfusion.

**Table 1.1:** Classification of shock (based on 70 kg patient). Adopted from Cheatham, et al. [1]

| | CLASS I | CLASS II | CLASS III | CLASS IV |
|---|---|---|---|---|
| Blood Loss (mL) | up to 750 | 750-1500 | 1500-2000 | 2000 or more |
| Blood Loss (%BV) | up to 15% | 15-30% | 30-40% | 40% or more |
| Pulse Rate | <100 | >100 | >120 | 140 or higher |
| Blood Pressure | Normal | Normal | Decreased | Decreased |
| Pulse Pressure | Normal/Increased | Decreased | Decreased | Decreased |
| Capillary Refill | Normal | Decreased | Decreased | Decreased |
| Respiratory Rate | 14-20 | 20-30 | 30-40 | >35 |
| Urine Output (mL/hr) | 30 or more | 20-30 | 5-15 | Negligible |
| CNS-Mental Status | Slightly anxious | Anxious | Anxious - confused | Confused -lethargic |
| Fluid Replacement | Crystalloid | Crystalloid | Crystalloid + blood | Crystalloid + blood |

# Chapter 2: Experimental Study

## Ch 2.1 Materials and Methods

### Ch 2.1.1 Surgical Operations and Tissue Collection

Male Wistar rats weighing between 240 and 300 grams were tranquilized with Xylazine (20 mg/ml, 200 μL / kg bodyweight i.m.) (MWI, Nampa, ID). After 20 minutes, general anesthesia was administered (Nembutal, 50 mg/ml, 1 ml / kg bodyweight, i.m.) (Pentobarbital Sodium Injection, Ovation Pharmaceuticals, Inc., Deerfield, IL). After 20 minutes, reflex level was tested with a toe pinch. Animals which were responsive were given an additional waiting period of 5 – 10 minutes and tested again. All of the rats utilized in this study were fully anesthetized and unresponsive to the toe pinch after this additional waiting period.

Following anesthesia, rats were secured to a cutting board resting atop a heating pad set at 37$^{o}$C. The fur was shaved and the left femoral artery and left femoral vein were cannulated. To the femoral vein catheter, a syringe with Nembutal was attached in order to provide anesthetic as necessary during the operations. Rats were tested periodically for consciousness with toe pinches and signs of awakening resulted in small (5 – 10 μL) doses of Nembutal delivered intravenously through the femoral vein. Arterial blood pressure of the rats was monitored via a pressure transducer attached to the femoral artery catheter. This served to monitor the delivery of intravenous Nembutal, the occlusion of the mesenteric and ciliac arteries in SAO shock, successful reperfusion, and any other irregularities that may occur.

Following the cannulation procedure, a midline incision was made on the rats, being careful not to injure any internal organs. In order to expose the peritoneum, the intestine was gently moved to a saline-dampened gauze pad lying immediately adjacent to the rat, and then covered with another saline-dampened gauze and plastic wrap to keep in heat and moisture. This process was done with care in order to avoid disturbing intestinal contents and/or inducing injury to the tissue.

In the control group, animals were then covered with plastic wrap and monitored for 90 minutes. After this time period, animals were euthanized through an intravenous injection of 200 μL Fatal Plus (390 mg/ml) (Pentobarbital Sodium, Vortech Pharmaceuticals, Ltd., Dearborn, MI) and the brain, kidney, heart, intestine, liver, pancreas, and lung were promptly harvested in whole and placed in saline for freezing.

In the ischemic group, following the exposure of the peritoneum, the superior mesenteric artery and the ciliac artery were isolated with umbilical tape and clamped. Successful clamping was confirmed by the observation of a large rise in blood pressure over the next 15 – 20 seconds. The animals were then covered with plastic wrap and monitored for 90 minutes. After this time period, animals were euthanized through an intravenous injection of 200 μL Fatal Plus and the organs were harvested as in the control group.

In the ischemia-reperfusion group, the superior mesenteric artery and the ciliac artery were isolated and clamped and monitored as in the ischemic group. Animals were covered with plastic wrap and monitored for 90 minutes. Following the ischemic period, the superior mesenteric and the ciliac arteries were unclamped in order to allow reperfusion of the digestive tract. Successful reperfusion was confirmed by the

observation of a large drop in blood pressure over the next 15 – 20 seconds. The animals were then re-covered with plastic wrap and monitored for an additional 30 minutes. After this time period, animals were euthanized by intravenous injection of 200 μL Fatal Plus, and organs were harvested as in the previous groups.

**Ch 2.1.2 Tissue Embedding and Sectioning**

*Tissue Preparation and Embedding*

After the organs were removed from the animals, they were cut and embedded in Tissue-Tek O.C.T. (Optimal Cutting Temperature) Compound (Sakura Finetek, Torrance, CA). A small portion of each organ was placed in O.C.T. Compound at room temperature and quickly frozen in 2-Methylbutane chilled in liquid nitrogen, then stored at -80 $^{o}$C to preserve enzymatic activity before sectioning. The preparation of each individual organ is as follows:

1. Brain: a small portion in the anterior portion, just posterior to the olfactory bulb, was cut with a scalpel and embedded (Figure 2.1A).
2. Heart: the distal portion of the ventricles was removed with a scalpel and embedded (Figure 2.1B).
3. Intestine: a 5 cm portion was removed from a segment 2 – 3” away from the cecum (Figure 2.1C). Intestinal contents were flushed with O.C.T. Compound and 1 cm of this flushed portion was cut and embedded longitudinally.
4. Kidney: a small portion in the radial direction, avoiding the ureter and major blood vessels, but including the cortex and medulla, was cut with a scalpel and embedded (Figure 2.1D).

5. Liver: a lobe was removed and a small section near the edge was removed with a scalpel and embedded (Figure 2.1E).

6. Lung: a small portion from the distal section of the right lobe was removed with a scalpel and embedded (Figure 2.1F).

7. Pancreas: a small portion was simply removed and embedded (Figure 2.1G). In this study, the collection of the pancreas for one ischemic-reperfusion group animal was unsuccessful and is excluded from the results.

*Sectioning*

Each tissue sample was sectioned using a Leica CM 3500 cryostat onto Fisherbrand Superfrost Plus Microscope Slides (Fisher Scientific, Pittsburgh, PA). Section thickness was fixed at 8 μm for all organs. Two consecutive sections were made on each slide, so that one could serve as a control to monitor auto-fluorescence of the tissues. Slides were then stored at -80 $^{o}$C until used for *in situ* zymography.

**Ch 2.1.3 *In situ* Zymography**

*Lipase Assay*

Before performing the *in situ* zymography, slides containing the tissue sections were placed in an oven at 37 $^{o}$C for 5 minutes to thaw and remove any visible residual moisture. After drying, 10 μL of an O-pivaloyloxymethyl umbellifoerone (C-POM) (Invitrogen, Eugene, OR) mixture (2 μL of 2 mM C-POM per 62 μL PBS) was applied to one section on the slide. In order to measure the auto-fluorescence in the tissues, 10 μL of PBS was applied to the other section. The sections were then coverslipped, and a coat of nail polish was painted around the border to prevent evaporation or movement of the coverslip and underlying substrate.

After a 10 minute waiting period at room temperature in dark conditions, slides were viewed under a fluorescent microscope. In each section to which substrate had been applied, four to five different bright field light images and their corresponding fluorescent images were recorded, in order to generate an "average" measurement of fluorescence for the section. Immediately following this, two to three different light images and their corresponding fluorescent images were taken of the control section, in order to generate an estimate of the tissue auto-fluorescence levels. Fluorescence for the C-POM was achieved through the use of a filtered mercury light source.

*Protease Assay*

Before performing the *in situ* zymography, slides containing the tissue sections were again placed in an oven at 37 $^{o}$C for 5 minutes to thaw and remove any visible residual moisture. After drying, 10 μL of protease substrate from the EnzChek Protease Assay Kit – red fluorescence (Invitrogen, Eugene, OR) was applied to one section on the slide. To the other section, the digestion buffer from the kit was applied. Coverslips were applied to the sections, and a coat of nail polish was painted around the border to prevent movement of the coverslip and underlying substrate, as well as evaporation of the substrate.

The incubation time of this assay was 60 minutes. Because of the longer duration of the incubation period, it was important to prevent evaporation of the substrate while maintaining a temperature of 37 $^{o}$C. Therefore, in addition to sealing with nail polish, slides were placed on inverted Petri dishes resting in a water bath at 37 $^{o}$C for the incubation period.

After incubation, slides were again viewed under a microscope. Similar to the lipase *in situ* zymography, four to five bright field images and their corresponding fluorescent images were recorded of each section to which substrate had been applied to generate an estimate of the "spatial average" fluorescence for each section. Immediately following this, two to three different light images and their corresponding fluorescent images were recorded of the control section, in order to generate an estimate of the tissue auto-fluorescence levels. Fluorescence for the protease substrate was achieved through the use of a filtered mercury light source.

**Ch 2.1.4 Data Analysis**

*Image Analysis*

The images of the sections were digitized and processed with Image J (NIH, http://rsbweb.nih.gov/ij/). Images were first split into RGB layers for analysis. For the lipase assay, the blue layer represented the fluorescent emission of the cleaved substrate and the other layers were discarded; for the protease assay, the red layer represented the fluorescent emission of the cleaved substrate and the other layers were discarded. The area over the tissue was carefully selected using the polygonal selection tool and the average grayscale value of the selection was measured with the program (scale of 0 to 255; 0 being pure black and 255 being pure white) (Figure 2.2). The thin border profile of the tissue was avoided due to excessive brightness resulting from border effects, presumably due to substrate mobility in the areas beyond the tissue (Figure 2.3). Measurements of the control sections were used to determine the auto-fluorescence (background fluorescence) of the tissue. The measured auto-fluorescence was compared to the measured signal to determine if there was a signal present.

The grayscale value of the protease substrate digested with 1 μg/ml purified chymotrypsin for 60 minutes was approximately 45, and the grayscale value of C-POM digested with 1 mg/ml porcine pancreatic lipase for 10 minutes was approximately 35.

*Statistical Analysis*

For both the lipase and protease *in situ* zymographies, two-tailed t-tests on groups of unknown variance were performed between the control and ischemic group, the control and ischemic-reperfusion group, and the ischemic and ischemic-reperfusion group. Since the sample size studied in this experiment was relatively small, comparisons which were close to being significant ($0.05<p<0.10$) were further tested with the Mann-Whitney test.

Finally, paired t-tests comparing the signal to the background were performed in order to determine if there was significant fluorescence above background.

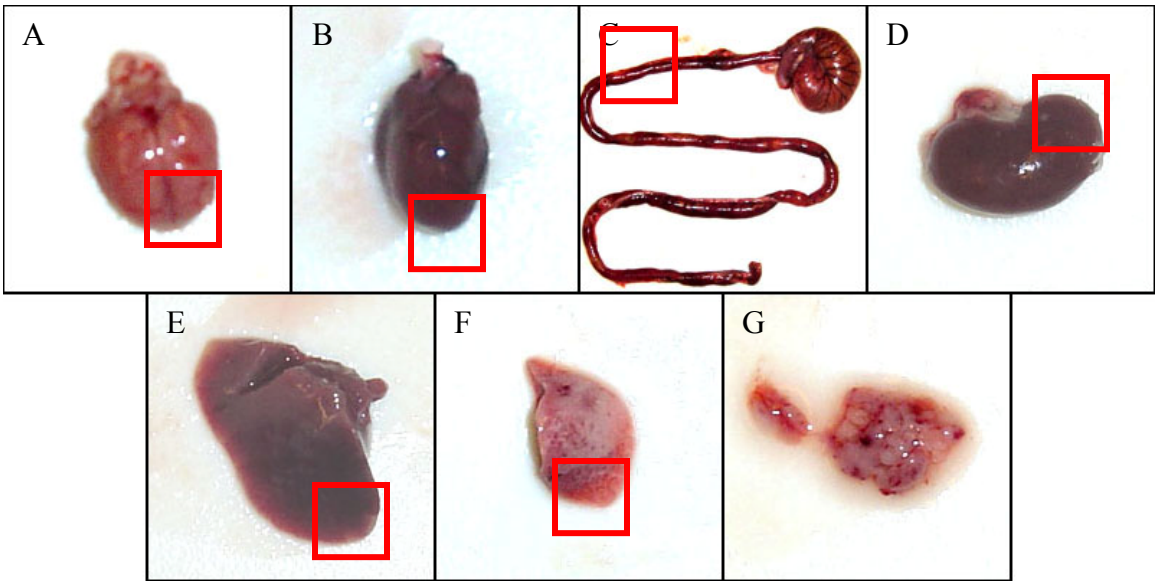


**Figure 2.1:** Photographs of freshly harvested rat organs. Red boxes indicate approximate area of tissue used for *in situ* zymography. For the pancreas, no specific area of the organ was collected. A. Brain, B. Heart, C. Intestine, D. Kidney, E. Liver, F. Lung, G. Pancreas

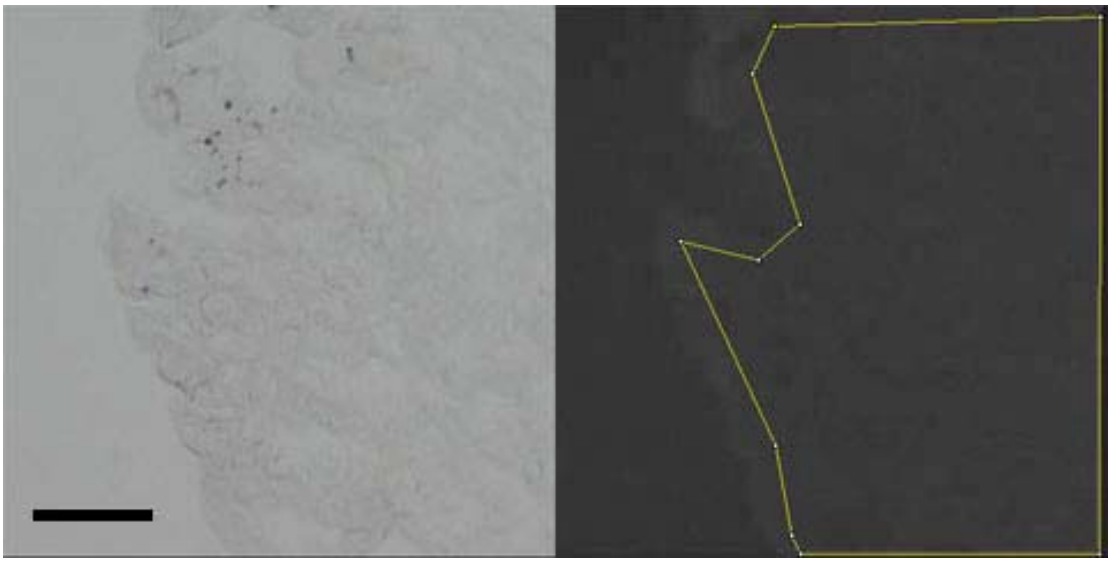

**Figure 2.2:** A representative fluorescent image (right) analyzed in this experiment. The bright field image (left) was used to aid the delineation of the border of the tissue. The tissue area was carefully selected (delineated by the yellow line in the right panel) and the average grayscale intensity of this area was measured with Image J. Scale bar indicates 50 μm.

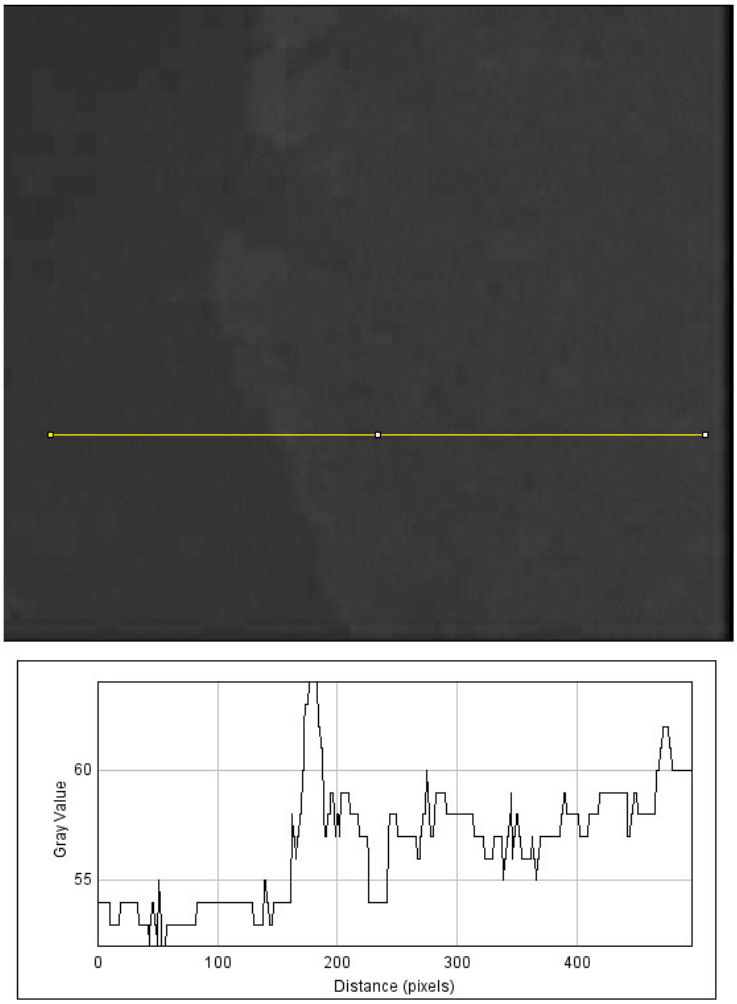


**Figure 2.3:** A fluorescent intensity profile (in units of grayscale) value along the line indicated in the blue layer of the fluorescent image. As can be seen, there is a spike in grayscale value along the border of the tissue as a result of border effects. This border area was avoided in the measurement of the average grayscale value.

