ERYTHROPOIETIN PROMOTES FUNCTIONAL RECOVERY VIA ANTI-APOPTOTIC MECHANISMS IN MICE UNILATERAL URETERAL OBSTRUCTION

by

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Erythropoietin promotes functional recovery via anti-apoptotic mechanisms in mice unilateral ureteral obstruction

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Abstract

The ureter transfers urine from the kidney through sequential contractions, called peristalsis. When obstructed, accumulated urine builds up high pressure which leads to dysfunction in the urological tracts. Our laboratory showed that, in mice, ureteral peristalsis is not recovered for 10 days after removing a 24-hour obstruction. Delayed ureteral recovery affects the kidney negatively as the ureter cannot transport urine properly. Studies have shown that erythropoietin (EPO), a hematopoietic hormone, protects different organs against various injuries mainly by suppressing apoptosis, via EPO receptor (EPOR) and β-common receptor (βCR) heterodimers. Our laboratory showed that prophylactic EPO treatment of obstructed mice accelerated recovery of the ureter and the kidney following the reversal of ureteral obstruction. We hypothesized that EPO treatment promotes functional recovery of the ureter and the kidney via anti-apoptotic mechanisms. The objective of this study was to investigate EPO-induced mechanisms in accelerating recovery from ureteral obstruction in 2 mice strains.

Unilateral ureteral obstruction was created for 24, 48, 72 hours using non-traumatic micro-clip (n=10). EPO was administered daily for 4 days either prophylactically or concomitantly with ureteral obstruction. TUNEL assay and immunohistochemistry with phospho-NF-κB p65 and phospho-STAT5 antibodies on ureteral tissues and qRT-PCR with primers specific to EPO, EPOR, βCR, STAT5A, BCL-2, BCL-XL, BAX and NF-κB on ureteral and renal tissues were performed. Our study showed that ureteral obstruction decreased ureteral peristalsis and increased apoptosis in 72-hour obstructed ureters. Ureteral obstruction decreased anti-apoptotic EPOR-βCR signaling and increased phospho-NF-κB p65. EPO treatment on ureteral obstruction

improved ureteral function and suppressed apoptosis in obstructed ureters, by suppressing NF-κB and decreasing apoptotic BAX. EPO treatment did not induce erythropoiesis in our study, which supports that EPO's protective effect is a separate mechanism from increased blood circulation by hematopoiesis. Also, EPO treatment without obstruction did not change EPOR-βCR signaling.

In conclusion, ureteral obstruction increased apoptosis in ureteral tissues and decreased anti-apoptotic EPOR signaling with increased phospho-NF-κB p65, along with obstruction induced ureteral dysfunction. EPO treatment improved ureteral peristalsis and suppressed ureteral apoptosis, via suppression of NF-κB activation and decreased expression of BAX that compensated for the decreased expression of BCL-2 and BCL-XL by obstruction.

Lay Summary

The ureters are actively moving tubes to transfer urine produced in the kidney to the bladder. When a ureter is blocked by kidney stones, enlarged prostate (BPH) or cancer etc., urine cannot be excreted from the kidneys and the ureters, which damages their normal functioning. Even a brief obstruction (24 hours) in mice requires 10 days recovery to regain normal function. Erythropoietin (EPO) is a hormone that increases red blood cells to help blood circulation. Additionally, EPO protects tissues against various adverse conditions. Our team previously showed that EPO treatment accelerated the recovery after removing ureteral blockage. Current study showed that ureteral cells actively die in a process called apoptosis when the ureter is blocked. Ureteral blockage also decreased EPO-related 'protector genes', which makes cells more prone to death. EPO treatment helped maintain the ureteral movement and rescued ureteral cells from active death during ureteral blockage by decreasing death-related factors.

Preface

This thesis was compiled under the guidance of Dr. Dirk Lange at The Stone Centre at Vancouver General Hospital. Dr. Claudia Janssen devised the original concept for the research. Under the supervision of Dr. Dirk Lange, I was responsible for designing the experiments, performing them, analyzing the results and writing the manuscripts.

All animal studies have been conducted under the close supervision of the University of British Columbia Animal Care Committee and under the protocol number A16-0254.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

α-SMA: α-Smooth Muscle Actin

βFGF: Basic Fibroblast Growth Factor

βCR: β Common Receptor

BAD: BCL-2-associated Death Promoter

BAK: BCL-2 Homologous Antagonist Killer

BAX: BCL-2-Associated X Protein

BCL-2: B-Cell Lymphoma 2

BCL-3: B-Cell Lymphoma 3

BCL-XL: B-Cell Lymphoma-Extra Large

CIAP: Cellular Inhibitor of Apoptosis Protein

ED-1: Cluster of Differentiation 68

p16INK4a: Cyclin Dependent Kinase Inhibitor 2A

EPO: Erythropoietin

EPOR: Erythropoietin Receptor

FLIP: FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein

GSK-3β: Glycogen Synthase Kinase 3β

GM-CSF: Granulocyte-Macrophage Colony-Stimulating Factor

IFN-γ: Interferon γ

IL: Interleukin

ICC-like cell: Interstitial Cells of Cajal-like cell

JAK 2: Janus Kinase 2

MCP-1: Monocyte Chemoattractant Protein-1

NF-κB: Nuclear Factor Kappa-light-chain-enhancer of Activated B cells

PI3-K: Phosphatidyl Inositol-4,5-bisphosphate 3-Kinase

RAS: Rat Sarcoma

SRC: Sarcoma

S100A4: S100 Calcium-binding Protein A4

STAT: Signal Transducer and Activator of Transcription

Smad: Homologues of the Drosophila protein, Mothers Against Decapentaplegic (Mad) and the Caenorhabditis Elegans Protein Sma

TUNEL assay: Terminal deoxynucleotidyl Transferase (TdT) dUTP Nick-End Labeling assay

TGF- β: Transforming Growth Factor β

TNF-α: Tumor Necrosis Factor-α

XIAP: X-Linked Inhibitor of Apoptosis Protein

Acknowledgements

I offer my sincere gratitude to my supervisor, Dr. Dirk Lange for taking on me and guiding me through the journey of science with a huge encouragement and enthusiasm.

I would like to extend my thanks to my committee member, Dr. Michael E. Cox and Dr. Ralph Buttyan, who always provided invaluable feedback and supported me throughout my research. I am greatly appreciative for my hard-working lab members at The Stone Centre at Vancouver General Hospital and incredible people at Vancouver Prostate Centre.

Special thanks are owed to my family and friends, who provided endless support throughout my journey.

Dedication

To the peaceful science and better world.

Chapter 1: Introduction

1.1 Ureteral obstruction

The ureter is a tube-like structure through which urine passes from the kidney to the bladder. Ureteral obstruction is a blockage in the ureter that prevents kidneys from excreting urine. Ureteral obstruction can be caused by various pathological causes including kidney stone diseases, tumor, fibrosis, benign prostate hyperplasia and strictures.

1.1.1 Kidney stone disease

Kidney stone disease is highly prevalent in both United States and Canada[1]. As many as 1 in 11 individuals in the United States will experience kidney stones in their lifetime[2]. Approximately 50% of these patients will have stone recurrence within 10 years[3]. Government reports that direct health care expenditures related to kidney stone management are as high as $5 billion USD[1]. Kidney stones present when crystal-forming substrates level in plasma and subsequently urine is too high, resulting in them not properly excreted. Accumulation of these substrates in urine results in crystal formation that cause blockages within the urinary tract. Currently, the exact mechanisms of stone formation in humans is still unknown. However, once blockage by kidney stones occurs, high pressure in the kidney is built up which damages its function. Untreated blockage can result in urosepsis and kidney failure.

1.1.2 Ureteral peristalsis

The ureters move urine from the kidneys into the bladder using a series of wave-like muscle contractions called peristalsis. Peristalsis also occur in the esophagus, stomach, and intestines to move food to different processing centres. Atypical smooth muscle cells located at the proximal renal pelvis function as autonomous pacemakers to start peristalsis throughout the ureters[4]. In addition, ICC-like cells (ICC-LCs), which works as a pacemaker in intestinal peristalsis, are also engaged in coordinating the ureteral peristalsis[5]. ICC-LCs are the primary pacemaker cells driving slow wave generation with the ability to start spontaneous calcium ion transient. ICC-LCs are located at the lamina propria of the renal pelvis and pelvi-calyceal junction in proximal ureter and more dispersed population are found in smooth muscle layer and lamina propria within the ureter[6] (Figure 1-1). The depolarizing pacemaker potentials generated within ICC-LC and atypical smooth muscle cells propagate to the neighboring typical smooth muscles and renal interstitial cells. It initiates vigorous contraction that is propagated to the uretero-vesical junction as a peristaltic wave, which were conducted by the smooth muscle cells within the ureters toward the bladder (reviewed by Lang et al.[7]).

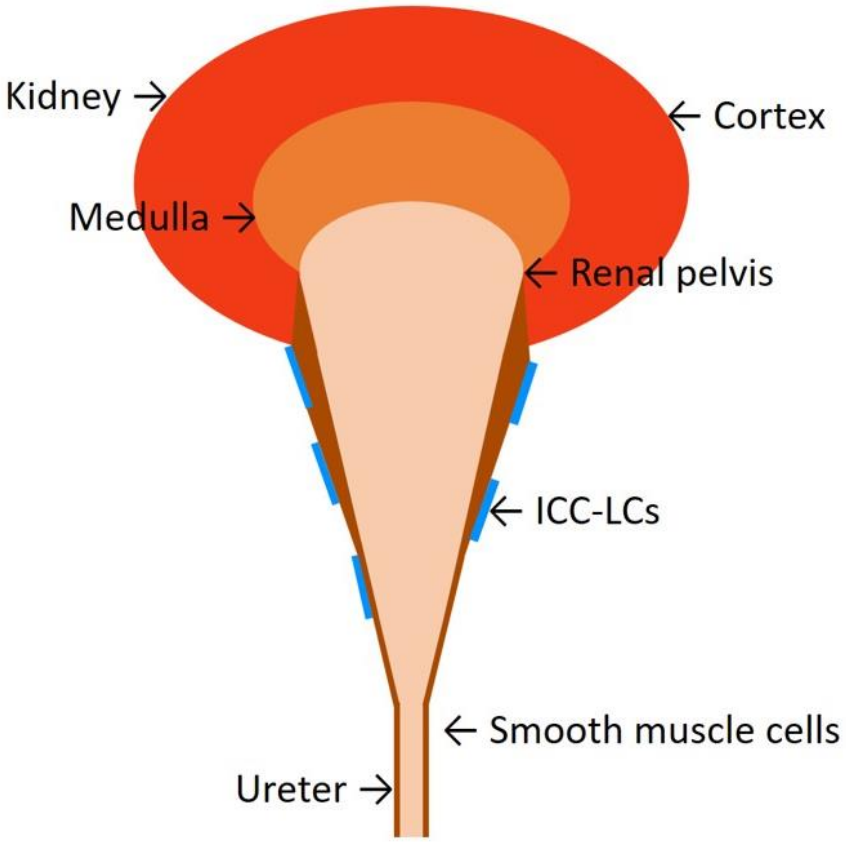


Figure 1-1 Location of ICC-like cells

ICC-LCs are located at the lamina propria of the renal pelvis and pelvi-calyceal junction in proximal ureter and more dispersed population are found in smooth muscle layer. Blue color represents ICC-LCs, Brown color represents smooth muscle cells. Red color represents renal cortex. Orange color represents renal medulla. Light orange color represents renal pelvis and the ureter.

1.1.3 Pathophysiology of ureteral obstruction

Ureteral obstruction results from various pathologies including tumor, fibrosis, benign prostate hyperplasia, and strictures as well as kidney stones. As the ureters are obstructed, ureteral and renal pressure rises quickly and leads to ureteral smooth muscle spasm and inflammation[8].

Ureteral obstruction disrupts ureteral peristaltic movement. Acute obstruction increases the amplitude of pressure waves and the frequency of contractions as a compensatory mechanism (Reviewed by Rose et al.[9]). However, prolonged obstruction eventually abolishes the contractility and the frequency of ureteral movement[10]. Even after the obstruction is removed, peristaltic activity is slow to return, resulting in prolonged damage to the upper urinary tract despite having resolved hydronephrosis[11]. Chronic ureteral obstruction changes the structure of the renal pelvis and ureter. If the obstruction persists, the ureteral wall deforms by increased thickness of the muscular layer with a corresponding increase of connective tissue[12]. The renal pelvis gets deformed because of hypertrophy and fibrosis of the muscular layer[13]. The tight junctions between ureteral smooth muscle cells are infiltrated by increasing amounts of connective tissues and collagens accompanying rarefication and disorientation of nerve fibres[14]. This structural deformation decreases the contractile properties of ureteral smooth muscles to continue its peristaltic movement, which worsens the condition in addition to the effects of obstruction blocking the flow of urine from kidney and disrupting peristaltic function of the ureter.

Ureteral obstruction accumulates the urine inside the upper urinary tracts which builds up high pressure leading to severe dilation of the lumen pressing the ureteral walls. The mechanical stretch on the dilated ureteral walls and renal tubules increases apoptosis via activating caspase 3[15]. Increased apoptosis leads to dysfunction of the urinary tracts. Ureteral obstruction activates multiple factors including angiotensin II, TGF-β, TNF-α, reactive oxygen species, epidermal growth factors (EGF) and several other cytokines (reviewed by Chevalier et al.[16]). These factors result in mitochondrial destabilization and the release of cytochrome C, which triggers the

caspase-mediated apoptotic pathway[17]. The release of cytochrome C is also promoted by the downregulation of anti-apoptotic protein BCL-2 from ureteral obstruction[18]. These studies shows that pathology of ureteral obstruction is related to apoptotic signaling.

1.1.4 Apoptosis in ureteral obstruction

Apoptosis refers to a process of programmed cell death that mediates the controlled deletion of unwanted cells. Increased cell proliferation and apoptosis in the obstructive nephropathy response has been shown in the studies[19]. Apoptosis contributes to atrophy and fibrosis in different types of renal diseases[20,21].

Studies using murine models of unilateral ureteral obstruction have shown apoptosis of renal tissue measured as early as 3 days of obstruction[19,22]. However, most the studies on apoptosis induced by ureteral obstruction focused only on the effects in renal tissue and there has only been one study to date looking at apoptosis in ureteral tissues after unilateral ureteral obstruction[23]. Similarly, functional studies on the effect of obstruction and its recovery after removal focused on the kidney, whereas no study was performed on the recovery of ureteral peristaltic function. Ureteral peristaltic function is very important in the recovery of kidney function after transient obstruction. Ureteral peristaltic activity decreases the pressure on the kidney after the removal of obstruction by excreting urine efficiently. And if the ureter cannot function properly, the kidney is subject to abnormal retrograde pressure. Increased apoptosis in ureteral tissues might be related to ureteral dysfunction in peristaltic activity.

1.1.5 Current drug of choice for ureteral obstruction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are most commonly used to decrease inflammation and pain resulting from ureteral obstruction. As the pain of obstruction is related to over-activated ureteral muscular contraction, drugs able to relax the smooth muscle are also used. These drugs have been also used to promote spontaneous stone passage[24]. Antispasmodic drugs such as anti-muscarinic medications are used for this therapeutic purpose[25]. Calcium is necessary to develop the action potential within muscular tissue and is therefore required for smooth muscle contraction of the ureter. Calcium-channel blockers prevent calcium influx and have an inhibitory effect on ureteral function which facilitates relaxation of the smooth muscle[26]. As the autonomic nervous system modulates ureteric activity, α-adrenergic antagonists are effective promoters of kidney stone passage[27,28].

The current drug regimen for ureteral obstruction is aimed at decreasing over-activated ureteral contraction to pass stones and relieve the pain, although the efficiency of these drugs in facilitating spontaneous stone passage is controversial[29]. Medications that are used to relax ureteral smooth muscle are not as effective as desired in passing stones and could negatively affect the recovery of normal ureteral peristaltic function after the obstruction is removed. Meanwhile, delayed recovery of ureteral peristaltic function after transient obstruction would provide negative pressure in normal functioning of the kidney. Currently, there is no drug of choice to accelerate the recovery of ureteral function after removing the obstruction. Previous study in our laboratory showed that erythropoietin (EPO) is effective in decreasing renal injury

and accelerating functional recovery of the ureter and the kidney after removal of obstruction in murine model of unilateral ureteral obstruction.

1.2 Erythropoietin

Erythropoietin (EPO) is a 165-amino-acid protein (MW 30.4 kDa) and a member of the type I cytokine superfamily. EPO is a hormone that induces hematopoiesis in our body. In addition, studies have shown that it has a non-hematopoietic function, which is decreasing the degree of injury and accelerating recovery following injuries in different organs.

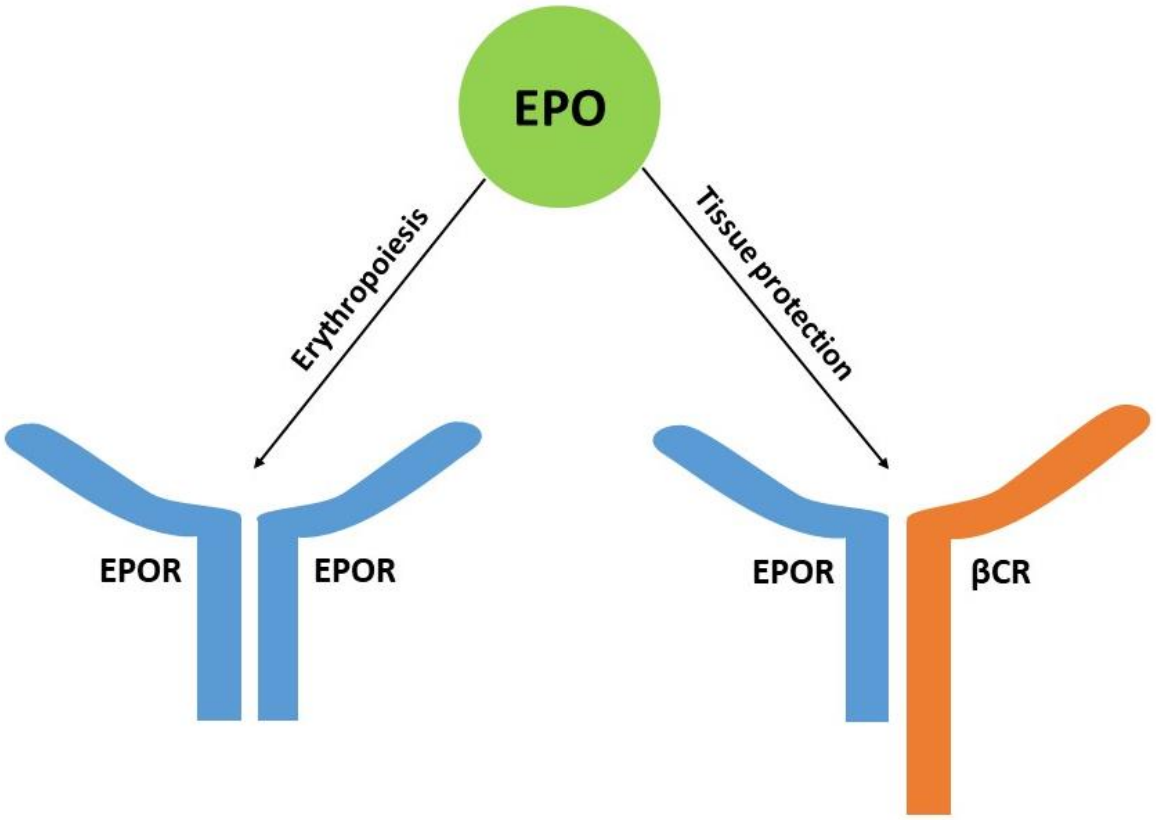


Figure 1-2 Erythropoietic and protective function of Erythropoietin (EPO) via 2 different receptor dimers

EPO has 2 different receptor dimers that it binds to induce different functions. When EPO binds to the EPO receptor homodimers, it induces erythropoiesis by suppressing apoptosis of erythroid progenitors as well as promoting differentiation and proliferation in these cells. When EPO binds to the EPO receptor and β common receptor (βCR) heterodimer, it induces protective function against various injuries in different cell types

1.2.1 Hematopoietic function of Erythropoietin

The kidney is the main organ that produces erythropoietin (EPO) to increase erythrocytes (red blood cells) in response to hypoxia in the blood stream. Peritubular capillaries in renal cortex senses the decreased oxygen concentration and produce EPO (reviewed by Bauer et al.[30]). EPO receptor binding leads to intracellular activation of JAK2[31], which transmits a signal to promote

cell survival, differentiation and proliferation, via phosphatidylinositol 3-kinase[32], STATs 1, 3, 5A, and 5B[33], RAS[34] and other transcriptional factors (reviewed by Fisher et al.[35])

EPO acts primarily to rescue erythroid progenitors (pre-erythrocyte) from apoptosis[35]. In the absence of EPO, erythroid progenitors accumulate DNA cleavage process, which leads to apoptosis[36]. EPO promotes the survival of erythroid progenitor and its differentiation to reticulocytes through increasing the expression of the anti-apoptotic proteins BCL-2 and BCL-XL[37-39].