# Ch 2.2 Results

### Ch 2.2.1 *In Situ* Zymography – Protease

*In situ* zymography was used to measure the presence of protease activity in the brain, heart, intestine, kidney, liver, lung, and pancreas (Figure 2.4).

In the intestine, there was protease activity in all three groups (control, ischemia, and ischemia-reperfusion). Additionally, the ischemia-reperfusion group had significantly elevated protease activity when compared to the control group (Figures 2.5, 2.6).

Similar to the intestine, the pancreas also had protease activity in all three groups, and the ischemia-reperfusion group had significantly elevated protease activity when compared to the control group. The Mann-Whitney test, but not the t-test, determined that the ischemic group had a significantly elevated protease activity compared to the control group (Figures 2.7, 2.8).

In the lung, all three groups were found to have protease activity. However, there was no significant difference between any of the three groups (Figures 2.9, 2.10).

In the heart, it was found that the control group had no protease activity, but the ischemia and ischemia-reperfusion groups had protease activity. Interestingly, when compared to each other, there were no significant differences between any of the three groups (Figures 2.11, 2.12).

The brain (Figures 2.13, 2.14), kidney (Figures 2.15, 2.16), and liver (Figures 2.17, 2.18) had no protease activity, and there was no significant difference between any of the three groups for these organs.

### Ch 2.2.2 *In Situ* Zymography – Lipase

*In situ* zymography (Figure 2.19) showed that there was lipase activity in all organs, but that there was no significant difference between the three groups in any of the organs—brain (Figures 2.20, 2.21), heart (Figures 2.22, 2.23), intestine (Figures 2.24, 2.25), kidney (Figures 2.26, 2.27), liver (Figures 2.28, 2.29), lung (Figures 2.30, 2.31), or pancreas (Figures 2.32, 2.33).

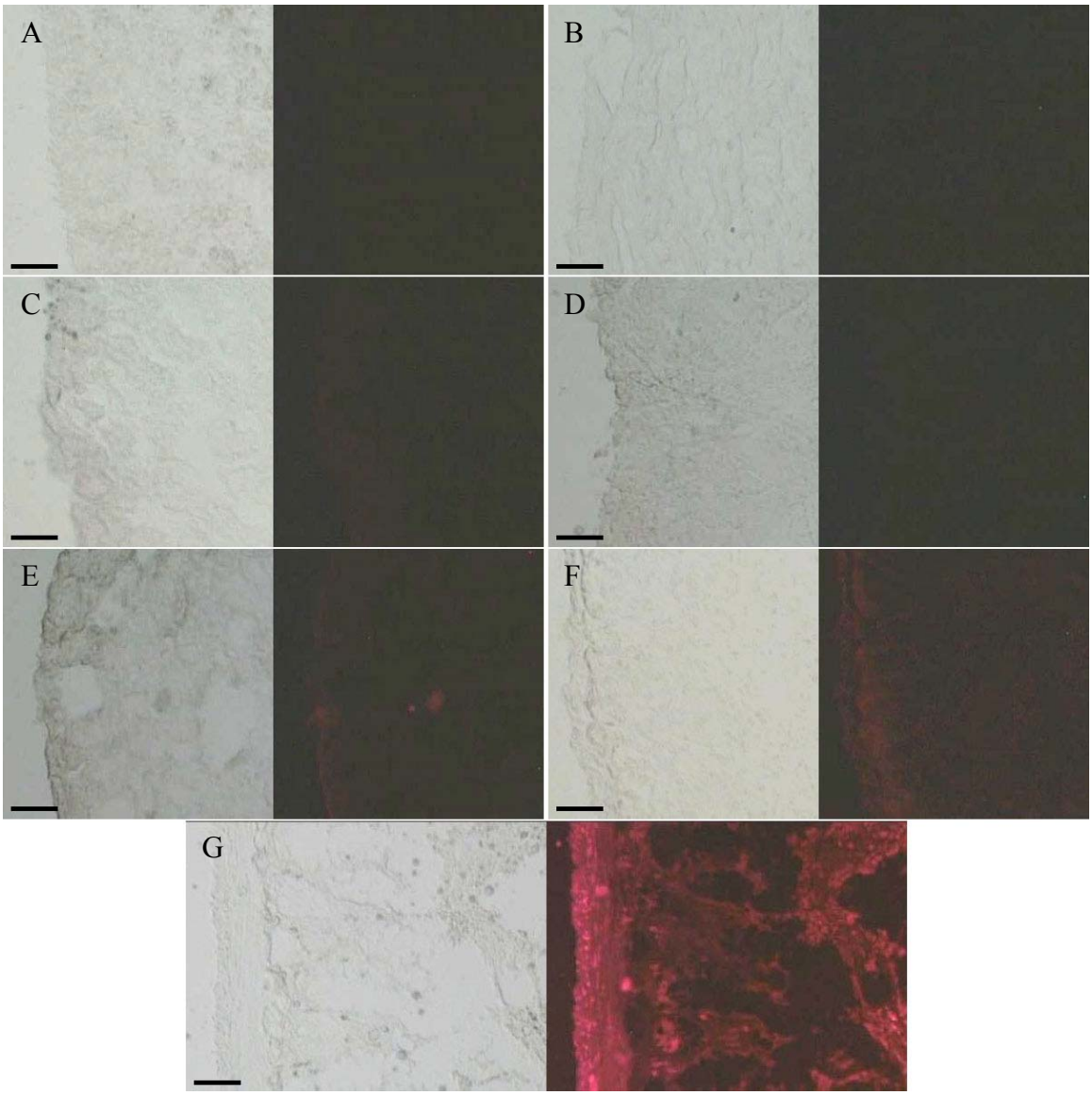


**Figure 2.4:** Representative images collected for protease *in situ* zymography. A. Brain, B. Heart, C. Kidney, D. Liver, E. Lung, F. Pancreas, G. Intestine. Brightfield images are on the left and original fluorescent images are on the right. Scale bar indicates 50 μm.

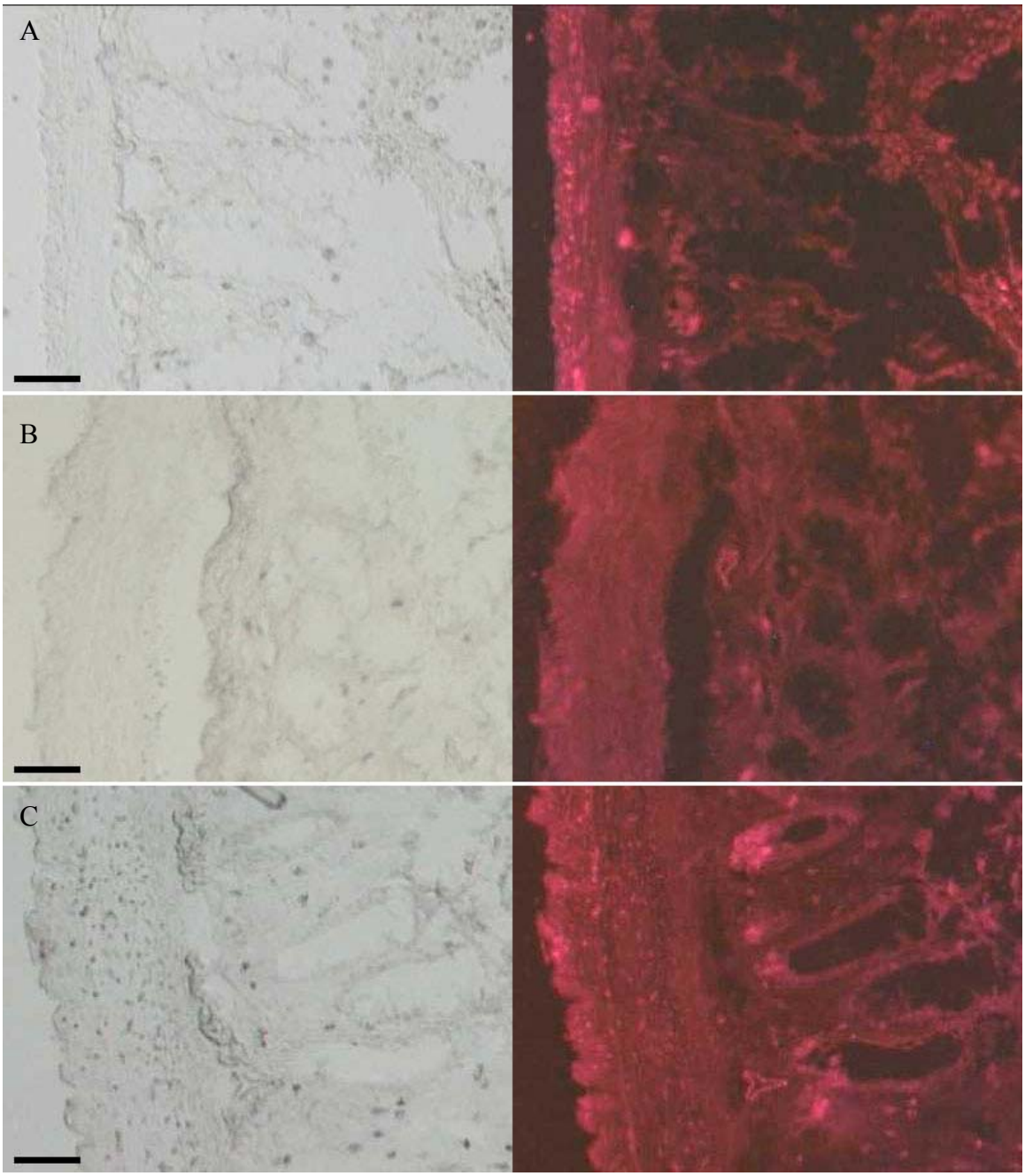


**Figure 2.5:** Representative images collected for protease *in situ* zymography of (A) control, (B) ischemic, and (C) ischemic-reperfusion intestinal tissue. Scale bar indicates 50 µm.

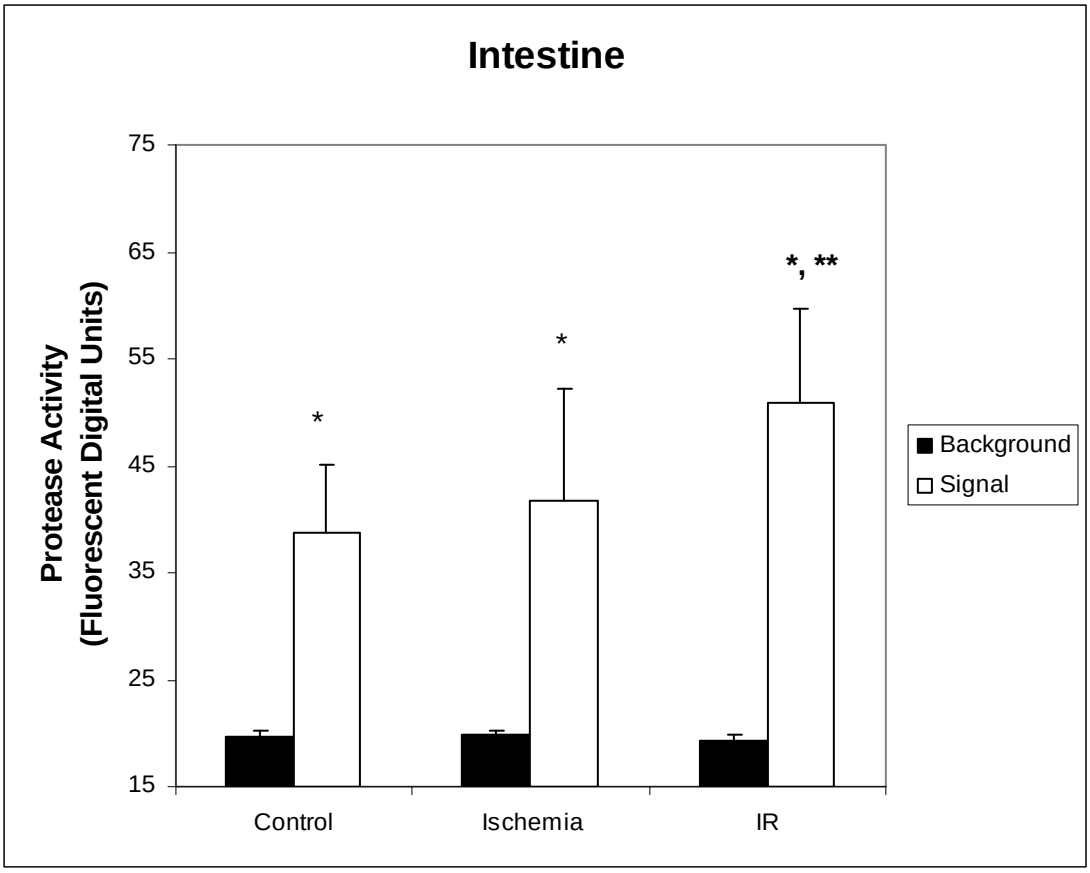


**Figure 2.6:** Protease activity, expressed in fluorescent digital units, of non-ischemic control rat intestine, ischemic intestine, and intestine exposed to ischemia followed by reperfusion. Error bars are mean ± SD, N = 5, * $p<0.0.5$ compared to background in a paired t-test, ** $p<0.05$ compared to control in an unpaired t-test

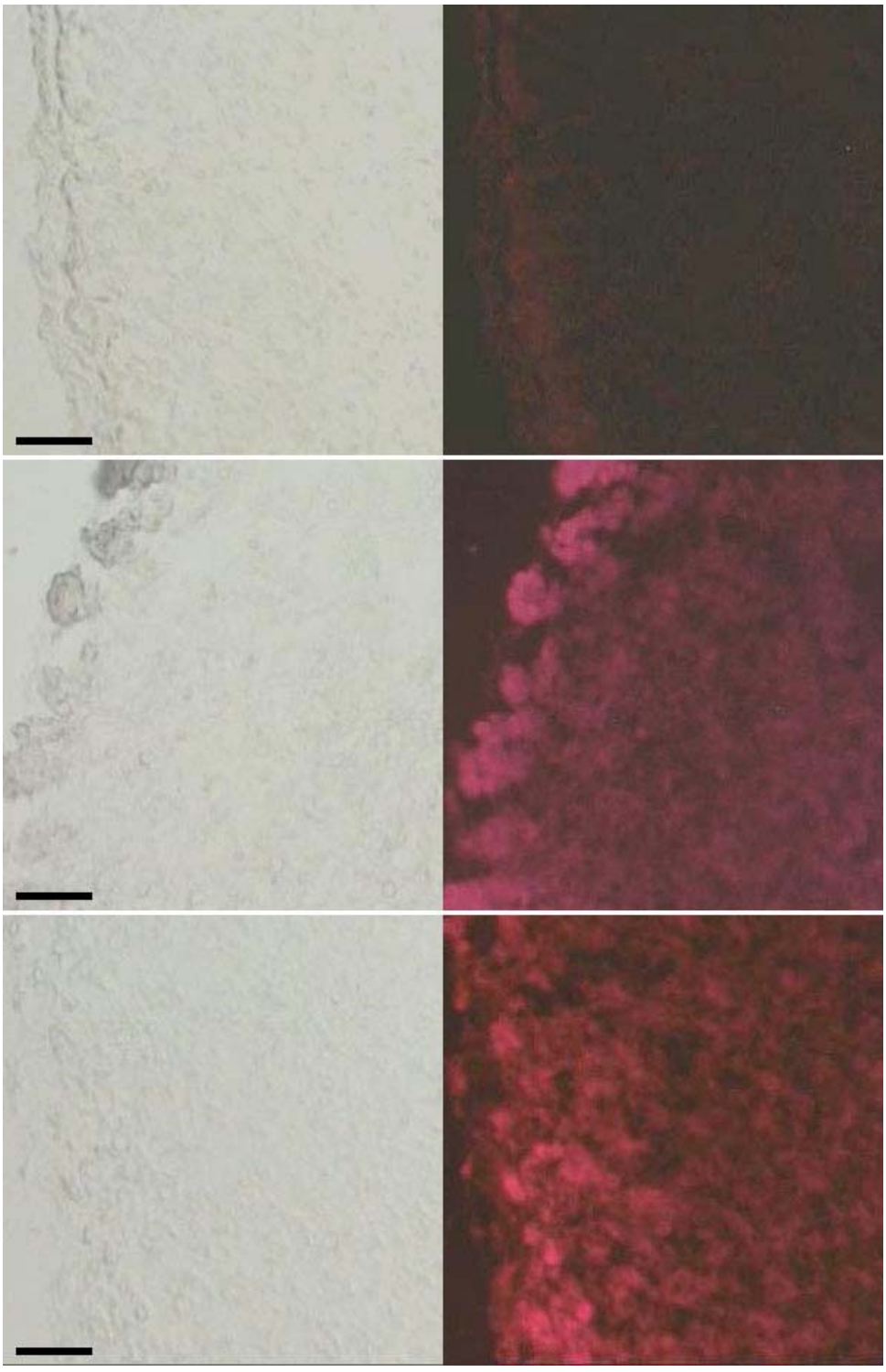

**Figure 2.7:** Representative images collected for protease *in situ* zymography of (A) control, (B) ischemic, and (C) ischemic-reperfusion pancreatic tissue. Scale bar indicates 50 µm.

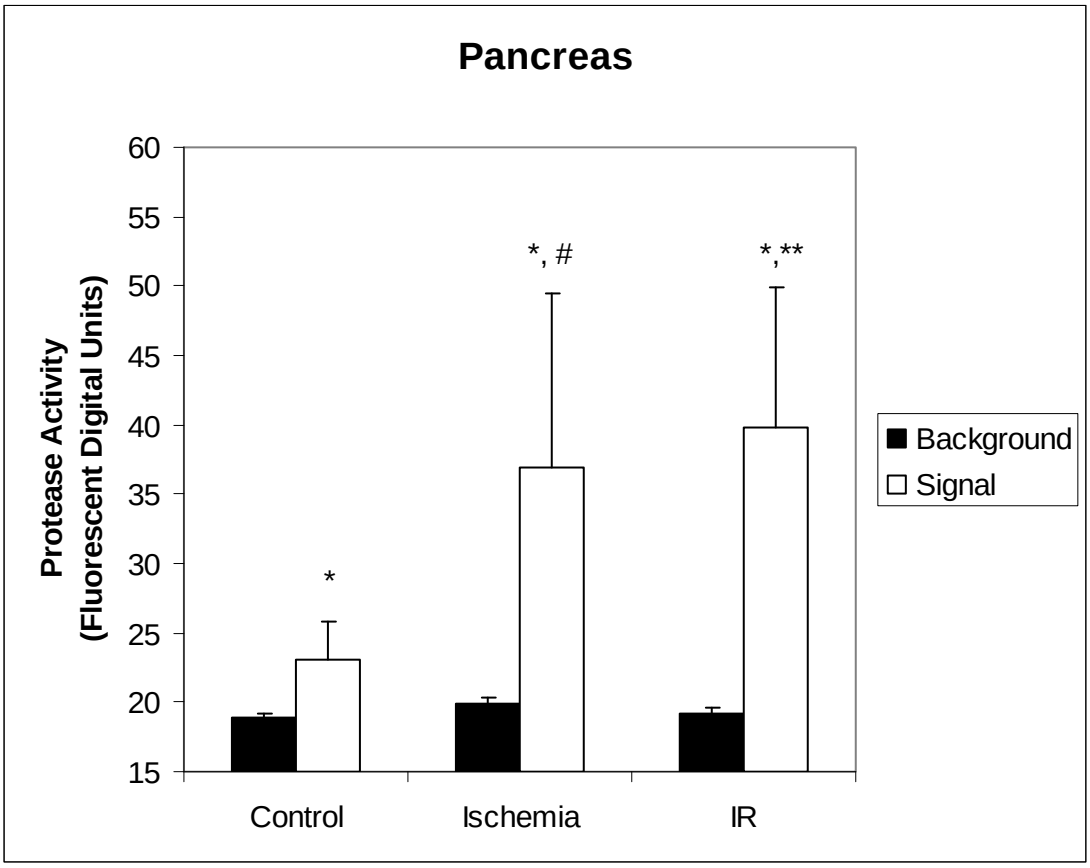


**Figure 2.8:** Protease activity, expressed in fluorescent digital units, of non-ischemic control rat pancreas, ischemic pancreas, and pancreas exposed to ischemia followed by reperfusion. Error bars are mean ± SD, N = 5, * $p<0.0.5$ compared to background in a paired t-test, ** $p<0.05$ compared to control in an unpaired t-test, # $p<0.05$ compared to control in Mann-Whitney test

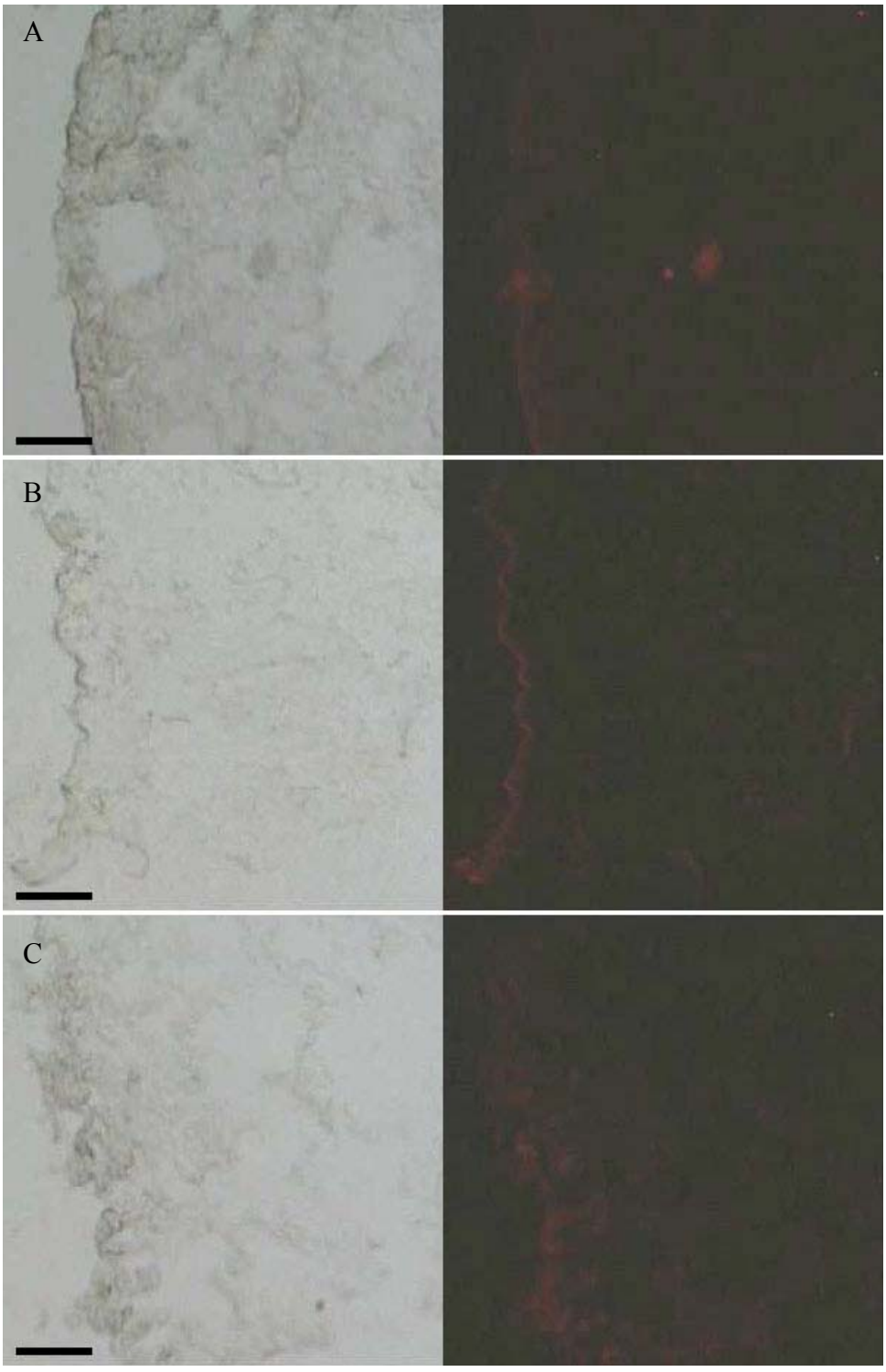


**Figure 2.9:** Representative images collected for protease *in situ* zymography of (A) control, (B) ischemic, and (C) ischemic-reperfusion lung tissue. Scale bar indicates 50 ɯm.

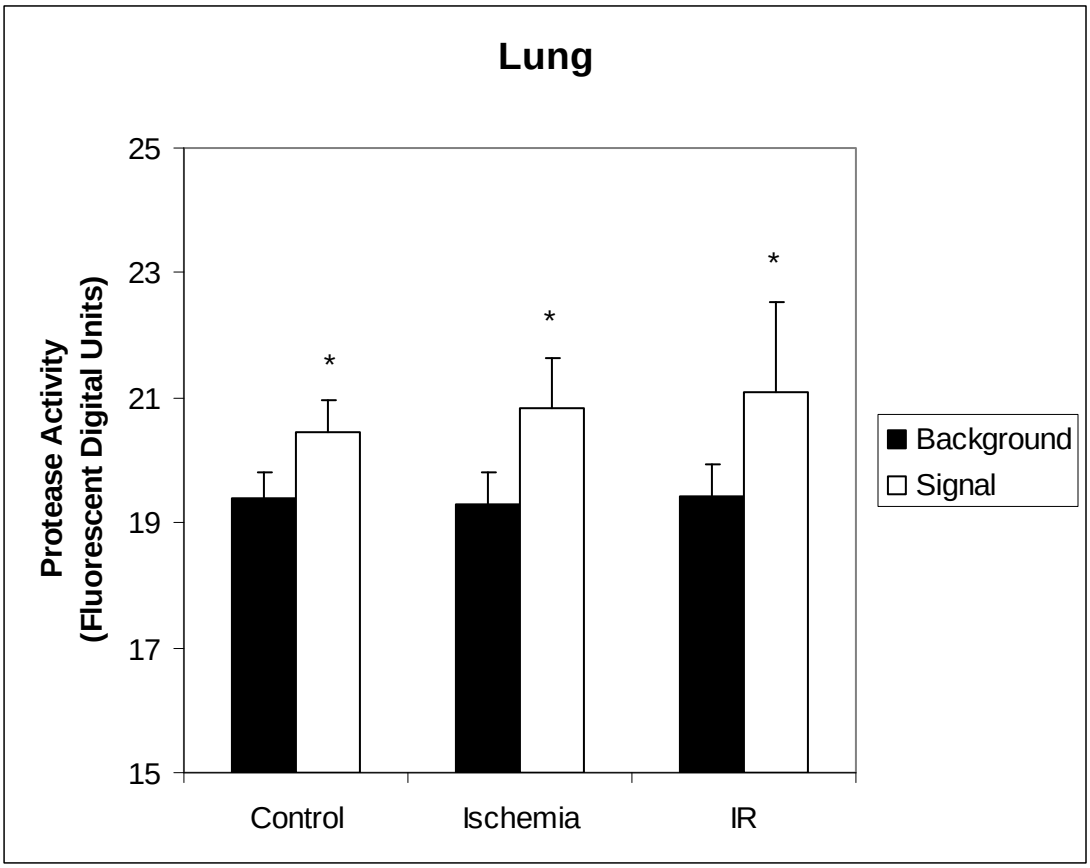


**Figure 2.10:** Protease activity, expressed in fluorescent digital units, of non-ischemic control rat lung, rat lung tissue exposed to intestinal ischemia, and rat lung tissue exposed to intestinal ischemia followed by reperfusion. Error bars are mean ± SD, N = 5, * $p<0.0.5$ compared to background in a paired t-test

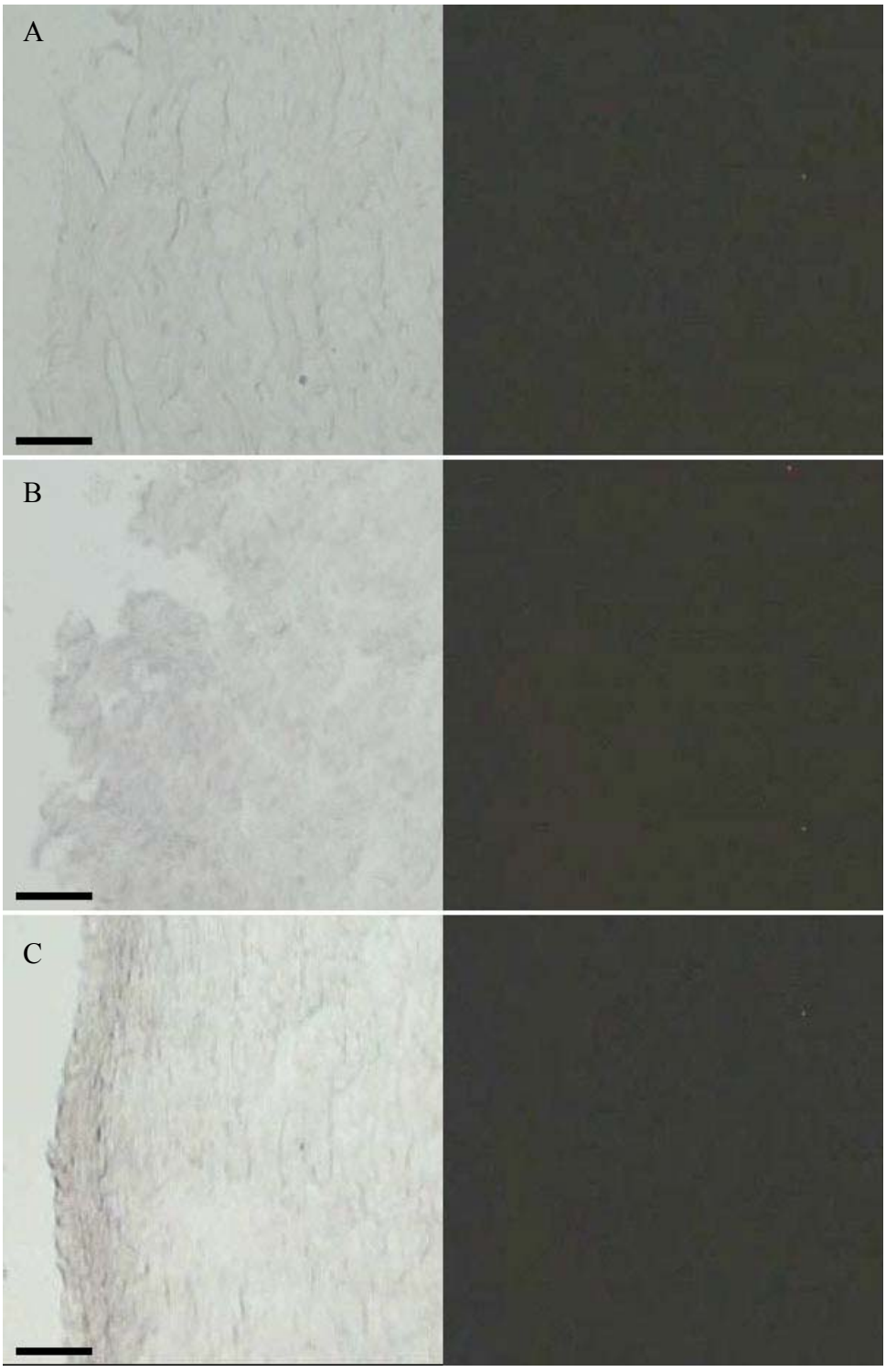


**Figure 2.11:** Representative images collected for protease *in situ* zymography of (A) control, (B) ischemic, and (C) ischemic-reperfusion heart tissue. Scale bar indicates 50 µm.

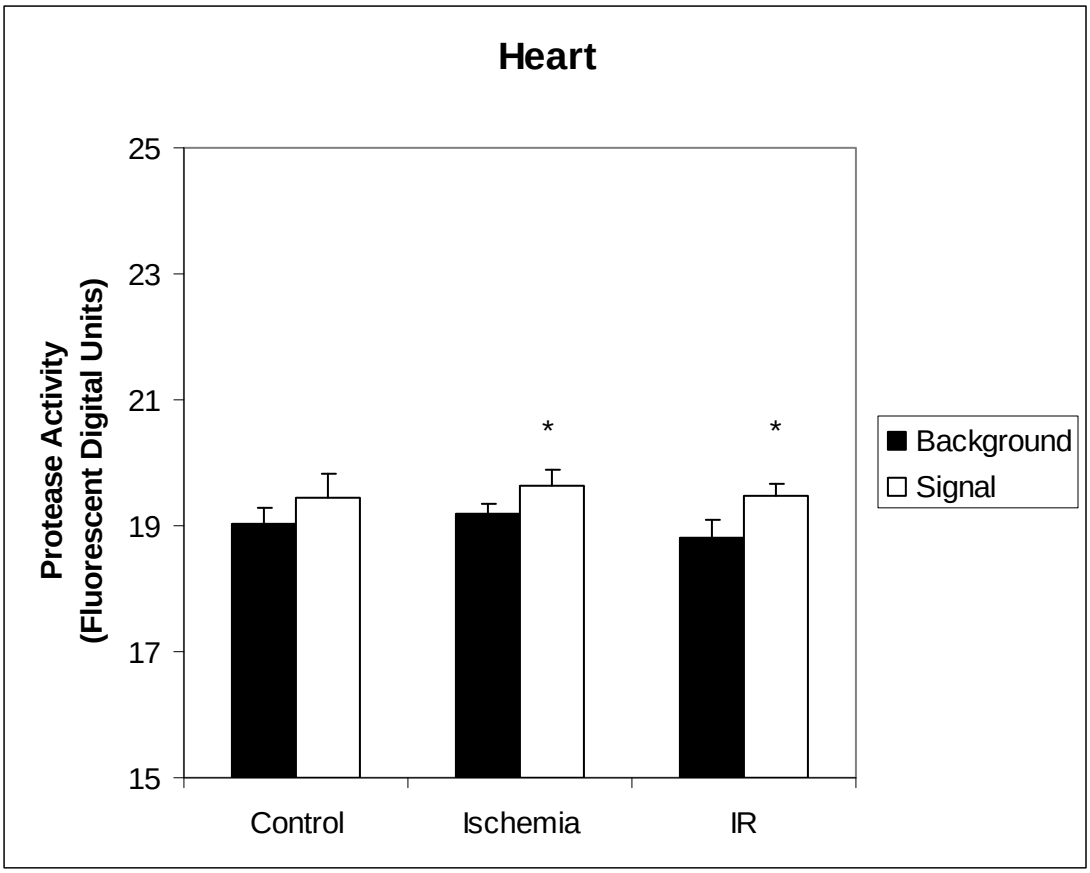


**Figure 2.12:** Protease activity, expressed in fluorescent digital units, of non-ischemic control rat heart tissue, rat heart muscle exposed to intestinal ischemia, and rat heart muscle exposed to intestinal ischemia followed by reperfusion. Error bars are mean ± SD, N = 5, * $p<0.0.5$ compared to background in a paired t-test