1.2.2 Non-hematopoietic function of erythropoietin

Recent evidence suggests that EPO has protective effects on nonhematopoietic tissues in different injuries. Early studies showed that mice lacking EPO or EPO receptor (EPOR) showed defects in brain, liver, heart and vascular network with increased apoptosis suggesting additional functions of EPO in normal development of different organs[40,41]. Later studies suggested that non-hematopoietic tissues express and/or produce EPO and EPOR including the brain[42], kidney, eyes[43], heart[44], smooth muscle cells[45] and endothelial cells[46]. Expression in non-hematopoietic tissues suggest EPO has a role as a paracrine and autocrine factor on the surrounding tissues beyond its hematopoietic function. Research has shown that EPO provides protection against various types of injuries including ischemia[47,48], toxic substances[49,50], diabetes[51], oxidative stress[52], inflammation[53], edema[54] and apoptosis[48]. Also, EPO showed additional function in normal tissues including angiogenesis[55], proliferation[56], calcium influx[57] and vasoconstriction[58].

EPO's protective function is mediated through EPOR and β common receptor (βCR) heterodimers whereas hematopoiesis is mediated through EPOR homodimers[59]. Knockdown of βCR abolishes the protective effects provided by EPO[59,60], while erythropoiesis is maintained[61]. Similarly, EPO derivatives that do not bind to EPOR homodimers still provide protection against injuries[62,63]. These studies support that EPO's protective function is mediated by EPOR-βCR heterodimers. (Figure 1-2)

EPO binds to EPOR-βCR heterodimers with different affinity from the EPOR homodimers. The EPOR homodimers has an affinity of 100-200 pmol/L[64], whereas EPOR-βCR has an affinity of 1-20 nmol/L[57]. This suggests that much higher concentration of EPO is needed to activate the EPOR-βCR heterodimers than for activation of the EPOR homodimers. Given that EPO's concentration in the blood stream is 2.0-8.3 pmol/L[65,66], EPOR-βCR heterodimers cannot be activated by EPO from the blood stream, but may require the production of EPO from the injured tissues themselves for self-protection. External EPO administration boosts this self-protective pathway to decrease the effects of injury.

1.2.3 Anti-apoptotic role of Erythropoietin

EPO's primary role is to block apoptosis of erythroid progenitor cells to increase erythroid cells in response to hypoxia[35]. Many studies have shown that the anti-apoptotic function of erythropoietin is not limited to erythropoiesis, but also provides a protective function against injuries in different tissues including brain[48,67,68], spinal cord[69], heart[70,71], skin[72,73], eye[74], intestine[75-77], muscles[78,79] and kidney[47,80]. This implies that EPO acts as an anti-apoptotic factor

in addition to its role in hematopoiesis. Cancer cells use EPO signaling to decrease apoptosis and increase proliferation to increase their survival (reviewed by Hardee et al.[81]). Blocking EPO signaling in these cells leads to increased apoptosis and delayed tumor growth[82-84].

EPO has been shown to block apoptosis by modulating the expression of the BCL-2 family of apoptosis-related regulators, which include both anti-apoptotic BCL-2, BCL-XL and pro-apoptotic BAX, BAD, BAK (reviewed by Ghezzi et al.[85]). The balance between anti-apoptotic regulators and pro-apoptotic regulators is an on/off switch to determine the propensity for apoptosis in a given cell. EPO has been shown to prevent injury-induced apoptosis via its effects on BCL-2 family of apoptosis-related genes in various diseased organs including erythroid progenitors[86], brain[87-89], heart[90,91], liver[92,93], intestine[77] and kidney[80,94]. (Figure 1-3)

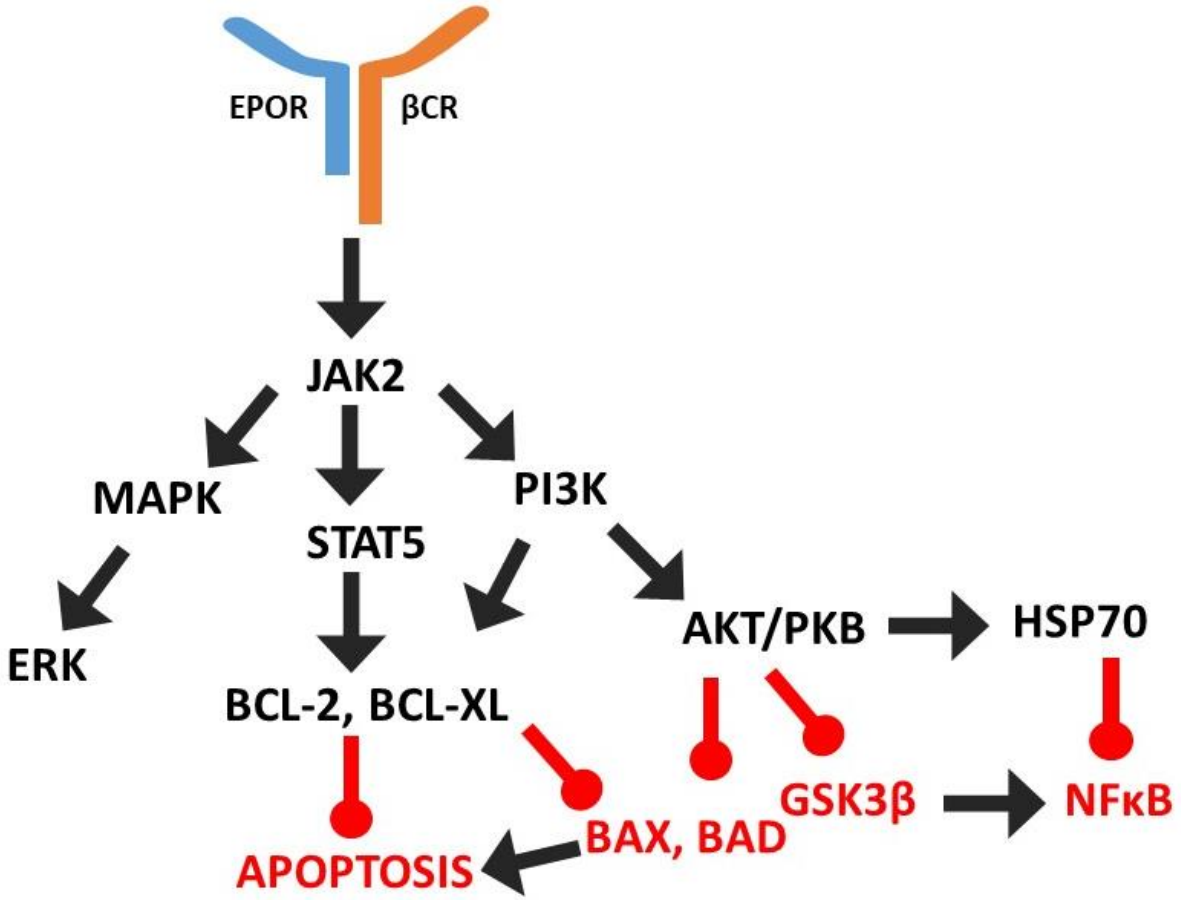


Figure 1-3 EPOR-βCR signaling pathway

When EPO binds to EPOR-βCR, it induces various signaling including JAK2, STAT5, PI3-K, MAPK. EPO's primary role in protecting tissues against various injury is by suppressing apoptosis. We have chosen to investigate EPO, EPOR, βCR, STAT5, BCL-2, BCL-XL, BAX and NF-κB. EPOR-EPOR signaling shows similar signaling pathway as EPOR-βCR signaling.

1.2.4 β common receptor (CD131) – EPO receptor heterodimer

β common receptor (also known as common β subunit) is a common subunit that forms a complex with the GM-CSF receptor, IL-3 receptor or IL-5 receptor. GM-CSF receptor, IL-3 receptor and IL-5 receptor, as well as EPOR belong to type 1 cytokine receptor. GM-CSF

receptor, IL-3 receptor and IL-5 receptor work as an α subunit of the complex that provides ligand specificity to the receptor complex, whereas βCR is a common subunit to all of the receptor complexes[95]. According to studies using EPO derivatives that do not bind to EPOR homodimers, EPOR-βCR heterodimer showed similar signaling pathways to those used in the hematopoietic system by EPOR homodimers[96-99].

1.2.5 Erythropoietin effects on peristaltic dysfunction

Peristalsis occurs as a series of constriction and relaxation of smooth muscle cells to transport the contents with wave-like contractions. Several studies have shown that EPO is effective against various injury models including inflammatory bowel disease[100], ischemia/reperfusion injury[75] and necrotizing enterocolitis[101] in the intestine, which is another organ where peristaltic activity exists. Moreover, EPO treatment was effective in restoring bowel damage and peristaltic activity in the hypocontractile intestine[102]. Our laboratory has previously shown that EPO accelerated the recovery of peristaltic activity of ureteral smooth muscle cells in the mouse ureters, as well as deceased renal injury after ureteral obstruction[11]. Additionally, EPO was shown to directly affect smooth muscle function in vascular cells[103] as well as renal mesangial cells[104]. This shows EPO's function on improving dysfunction of peristalsis in the smooth muscles of different organs.

1.2.6 Erythropoietin and ureteral obstruction

Several studies have shown that EPO protects the kidney against the injury from ureteral obstruction by decreasing inflammation, interstitial fibrosis, and tubular apoptosis. Srisawat et al.

showed that EPO decreased apoptotic cell death in the renal cortical interstitium shown by TUNEL assay, collagen, α-SMA (a marker for renal dysfunction and fibrogenesis), ED1-positive cells and TGF-β1 after 14 days of ureteral obstruction[105]. Kitamura et al. used carbamylated EPO, non-hematopoietic EPO analog that can only bind to EPOR-βCR, and carbamylated EPO treatment was shown to decrease tubular apoptosis and α-SMA as similarly as EPO treatment, which were increased after 7 days of ureteral obstruction[106]. Chang et al. showed that EPO decreased apoptotic cell death shown by TUNEL assay, TGF-β, TNF- α, MCP-1, osteopontin, FAS, caspase-3 and increased BCL-2 after 3 days of ureteral obstruction[107]. Park et al. showed that EPO decreased f TGF- β1 and α-SMA and E-cadherin after 14 days of ureteral obstruction[108]. Acikgoz et al. showed that EPO decreased TGF-β, fibronectin, NF-κB and β-FGF after 14 days of ureteral obstruction[109]. Tasanarong et al. showed that EPO decreased TGF-β, Smad2/3, S100A4, and p16INK4a[110]. These studies demonstrate the protective effect of EPO on decreasing inflammatory and apoptotic signaling against obstructive nephropathy. Previous study in our laboratory is the first study examining the effect of EPO on ureteral dysfunction[11].

1.3 Hypothesis

Previous studies from our laboratory[11] showed that EPO treatment accelerated the recovery of ureteral peristaltic function and hydronephrosis as well as decreased the degree of hydronphrosis following the reversal of ureteral obstruction. Studies have shown that EPO's primary role in protecting tissues against a variety of injuries is via decreasing apoptosis from the injury. In addition, previous research has shown that ureteral obstruction leads to apoptosis in the kidney and the ureters. *We hypothesized that EPO administration promotes ureteral peristaltic recovery*

from the obstruction via activation of anti-apoptotic mechanisms. The objective of this study was to investigate the EPO-induced mechanisms that promote accelerated recovery following obstruction with specific emphasis on its role as a regulator of apoptotic genes. EPO administration accelerated the functional recovery of ureters and kidneys from injury caused by ureteral obstruction.

1.4 Aims

Aim 1: Evaluate apoptosis of cellular components in the ureter after ureteral obstruction and studying the effect of EPO treatment on suppressing apoptosis following ureteral obstruction.

Aim 2: Elucidate EPOR-βCR signaling following EPO treatment and the possible link to protection of ureteral and renal tissues against the injuries from ureteral obstruction.

Aim 3: Investigate EPOR-βCR signaling in the ureteral and renal tissues after ureteral obstruction and compare the difference in genetic response in 2 strains of mice.

Aim 4: Evaluate the effect of prophylactic EPO treatment on pre-conditioning without potential injury.

Chapter 2: Ureteral obstruction increased apoptosis with decreased EPO receptor signaling

2.1 Introduction

In order to study the effect of obstruction on EPOR-βCR signaling in the ureters without EPO treatment, we investigated EPOR signaling and ureteral apoptosis in 24, 48, 72-hour ureteral obstruction without EPO treatment. We examined the role of endogenous EPOR signaling and its physiological role in the ureter in response to ureteral obstruction. Following the finding of suppressed EPOR anti-apoptotic pathway by obstruction, apoptosis in the ureteral tissues by obstruction was evaluated. *We hypothesized that ureteral obstruction activates apoptotic signaling and increases apoptosis in the ureter.*

2.2 Methods

2.2.1 Animals

Wild-type female mice between 8- and 10-week of age were used for the experiment. CD-1 mice were purchased from Harlan laboratories (Indianapolis, IN, USA) and C57BL/6 were from a breeding protocol (protocol number: A14-0093) approved by University of British Columbia. The experimental protocols were approved by the institutional animal care and use committee. All animals were housed in the Jack Bell Research Centre animal facility (Vancouver, BC,

Canada) and nursed under supervision of a veterinarian. A total of 60 mice were used in this experiment. The experiment was divided into 2 independent sets using 5 mice per each set.

2.2.2 Unilateral ureteral obstruction model

Mice received inhalational anesthesia with 2 % isoflurane and was placed supine on a heating pad. Limbs were fixed with laboratory tape followed by preoperative administration of 5 mg/kg meloxicam, 0.05 mg/kg bupivacaine, 6 mg/kg buprenorphine subcutaneously for pain management. After confirming the proper depth of anesthesia, a median laparotomy was performed under sterile conditions. The intestine was moved to the side onto sterile gauze soaked in warm saline using sterile cotton swabs. After exposing the ureters, peristaltic activity was examined with a MZ6 microscope (Leica Microsystems, Concord, ON, Canada) and quantified bilaterally 1-minute intervals each before placing a nontraumatic RS-6470 vascular clip (Roboz Surgical Instrument Co., Gaithersburg, MD, USA) on the distal end of 1 ureter (Figure 2-1). Care was taken not to manipulate the ureter before clip placement to eliminate negative effects on peristalsis before unilateral ureteral obstruction. The intestine was replaced and the abdomen was closed in 2 layers with 4-zero Vicryl absorbable sutures (Ethicon Inc., Somerville, NJ, USA). Virox was applied to the abdominal wound. The same surgical approach was performed to remove the clip at 24, 48 and 72 hours in 10 mice per each strain.

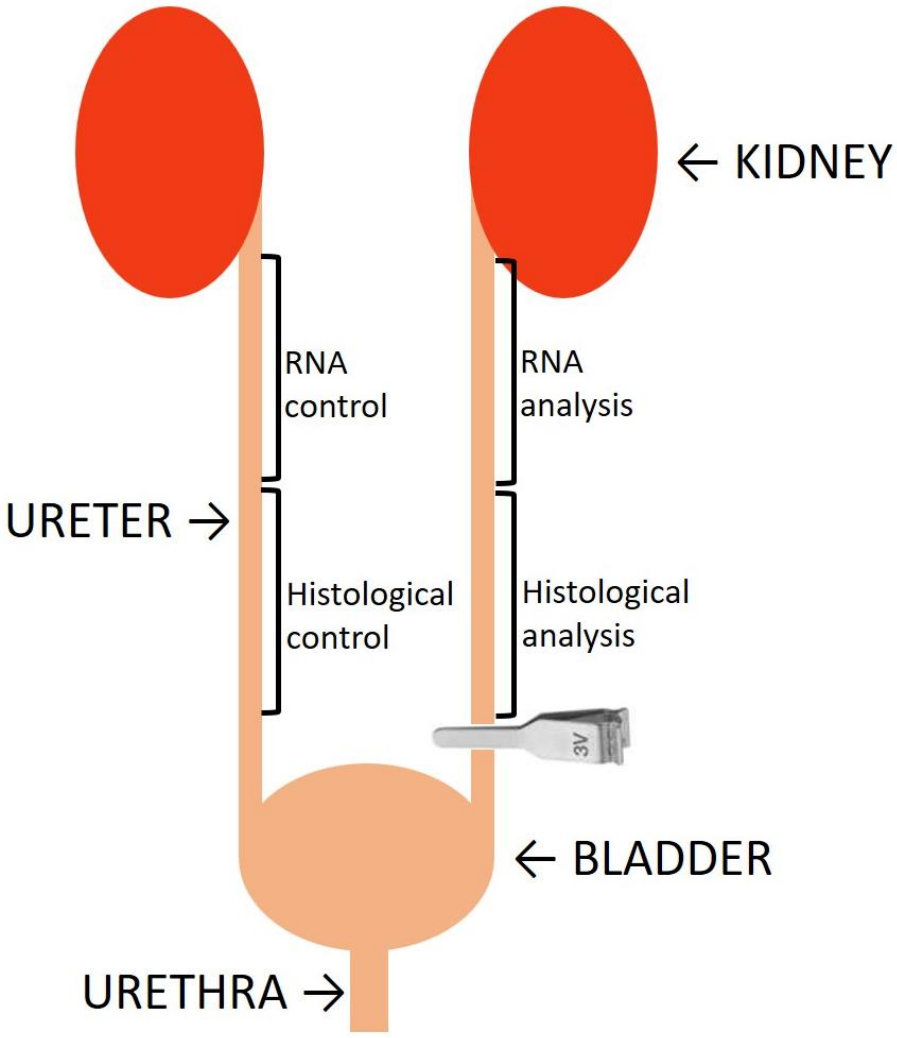


Figure 2-1 Unilateral ureteral obstruction model using non-traumatic vascular micro-clip

Anatomy of upper urological tract and the location of non-traumatic vascular micro-clip on the distal end of left ureter. Location of samples harvested for RNA and histological analysis due to technical difficulty in RNA extraction. Samples harvested were from same location in the contralateral ureters.

2.2.3 Ureteral function evaluation

After ureteral obstruction for 24, 48, 72 hours, non-recovery laparotomy was performed to expose the ureters for final bilateral microscopic assessment of ureteral peristaltic activity. Peristaltic movement of ureters were counted for 1 minute under the microscope.

2.2.4 Quantitative Real-Time PCR

Entire ureters and kidneys were harvested bilaterally, from mice sacrificed after unilateral ureteral obstruction. Kidneys and ureters in TRIzol (Invitrogen, Carlsbad, CA, USA) were homogenized using a Precellys 24 lysis/homogenizer (Bertin Technologies, Sweden) and RNA was isolated according to product specifications. RNA concentrations diluted in DEPC-treated water (Invitrogen, Carlsbad, CA, USA) were measured using a Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). cDNA was synthesized using total RNA (1000 ng for kidney samples, 50 ng for ureter samples) using Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland) with random hexamer primer under the following condition: 10 min at 25 °C, 55 C for 30 min followed by 85 °C for 10 min for inactivation. qPCR was performed using FastStart Universal SYBR Green Master (Rox) (Roche, Basel, Switzerland). Experimentally validated primers specific to mouse Epor, Csf2rb1 (βcr), Bcl2 (Bcl-2), Bcl2Ll (Bcl-xl), Bax, Nfkb1 (Nf-κb p105), Stat5a (Stat5) and Actb (β-actin)[i] were purchased from Genecopoeia (Genecopoeia Inc, Rockville, MD, USA) (Table 2-1). Experimentally validated primers specific to mouse EPO were purchased from Qiagen (Qiagen, Hilden, Germany). qPCR assays were performed on a Life Technologies ViiA 7 Real-Time PCR System (Thermo Fisher Scientific,

[i] Mouse gene nomenclature will be replaced to all capital letters to minimize confusion in this paper.

Waltham, MA, USA) using the following conditions: 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Melt curve assay was performed using the following conditions: 95 °C for 15 s and 60 °C for 1 min and 95 °C for 15 s. All assays were performed in triplicates. CT values were determined using the Quantstudio Realtime PCR V1.2 software with automatic baseline and threshold settings. Triplicate CT values were averaged and normalized to β-ACTIN, which was selected as an endogenous control.

Gene	Catalog #	Forward primer	Backward primer
ACTB	MQP026493	ctaaggccaaccgtgaaaag	accagaggcatacagggaca
CSF2RB1	MQP026868	gcccatagcatggacctctc	actcttcgctccacttgctc
EPOR	MQP029111	ccgtctgacttggcctcaaa	tcaagtgaggtggagtggga
BCL2	MQP028734	gtacctgaaccggcatctg	gctgagcagggtcttcagag
BCL2L1	MQP078883	gcaggtattggtgagtcgga	ctgctgcattgttcccgtag
BAX	MQP026624	gtgagcggctgcttgtct	gaggactccagccacaaaga
NFKB1	MQP027731	caatagcctgccatgtctgc	atgttgtcgtactccacggc
STAT5A	MQP030266	ggagctggttcgctgtatcc	tctgggacatggcgtcaac
EPO	QT00170331	Contract Qiagen for more information	

Table 2-1 Primer sequences for qRT-PCR

Forward and backward primer sequences for the primers that are used in this experiment. Experimentally validated primers were purchased and sequence are provided upon request. Gene represents primary gene name for each primer set.

2.2.5 TUNEL assay

C57BL/6 mouse kidneys and middle ureters were removed after sacrifice and fixed in 10% phosphate buffered formalin (pH 7.1) for 48 hours before transfer to 70% ethanol. After embedding the samples in paraffin 4 mm sections were cut and stained with hematoxylin and eosin to analyze potential tissue damage. Slides were stained to localize apoptosis in ureteral tissues. All slides were scanned with a scanner (Leica Microsystems, Concord, ON, Canada) and digital images were analyzed by a blinded pathologist (Dr. Ladan Fazli).

The immunolabeling was examined by a Zeiss light microscope (Axioplan 2 imaging; Carl Zeiss, Jena, Germany) or a Leica DM IRB inverted microscope (Leica Microsystems, Wetzlar, Germany) equipped with a CoolSNAP HQ camera (Photometrics, Tucson, AZ, USA).
TUNEL staining was performed using Terminal transferase (Cat #: 03 333 566 001, Roche, Basel, Switzerland), Digoxigenin-11-dUTP, alkali-stable (Cat #: 11 093 088 910, Roche, Basel, Switzerland) according to the manufacturer's instructions. Briefly, sections were deparaffinized and subjected to antigen retrieval in preheated 10 mmol/L sodium citrate (pH 7). They were then incubated with 3 % H_2O_2 for 10 min, followed by incubation with a TdT enzyme solution for 90 min at 37 °C. The reaction was terminated by incubation in stop/wash buffer for 30 min at 37 °C. The number of TUNEL-positive staining cells was counted in 4 random areas and averaged.

2.2.6 Immunohistochemistry

C57BL/6 mouse kidneys and middle ureters were removed after sacrifice and fixed in 10% phosphate buffered formalin (pH 7.1) for 48 hours before transferred to 70% ethanol. After embedding the samples in paraffin 4 mm sections were cut and stained with hematoxylin and eosin to analyze potential tissue damage. Paraffin embedded ureters and kidneys that were harvested bilaterally from the mice after unilateral ureteral obstruction. Slides were stained with either anti-NF-κB p65 (phospho S536) antibody or anti-STAT5 (phospho Y694) in ureteral tissues. All slides were scanned with a scanner (Leica Microsystems, Concord, ON, Canada) and digital images were analyzed by a blinded pathologist (Dr. Ladan Fazli). The immunolabeling was examined by a Zeiss light microscope (Axioplan 2 imaging; Carl Zeiss, Jena, Germany) or a Leica DM IRB inverted microscope (Leica Microsystems, Wetzlar, Germany) equipped with a CoolSNAP HQ camera (Photometrics, Tucson, AZ, USA).

Immunohistochemistry staining was performed using either 1:400 anti-NF-κB p65 (phospho S536) antibody (Cat #: Ab86299, Abcam, Cambridge, UK) or 1:50 anti-STAT5 (phospho Y694) antibody (Cat #: Ab32364, Abcam, Cambridge, UK) according to the manufacturer's instructions. The number of positive staining cells per area was counted in 3 random areas and averaged.

2.2.7 Statistical analysis

Statistical analysis was performed with GraphPad Prism 8. Mean was analyzed using either t-test (paired or unpaired) or one-way parametric ANOVA test followed by Turkey's multiple comparison test as appropriate. Unpaired t-test and F test was used to compare the mean and variance of basal peristaltic activity between 2 strains of mice. Paired t-test was used to compare

qRT-PCR results and immunohistochemistry results of the obstructed side to its own contralateral side. Matched one-way ANOVA test followed by Turkey's multiple comparison test was used to compare ureteral peristalsis after obstruction in 3 groups. Data are represented as mean ± SEM. $p < 0.05$ was considered statistically significant.

2.3 Results

2.3.1 Ureteral function evaluation

The ureters move down the urine from kidney to bladder using series of wave-like muscle contractions, which is called peristalsis. Peristaltic activity of the ureter represents the efficiency of its transporting function. Therefore, ureteral function was measured by its peristaltic activity in our study.

2.3.1.1 Basal ureteral peristaltic activity in mice

Before measuring the effect of obstruction on ureteral peristaltic function, we first measured the basal peristaltic activity. Murine ureters showed peristaltic movement at the rate of 10 to 20 times per minute in our experiment. To our knowledge, it is the first experiment measuring and comparing ureteral peristaltic frequency in 2 mice strains. Interestingly, CD-1 strain had more peristaltic movement per minute in the ureters compared to C57BL/6 strain in the basal level (1.65-fold, $p < 0.0001$) (Figure 2-2). The ureteral peristaltic frequency of CD-1 showed more dispersed distribution than C57BL/6, although it was not statistically significant ($F = 1.654$, $p =$

0.1899). The overall peristaltic counts in both stains showed normal distribution curve, which showed the confidence in our method. (Figure 2-2). Different peristaltic frequency in the ureters between 2 mice strains showed physiological difference in basal ureteral function.

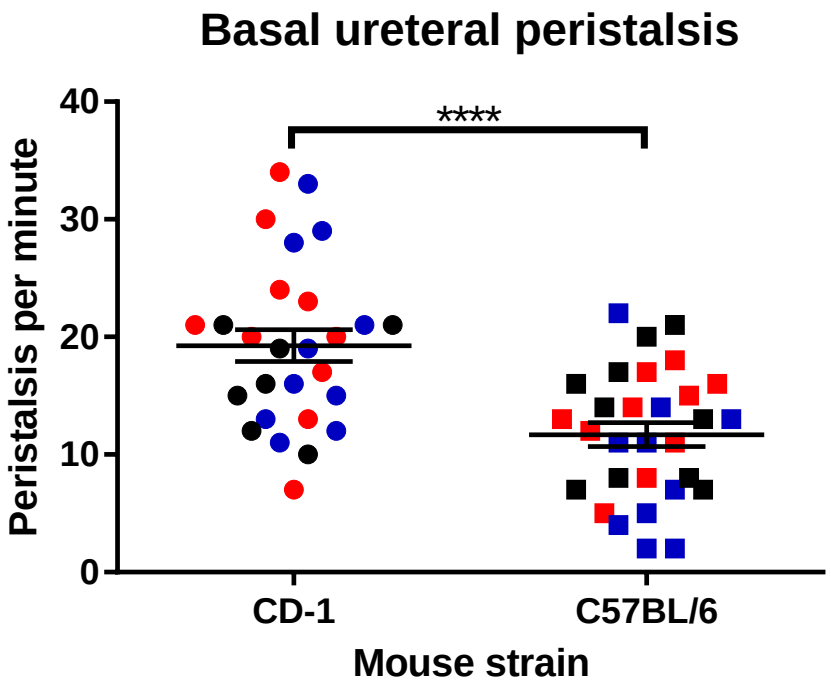


Figure 2-2 Basal ureteral peristaltic frequency in 2 mice strains

Ureteral peristaltic count per minute. Each dot represents individual sample. Data was pulled from obstruction experiments before inducing obstruction. Different color represents different experimental set which were performed in 3 experimental sets (red = 24-hour obstruction, blue = 48-hour obstruction, black = 72-hour obstruction). Data are represented as mean ± SEM.

2.3.1.2 Effect of obstruction on ureteral peristaltic function

Based on basal ureteral peristaltic count, we decided to look at the effect of obstruction on the ureteral peristaltic frequency and compare it to basal level. Our results showed that after 24-hour

obstruction 70 % mice of both stains lost ureteral peristaltic activity. None of mice ureters obstructed for 48, 72-hour retained peristaltic function. (Figure 2-3-3)

The peristaltic count was used to measure ureteral function. CD-1 was used as it is the strain that EPO was shown to be protective against obstructive uropathy in our previous work[11]. After 24-hour obstruction, ANOVA test showed that there was difference among the groups ($p < 0.05$). However, Turkey's multiple comparison test did not show difference between the groups. Ureteral peristaltic count after 24-hour obstruction compared to that of before obstruction in CD-1 could not be distinguished from one another (0.40-fold, $p = 0.0605$). Ureteral peristaltic count in the obstructed ureters and contralateral ureters after 24-hour obstruction in CD-1 could not be distinguished from one another (0.44-fold, $p = 0.0593$). Peristaltic count between contralateral ureters after 24-hour obstruction and that of before obstruction in CD-1 could not be distinguished from one another (0.91-fold, $p = 0.8416$) (Figure 2-3-1A). Although there was no statistically significant difference in the results because of high variance in the groups, the results showed that majority of mice lost ureteral peristaltic function. 70% of mice obstructed for 24 hours has lost its peristaltic activity, which shows rapid response of ureteral function following obstruction. 30% of mice retained peristaltic activity, showing biological variability in response against the obstructive injury.

We then decided to look at longer obstruction than 24 hours to see if it disrupts ureteral function further. Interestingly, 48-hour obstruction led to aperistalsis in all mice ureters. Ureteral peristaltic count was decreased after 48-hour obstruction compared to that of before obstruction in CD-1 ($p < 0.0001$). Ureteral peristaltic count was decreased after 48-hour obstruction

compared to that of contralateral after obstruction in CD-1 ($p < 0.001$). Ureteral peristaltic count of contralateral ureters after 48-hour obstruction was decreased compared to that of before obstruction in CD-1 (0.46-fold, $p < 0.005$) (Figure 2-3-1B). None of mice ureters obstructed for 48-hour retained peristaltic function, compared to 30% of mice ureter after 24-obstruction. Decreased peristalsis on unobstructed contralateral ureters after obstruction could be caused by functional response to the stress following more pressure given by non-functioning urological tracts on the other side.

As we expected from the effect of 48-hour obstruction on peristaltic activity, none of mice ureters obstructed for 72-hour retained peristaltic function. Ureteral peristaltic count was decreased after 72-hour obstruction compared to that of before obstruction in CD-1 ($p < 0.001$). Ureteral peristaltic count was decreased after 72-hour obstruction compared to that of contralateral after obstruction in CD-1 ($p = 0.001$). Peristaltic count between contralateral ureters after 72-hour obstruction to that of before obstruction in CD-1 could not be distinguished from one another (1.3-fold, $p = 0.3477$) (Figure 2-3-1C).

Considering physiological difference in physiological and pathological difference between different mice strains, we decided to look at C57BL/6 strain to confirm that ureteral dysfunction from obstruction is not CD-1 strain specific. As shown in figure 2-2, C57BL/6 presents physiological difference in ureteral function from CD-1. Despite the difference, we observed the same trend in C57BL/6 as CD-1, which shows that ureteral response to obstruction is more likely the universal change across the species.

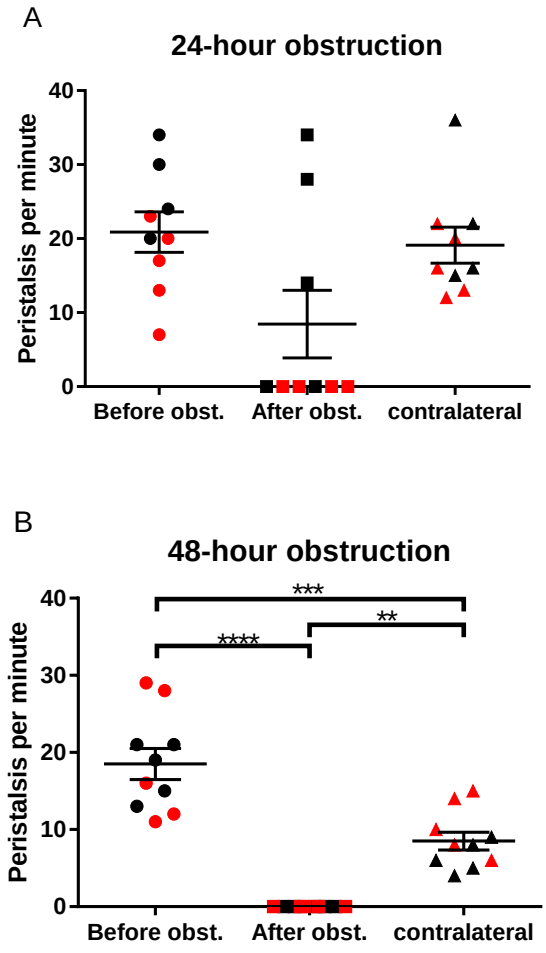
Same as we observed in CD-1 strain, 70% of mice ureters obstructed for 24 hours has lost its peristaltic activity. Ureteral peristaltic count was decreased after 24-hour obstruction compared to that of before obstruction in C57BL/6 (0.16-fold, $p < 0.001$). Ureteral peristaltic count was decreased after 24-hour obstruction compared to that of contralateral after obstruction in C57BL/6 (0.2-fold, $p < 0.01$). Peristaltic count between contralateral ureters after 24-hour obstruction to that of before obstruction in C57BL/6 could not be distinguished from one another (0.81-fold, $p = 0.4863$) (Figure 2-3-2A).

As we observed in CD-1 strain, ureters obstructed for 48 hours did not retain peristaltic function. Ureteral peristaltic count was decreased after 48-hour obstruction compared to that of before obstruction in C57BL/6 ($p < 0.01$). Ureteral peristaltic count was decreased after 48-hour obstruction compared to that of contralateral after obstruction in C57BL/6 ($p < 0.05$). Peristaltic count between contralateral ureters after 48-hour obstruction and that of before obstruction in C57BL/6 could not be distinguished from one another (0.48-fold, $p = 0.2378$) (Figure 2-3-2B). Results from both mice strains suggests that between 24 to 48 hours of obstruction, ureteral peristaltic activity is completely lost in mice.

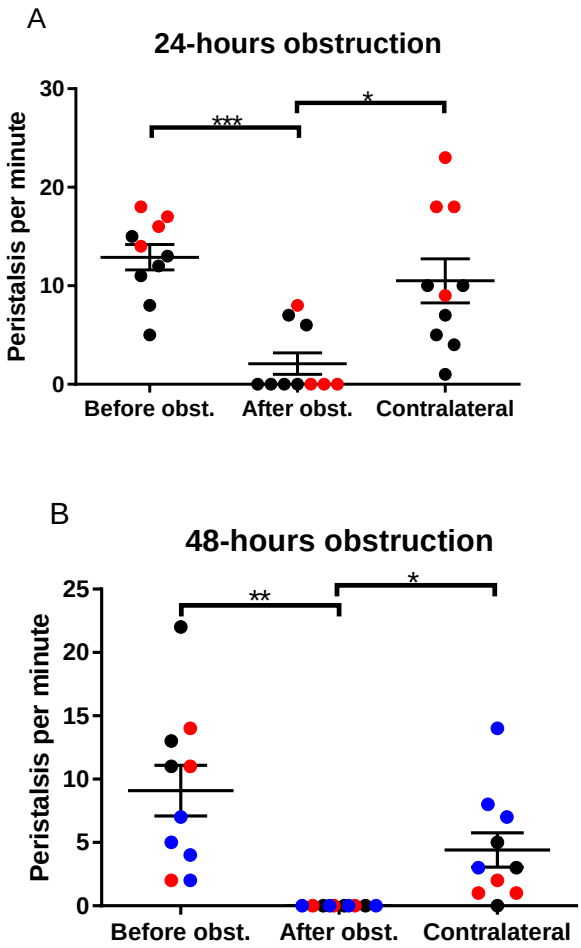
As we expected from the previous results, none of mice ureters obstructed for 72-hour retained peristaltic function. Ureteral peristaltic count was decreased after 72-hour obstruction compared to that of before obstruction in C57BL/6 ($p < 0.0001$). Ureteral peristaltic count was decreased after 72-hour obstruction compared to that of contralateral after obstruction in C57BL/6 ($p < 0.001$). Peristaltic count between contralateral ureters after 72-hour obstruction and that of before obstruction in C57BL/6 could not be distinguished from one another (0.72-fold, $p = 0.2192$)

(Figure 2-3-2C). The effect of 72-hour obstruction on the upper urological tract is leads to high pressure on the ureteral wall and the kidneys, which leads to massive expansion with stretch of the organs. CD-1 strain showed the similar appearance after ureteral obstruction as C57BL/6 (data not shown). The stretch of the cells in the kidneys was shown to induce apoptotic cell death[15], which suggests that stretch on ureteral cells following obstruction may lead to apoptosis as well. (Figure 2-5)

1. CD-1



2. C57BL/6



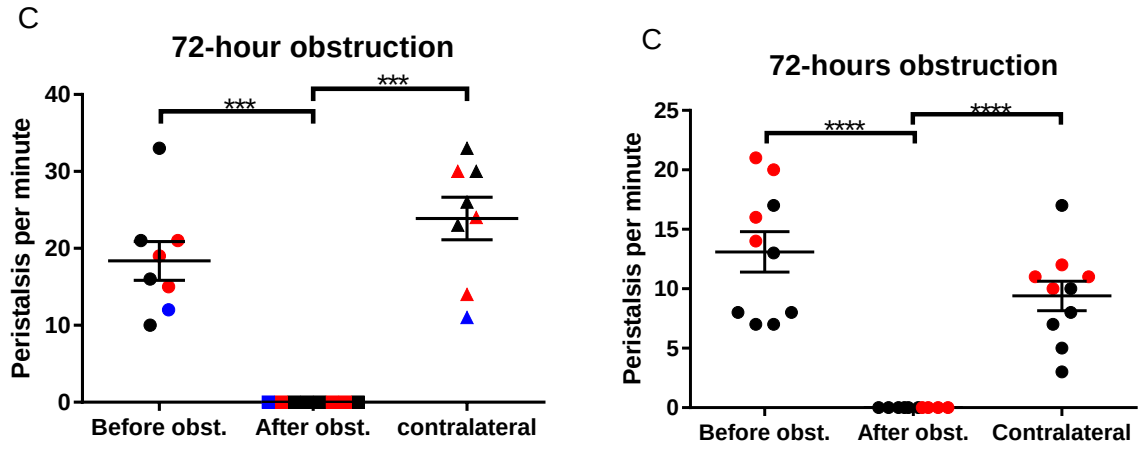


Figure 2-3 Effect of obstruction in ureteral peristaltic activity

Ureteral peristaltic activity after 24, 48, 72-hour unilateral ureteral obstruction of ureters before and after obstruction and its unobstructed contralateral ureter after obstruction in CD-1 (1) and C57BL/6 (2) strain. Different color of dots indicate independent experiments (black, red and blue). Asterisk indicates $p < 0.05$. Double asterisks indicate $p < 0.01$. Triple asterisks indicate $p < 0.001$. Quadruple asterisks indicate $p < 0.0001$. *Obst*, obstruction.