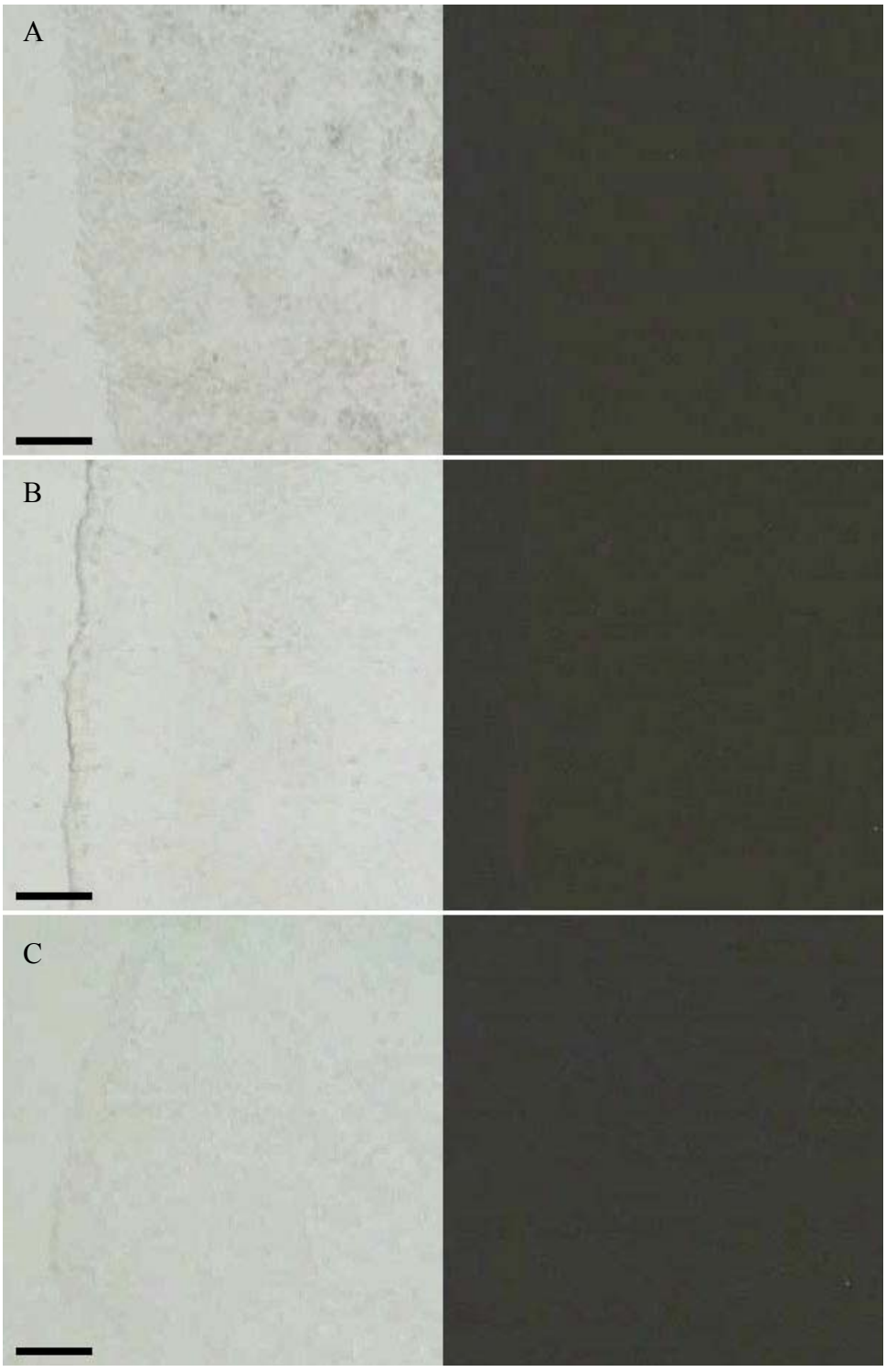


**Figure 2.13:** Representative images collected for protease *in situ* zymography of (A) control, (B) ischemic, and (C) ischemic-reperfusion brain tissue. Scale bar indicates 50 µm.

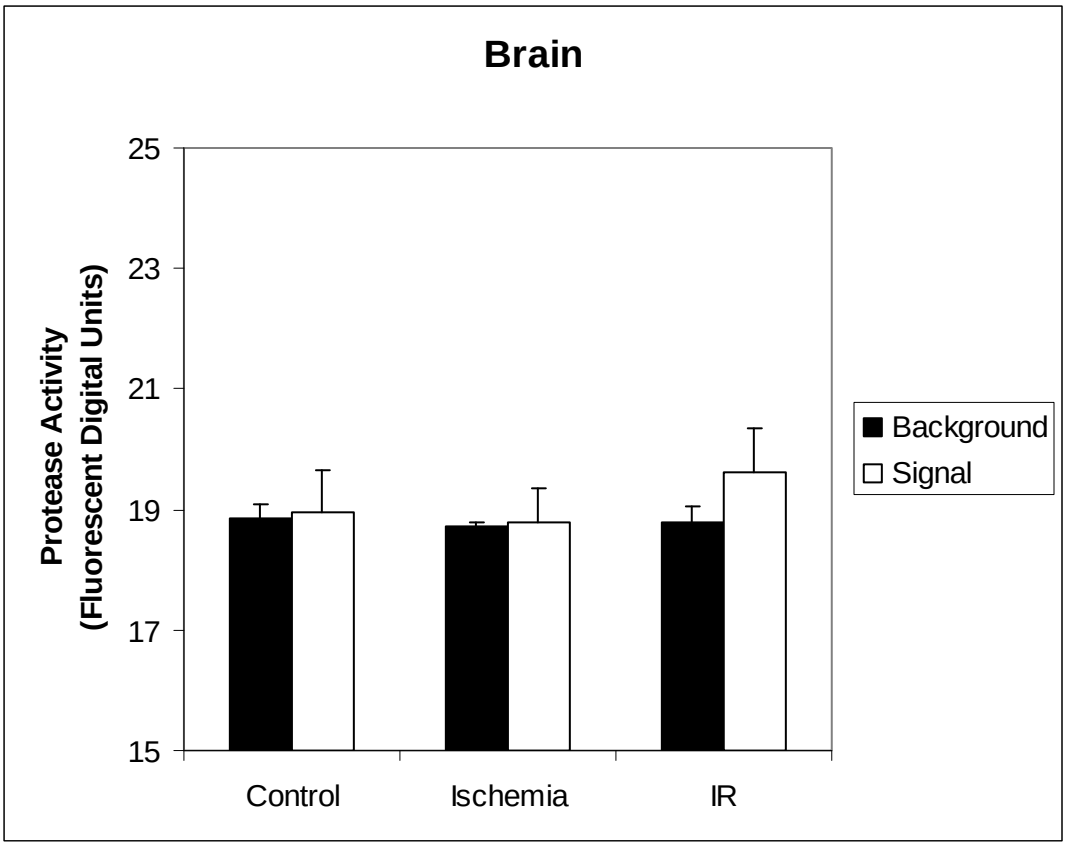


**Figure 2.14:** Protease activity, expressed in fluorescent digital units, of non-ischemic control rat brain tissue, rat brain tissue exposed to intestinal ischemia, and rat brain tissue exposed to intestinal ischemia followed by reperfusion. Error bars are mean ± SD, N = 5

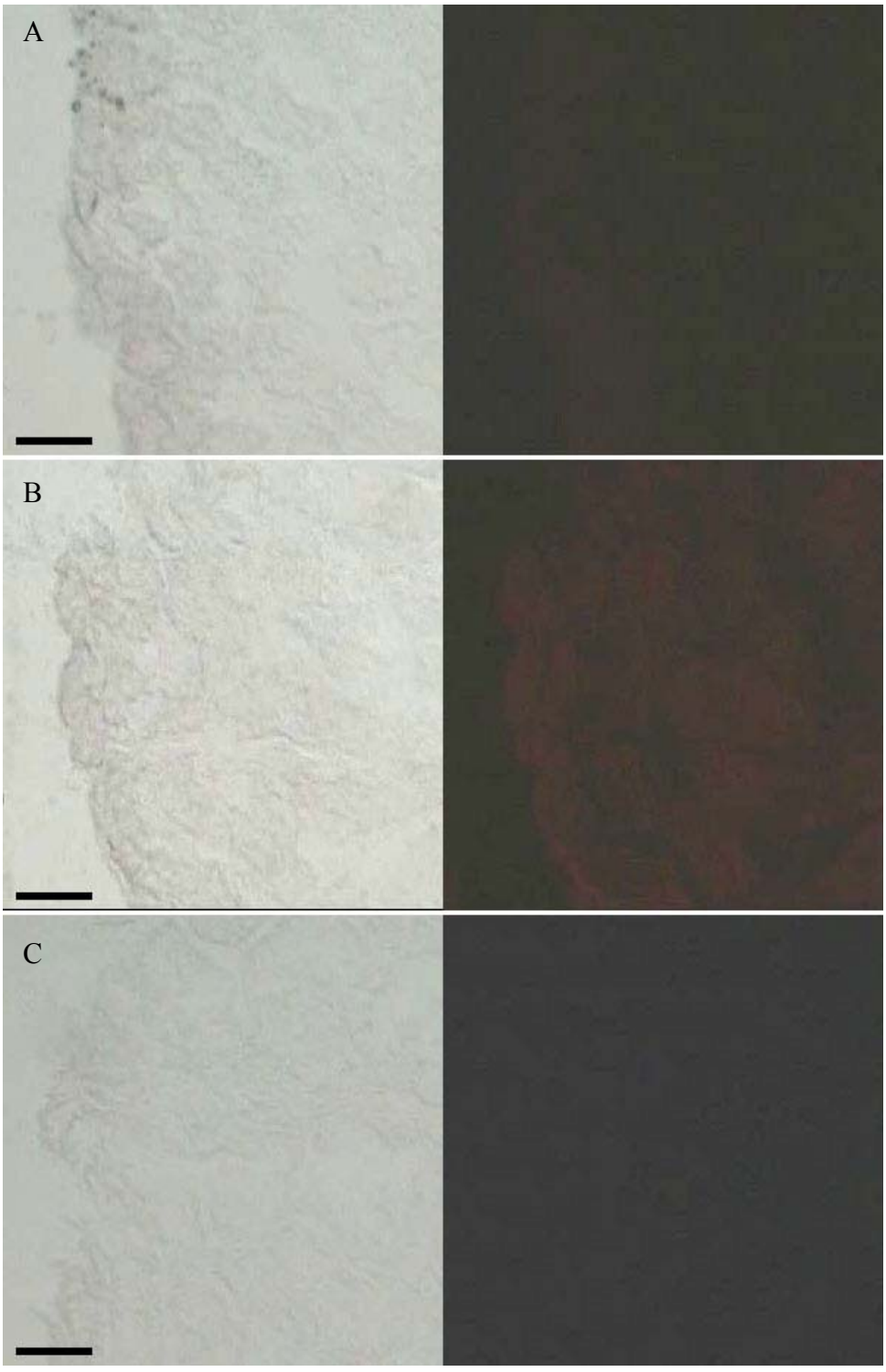


**Figure 2.15:** Representative images collected for protease *in situ* zymography of (A) control, (B) ischemic, and (C) ischemic-reperfusion kidney tissue. Scale bar indicates 50 µm.

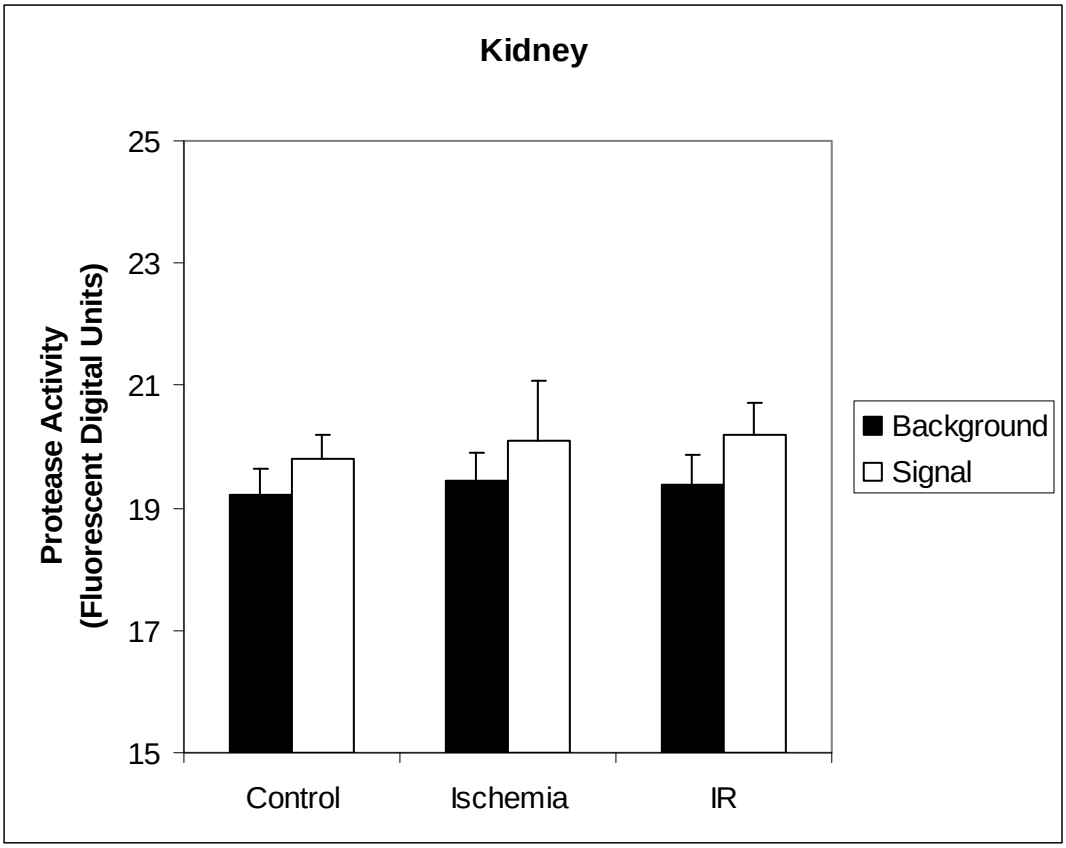


**Figure 2.16:** Protease activity, expressed in fluorescent digital units, of non-ischemic control rat kidney tissue, rat kidney tissue exposed to intestinal ischemia, and rat kidney tissue exposed to intestinal ischemia followed by reperfusion. Error bars are mean ± SD, N = 5

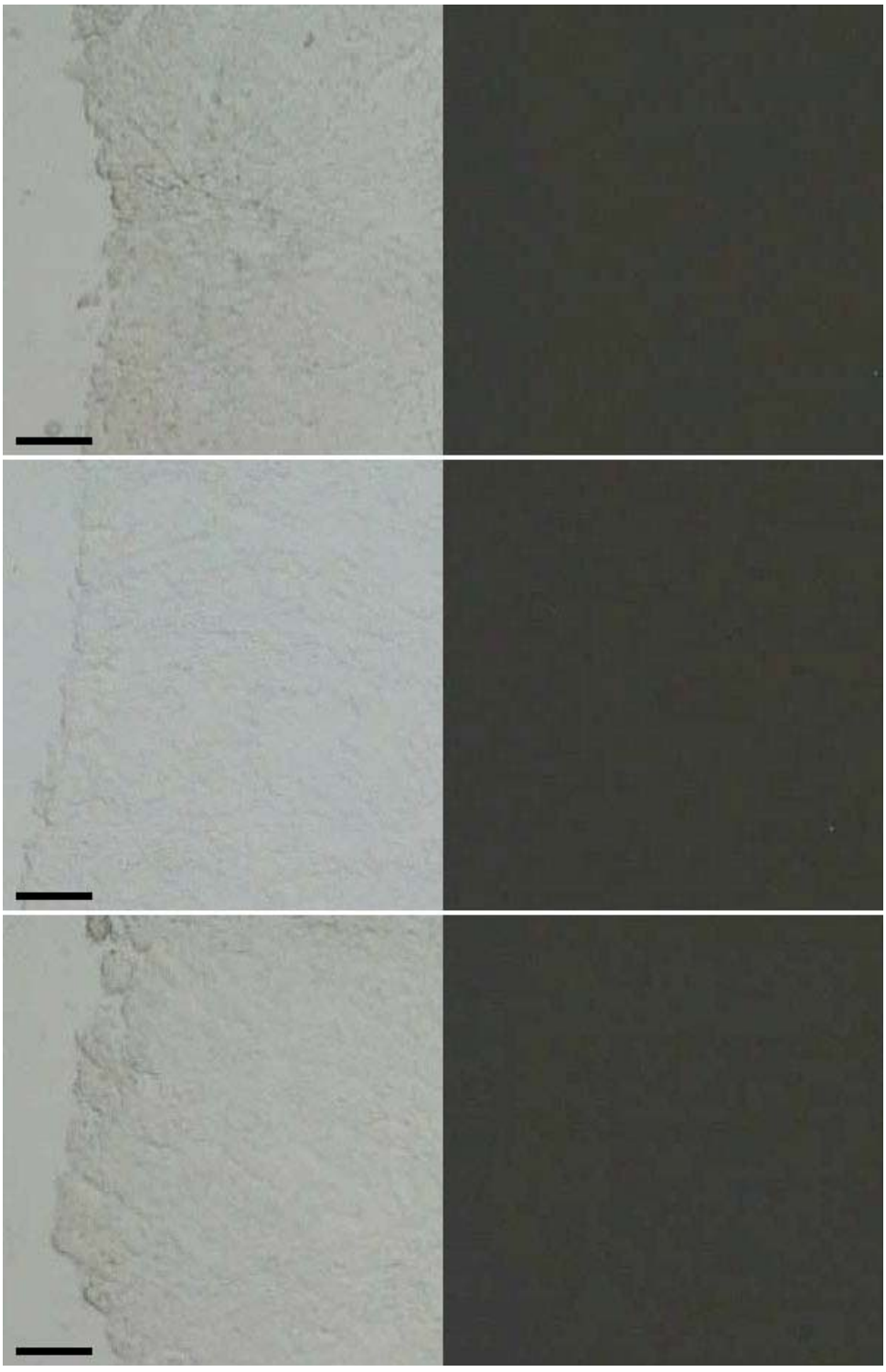

**Figure 2.17:** Representative images collected for protease *in situ* zymography of (A) control, (B) ischemic, and (C) ischemic-reperfusion liver tissue. Scale bar indicates 50 ɯm.