Taken together, ureteral obstruction decreased peristaltic activity as early as 24-hours after obstruction. Due to physiological difference, CD-1 and C57BL/6 showed difference in basal ureteral peristalsis. However, similar slope in decreasing peristalsis is observed in both strains after 24-hour obstruction. Peristaltic activity of contralateral ureters remained consistent after 24-hour obstruction. After 48-hour obstruction, obstructed ureters of both mice strains showed aperistalsis. Peristaltic activity of contralateral ureters showed slight decrease after 48-hour obstruction in both strains. One of the reasons of decreased peristaltic activity in the contralateral ureters can be an acute stress signal released from the obstructed ureters and kidneys to the body. It is also shown to be transient, as its function returns to the basal state following 72-hour obstruction. Transient stress signal could be involved in inflammation, as ureteral obstruction has been shown to induce inflammation (reviewed by Jay et al.[111]). Contralateral ureters may

respond to the cytokines released from the obstructed urological tracts. Apart from obstruction, there has been no study showing the factors that directly disrupt peristaltic activity. Further work will be needed to investigate the effect of obstruction on contralateral ureteral peristalsis. Similar to 48-hour obstruction, ureters obstructed for 72 hours did not show any peristaltic activity in both mice strains. Peristaltic activity of contralateral ureters has recovered to basal level in C57BL/6. In CD-1, peristaltic activity of contralateral ureters has slightly increased compared to basal level. It may show the overload on the contralateral ureters and kidneys to compensate dysfunction in the obstructed kidneys, which could lead to damage in the contralateral ureter if the obstruction is not resolved for prolonged time. (Figure 2-4)

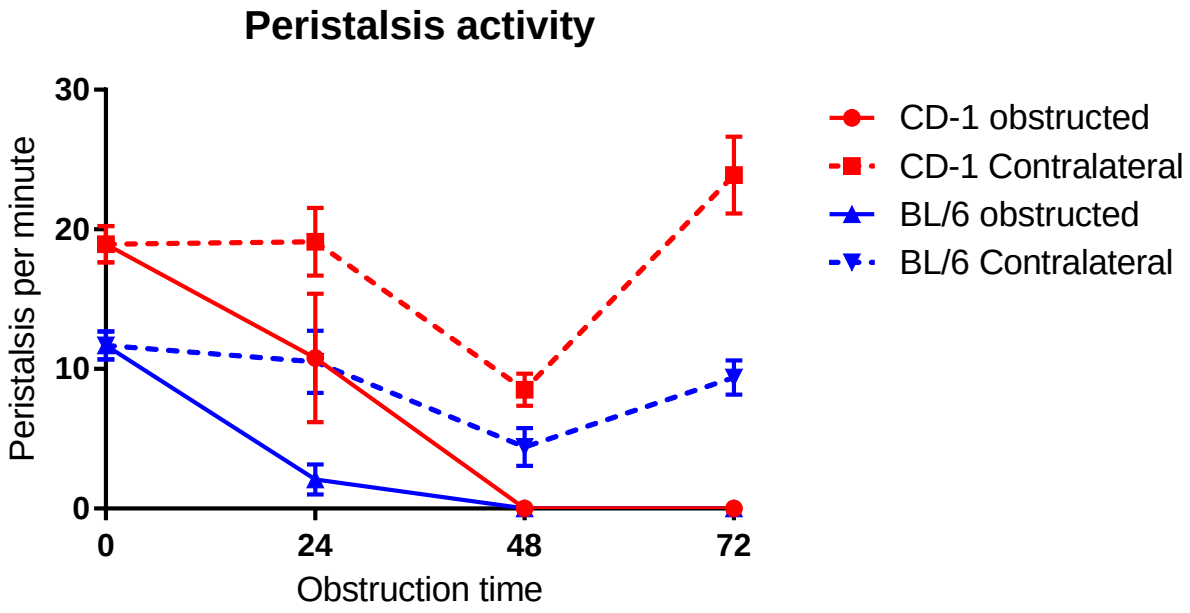


Figure 2-4 Time course of the effect of obstruction in ureteral peristaltic activity

Ureteral peristaltic activity after obstruction over the time course. Red color represents peristalsis of CD-1 strain. Blue color represents peristalsis of C57BL/6 strain. Plain lines represent obstructed ureters. Plural dotted lines represent unobstructed contralateral ureters. *BL/6*, C57BL/6 strain

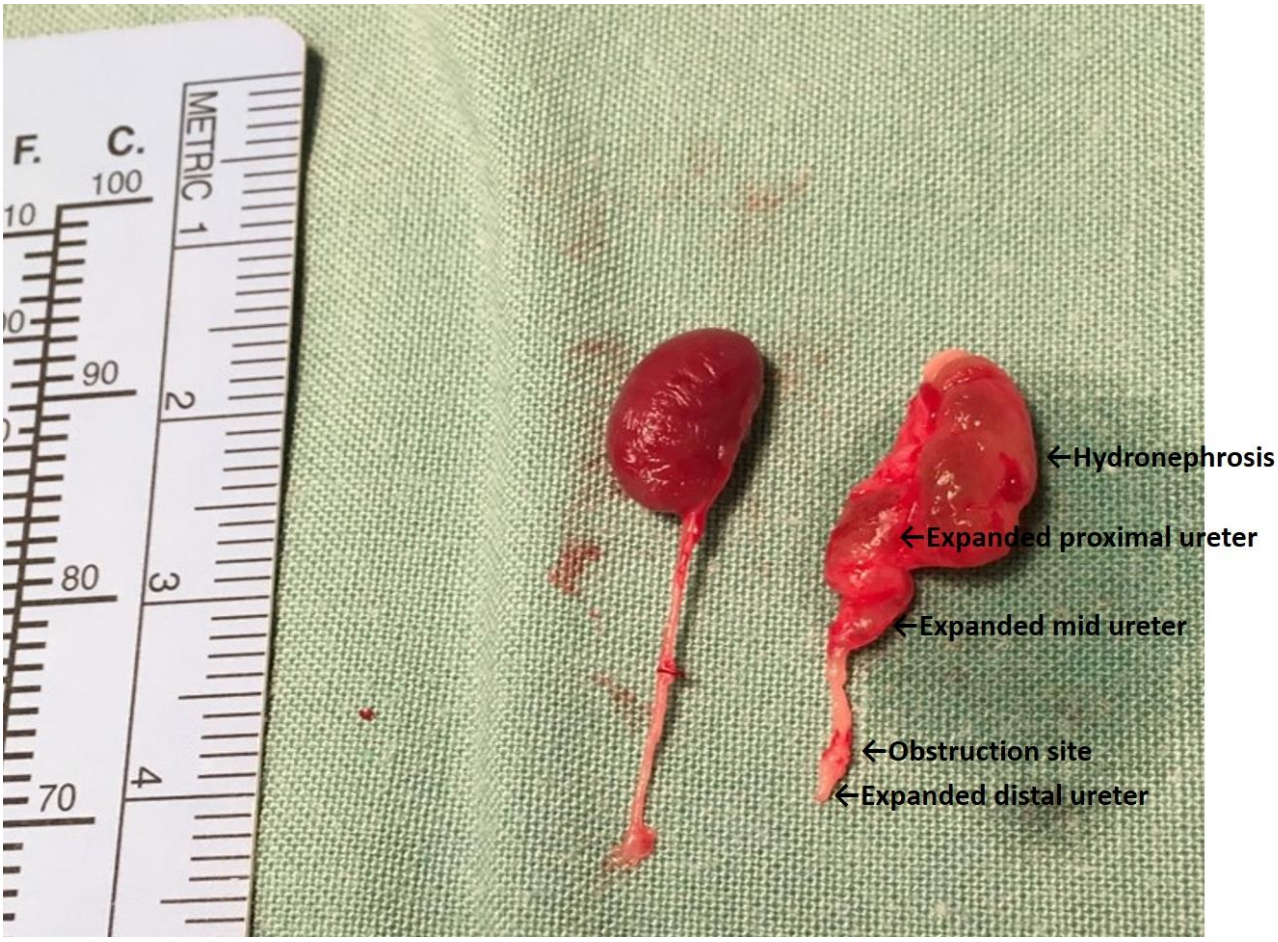


Figure 2-5 Representative photos of upper urinary tract after unilateral ureteral obstruction

Effect of obstruction on the dilation of upper urinary tracts in C57BL/6 strain. Contralateral unobstructed upper urinary tract on the left and upper urinary tract obstructed for 72 hours on the right.

2.3.2 Effect of obstruction on RNA expression

The expression of genes in the obstructed ureters were compared to the contralateral side which served as the control.

2.3.2.1 Effect on EPO, EPOR and βCR expression by ureteral obstruction

Previous results in our laboratory showed the protective effect of EPO on accelerating functional recovery of the ureters and the kidneys from transient ureteral obstruction. Therefore, we investigated the existence of EPO and EPOR-βCR expression on the ureters and kidneys that led to protective signaling upon EPO administration. To our knowledge, the expression of EPO and EPOR-βCR on the ureters was only shown in our previous work, for which the sample size was 3. To verify the expression of EPO and EPOR-βCR on the ureters, we examined the expression with increased sample size to validate the results (n = 10). We further investigated the effect of obstruction on EPO and EPOR-βCR expression in the ureters and kidneys to test if ureteral obstruction modulates endogenous EPOR signaling in the ureters and the kidneys.

2.3.2.1.1 Effect on EPO, EPOR and βCR expression by ureteral obstruction in CD-1 strain

We were able to detect the expression of EPO and EPOR-βCR in the ureteral tissues confirming our previous work. EPO expression was shown to be decreased on obstructed kidneys, however EPO expression on the ureters was not shown to be affected by obstruction. EPO expression was decreased in 24-hour obstructed ureters compared to contralateral ureters ($p < 0.01$). EPO expression of 48 or 72-hour obstructed ureters and their own contralateral ureters could not be distinguished from one another (Figure 2-6-1A). EPO expression was decreased in 24, 48 and 72-hour obstructed kidneys compared to contralateral kidneys ($p < 0.0001$, $p < 0.0001$, $p < 0.0001$) (Figure 2-6-2A). EPOR expression was shown to be decreased on the ureters, however

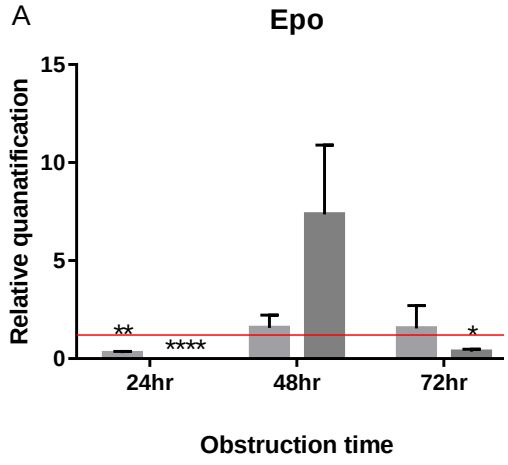
the EPOR expression of kidney was not shown to be affected by obstruction. Although we could observe decreasing trends of EPOR expression there was no statistically significant difference in EPOR expression in 24, 48-hour obstructed ureters compared to contralateral ureters. EPOR expression was decreased in 72-hour obstructed ureters ($p < 0.001$) (Figure 2-6-1B). EPOR expression of 24, 72–hour obstructed kidneys and their own contralateral kidneys could not be distinguished from one another. EPOR was decreased in 48-hour obstructed kidneys compared to contralateral kidneys ($p < 0.0001$) (Figure 2-6-2B). βCR expression was shown to be increased on the kidneys starting from 48-hour obstruction, however the βCR expression of ureters was not shown to be affected by obstruction. βCR expression of 24, 48, 72-hour obstructed ureters and contralateral ureters could not be distinguished from one another (Figure 2-6-1C). βCR expression of 24-hour obstructed kidneys compared to contralateral kidneys could not be distinguished from one another. βCR expression was increased in 48, 72-hours obstructed kidneys compared to contralateral kidneys ($p < 0.001$, $p < 0.01$) (Figure 2-6-2C). Though there was difference in degree of changes between the expression of the kidneys and ureters, the trend of decreased EPO and EPOR was observed in both organs following obstruction. Prophylactic EPO treatment may work by compensating the decreased EPO and EPOR expression. In addition, decreased EPOR expression and increased βCR expression following the obstruction suggest us increased EPOR-βCR signaling following the injury, as a self-protective mechanism.

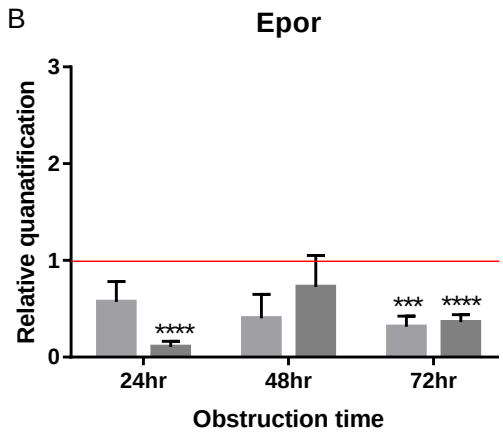
2.3.2.1.2 Effect on EPO, EPOR and βCR expression by ureteral obstruction in C57BL/6 strain

To verify our results on CD-1 strain, the expression of EPO and EPOR-βCR following obstruction on C57BL/6 strain was investigated. EPO expression was shown to be decreased on both ureters and kidneys. EPO expression was decreased in 24, 72-hour obstructed ureters compared to contralateral ureters ($p < 0.0001$, $p < 0.05$). EPO expression of 48-hour obstructed ureters and contralateral ureters could not be distinguished from one another, possibly due to high variance (Figure 2-6-1A). EPO expression was also decreased in 24, 48, 72-hour obstructed kidneys compared to contralateral kidneys ($p < 0.0001$, $p < 0.0001$, $p < 0.0001$) (Figure 2-6-2A). EPO expression was shown to be decreased on both ureters and kidneys. EPOR expression was decreased in 24 and 72-hour obstructed ureters compared to contralateral ureters ($p < 0.0001$, $p < 0.0001$). EPOR expression of 48-hour obstructed ureters and contralateral ureters could not be distinguished from one another (Figure 2-6-1B). EPOR expression was decreased in 24, 48 and 72-hour obstructed kidneys compared to contralateral kidneys ($p < 0.05$, $p < 0.001$, $p < 0.001$) (Figure 2-6-2B). Same as we observed in CD-1 strain, βCR expression was shown to be increased on the kidneys, and its expression of ureters was not shown to be affected by obstruction. Initially, βCR was decreased in 24-hour obstructed ureters compared to contralateral ureters ($p < 0.01$). However, βCR expression of 48, 72-hour obstructed ureters and contralateral ureters could not be distinguished from one another (Figure 2-6-1C). βCR expression of 24-hour obstructed kidneys and contralateral kidneys could not be distinguished from one another. βCR expression was increased in 48 and 72-hour obstructed kidneys compared to contralateral kidneys ($p < 0.01$, $p < 0.01$) (Figure 2-6-2C). Expression on C57BL/6 suggests decreased EPO, EPOR and increased βCR in the kidneys from ureteral obstruction, which was also observed in CD-1 strain. Interestingly, C57BL/6 showed more sensitive response to the obstruction compared to CD-1, which might be due to innate physiological difference between strains that

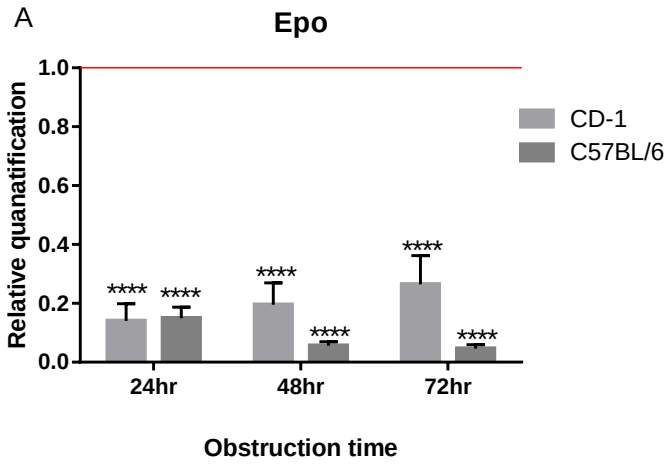
we also observed in basal peristaltic frequency (Figure 2-2). Taken together, we confirmed that ureteral obstruction decreased EPO, EPOR in the ureters and kidneys and increased βCR in the kidneys.

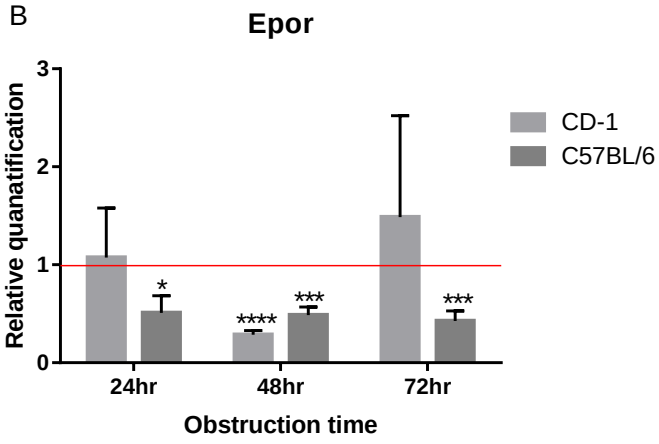
1. **Ureter**





2. **Kidney**





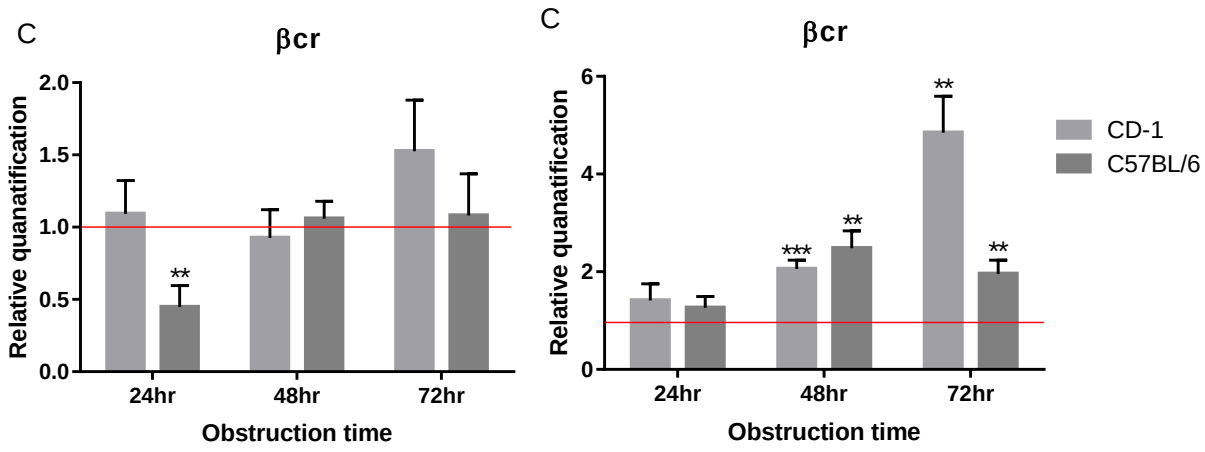


Figure 2-6 Effect of obstruction on RNA expression of EPO, EPOR, βCR in CD-1, C57BL/6 strains

Real-time quantitative PCR analysis in 24, 48, 72-hour obstructed ureters and kidneys and compared to those in unobstructed contralateral side per each animal. RNA expression of EPO, EPOR, βCR in ureters and kidneys after unilateral ureteral obstruction was analyzed by qRT-PCR (A to F). Light gray bars represent RNA expression in CD-1 strain. Dark gray bars represent RNA expression in C57BL/6 strain. Red line represents contralateral side. Data are presented as mean ± SEM. Asterisk indicates $p < 0.05$. Double asterisks indicate $p < 0.01$. Triple asterisks indicate $p < 0.001$. Quadruple asterisks indicate $p < 0.0001$.

2.3.2.2 Effect on BCL-2, BCL-XL, BAX, NF-κB p105, STAT5A expression by ureteral obstruction

Our previous results showed decreased EPO and EPOR expression following the injury. It led us to investigate downstream genes of EPOR-βCR, which were expected to be also decreased. Among multiple signaling that EPO activates, we focused on apoptosis-related genes as studies have shown that EPO's primary role in protecting tissues against injuries is via decreasing apoptosis from the injury. Previous studies have shown that ureteral obstruction leads to apoptosis in the kidney and the ureters (detailed in section 1.1.4). In addition, we observed

severe expansion of upper urological tracts following obstruction, which is related to apoptosis in surrounding cells (detailed in section 2.3.1.2). Therefore, we investigated apoptosis-related regulators in EPOR-βCR signaling, which were BCL-2, BCL-XL, BAX, NF-κB1 (p105) and STAT5A (Figure 1-3). STAT5 was chosen to represent the upstream of anti-apoptotic EPOR signaling that is activated upon JAK2 activation. BCL-2, BCL-XL, BAX, NF-κB p105 represent the downstream signaling that directly modulates apoptosis.

2.3.2.2.1 Effect on BCL-2, BCL-XL, BAX, NF-κB p105, STAT5A expression by obstruction on CD-1 strain

The expression of genes downstream of EPOR-βCR was examined in CD-1 strain. BCL-2 expression was shown to be decreased on the ureters, however BCL-2 expression of kidneys was not shown to be affected by obstruction. BCL-2 expression of 24, 48-hour obstructed ureters and contralateral ureters could not be distinguished from one another. BCL-2 expression was decreased in 72-hour obstructed ureters ($p < 0.01$) (Figure 2-7-1A). BCL-2 expression of 24, 72-hour obstructed kidneys and contralateral kidneys could not be distinguished from one another. BCL-2 expression was decreased in 48-hour obstructed kidneys ($p < 0.0001$) (Figure 2-7-2A). BCL-XL expression was shown to be decreased on the kidneys, as well as long-term obstructed ureters. BCL-XL expression of 24, 48-hour obstructed ureters and contralateral ureters could not be distinguished from one another. Longer obstruction for 72 hours decreased BCL-XL expression in obstructed ureters ($p < 0.001$) (Figure 2-7-1B). BCL-XL expression in kidneys was initially increased in 24-hour obstruction but its expression dramatically decreased following longer injury of 48 and 72-hour obstruction ($p < 0.05$, $p < 0.01$, $p < 0.0001$) (Figure 2-7-2B).

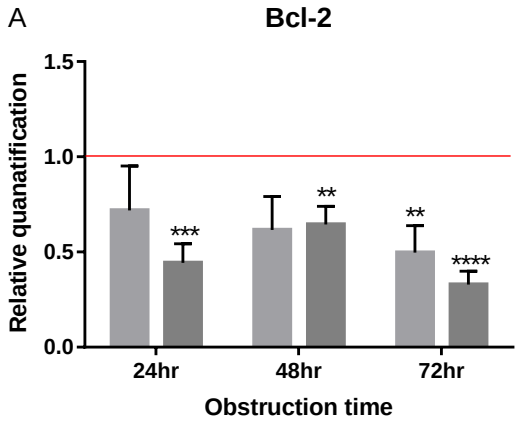
BAX expression following obstruction in both ureters and kidneys could not be distinguished from contralateral side. BAX expression of 24, 48, 72-hour obstructed ureters and contralateral ureters could not be distinguished from one another (Figure 2-7-1C). BAX expression of 24, 48, 72-hour obstructed kidneys and contralateral kidneys could not be distinguished from one another (Figure 2-7-2C). To examine transcriptional NF-κB activation, NF-κB p105 was used in this experiment. NF-κB p105 is a precursor of NF-κB p50, which binds to NF-κB p65 to induce NF-κB activation. Tan et al. showed that NF-κB p50 is regulated in a transcriptional level in the intestines, which exhibit peristaltic function[112]. NF-κB p105 expression was shown to be increased on the kidneys, however NF-κB p105 expression on the ureters was not shown to change following obstruction. NF-κB p105 expression of 24, 48, 72-hour obstructed ureters and contralateral ureters could not be distinguished from one another (Figure 2-7-1D). Though we could see increasing trend of NF-κB p105 expression, NF-κB p105 expression of 24, 48-hour obstructed kidneys and contralateral kidneys could not be distinguished from one another. NF-κB p105 was increased in 72-hour obstructed kidneys ($p < 0.05$) (Figure 2-7-2D). STAT5A expression was shown to be dramatically decreased on both ureters and kidneys. STAT5A expression was decreased in 24, 48, 72-hour obstructed ureters ($p < 0.05$, $p < 0.0001$, $p < 0.01$) (Figure 2-7-1E). STAT5A expression was decreased in 24, 48, 72-hour obstructed kidneys ($p < 0.001$, $p < 0.001$, $p < 0.0001$) (Figure 2-7-2E). RNA expression results showed decreased expression of EPOR-βCR downstream survival genes (BCL-2, BCL-XL, STAT5A), as we expected from decreased EPO and EPOR expression. Decreased anti-apoptotic genes would lead the cells prone to apoptosis. Interestingly, expression of pro-apoptotic BAX gene was not affected by the obstruction.

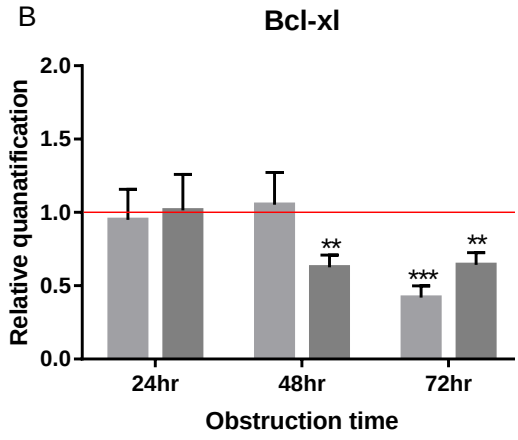
2.3.2.2.2 Effect on BCL-2, BCL-XL, BAX, STAT5A expression by obstruction on C57BL/6 strain

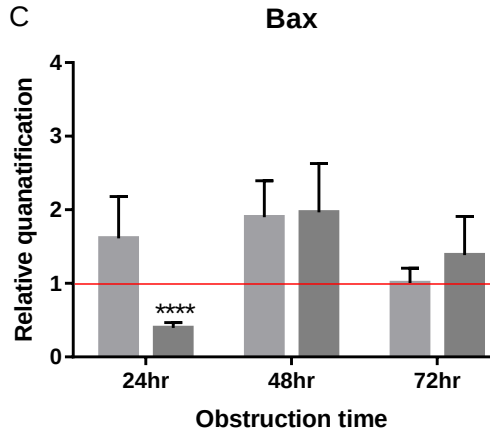
To verify our results observed in CD-1 strain, we investigated the expression of EPOR downstream genes of C57BL/6 strain following obstruction. BCL-2 expression was shown to be decreased on the ureters, however the BCL-2 expression of kidneys was not shown to be affected by obstruction, which was similar to what we observed in CD-1. BCL-2 expression was decreased in 24, 48 and 72-hour obstructed ureters ($p < 0.001$, $p < 0.01$, $p < 0.0001$) (Figure 2-7-1A). BCL-2 expression was initially decreased in 24-hour obstructed kidneys ($p < 0.05$). But in longer obstruction for 48, 72 hours, BCL-2 expression of obstructed kidneys and contralateral kidneys could not be distinguished from one another (Figure 2-7-2A). BCL-XL expression was shown to be decreased on long-term obstructed ureters and kidneys. BCL-XL expression of 24-hour obstructed ureters and contralateral ureters could not be distinguished from one another. BCL-XL expression was decreased in 48 and 72-hour obstructed ureters ($p < 0.01$, $p < 0.01$) (Figure 2-7-1B). BCL-XL expression of 24, 48-hour obstructed kidneys and contralateral kidneys could not be distinguished from one another. BCL-XL expression was decreased in 72-hour obstructed kidneys ($p < 0.01$) (Figure 2-7-2B). There was no distinguishable change in BAX expression following obstruction in both ureters and kidneys, as similar to what we found in CD-1 strain. BAX expression was initially decreased in 24-hour obstructed ureters ($p < 0.0001$). But BAX expression of 48, 72-hour obstructed ureters and contralateral ureters could not be distinguished from one another (Figure 2-7-1C). BAX expression of 24, 48, 72-hour obstructed kidneys and contralateral kidneys could not be distinguished from one another (Figure 2-7-2C). NF-κB p105 expression was shown to be increased on the kidneys, whereas decreased

NF-κB p105 expression is found in the ureters. NF-κB p105 expression was decreased in 24 and 72-hour obstructed ureters ($p < 0.001$, $p < 0.05$). NF-κB p105 expression of 48-hour obstructed ureters and contralateral ureters could not be distinguished from one another (Figure 2-7-1D). There was initially no distinguishable difference in NF-κB p105 expression after 24-hour obstruction between obstructed kidneys and contralateral kidneys. However, NF-κB p105 expression was increased in 48, 72-hour obstructed kidneys ($p < 0.05$, $p < 0.05$) (Figure 2-7-2D). STAT5A expression was shown to be decreased on both ureters and kidneys, as we observed in CD-1. STAT5A expression of 24-hour obstructed ureters and contralateral ureters could not be distinguished from one another. However, STAT5A expression was decreased in longer obstruction for 48 and 72-hour in the ureters ($p < 0.0001$, $p < 0.0001$) (Figure 2-7-1E). STAT5A expression was decreased in 24 and 72-hour obstructed kidneys ($p < 0.0001$, $p < 0.0001$). STAT5A expression of 48-hour obstructed kidneys contralateral kidneys could not be distinguished from one another (Figure 2-7-2E). Results from 2 mice strains showed decreased expression of EPOR-βCR downstream survival genes (BCL-2, BCL-XL, STAT5A). In addition to similar trend of EPOR-βCR downstream expression in both mice strains, we observed more sensitive response to obstructive injury in C57BL/6 strain compared to CD-1, which also corresponds to EPO and EPOR expression of both strains. Same trend with more sensitive response of C57BL/6 compared to CD-1 led us to examine further assays with C57BL/6 to represent both strains.

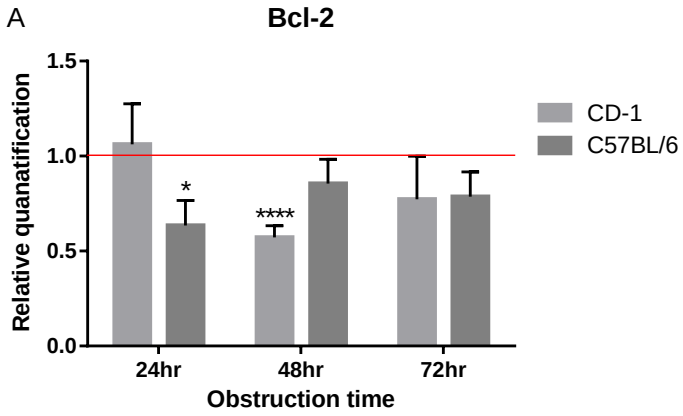
1. **Ureter**

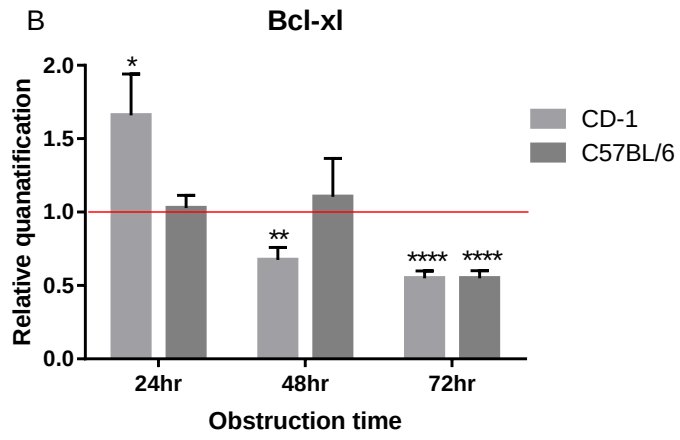


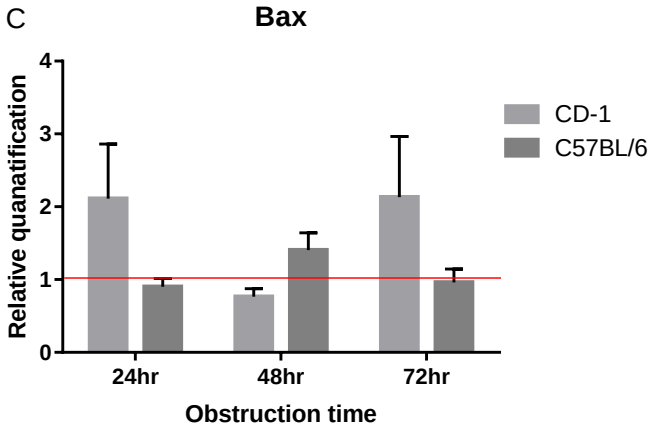




2. **Kidney**







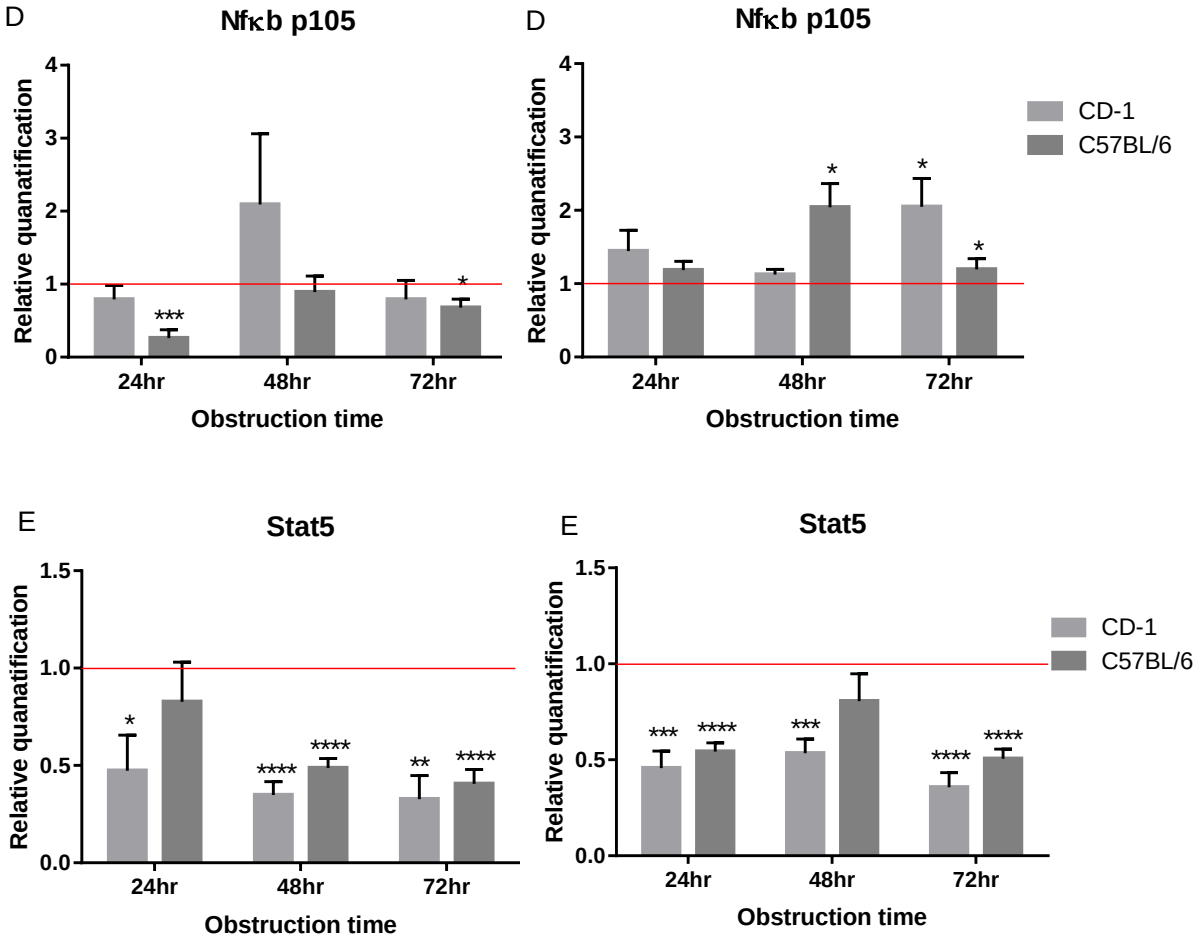


Figure 2-7 Effect of obstruction on RNA expression of BCL-2, BCL-XL, BAX, NF-κB p105, STAT5A in CD-1, C57BL/6 strains

Real-time quantitative PCR analysis in 24, 48, 72-hour obstructed ureters and kidneys and compared to those in unobstructed contralateral side per each animal. RNA expression of BCL-2, BCL-XL, BAX, NF-κB p105, STAT5A in ureters and kidneys after unilateral ureteral obstruction was analyzed by qRT-PCR (A to F). Light gray bars represent RNA expression in CD-1 strain. Dark gray bars represent RNA expression in C57BL/6 strain. Red line represents contralateral side. Data are presented as mean ± SEM. Asterisk indicates $p < 0.05$. Double asterisks indicate $p < 0.01$. Triple asterisks indicate $p < 0.001$. Quadruple asterisks indicate $p < 0.0001$.

2.3.3 Immunohistochemistry

Ureteral epithelium on C57BL/6 was used to analyze the immunohistochemical staining and overall ureteral tissue except connective tissue was used to analyze TUNEL assay. Ureter is divided into 3 layers, inner layer called mucosa, middle layer called muscularis and outer layer called aventitia. Lumen is the inside space of the ureter that urine is transported. Ureteral epithelium has transitional epithelial cells located in inner layer, that are specialized to rapidly stretch according to varying volume of urine as well as protecting other cells from toxins. Muscularis is a muscular coat, consisting of inner circular and outer longitudinal smooth muscle. Its function is peristalsis consisting of contraction and relaxation in response to signal from pacemarker cells located in renal pelvis and proximal ureters. The aventitia consists of connective fibrous tissues and adipose tissues to protect the ureter and connected to the vessel and the nerve. (Figure 2-8) (reviewed by Bergman et al.[113]) Although smooth muscle cells are responsible for peristaltic function that we are interested, transitional epithelium interacts and affects smooth muscle function including peristalsis in response to several cytokines in the intestines (reviewed by McKay et al.[114]). Also, transitional epithelium in urological tract was shown to exhibit rapid and sensitive signaling to the different conditions as well as carrying

significant amount of genes[113]. Therefore, we decided to investigate uroepithelium in our immunohistochemistry assay.

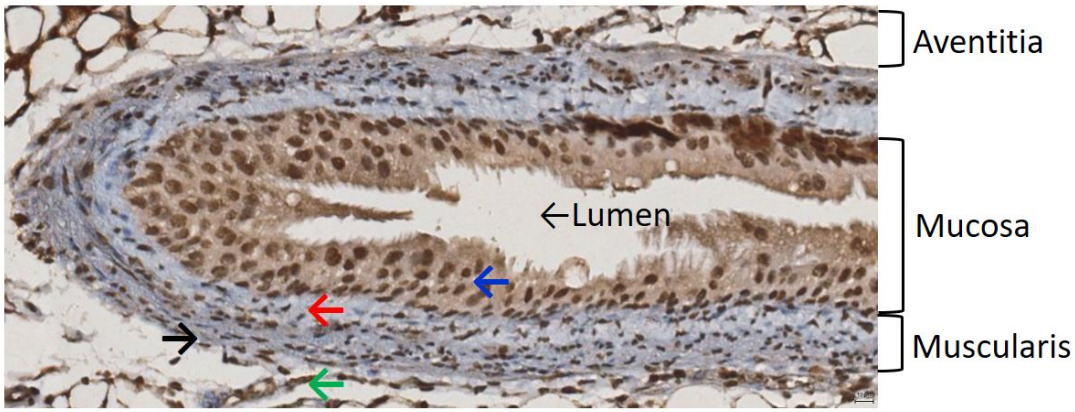


Figure 2-8 Structure of the ureter

Microscopic structure of the ureter. Unobstructed ureter stained for immunohistochemistry with phospho-STAT5 antibody was used in this image as an example. The substructure is described as letters with black color. Cells comprising the substructure is described as letters with blue color. Blue arrow indicates transitional epithelium. Red arrow indicates longitudinal muscle. Black arrow indicates circular muscle. Green arrow indicates connective tissues consisting of fibrous and adipose cells.

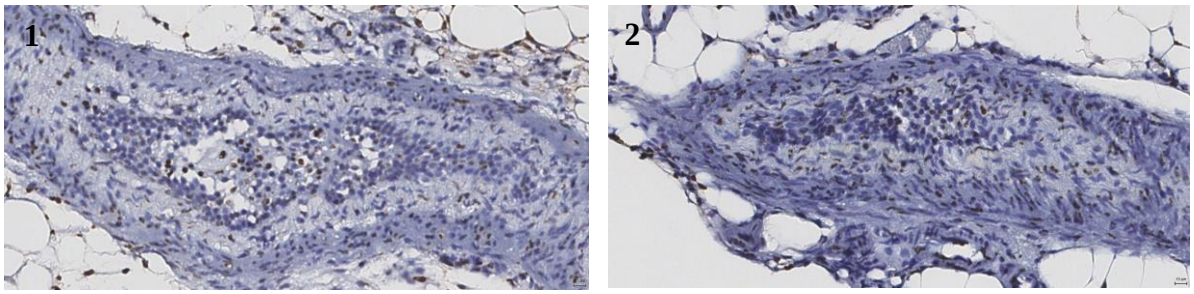
2.3.3.1 Effect of 72-hour obstruction on apoptosis detected by TUNEL assay

We then focused on apoptosis in the ureters induced by obstruction. Decreased in expression of EPOR-βCR downstream anti-apoptotic genes led us to investigate cellular apoptotic cell death, which may also be related to ureteral dysfunction following obstruction. Although we observed aperistalsis in obstructed ureters after as early as 48-hour obstruction (Figure 2-3), we decided to investigate apoptosis in 72-hour obstructed ureters, considering more dramatic apoptotic

signaling occurs after 72-hour obstruction according to RNA expression results (Figure 2-7). TUNEL assay was used to assess apoptosis, as it is shown to be most trusted method to validate apoptosis in the literature. Apoptosis in unobstructed ureters was compared to unobstructed contralateral ureters. Apoptotic cells were observed in both obstructed ureters and unobstructed contralateral ureters (Figure 2-9A). There was increased apoptosis in 72-hour obstructed ureters compared to contralateral ureters (2.71-fold, $p < 0.05$) (Figure 2-9B). Interestingly, we could find variance in immunohistochemical staining between 2 experimental replicates. It could be resulted from unknown factors that cannot be controlled, although they were treated in the same methods. Additional experimental replicates would be needed to validate the difference. Despite of the difference in staining amount, we could find similar trend of increased apoptosis in the obstructed ureters.