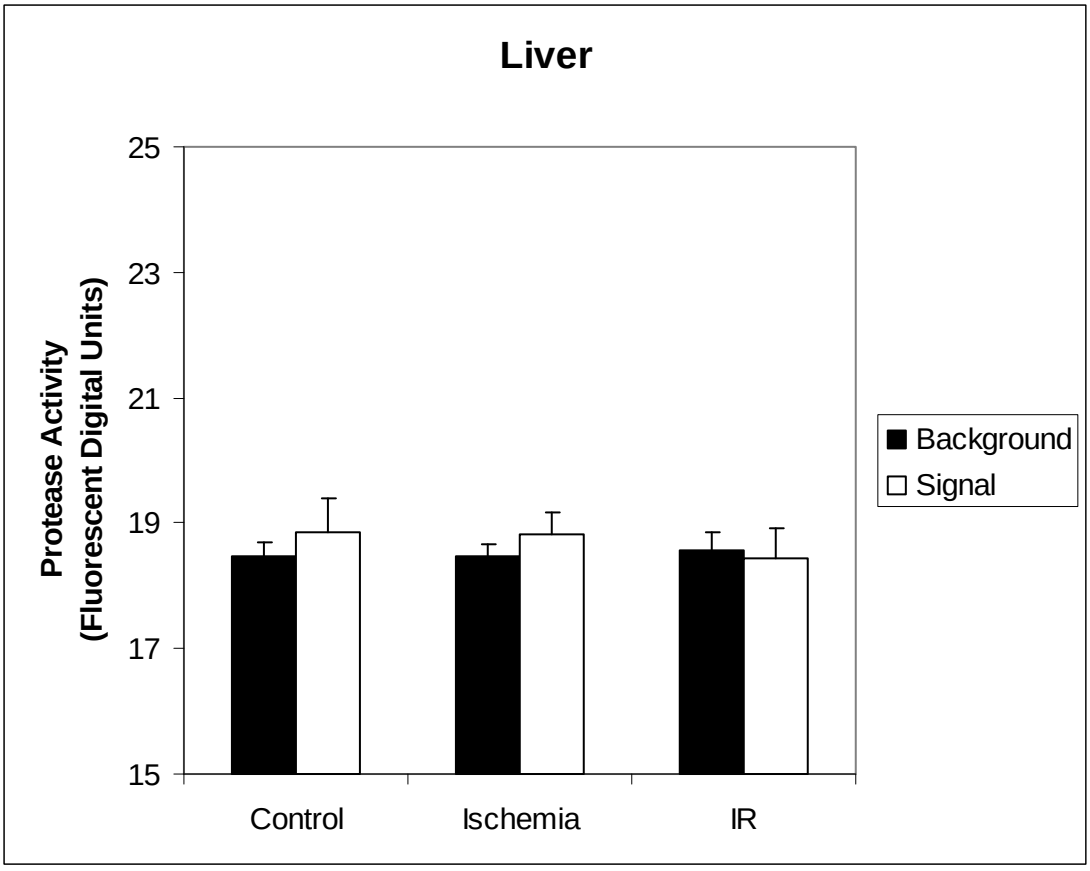


**Figure 2.18:** Protease activity, expressed in fluorescent digital units, of non-ischemic control rat liver tissue, rat liver tissue exposed to intestinal ischemia, and rat liver tissue exposed to intestinal ischemia followed by reperfusion. Error bars are mean ± SD, N = 5

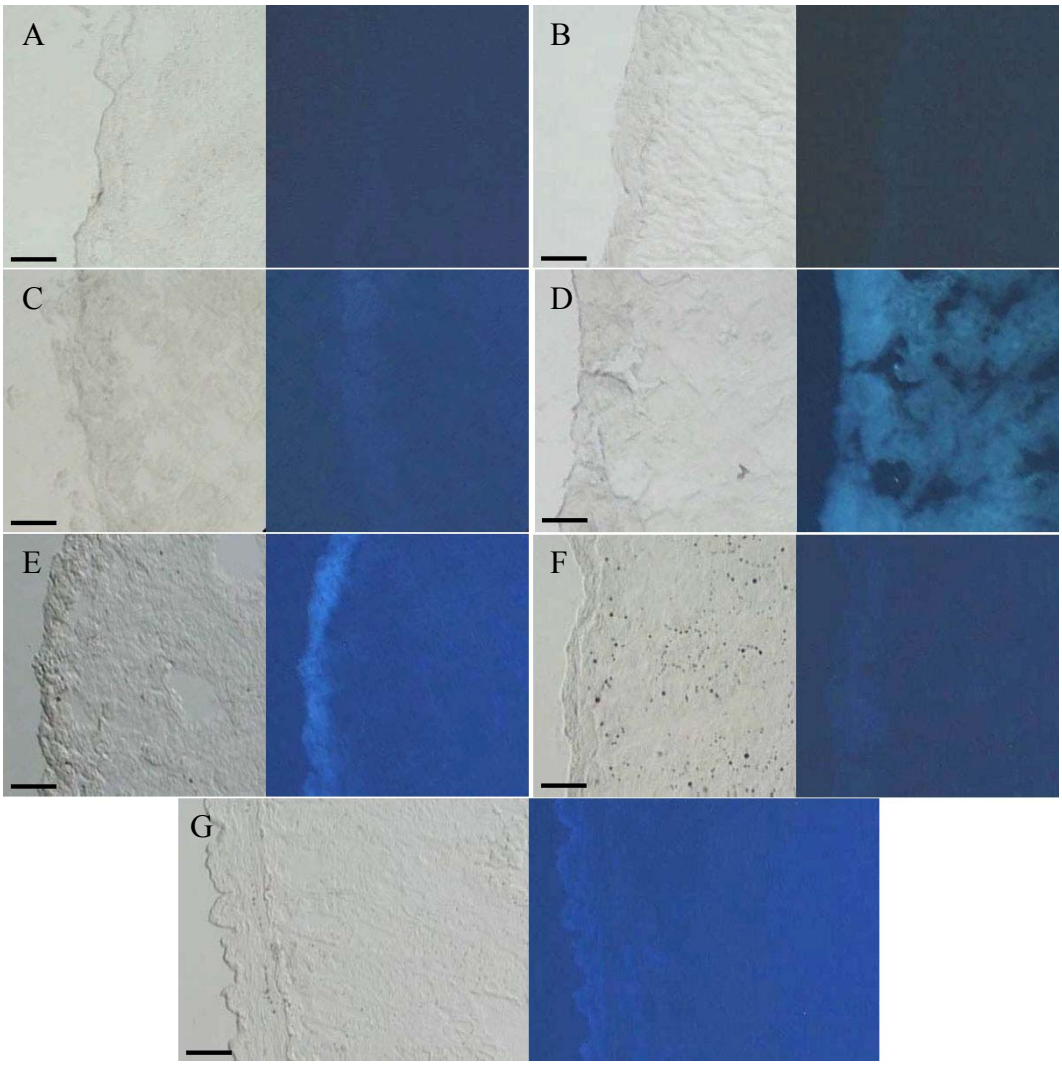


**Figure 2.19:** Representative images collected for lipase *in situ* zymography. A. Brain, B. Heart, C. Kidney, D. Liver, E. Lung, F. Pancreas, G. Intestine. Bright field images are on the left and original fluorescent images are on the right. Scale bar indicates 50 μm.

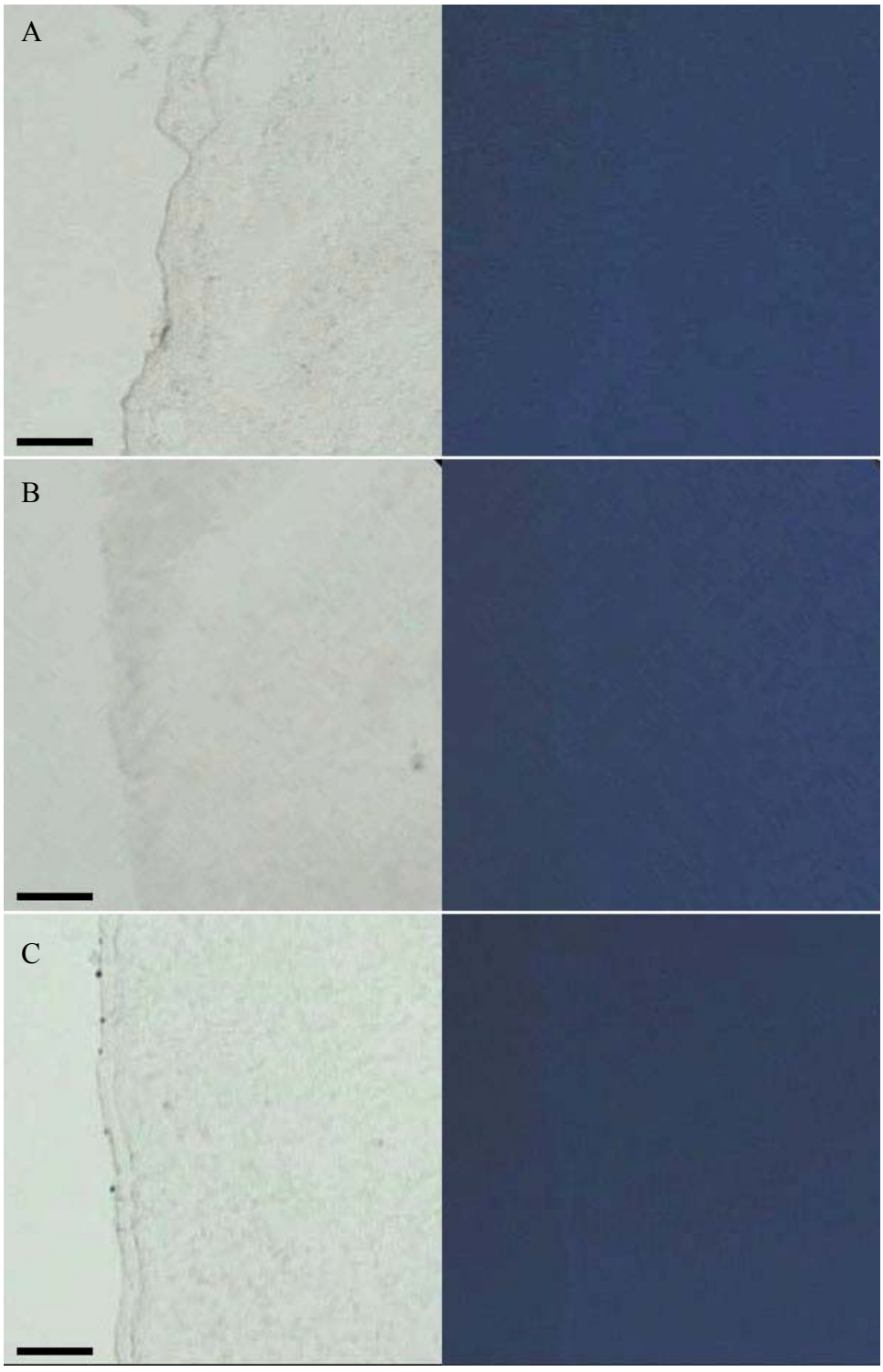


**Figure 2.20:** Representative images collected for lipase *in situ* zymography of (A) control, (B) ischemic, and (C) ischemic-reperfusion brain tissue. Scale bar indicates 50 ɯm.

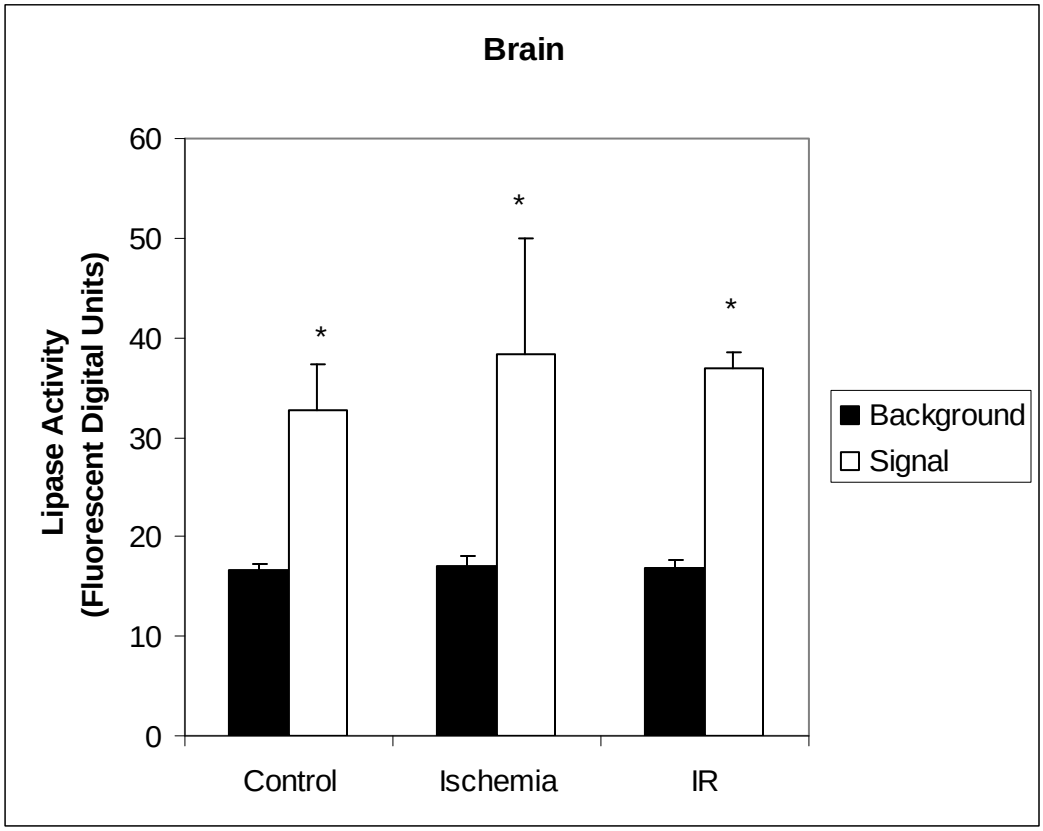


**Figure 2.21:** Lipase activity, expressed in fluorescent digital units, of non-ischemic control rat brain tissue, rat brain tissue exposed to intestinal ischemia, and rat brain tissue exposed to intestinal ischemia followed by reperfusion. Error bars are mean ± SD, N = 5, * $p<0.0.5$ compared to background in a paired t-test

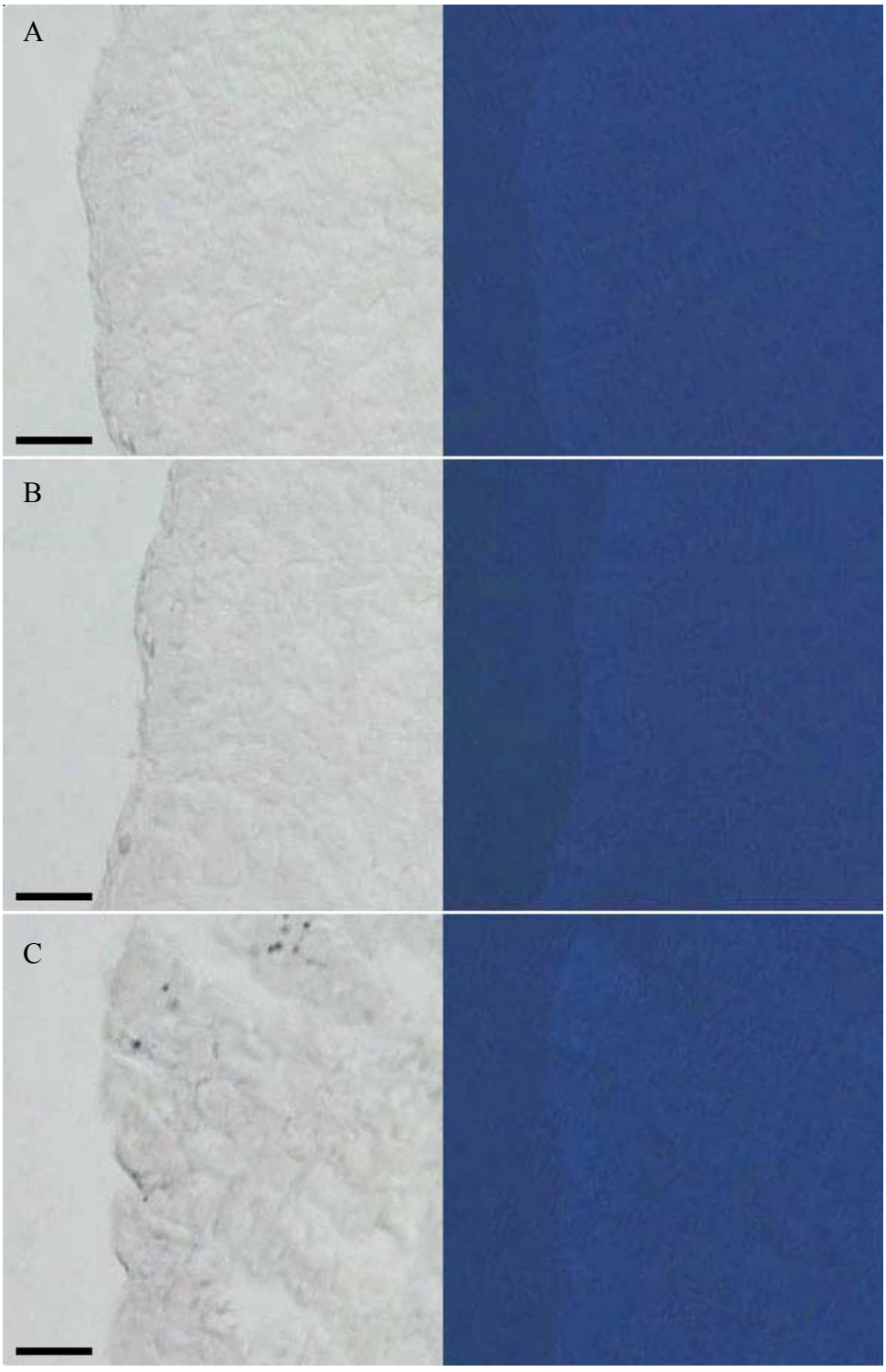


**Figure 2.22:** Representative images collected for lipase *in situ* zymography of (A) control, (B) ischemic, and (C) ischemic-reperfusion heart tissue. Scale bar indicates 50 µm.