A. Paraffin-embedded ureteral tissues stained for TUNEL assay

72-hour obstructed **Contralateral**



B. Quantitative analysis of apoptotic cell death

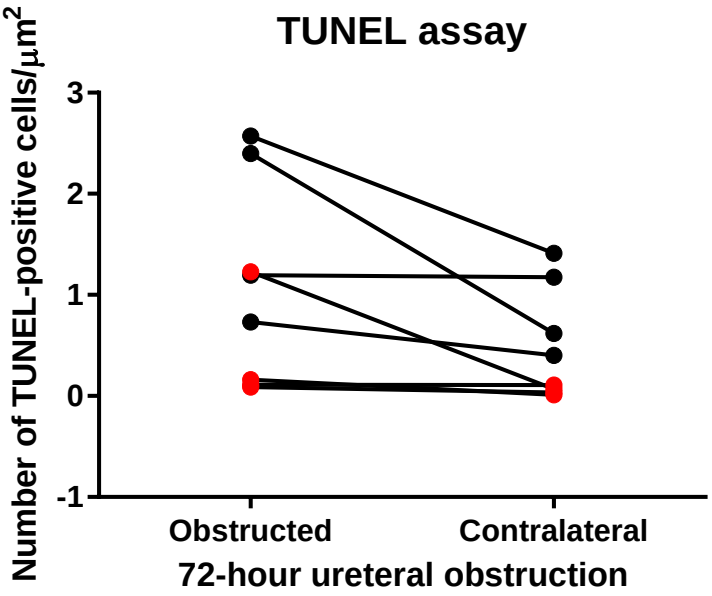


Figure 2-9 Effect on apoptosis in 72-hour obstructed ureteral tissues

A. Representative photomicrographs showing terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP) nick-end labeling (TUNEL) staining in the 72-hour obstructed ureter and its unobstructed contralateral ureter. Cells with brown staining represent strong positive staining and cells with blue staining represent negative staining. **B.** Quantitative analysis of apoptotic cells in 72-hour obstructed ureters and unobstructed contralateral ureters. The number of TUNEL-strong positive staining pixels counted in four random high power fields of the obstructed or non-obstructed contralateral ureters of 72-hour ureteral obstruction. A line connected with 2 dots represents paired data per each animal. Different color of dots indicates independent experiments (black and red). Data are presented for individual value. Asterisk indicates $p < 0.05$.

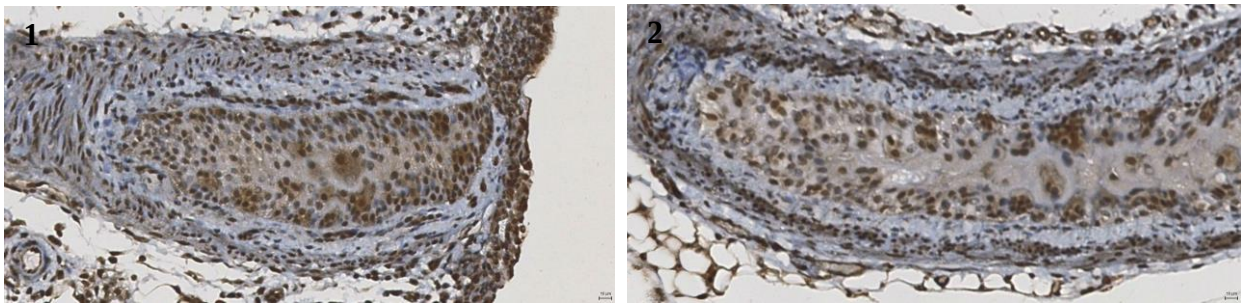
2.3.3.2 Effect of 72-hour obstruction on phospho-NF-κB p65 staining in the ureters

NF-κB activation is shown to be regulated by post-translational modification, which shows that RNA expression of NF-κB p105 may not represent the activation of NF-κB. Phosphorylated

(Phospho-) NF-κB p65 was selected to examine its activity, as phosphorylation of p65 decreases its affinity for IκBα, resulting in nuclear translocation and DNA binding of p65[115]. Immunohistochemical analysis was performed using anti-phospho-NF-κB p65 monoclonal antibody. Following 72-hour ureteral obstruction, phospho-NF-κB p65 immunoreactivity was observed in both unobstructed and obstructed ureters (Figure 2-10A). The immunoreactivity of phospho-NF-κB p65 in obstructed ureters was increased compared to that in contralateral ureters (1.19-fold, $p < 0.05$) (Figure 2-10B). Compared to what we observed on RNA expression of obstructed ureters where we found no distinguishable change in NF-κB p105 (Figure 2-7-1D), we found increased NF-κB p65 activation following 72-hour obstruction. NF-κB p105 generates NF-κB p50, which not only binds to p65, but also has separate function as a homodimer (reviewed by Yu et al.[116]). Therefore, different results may result from the distinct function of p50. The difference can also be caused by mechanisms that regulated post-translational modification of p65.

A. Paraffin-embedded ureteral tissues immunohistochemically stained for phospho-NF-κB p65

72-hour obstructed **Contralateral**



B. Quantitative analysis of phospho-NF-κB p65 staining

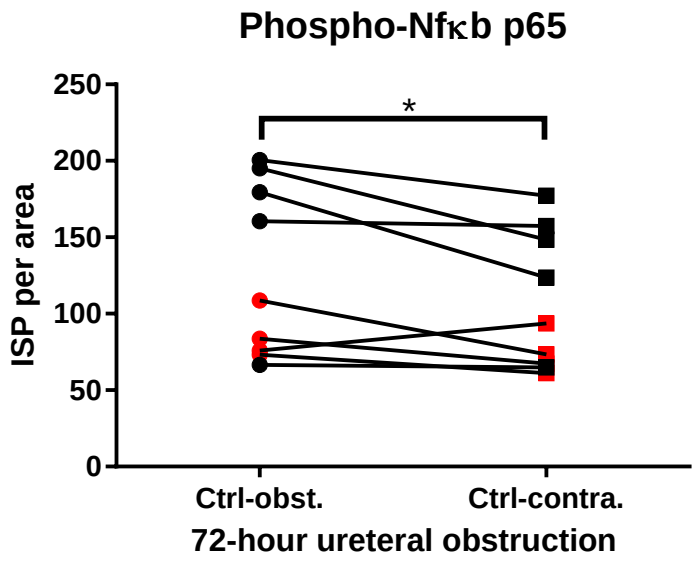


Figure 2-10 Effect on phospho-NF-κB p65 expression in 72-hour obstructed uroepithelial tissues

A. Representative pictures showing immunohistochemistry with antibody for phospho-NF-κB p65 in the 72-hour obstructed ureters. Cells with brown staining represent positive staining and cells with blue staining represent negative staining. **B.** Quantitative analysis of phospho-NF-κB p65 expression in uroepithelium in 72-hour obstructed ureters. The intense strong positive staining counts per area was counted in three random high power fields of the obstructed or non-obstructed contralateral ureters of 72-hour unilateral ureteral obstruction. A line connected with 2 dots represents paired data per each animal. Different color of dots indicates independent experiments (black and red). Data are presented for individual value. Asterisk indicates $p < 0.05$.

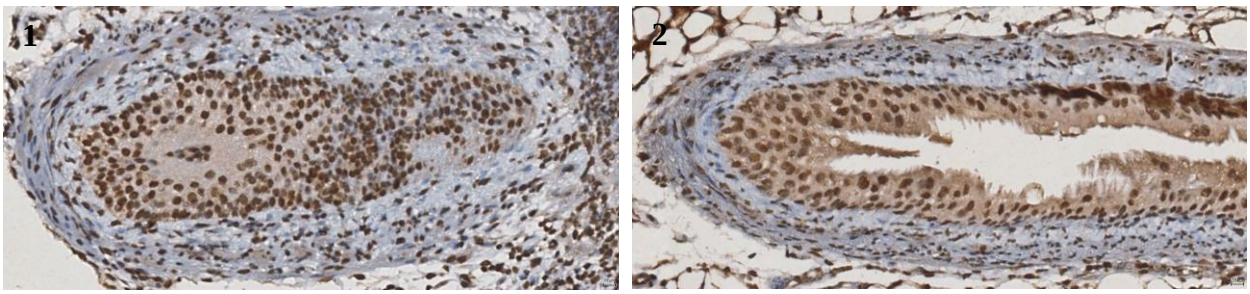
2.3.3.3 Effect of 72-hour obstruction on phospho-STAT5 staining in the ureters

Similar to the activation of NF-κB, STAT5 activation is shown to be regulated by post-translational modification on its tyrosine residues after stimulation by cytokines (i.e. EPO) (reviewed by Bunting et al.[117]). To examine phospho-STAT5 in obstructed ureters, immunohistochemical analysis was performed using anti-phospho-STAT5 monoclonal antibody. Following 72-hour ureteral obstruction, phospho-STAT5 immunoreactivity was observed in both

obstructed ureters and unobstructed contralateral ureters (Figure 2-11A). There was no significant difference between obstructed ureters compared to contralateral ureters in the immunoreactivity of phospho-STAT5 (0.95-fold, p = 0.9688) (Figure 2-11B). It was interesting to compare immunohistochemistry results and RNA expression results, where we found downregulated STAT5A in both ureters and kidneys (Figure 2-7-1E, 2-7-2E). There is a possibility that the total amount of STAT5 protein gets downregulated, however increased phosphorylation on STAT5 may lead to compensate decreased total STAT5 protein. To elucidate it, future works are required to perform IHC to measure the total STAT5 protein.

A. Paraffin-embedded ureteral tissues immunohistochemically stained for phospho-STAT5

72-hour obstructed **Contralateral**



B. Quantitative analysis of phorpho-STAT5 staining

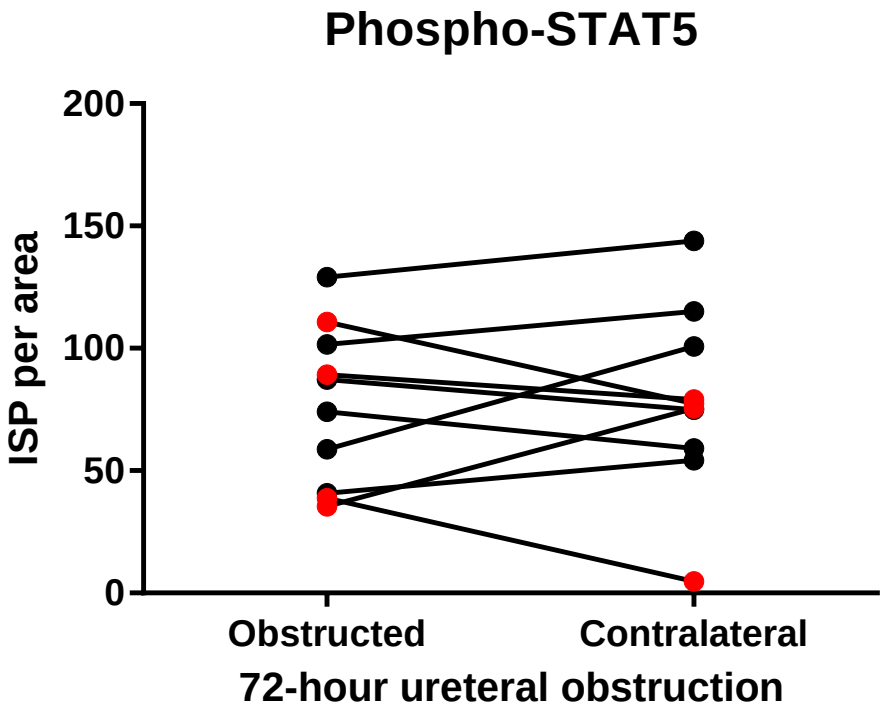


Figure 2-11 Effect on phospho-STAT5 expression in 72-hour obstructed uroepithelium

A. Representative pictures showing immunohistochemistry with antibody for phospho-Stat5 in the 72-hour obstructed ureters. Cells with brown staining represent positive staining and cells with blue staining represent negative staining. **B.** Quantitative analysis of phospho-STAT5 expression in uroepithelium in 72-hour obstructed ureters. Intense strong positive staining counts per area was counted in three random high power fields of the obstructed or non-obstructed contralateral ureters of 72-hour unilateral ureteral obstruction. A line connected with 2 dots represents paired data per each animal. Different color of dots indicates independent experiments (black and red). Data are presented for individual value. Asterisk indicates $p < 0.05$.

2.4 Discussion

Here, we showed that ureteral obstruction alters that EPOR signaling in the ureters, which leads to apoptosis and peristaltic dysfunction. Previous study in our laboratory showed that obstructed

ureters exhibit EPO and EPOR-βCR RNA expression and EPO protein expression in ureteral muscle layer using 3 mice[11]. Chuang et al. showed BCL-2, BCL-XL, BAX expression in obstructed ureteral muscle layer in a rat model[118]. Our study is the first study to show RNA expression of EPOR-βCR signaling in obstructed ureters over the time course using 2 mice strains. We first focused on studying the effects in CD-1 strain as that is the mouse strain that the original observations were made in[11]. Several studies have shown that difference in mice strains leads to different physiological responses which can sometimes be even opposite[119]. To verify that our findings on gene expression were not CD-1 strain specific and also to compare the responses between the mice strains, we investigated EPOR signaling in C57BL/6 mice. C57BL/6 was chosen as it is the most widely used experimental mouse model and therefore a very well-characterized laboratory mice strain. We could observe inter-strain difference, as ureteral tissues of CD-1 started to show decreased BCL-2 and BCL-XL expression from 72-hour obstruction whereas ureteral tissues of C57BL/6 showed decreased expression of BCL-2 from 24-hour obstruction and decreased expression of BCL-XL from 48-hour obstruction. This result suggests that C57BL/6 strain is more sensitive to obstructive injury than the CD-1 strain. It is supported by other studies where C57BL/6 strain shows more sensitive responses including more apoptosis to the various injury than CD-1 strain. Madri et al. showed that C57BL/6 strain exhibited higher number of apoptotic cells than CD-1 strain in CNS injury model[120]. Using streptozotocin-induced diabetic model, Li et al. showed that C57BL/6 strain presented higher number of apoptotic cells in islet than CD-1 strain[121], and Rossini et al. showed that C57BL/6 strain exhibited more sensitive hyperglycemic response than CD-1 strain[122]. Masubuchi et al. also showed that C57BL/6 strain presented more sensitive toxic responses in acetaminophen-induced liver injury than BALB/c strain[123]. This is the first study to directly compare the genetic

responses to ureteral obstruction between C57BL/6 and CD-1 mice. And we observed that C57BL/6 showed more sensitive response with the same trend in expression as CD-1 to obstructive injury, which lead us to pursue further studies in C57BL/6 strain.

Our results showed that ureteral peristaltic counts showed diminished or abolished ureteral function after ureteral obstruction. Previous study in our laboratory showed that peristaltic function is lost in 24-hour obstructed ureters using 3 mice per group[11]. With increased sample size in our experiment (n = 10, 2 strains), we could find that 70% of mice lost peristaltic function. Ureteral obstruction disrupted ureteral peristaltic movement, which can further damage renal and ureteral function. Upon acute ureteral obstruction, the peristaltic rate and contractile force of the ureter smooth muscles initially increased[124]. However, after prolonged obstruction peristaltic activity and contractile force continually decreased until it was completely lost. Our results showed that after 24-hour obstruction, 70 % of mice had completely lost peristaltic function whereas 30 % of mice retained peristaltic function but with decreased activity. These findings also suggest that there are individual variances, in regards to losing peristaltic movement after ureteral obstruction. Reduced peristaltic activity was observed at 24-hour obstruction and obstructed ureters had completely lost their peristaltic movement following 48 and 72-hour obstruction. Our findings are consistent with a previous study from our laboratory where ureteral obstruction for 24 hours decreased ureteral peristaltic activity and its activity was not recovered until 10 days following the release of the obstruction. Prolonged obstruction in the ureteral tissues induces ureteral fibrosis, hypertrophy of smooth muscles, proliferation of intracellular collagen fibers and submucosal connective tissues[125,126].These structural changes

following obstruction might increase the stiffness of the ureteral wall and impede peristalsis in the same way as shown in gastrointestinal tract of patients with progressive systemic sclerosis[127].

Previous studies have shown that EPOR signaling plays an additional important role including pleiotropic roles apart from its hematopoietic function. Current findings from this project suggest that EPOR signaling has modulatory effects in obstructive uropathy (detailed in Figure 2-12). Decreased EPOR signaling after ureteral obstruction leads to dysfunction of EPO's regulatory mechanisms that are essential for maintaining normal function of upper urinary tracts. Under healthy conditions, EPO may work as a paracrine factor to regulate proper function in upper urinary tract. However following injury, decreased EPO signaling results in loss of normal functioning. This is supported by earlier studies which have shown that mice lacking EPO fail to show the normal development of brain, liver and heart[40,41].

Ureters share similar peristaltic function to that of the intestine in the gastrointestinal system, which uses a highly conserved apoptotic process to delete damaged and deleterious cells to support its high turn-over rate. Dysregulation of apoptosis in gastrointestinal system is implied in different pathophysiological conditions including inflammatory bowel disease[128], malabsorption syndrome[129], colon cancer[130] and injuries from radiation exposure[131]. The observation that apoptosis promoted injury of ureteral tissue during obstruction suggests that a similar apoptotic dysregulation as seen in gastrointestinal disease may also be applied to pathophysiology of obstruction induced ureteral dysfunction.

Our results from this study of decreased EPOR signaling pathway after ureteral obstruction is also supported by other studies where injuries induced by exposure to toxic substances or pro-inflammatory cytokines decreased endogenous EPO and EPOR expression[132-135]. Reduced endogenous EPO and EPOR by ureteral obstruction impairs EPOR signaling that leads to decreased expression of genes that regulates anti-apoptosis, which are BCL-2, BCL-XL and STAT5. As a result of decreased anti-apoptotic signaling, apoptosis occurs without inhibition (Figure 2-9). As the main regulatory hormone of red blood cell production, EPO promotes erythroid progenitor survival by rescuing these progenitors from committing apoptosis, primarily through anti-apoptotic BCL-XL and BCL-2[37].

Upon ureteral obstruction, the renin-angiotensin system (RAS) is activated, and therefore angiotensin II is increased. Angiotensin II constricts the blood vessels to decrease the blood flow that has been increased in response to acute ureteral obstruction for as early as 24 hours[136]. Angiotensin II plays a central role in recruiting inflammatory factors including NF-κB in the obstructed kidneys[137]. Some studies have shown that angiotensin II in the normal kidneys is related to increased EPO expression[138]. Our results of decreased EPO and EPOR signaling showed that there is other signaling apart from RAS involved in ureteral obstruction that leads to decreased EPOR signaling. However, further work will be needed to measure angiotensin II level in our model to validate the relation between renin-angiotensin system (RAS) signaling and EPOR signaling in our model.

It is important to note the increased BCL-XL expression in 24-hour obstructed kidneys of CD-1 strain, compared to decreased BCL-XL expression in longer obstructed kidneys for 48 and 72

hours. This suggests a self-protective mechanism against the mild injury, where cells promote healing and survival by increasing anti-apoptotic signaling. However, as the obstructive injury is prolonged, this self-protective resistance fails and apoptotic signaling starts to increase as shown in 48, 72-hour obstructed kidneys. Similarly, self-protective resistance against mild injury is also observed in decreased apoptotic BAX in 24-hour obstructed ureters in C57BL/6 strain, whereas this decrease is abolished after longer obstruction for 48 and 72 hours. This is supported by the study from Chaung et al. that ureteral tissues exhibit a self-protective mechanism via modulating BCL-2 and BCL-XL expression against long-term ureteral obstruction in a rat model[118].

Immunohistochemistry results showed that ureteral obstruction increased phospho-NF-κB p65 in murine ureteral epithelium. Ureteral epithelium is translational epithelium that responses to rapid change of environment (section 2-3-3). Epithelium is shown to regulate smooth muscle function in the vessel (reviewed by Spina et al.[139]) and trancheal smooth muscle[140]. NF-κB is a transcriptional factor that responses to stress in the cells. NF-κB cooperates with multiple signaling pathways to regulate inflammation and apoptosis[141]. Studies have shown the detrimental roles of NF-κB in obstructed kidneys. NF-κB was shown to play a major role in increasing renal fibrosis, inflammation and oxidative stress following ureteral obstruction, and inhibition of NF-κB decreases the degree of the renal injuries[142-145]. Our study is the first study to demonstrate the activation of NF-κB by 72-hour ureteral obstruction in the ureteral epithelium, as well as showing the relatively short-term effect of ureteral obstruction as most studies have used longer duration of ureteral obstruction. The role of NF-κB in ureteral obstruction in the ureters could be similar to that in the kidney, which would be increasing fibrosis and deformation of smooth muscles that would be detrimental to peristaltic function. It is noteworthy to compare

translational expression of phospho-NF-κB p65 to transcriptional expression of NF-κB p105. RNA expression of NF-κB p105 was decreased in 24, 72-hour obstructed ureters in C57BL/6 and was increased in 48 and 72-hour obstructed kidneys in CD-1 and C57BL/6. NF-κB p105 is degraded by 26S proteasome to produce NF-κB p50. p50 binds to p65 and translocate to nucleus upon activation to induce NF-κB signaling. p50 can also form homodimer to induce distinct function that are regulates apoptosis, cell proliferation and inflammation (reviewed by Yu et al.[116]). Since p50 exerts distinct mechanisms without binding to p65, activation of phospho-NF-κB p65 and RNA expression of NF-κB p105 may not align together. In addition, different locations of the ureteral samples for each assay may have affected the expression. Since RNA extraction from the tissues requires homogenization, we could not use the identical samples for RNA expression and histology (Figure 2-1). The samples used for analyzing RNA expression was collected from proximal to mid ureters, and the samples for histology was collected from mid to distal ureters. As the obstruction was induced on the lower part of distal ureters, we confirmed that collected ureteral samples were obstructed. Though there could be difference in sensitivity according to the location in the ureters, we hypothesized that the expression pattern to the obstruction would be identical along the ureters. Future work may be needed to sample the entire ureters for each assay by using different animal to validate our finding.

STAT5 mediates the anti-apoptotic response to EPO by directly binding to the promoter of the BCL-XL gene in erythroid cells[146,147]. Our finding from this study showed STAT5A expression was reduced after 24, 48, 72-hour ureteral obstruction in both kidneys and ureters from two different strains. It suggests that STAT5 may also be regulated at the transcriptional level, in addition to alteration of its function via phosphorylation of the protein. Nakamura et al. also

showed that STAT5 is regulated in transcriptional level in hematopoietic cells[148]. Interestingly, immunohistochemistry results showed that there was no significant difference in phospho-STAT5 staining between 72-hour obstructed ureters and its contralateral unobstructed ureters. Effects on translational level by decreased transcriptional changes may be delayed, which could not be observed in 72 hours of our experiment. In addition, we examined phospho-STAT5 expression through immunohistochemistry. Post-translational modification of STAT5 is affected by more complicated signals including FLT3, SRC[149,150]. SRC expression was shown to be increased in the obstructed kidneys[151]. Increased signaling that affects phosphorylation of STAT5, such as SRC, might compensate decreased STAT5 expression. This suggests that ureteral obstruction induced complex signaling in addition to EPOR signaling, that are interactive to each other. And our study is the first study to look at STAT5 expression after ureteral obstruction.

Chuang et al. showed that ureteral obstruction increased BCL-2, BCL-XL and BAX expression and increased apoptosis in ureteral tissues, using ligation-induced ureteral obstruction in a rat model[118]. They showed that BAX protein was increased in 14, 21-day obstruction and BCL-2, BCL-XL protein was increased in 21, 28-day obstruction accompanying increased apoptotic cells shown as TUNEL-positive cells in 14, 21, 28-day obstruction in ureteral tissues[118]. In their model, BAX expression was increased at 14-day obstruction accompanied with increased apoptosis, and anti-apoptotic BCL-2, BCL-XL started to increase at 21-day obstruction to compensate for the increased apoptotic signaling in obstructive uropathy. This suggests that BAX/BCL-2 and BCL-XL proteins have cell survival mediating roles in ureteral smooth muscles during obstructive uropathy. In support of this prior work, our study also showed that the

expression of apoptosis-related regulators including BCL-2, BCL-XL and apoptosis was associated with short-term obstructive uropathy. Interestingly, Chaung et al. showed no change in BCL-2 and BCL-XL protein following ureteral obstruction shorter than 14 days, whereas our data showed a significant decrease in anti-apoptotic genes including BCL-2 and BCL-XL after 3-day obstruction. This may suggest that transcriptional signaling might be more sensitive than translational signaling for detecting milder changes in short-term ureteral obstruction[152]. Regardless of these varying time points between our study and that of Chaung et al., both studies detected apoptosis in ureteral tissues at the same time as detecting changes in apoptotic signaling.

βCR is a common subunit that forms a dimer with α subunits of IL-3, IL-5 and GM-CSF to initiate intracellular signaling. IL-3, IL-5 and GM-CSF have overlapping pleiotropic functions on hematopoietic cells by inducing inflammatory pathways and survival of these cells[153]. Increased βCR in the kidney after ureteral obstruction may be related to the inflammation induced by ureteral obstruction. Ureteral obstruction results in inflammation accompanying increased infiltration and activation of macrophages by recruiting the chemokines in the kidneys[107,154-156]. Increased βCR with decreased EPOR promotes EPO's susceptibility to bind EPOR-βCR heterodimer rather than EPOR homodimer, increasing the chance of EPO to induce tissue-protective function rather than hematopoietic function. This is suggestive of an innate self-protective mechanism, which becomes insufficient when EPO level decreases. To promote this protective mechanism, intracellular administration of supplemental EPO could boost EPOR-βCR signaling.

TUNEL assays performed in our study showed that ureteral obstruction induced apoptosis in ureteral tissues. This finding aligns well with our results on decreased anti-apoptotic gene expression by obstruction. Many studies have shown that ureteral obstruction induces apoptosis in the kidney[16,157-160], however the current work of this project is the first study that demonstrates the occurrence of apoptosis in ureteral tissues in a short-term unilateral ureteral obstruction model. Chuang et al. showed that 14-day ureteral obstruction increased apoptosis in myocyte of ureteral smooth muscle accompanying fibrosis and hypertrophic uromyopathy[23]. Using 6 rats per group, they found that apoptosis in ureteral myocytes start to increase after 14 days of obstruction. They could not find TUNEL-positive cells on 1, 3, 7, 10-day obstructed group. Our results show that apoptosis increased after 3 days of obstruction, which could be explained by inter-species variability and different sensitivity of TUNEL assays. Ureteral apoptosis has also been shown in clinical cases of congenital obstructive uropathy. Kang et al. compared the apoptosis of obstructive megaureters and refluxing megaureter in pediatric patients[161]. Obstructive megaureters showed higher apoptosis compared to refluxing megaureter, although this study did not have a control group to compare with. Kajbafzadeh et al. investigated smooth muscle apoptosis on ureteropelvic junctions in congenital obstructive patients compared to age-matched normal patients[162]. They found increased smooth muscle apoptosis and decreased nerve terminal in ureteropelvic junctions where kidney and ureter are connected. And our results showed increased apoptosis in 72-hour obstructed ureters accompanying decreased BCL-2, BCL-XL and STAT5 expression. Future work will be needed to support our finding by examining pro-apoptotic genes such as BAD, BIK and BIM, as well as their activation in mitochondria.

2.5 Conclusion

We demonstrated that ureteral peristaltic function is lost by ureteral obstruction for as early as 24 hours. And increased apoptosis in the ureters was found in 72-hour obstructed ureters, which might cause dysfunction of ureteral peristalsis. Ureteral obstruction decreased EPOR signaling genes that regulates apoptosis. Decreased anti-apoptotic downstream genes including BCL-2, BCL-XL and STAT5A, with NF-κB p65 activation was followed by decreased EPO and EPOR expression in obstructed ureters and kidneys. (Figure 2-12)

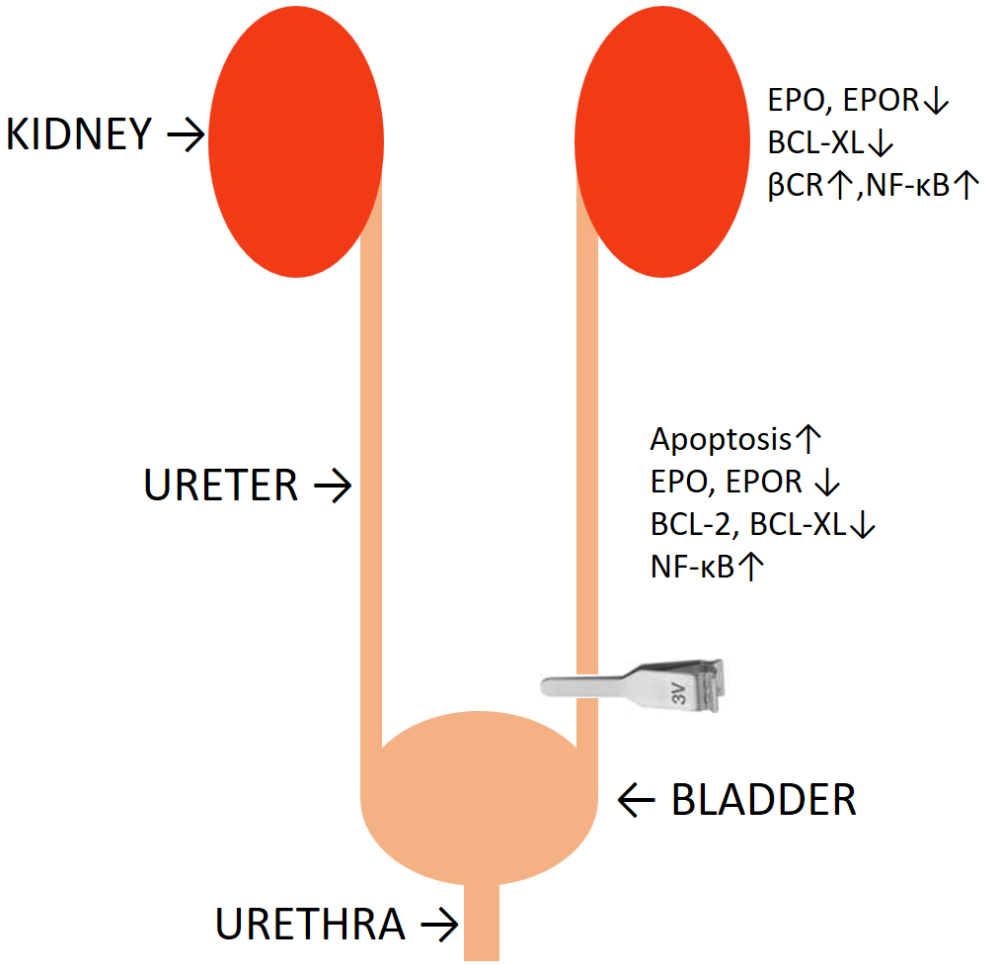


Figure 2-12 Overview on the effect of ureteral obstruction on EPOR-βCR signaling

Representative diagram of EPOR signaling in the ureters and the kidneys after ureteral obstruction in 2 strains of rodents. ↑ represents increased expression or immunoreactivity. ↓ represents decreased expression or immunoreactivity. Clip represents the unilateral ureteral obstruction.

Chapter 3: Prophylactic EPO rescued 72-hour obstructed ureters from apoptosis via suppressing NF-κB activation and BAX

3.1 Introduction

In order to study the mechanisms of prophylactic EPO treatment on accelerating recovery from ureteral dysfunction, we examined EPOR signaling and ureteral apoptosis in ureteral obstruction with prophylactic EPO treatment and compared it to that of without EPO treatment (Chapter 2). *We hypothesized that prophylactic EPO treatment accelerates the functional recovery of ureteral peristalsis from ureteral obstruction via anti-apoptotic mechanisms*[11].

In addition, some studies, mostly in brain research area, have shown that EPO treatment in non-injured organs activates anti-apoptotic EPOR signaling to cope with potential injuries, which is called pre-conditioning. However, the pre-conditioning effect varied depending on experimental conditions and the organs studied, as other studies shows that EPO treatment itself without injury does not induce any change in gene expression. *We hypothesized that prophylactic EPO treatment induces pre-conditioning effect on EPOR signaling apoptosis-related genes on murine ureteral and renal tissues.*

3.2 Methods

3.2.1 Animals

A total of 20 C57BL/6 were used in this experiment. Animals were treated as detailed in section 2.2.1.

3.2.2 Unilateral ureteral obstruction model

Unilateral ureteral obstruction was induced as detailed in section 2.2.2.

3.2.3 EPO treatment

20 IU of Human biosynthetic epoetin alfa (Eprex, 200 µl) diluted in saline (100 IU/ml) was administered once daily intraperitoneally for 4 consecutive days before and on the day of ureteral obstruction. The results were compared to those from non-treated animal (Chapter 2). For pre-conditioning experiment, control animals received 0.2 ml of saline intraperitoneally.

3.2.4 Complete blood cell count analysis

Mice received 0.2 ml of EPO (20 IU) or saline intraperitoneally once daily for 4 days. On day 4, 100 µl blood was collected via intracardiac or renal vena cava just prior to euthanasia. Complete blood count analysis was performed with VetScan VS2 (Abaxis, Union City, CA, USA) to test whether EPO administration triggered hematopoiesis by the end of prophylactic EPO treatment.

3.2.5 Ureteral function evaluation

After prophylactic EPO treatment, ureteral function with or without obstruction for 24, 48, 72 hours was assessed as detailed in section 2.2.3.

3.2.6 TUNEL assay

TUNEL assay was performed as detailed in section 2.2.5.

3.2.7 Immunohistochemistry

Immunohistochemistry with anti-NF-κB p65 (phospho S536) antibody or anti-STAT5 (phospho Y694) on ureteral tissues was performed as detailed in section 2.2.6.

3.2.8 Quantitative Real-Time PCR

Quantitative real-time PCR was performed as detailed in section 2.2.4.

3.2.9 Statistical analysis

To analyze the effect of EPO on ureteral peristaltic function, Fisher's exact test was used to compare the proportions of aperistlatic ureters according to different treatment groups, as the data was nonparametric. To analyze TUNEL assay, paired t-test was used to compare obstructed

ureters to contralateral ureters and one-way ANOVA test followed by Turkey's multiple comparison test was used to compare between different treatment groups in TUNEL assay. Statistical analysis was performed as detailed in section 2.2.7..

3.3 Results

3.3.1 Ureteral function evaluation

3.3.1.1 Effect of prophylactic EPO treatment on ureteral peristaltic activity in normal ureters

Hefer et al. showed that 90-minute incubation in EPO 50 IU/ml increased the contractile force of murine myocardial smooth muscles strips[163]. To examine if EPO can directly affect contractility and peristalsis of the ureter in our model, we looked at the peristalsis after treating with EPO. Our results showed that there was no distinguishable difference in peristaltic count of ureters in the EPO-treated group compared to control treated group without obstruction (0.88-fold, $p = 0.5451$) (Figure 3-1). The peristaltic count ranges from 5 to 20 in both groups, which was under a range that we observed in our previous experiment (Figure 2-2). Our results showed that ureteral peristaltic function is not directly affected by EPO treatment without injury. It suggests that improved peristaltic activity following EPO treatment is caused by its protective function against the injury, rather than directly inducing peristalsis in the obstructed ureters. Literatures about EPO's effect on muscle contractility is limited and controversial. In contrast to the study by Hefer et al., previous work in our lab showed that incubation in EPO decreased the contractile

force of smooth muscle in human distal ureteral tissues (unpublished data). In addition, peristalsis is harmonization of contraction and relaxation, which is regulated by complex system including pacemarker activity. Also, there could be variance between different sample types and experimental setting. However, we cannot ignore that EPO treatment could have affected contractile force of ureteral smooth muscle cells, not peristaltic frequency. Further investigation about the effect of EPO on the contractile force of ureteral muscles in the entire organism is warranted in the future work.

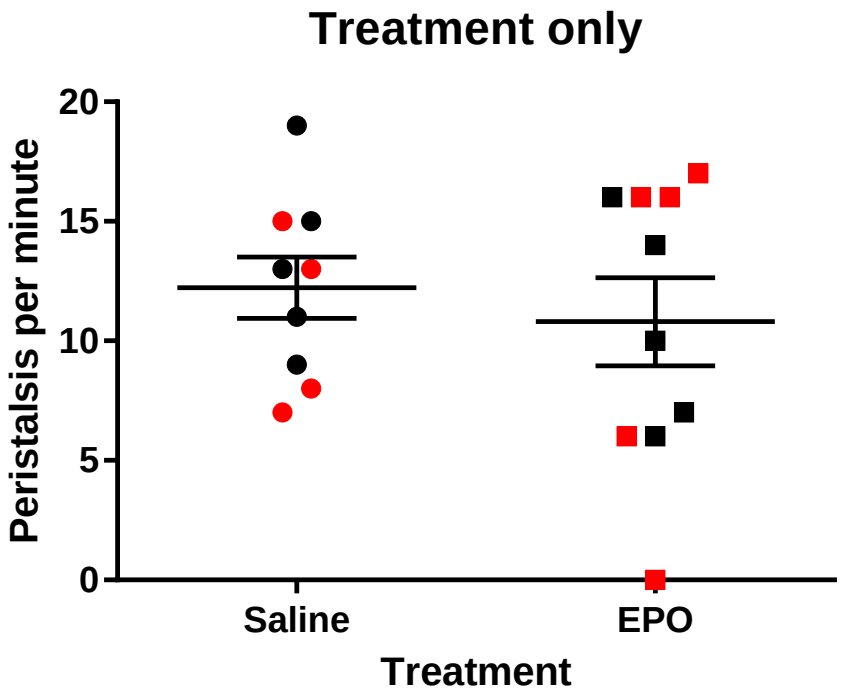


Figure 3-1 Effect of EPO treatment on ureteral peristaltic activity

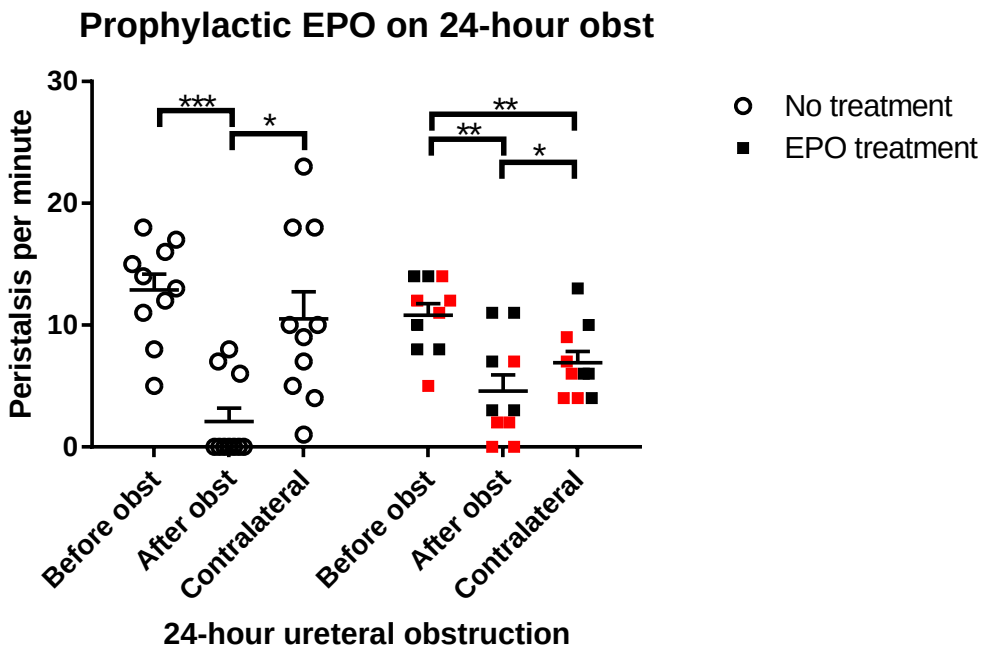
Ureteral peristaltic activity per minute of the ureters in either prophylactic EPO or control (saline)-treated mice. Different color of dots indicates independent experiments (black and red). Data are presented as mean ± SEM.