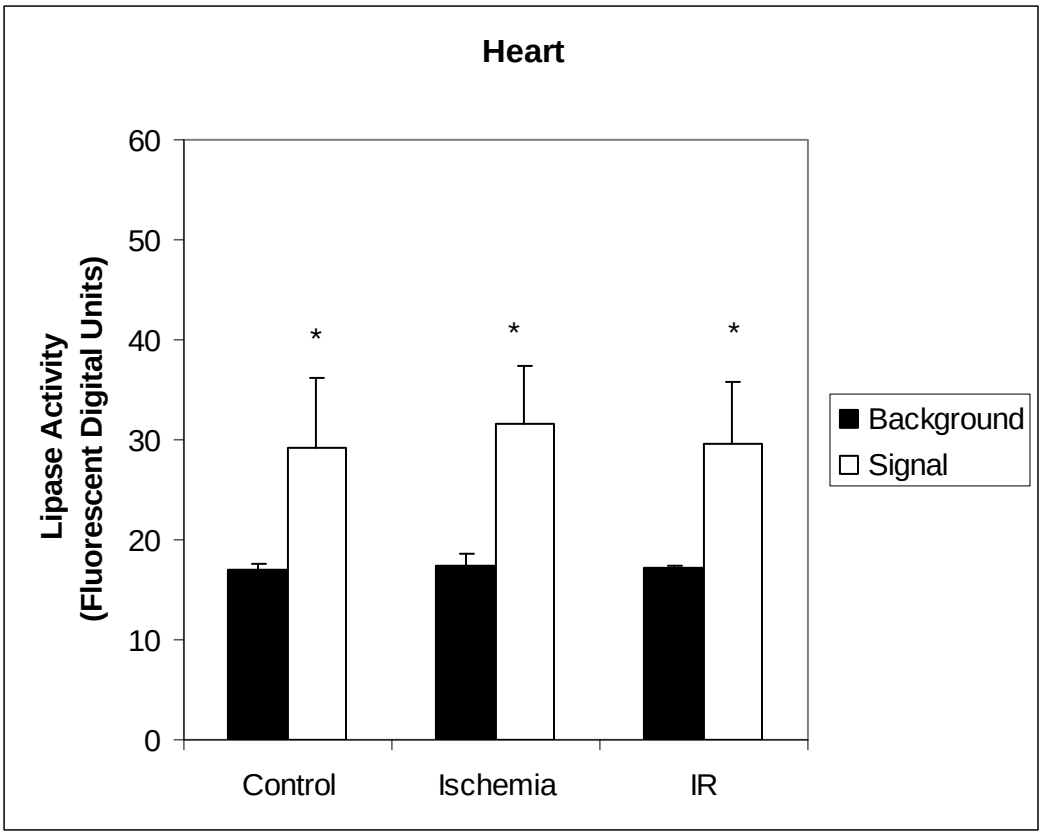


**Figure 2.23:** Lipase activity, expressed in fluorescent digital units, of non-ischemic control rat heart tissue, rat heart muscle exposed to intestinal ischemia, and rat heart muscle exposed to intestinal ischemia followed by reperfusion. Error bars are mean ± SD, N = 5, * $p<0.0.5$ compared to background in a paired t-test

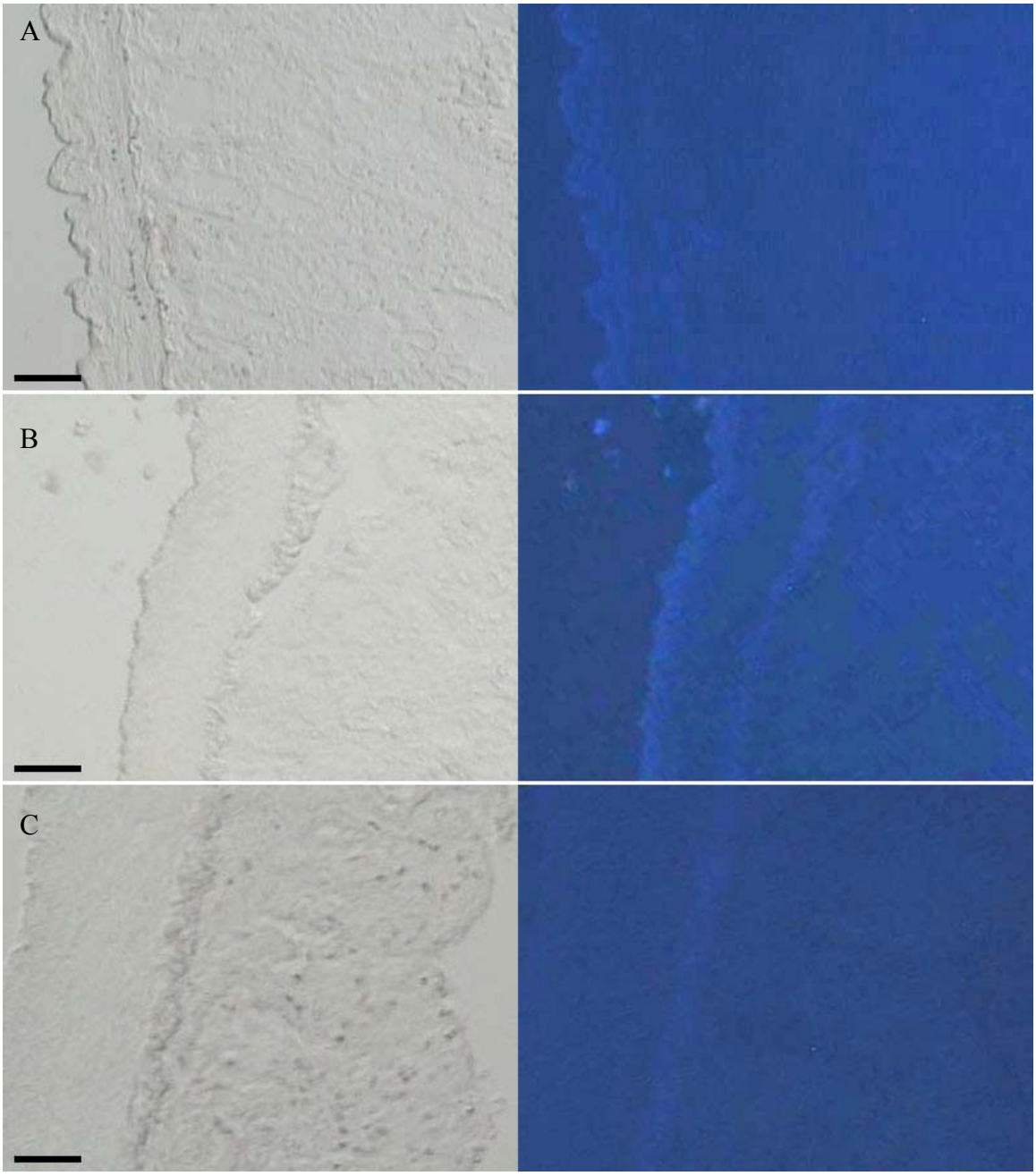


**Figure 2.24:** Representative images collected for lipase *in situ* zymography of (A) control, (B) ischemic, and (C) ischemic-reperfusion intestinal tissue. Scale bar indicates 50 μm.

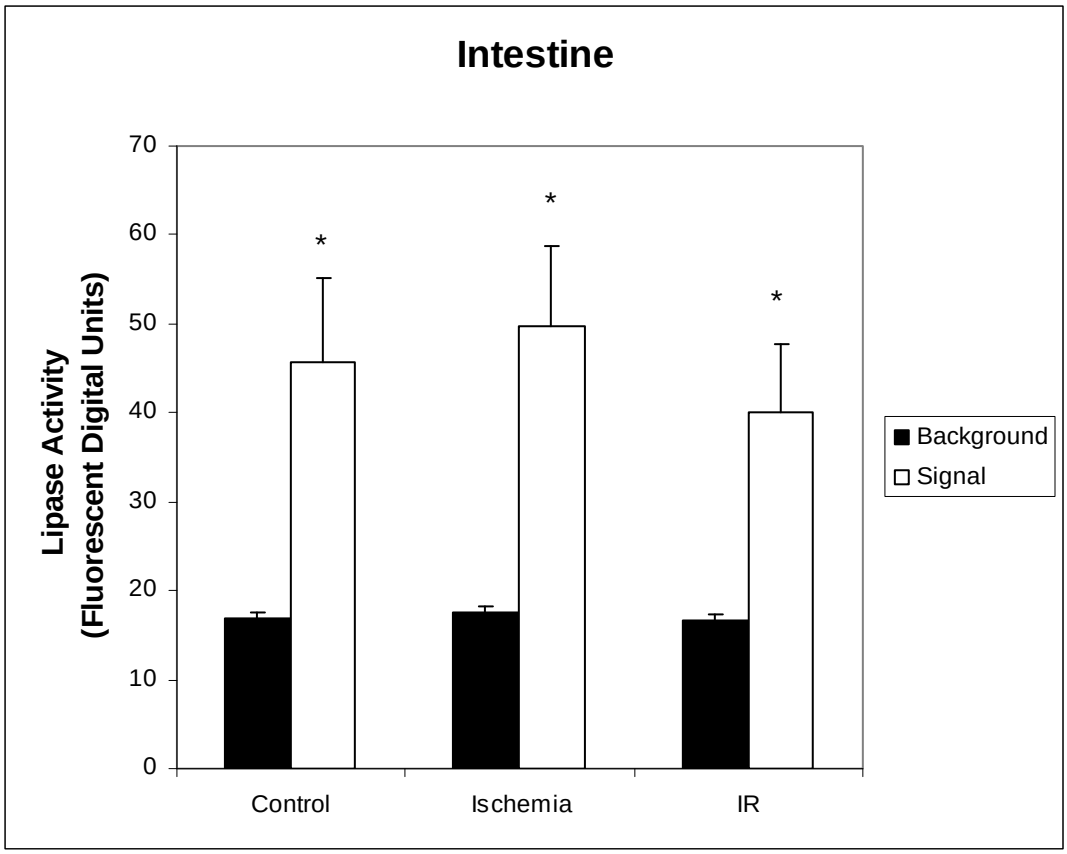


**Figure 2.25:** Lipase activity, expressed in fluorescent digital units, of non-ischemic control rat intestine, ischemic intestine, and intestine exposed to ischemia followed by reperfusion. Error bars are mean ± SD, N = 5, * $p<0.0.5$ compared to background in a paired t-test

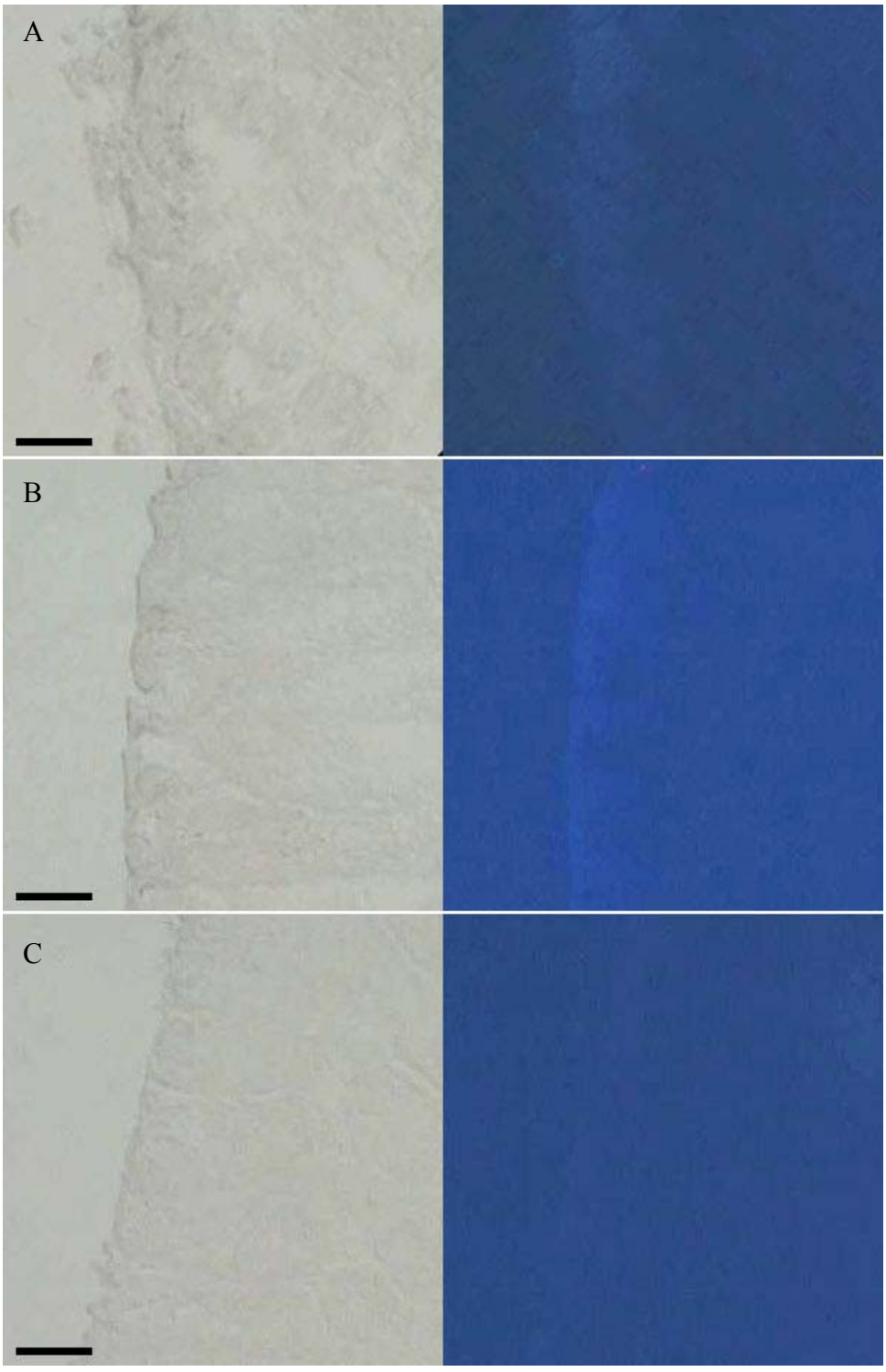


**Figure 2.26:** Representative images collected for lipase *in situ* zymography of (A) control, (B) ischemic, and (C) ischemic-reperfusion kidney tissue. Scale bar indicates 50 µm.

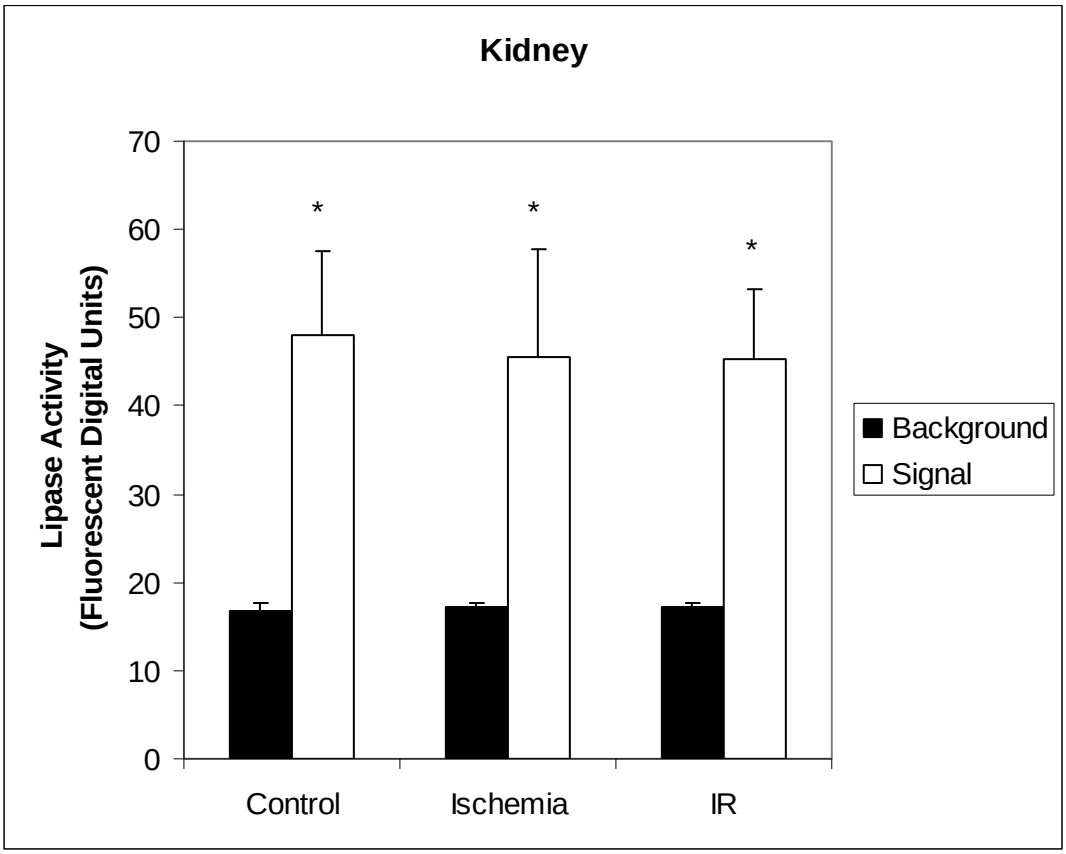


**Figure 2.27:** Lipase activity, expressed in fluorescent digital units, of non-ischemic control rat kidney tissue, rat kidney tissue exposed to intestinal ischemia, and rat kidney tissue exposed to intestinal ischemia followed by reperfusion. Error bars are mean ± SD, N = 5, * $p<0.0.5$ compared to background in a paired t-test

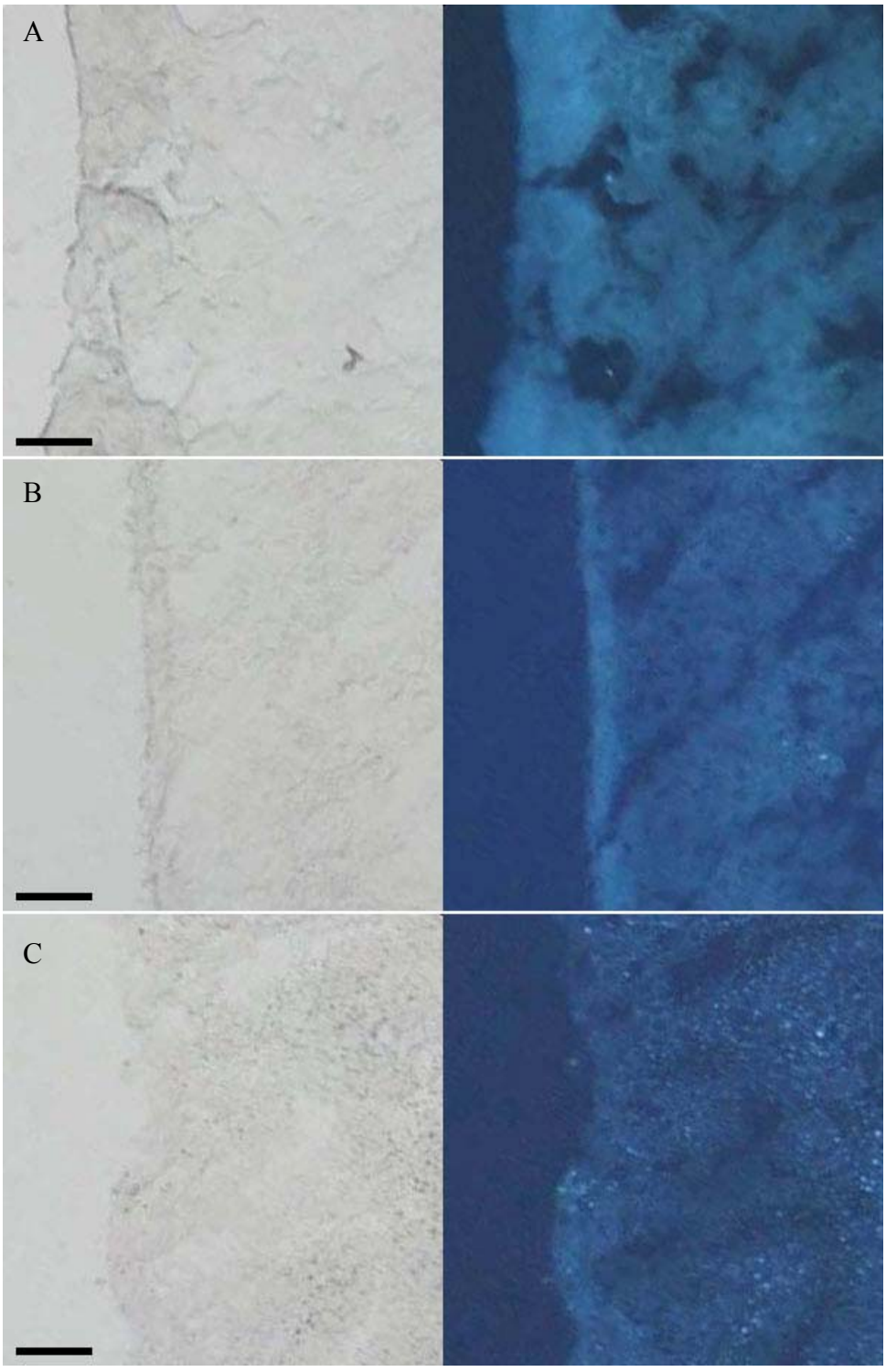


**Figure 2.28:** Representative images collected for lipase *in situ* zymography of (A) control, (B) ischemic, and (C) ischemic-reperfusion liver tissue. Scale bar indicates 50 ɯm.

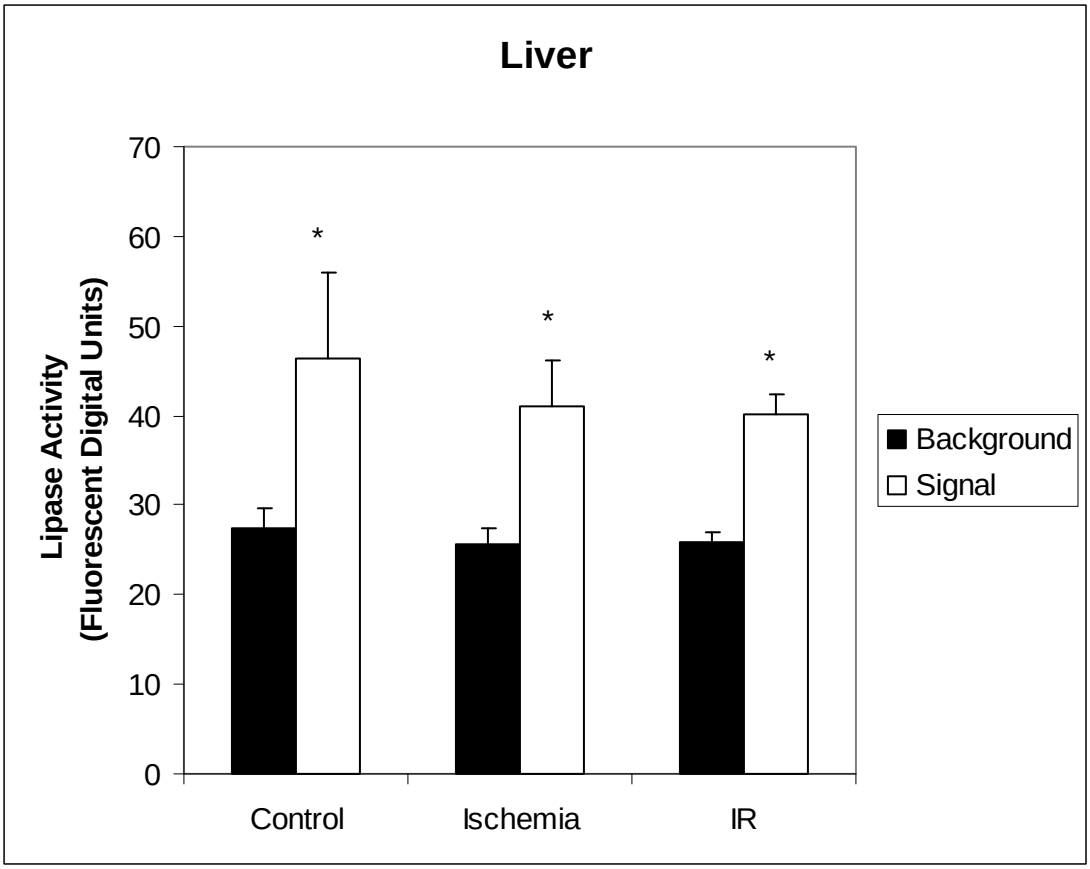


**Figure 2.29:** Lipase activity, expressed in fluorescent digital units, of non-ischemic control rat liver tissue, rat liver tissue exposed to intestinal ischemia, and rat liver tissue exposed to intestinal ischemia followed by reperfusion. Error bars are mean ± SD, N = 5, * $p<0.0.5$ compared to background in a paired t-test

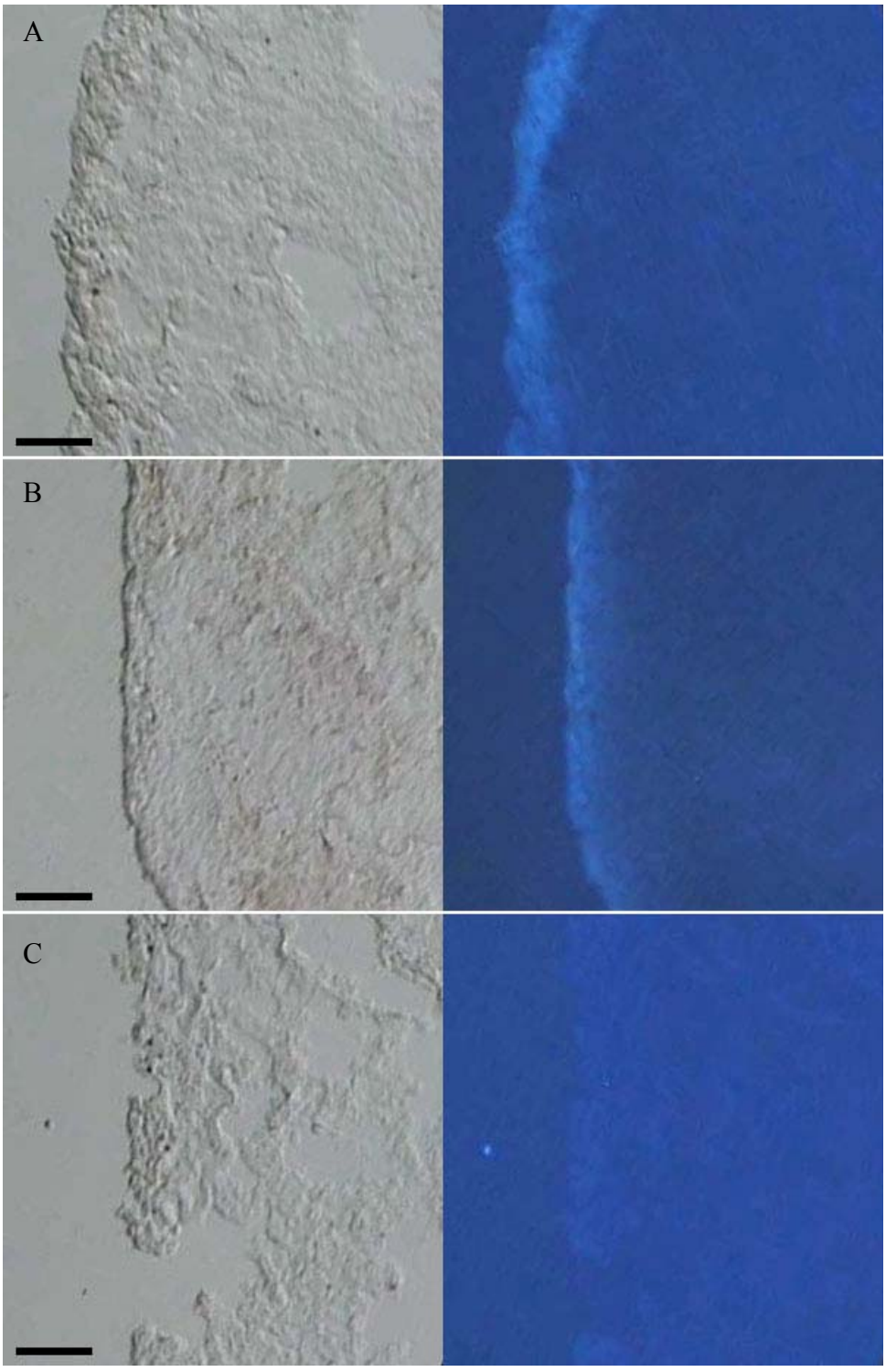


**Figure 2.30:** Representative images collected for lipase *in situ* zymography of (A) control, (B) ischemic, and (C) ischemic-reperfusion lung tissue. Scale bar indicates 50 ɯm.

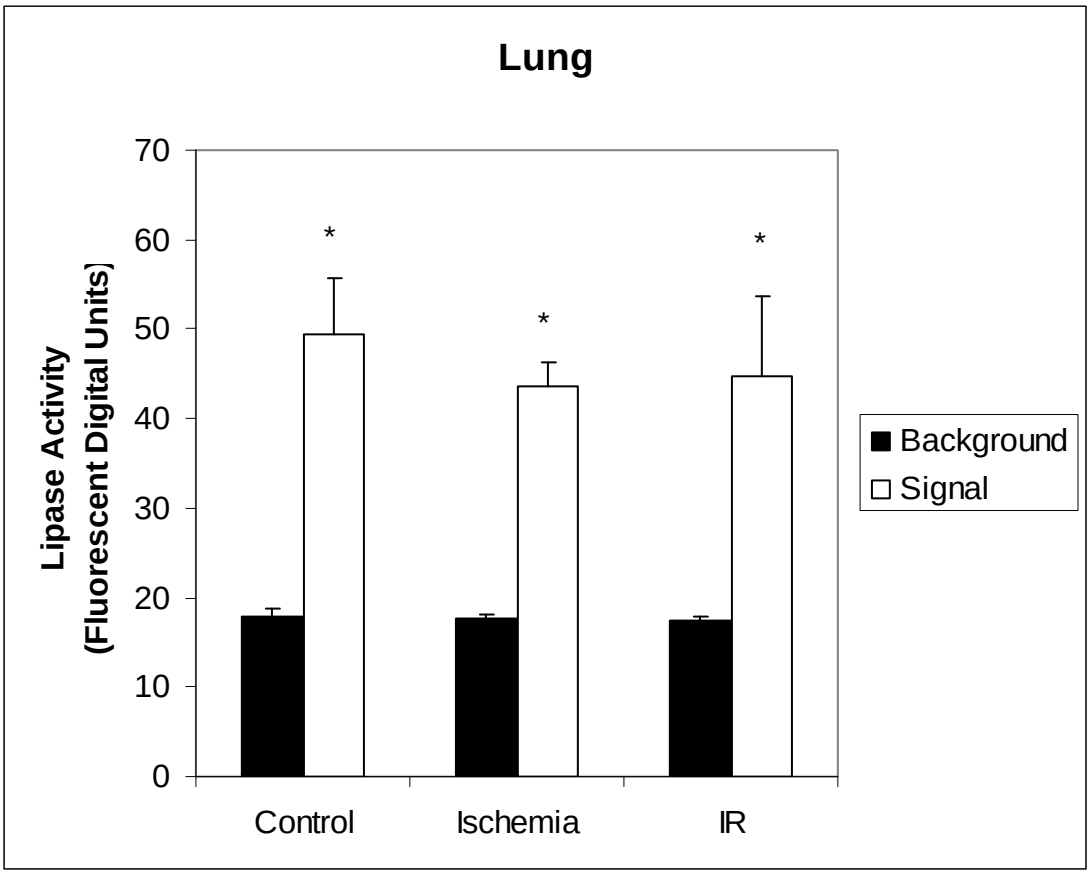


**Figure 2.31:** Lipase activity, expressed in fluorescent digital units, of non-ischemic control rat lung tissue, rat lung tissue exposed to intestinal ischemia, and rat lung tissue exposed to intestinal ischemia followed by reperfusion. Error bars are mean ± SD, N = 5, * $p<0.0.5$ compared to background in a paired t-test

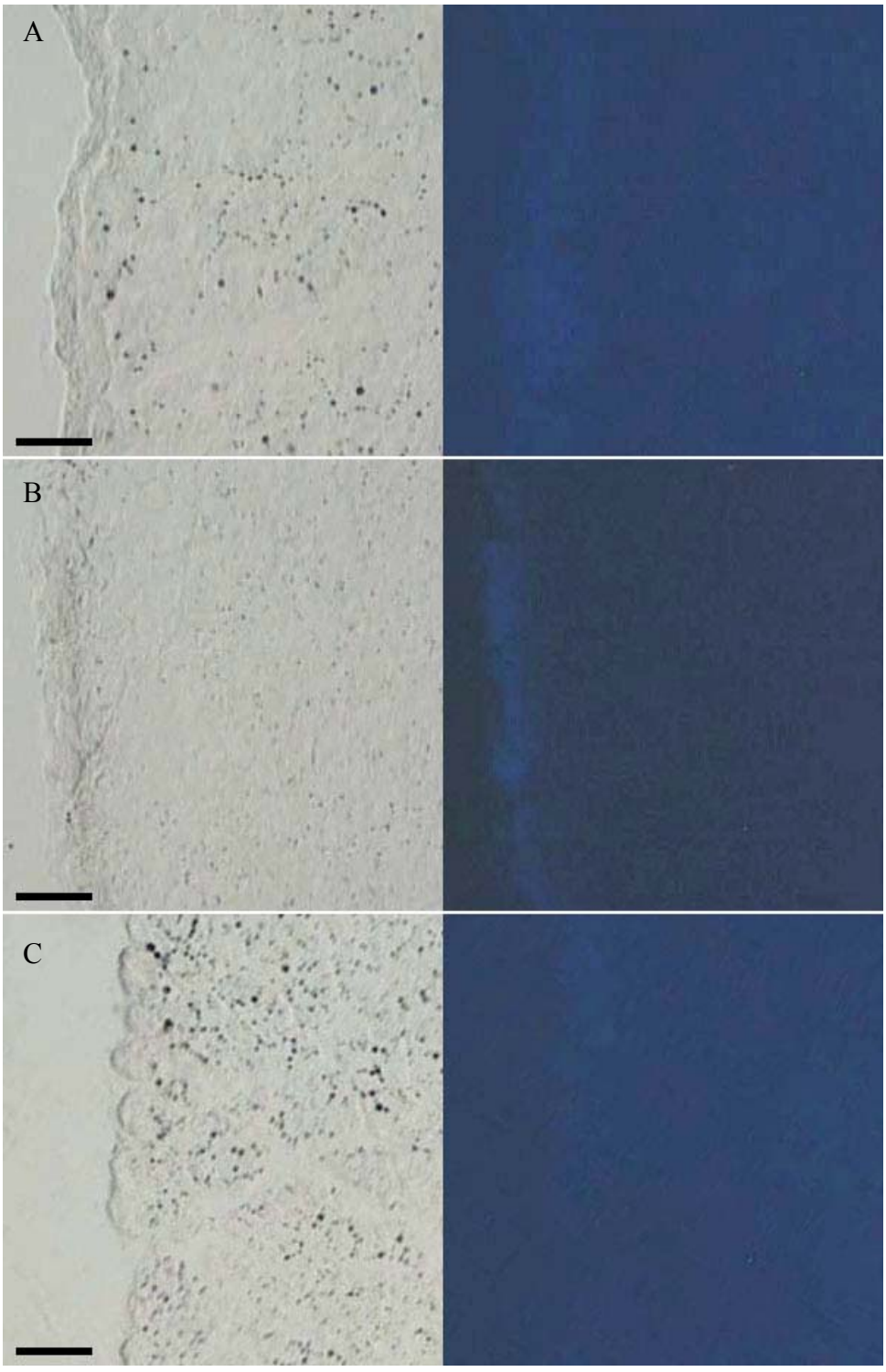


**Figure 2.32:** Representative images collected for lipase *in situ* zymography of (A) control, (B) ischemic, and (C) ischemic-reperfusion pancreatic tissue. Scale bar indicates 50 µm.

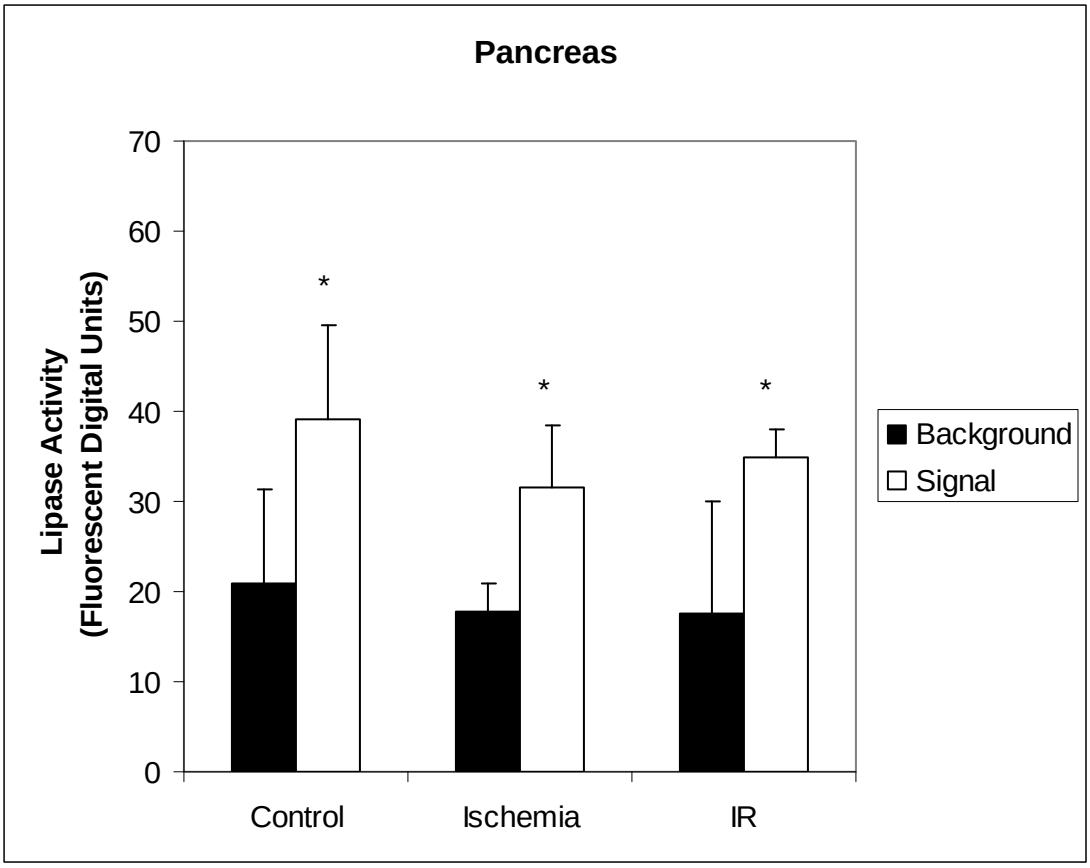


**Figure 2.33:** Lipase activity, expressed in fluorescent digital units, of non-ischemic control rat pancreas, ischemic pancreas, and pancreas exposed to ischemia followed by reperfusion. Error bars are mean ± SD, N = 5 for control and ischemia group, N = 4 for ischemia-reperfusion group, * $p<0.0.5$ compared to background in a paired t-test

# Ch 2.3 Discussion

### Ch 2.3.1 *In Situ* Zymography – Protease

It can be seen that following ischemia-reperfusion of the splanchnic region, there is indeed an increase of average protease activity in the pancreas and wall of the intestine. This result is consistent with previously published results[32]. However, the increase in protease activity is not statistically significant in the intestine during ischemia. This may be explained because the segment of intestine chosen was near the distal end of the small intestine, so proteases in the lumen may have less activity compared to more proximal segments due to autodigestion of proteases over time. Thus, during ischemia, fewer active proteases might enter the intestinal wall in a distal segment versus a proximal one.

In the pancreas, protease activity during ischemia was found to be significantly upregulated using the Mann-Whitney test, but not the t-test. The Mann-Whitney test is probably more accurate for this sample size, but this discrepancy is still indicative of the large standard deviation of protease activity in the pancreas in this set of measurements. This could be due to the large spatial heterogeneity of the pancreas and the anatomical differences in blood flow to the pancreas from animal to animal. Thus, some tissue regions in the pancreas had activated protease activities, while others remained in an inactivated form similar to that of the control state.

Both the intestine and pancreas have constitutive protease activity even under control situations. This indicates that if enzymatic activity plays an important role in the development of multiple organ failure, the upregulation, rather than the mere presence, of this activity is central to the process. The significant increase in protease activity

following reperfusion, however, provides supporting evidence for the hypothesis that inflammatory mediators entering the bloodstream through the splanchnic circulation may be protease-generated.

The current study also revealed that the lung had constitutive protease activity. However, there was no significant difference in this activity between any of the three groups. This provides evidence that protease activity may not be critical to the damage of the lung during ischemia-reperfusion injury. To check for lung injury, hematoxylin and eosin staining of lung tissue from each of the three groups was performed. Damage can be observed by way of the increased pink eosin staining for cell cytoplasm in both the ischemic and ischemia-reperfusion group tissues, indicating a swelling of alveolar cells (Figure 2.34).

In the heart, it was seen that the ischemic and ischemia-reperfusion group had protease activity, but the control group had no protease activity. However, there was no significant difference between these three groups. This somewhat contradictory result is indicative of borderline significance and might be improved with greater statistical power in future studies.

Finally, the brain, kidney, and liver, did not exhibit protease activity, and there was no difference between any of the three groups for these organs. This indicates that if protease activity is present in these tissues, it is not detectable using the chosen enzyme substrate (matrix metalloproteinases, for example, are less likely to cleave casein as a substrate than other substrates, like gelatin). More likely, localized protease activity in these organs does not play a significant role in their injury during ischemia-reperfusion injury.

These results are consistent with the observation that the inhibition of pancreatic enzymes in the bloodstream is not effective in preventing the symptoms of shock resulting from splanchnic arterial occlusion[34]. If protease activity in the bloodstream is not significantly elevated in the escalation of the inflammatory cascade, then inhibition of protease activity in the bloodstream would likely produce minimal effects.

**Ch 2.3.2 *In Situ* Zymography – Lipase**

The lipid fraction of intestinal homogenate is cytotoxic, and the cytotoxic mediators are free fatty acids released from their esterified states by lipases[38]. Therefore, determining if lipase activity in secondary organs is affected by splanchnic ischemia-reperfusion injury is important. If lipase activity levels were increased following splanchnic ischemia-reperfusion, it would provide evidence that an increased level of free fatty acids is generated following splanchnic ischemia-reperfusion, which, in the presence of proteases, may result in cytotoxicity and organ failure. However, my study found no change in lipase activity in any of the studied organs as a result of splanchnic ischemia-reperfusion. It would therefore seem that a change in lipase activity in either splanchnic or secondary organs may not be important to the progression of inflammation and multiple organ failure in intestinal ischemia produced by superior artery occlusion.

The results also show that there is the potential for significant constitutive lipase activity in all of the organs. This constitutive lipase activity may indicate that free fatty acids may be constantly produced, regardless of the physiologic state of the organ. Esterified fatty acids are probably compartmentalized separately from the lipases, though it was difficult to see any potential compartmentalization of lipase due to diffusion of the lipase substrate after cleavage. Also, to prevent free fatty acid cytotoxicity, free fatty

acids are also bound to proteins such as albumin under normal physiologic conditions. It has been shown that when albumin is combined with a lipid fraction, the cytotoxic effects of the lipid fraction are attenuated[39]. Hence, if free fatty acids do indeed play an important role in cell death, shock, it may not be their creation, but their distribution within the body and state of binding to proteins that is important.

Another possibility is that while there is the potential for significant constitutive lipase activity in the organs, under normal physiologic conditions, there is a lack of esterified fatty acids for these lipases to act on. It is possible that splanchnic ischemia-reperfusion injury may increase the availability of esterified fatty acids, allowing lipases to generate free fatty acids and induce cell death.

**Ch 2.3.3 Experimental Procedure**

While the results in this experiment cannot be discounted, the experimental procedure must be taken into account when evaluating these findings. As the goal of this investigation was to discover whether enzymatic activity in secondary organs is an early trigger of the inflammatory cascade, the period of reperfusion before organ harvest was 30 minutes. However, death following a 90 minute ischemic period has been observed by others in the lab to occur 1-3 hours following the start of reperfusion. Hence, these findings pertain to the early stages of reperfusion before cytokine and endotoxin release[40].

Complete occlusion of the splanchnic region through splanchnic arterial occlusion, while not representative of every type of shock, is representative of certain situations, such as transplant, surgery, and trauma to the splanchnic region. This shock model, therefore, represents a severe form of shock.

The sensitivity of *in situ* zymography must be taken into effect. An estimate of the sensitivity of the protease assay with the addition of pure chymotrypsin indicates that fluorescence disappears between 0.1 – 0.01 μg/ml purified chymotrypsin. Hence, protease activity below this level would be undetectable with the current technique.

Finally, the areas of tissue harvest for the organs tested in this study were chosen for consistency. It is possible that untested areas of the organs in this study have changes in enzymatic activity following splanchnic ischemia-reperfusion.

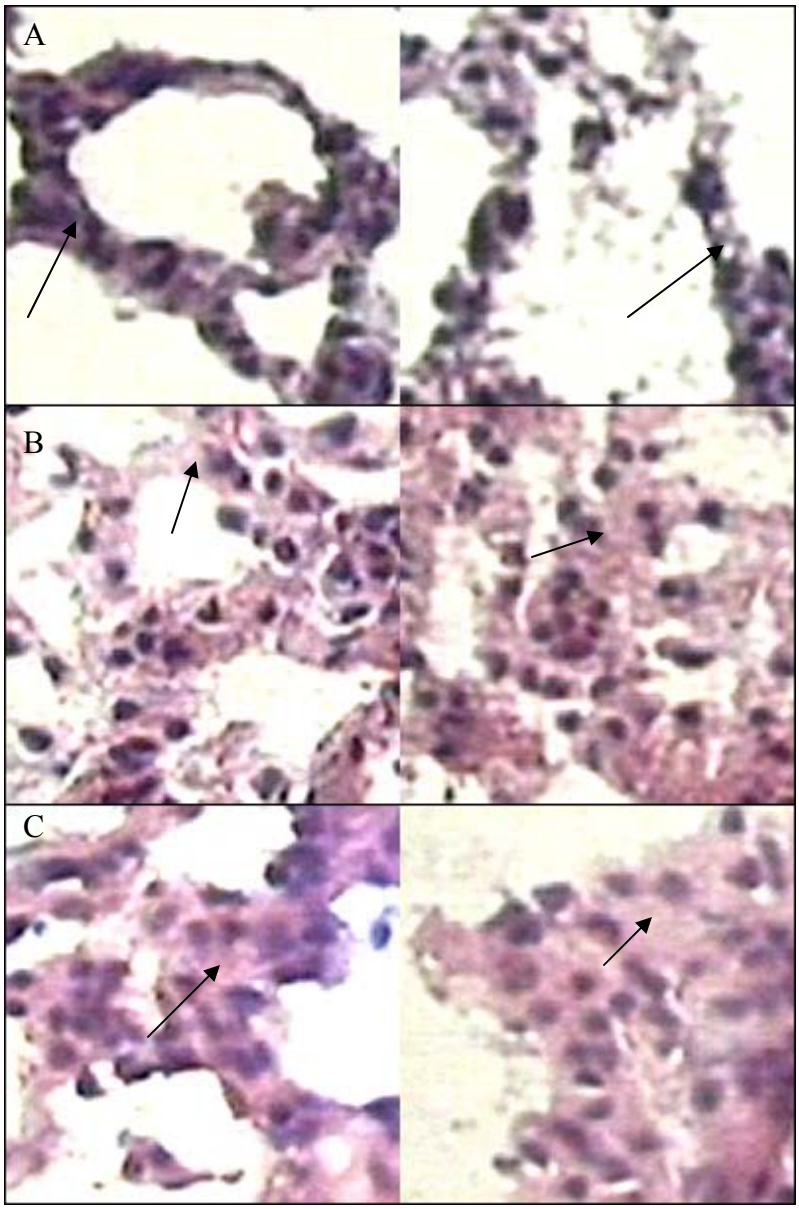


**Figure 2.34:** Hematoxylin and eosin stain of lung tissue from control (A), ischemia (B), and ischemia-reperfusion (C) groups. Arrows indicate alveolar cells; it can be noted that in the ischemia and ischemia-reperfusion groups, there is an increase in eosin staining, indicating swelling and injury of these cells.

## Ch 2.4 Conclusion

This study suggests that ischemia-reperfusion by occlusion of the superior mesentery and ciliac arteries does in fact enhance enzymatic activity in the intestine and the pancreas but does not significantly change the enzymatic activity in secondary, non-ischemic organs. Lipase activity is not changed in any organ following ischemia-reperfusion, and protease activity is only upregulated in the intestine and pancreas following ischemia-reperfusion, but has variable levels after ischemia. These findings contradict our hypothesis that ischemia-reperfusion may alter the enzymatic activity of some secondary organs, inducing local tissue damage and inflammation. The findings suggest that damage to secondary organs following splanchnic ischemia-reperfusion may be due to exogenous inflammatory mediators likely produced in the splanchnic region, rather than by localized protease or lipase activity.

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