3.3.1.2 Effect of prophylactic EPO treatment on ureteral peristaltic activity in obstructed ureters

Previous work in our laboratory showed that prophylactic EPO treatment accelerated the recovery of ureteral function from obstruction[11]. And our work has shown that even 24-hour obstruction led to aperistalsis in the majority of mice ureters (Figure 2-4). To examine the effect of prophylactic EPO treatment on ureteral function after obstruction, we examined the peristaltic function of the obstructed ureters with prophylactic EPO treatment. After 24-hour obstruction, 80% of mice retained ureteral peristaltic activity with prophylactic EPO treatment compared to 30% without treatment (2.2-fold, $p = 0.0698$). Average of peristaltic count after obstruction was also higher after prophylactic EPO treatment compared to no treatment (Figure 3-2A). Since prophylactic EPO without injury did not affect ureteral peristaltic activity (Figure 3-1), the effect of prophylactic EPO treatment came from protecting the ureteral function from obstructive injury. Ureteral peristaltic count was decreased in 24-hour obstructed ureters compared to that of before obstruction with prophylactic EPO treatment (0.43-fold, $p < 0.05$). Ureteral peristaltic count was decreased in 24-hour obstructed ureters compared to that of contralateral after obstruction with prophylactic EPO treatment (0.67-fold, $p < 0.05$). Ureteral peristaltic count was decreased in contralateral ureters after obstruction compared to that of before obstruction (0.64-fold, $p < 0.05$).

In 72-hour obstruction group, we found interesting results where 1 mouse still showed peristaltic activity with prophylactic EPO treatment. It shows individual variance in ureteral functional recovery that we also observed in losing ureteral peristaltic function after 24-hour obstruction (Figure 2-3), as well as regaining ureteral peristaltic function after EPO treatment. Still, increased sample size is needed to validate EPO's protective effect on 72-hour obstructed ureteral function. Ureteral peristaltic count was decreased in 72-hour obstructed ureters compared to that of before obstruction with prophylactic EPO treatment (0.14-fold, $p < 0.01$). Ureteral peristaltic count was decreased in 72-hour obstructed ureters compared to that of contralateral after obstruction with prophylactic EPO treatment (0.319-fold, $p < 0.05$). Peristaltic activity after 72-hour obstruction in contralateral ureters from that of before obstruction could not be distinguished from one another (0.72-fold, $p = 0.2804$). Peristaltic activity in 72-hour obstructed ureters with or without prophylactic EPO treatment could not be distinguished from one another ($p = 0.8226$). (Figure 3-2B).

A. 24-hour obstruction with EPO treatment



B. 72-hour obstruction with EPO treatment

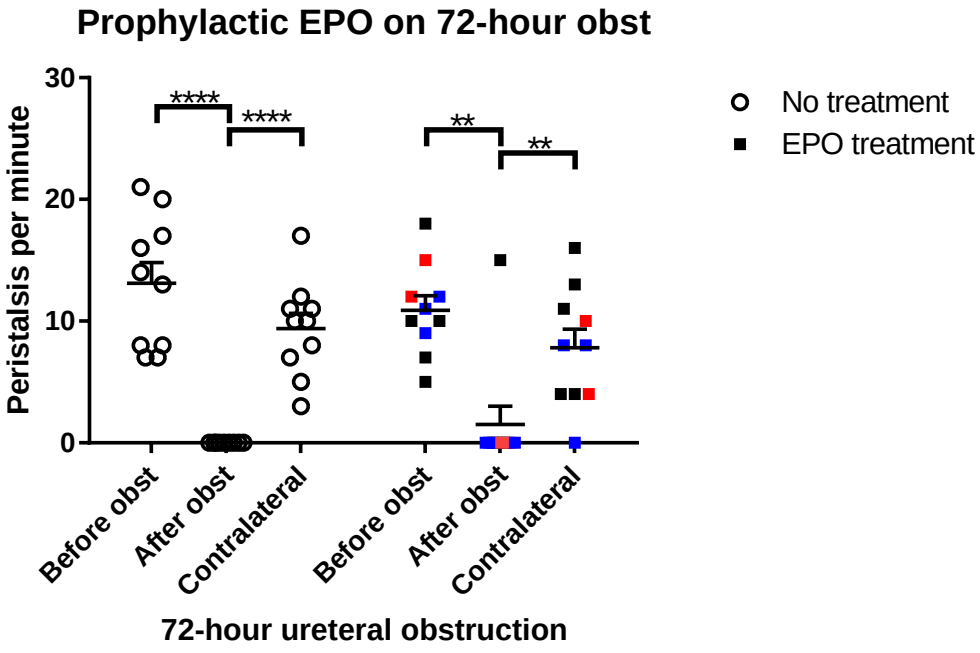


Figure 3-2 Effect of prophylactic EPO treatment on ureteral peristaltic activity after obstruction

Ureteral peristaltic activity per minute under surgical microscope after 24, 72-hour ureteral obstruction in C57BL/6 murine ureters. Ureteral peristaltic activity after 24, 72-hour unilateral ureteral obstruction of ureters before and after obstruction and its unobstructed contralateral ureter after obstruction with or without prophylactic EPO treatment. Different color of dots indicates independent experiments (black, red and blue). Asterisk indicates $p < 0.05$. Double asterisks indicate $p < 0.01$. Triple asterisks indicate $p < 0.001$. Quadruple asterisks indicate $p < 0.0001$. *Obst*, obstruction.

Taken together, prophylactic EPO treatment improved ureteral peristalsis in 24-hour obstructed ureters. Average ureteral peristaltic activity was higher than the untreated group, as less number of mice ureters was aperistaltic after prophylactic EPO treatment. EPO treatment did not change the peristaltic activity of contralateral ureters after 24-hour obstruction, although there was a slight decrease in peristaltic activity observed in EPO treated ureters. After 72-hour obstruction, the average ureteral peristaltic activity was increased with prophylactic EPO treatment, whereas there was no peristaltic movement in the untreated group. After 72-hour obstruction, ureteral peristaltic activity in contralateral ureters with or without prophylactic EPO treatment was not distinguishable between one another. (Figure 3-3) Overall trend of peristaltic activity in obstructed ureters showed improved function with prophylactic EPO treatment. Our results showed that EPO treatment protected ureteral peristaltic function against obstructive injury, in addition to accelerating the recovery of ureteral function shown in the previous work.

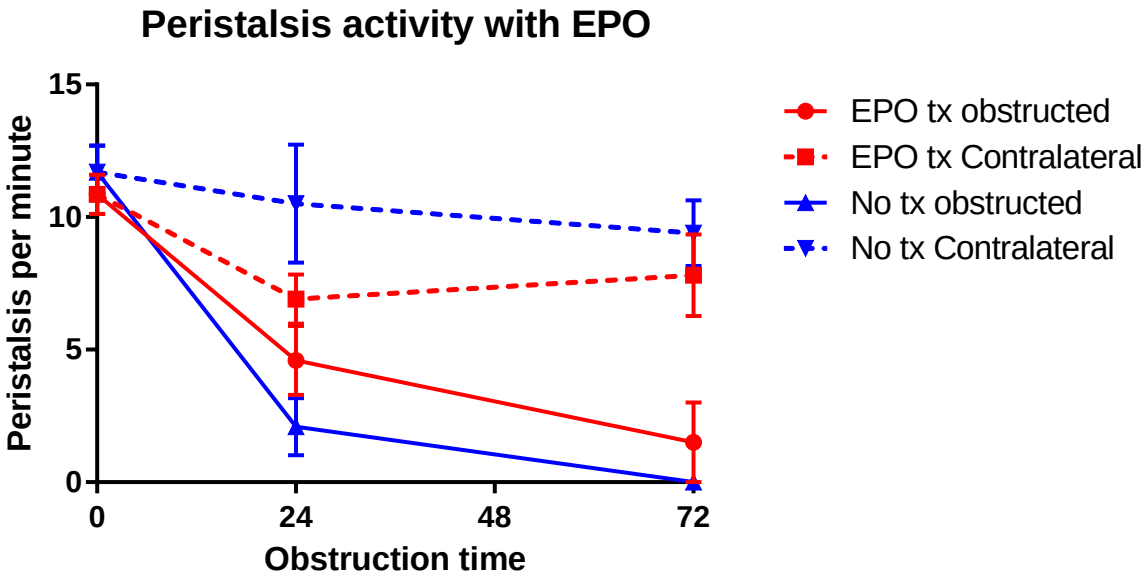


Figure 3-3 Time course of the effect of prophylactic EPO treatment on ureteral peristaltic activity after obstruction

Ureteral peristaltic activity after obstruction over the time course. Red color represents peristalsis of prophylactic EPO-treated group. Blue color represents peristalsis of the none-treated group (Chapter 2). Plain lines represent obstructed ureters. Plural dotted lines represent unobstructed contralateral ureters. *Tx*, treatment.

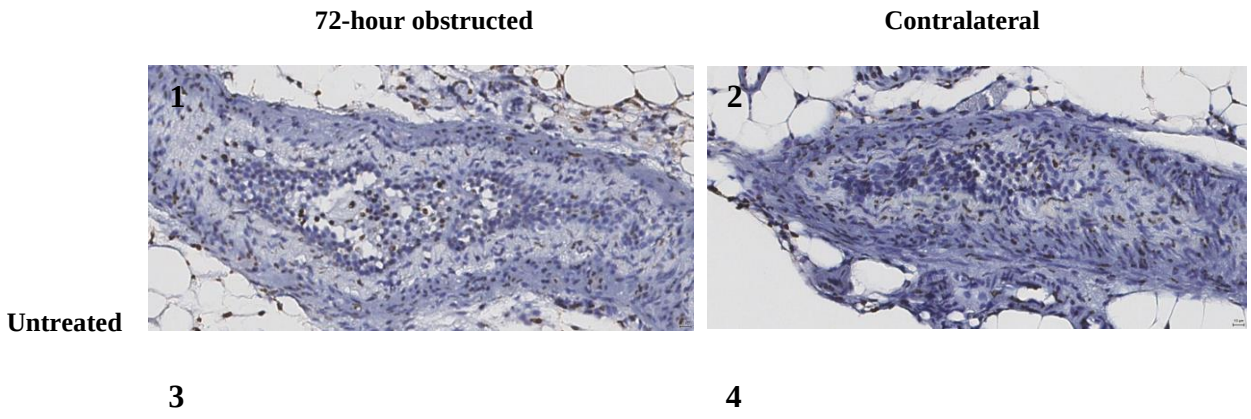
3.3.2 Immunohistochemistry

3.3.2.1 Effect of prophylactic EPO treatment on TUNEL assays in 72-hour obstructed ureters

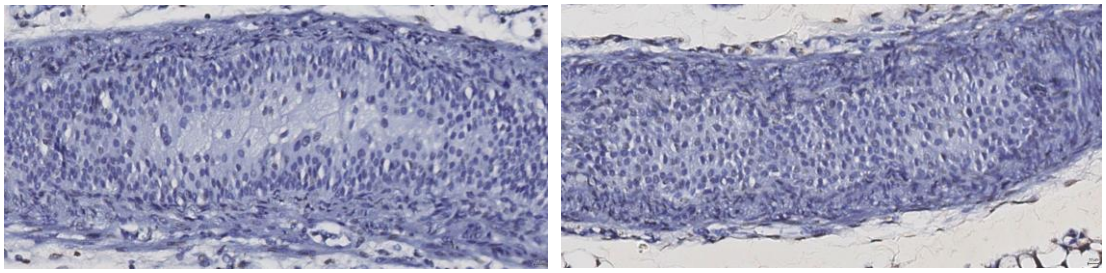
EPO was shown to exhibit protective function against the injury in various disease models mainly by decreasing apoptosis. In addition, our work showed that ureteral obstruction increased apoptosis in the ureters (Figure 2-8) and RNA expression data showed us anti-apoptotic signaling with prophylactic EPO treatment. Therefore, we investigated apoptosis in obstructed

ureters after prophylactic EPO treatment to see if the EPO treatment can attenuate apoptosis in the ureters against 72-hour obstruction. Obstructed ureters treated with prophylactic EPO treatment was compared to obstructed ureters without EPO treatment (Chapter 2). Our results showed suppressed apoptosis in obstructed ureters following prophylactic EPO treatment. We could observe much less apoptosis in EPO-treated ureters, and its degree was shown to be similar to contralateral unobstructed ureters (Figure 3-4A). Prophylactic EPO treatment decreased the number of apoptotic cells by 72-hour ureteral obstruction in the ureteral tissues (7.39-fold, $p < 0.05$). 72-hour obstructed ureters without EPO treatment showed increased apoptosis compared to prophylactic EPO treated contralateral ureters (8.49-fold, $p < 0.05$). In contrast, obstructed ureters and contralateral ureters after 72-hour obstruction with the prophylactic EPO treatment could not be distinguished from one another (0.87-fold, $p = 0.7008$) (Figure 3-4B). In conclusion, our results showed that EPO treatment suppressed apoptosis on 72-hour obstructed ureters, suggesting protective mechanisms of EPO against obstruction to protect ureteral function.

A. Paraffin-embedded ureteral tissues stained for TUNEL assay



EPO treated



B. Quantitative analysis of apoptotic cell death

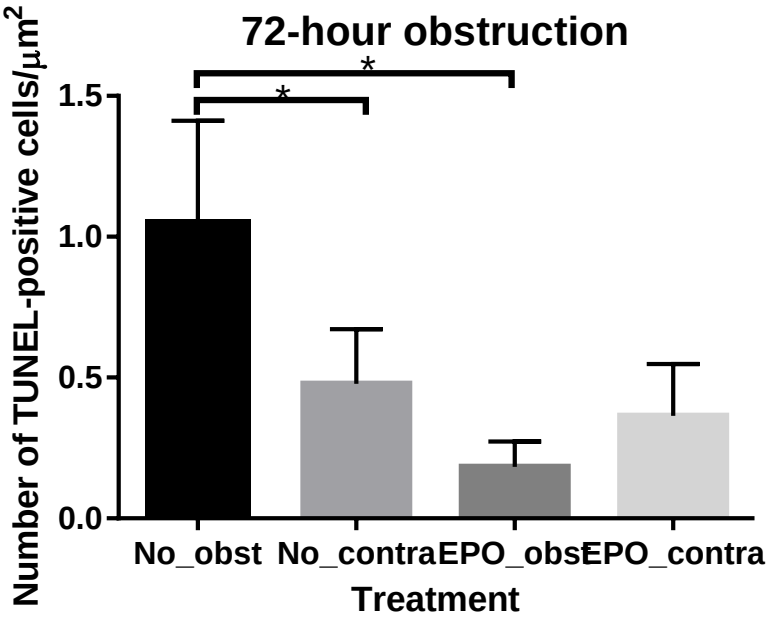


Figure 3-4 Effect on apoptosis in 72-hour obstructed ureters after prophylactic EPO treatment

A. Representative photomicrographs showing terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP) nick-end labeling (TUNEL) staining in the 72-hour obstructed ureter and its unobstructed contralateral ureter with or without prophylactic EPO treatment. Cells with brown staining represent positive staining and cells with blue staining represent negative staining. **B.** Quantitative analysis of apoptotic cells in 72-hour obstructed ureters and unobstructed contralateral ureters with or without prophylactic EPO treatment. The number of TUNEL-strong positive staining pixels counted in four random high power fields of the obstructed or non-obstructed contralateral ureters of 72-hour ureteral obstruction. Paired t-test was used for paired obstructed ureters and contralateral ureters in each treatment group. ANOVA followed by Turkey's multiple comparison test

was used between the groups. Data are presented as mean ± SEM. Asterisk indicates $p < 0.05$. *Obst*, obstruction. *Contra*, contralateral.

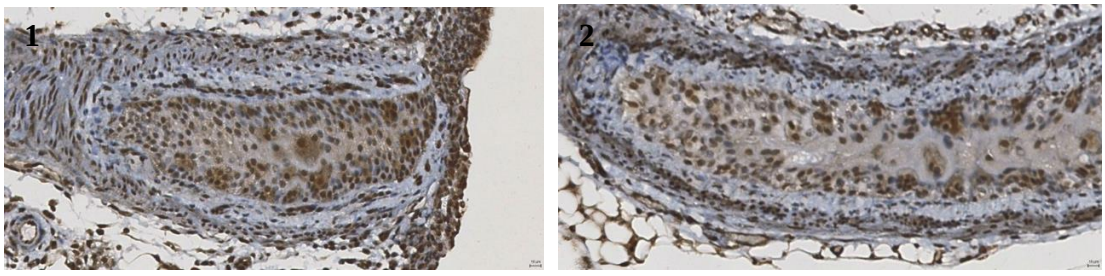
3.3.2.2 Effect of prophylactic EPO treatment on phospho-NF-κB p65 staining in 72-hour obstructed ureters

Our immunohistochemistry work showed that ureteral obstruction activated NF-κB p65 (Figure 2-8). Therefore, we decided to test whether EPO treatment affects the activation of NF-κB by obstruction. Obstructed ureters treated with EPO was compared to obstructed ureters without treatment. NF-κB activation by 72-hour obstruction was suppressed by prophylactic EPO treatment. The immunoreactivity of phospho-NF-κB p65 in the 72-hour obstructed ureters was increased compared to the contralateral ureters (Chapter 2). Although we still detected phospho-NF-κB p65 on EPO-treated ureters, the signal was reduced compared to untreated ureters (Figure 3-5A). The prophylactic EPO treatment decreased the activation of phospho-NF-κB p65 compared to no treatment (0.75-fold, $p < 0.05$). Phospho-NF-κB p65 activity in the 72-hour obstructed ureters and contralateral ureters with prophylactic EPO treatment could not be distinguished from one another (0.89-fold, $p = 0.2035$). (Figure 3-5B) In conclusion, prophylactic EPO treatment suppressed NF-κB p65 activation in the ureters induced by ureteral obstruction.

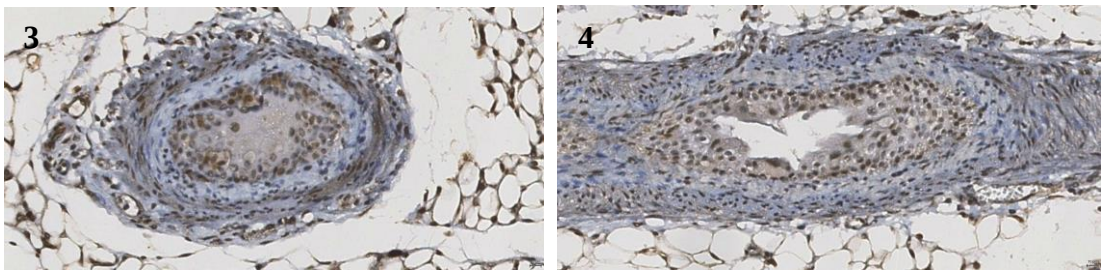
A. Paraffin-embedded ureteral tissues immunohistochemically stained for phospho-NF-κB p65

72-hour obstructed **Contralateral**

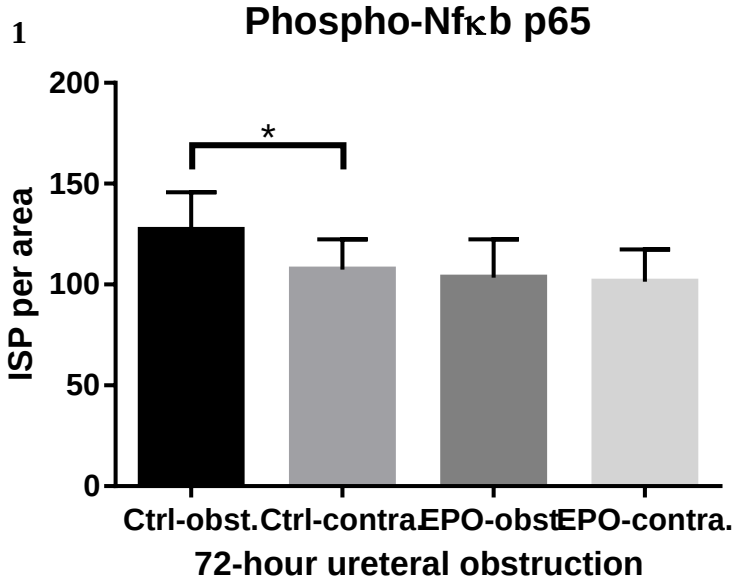
Untreated



EPO treated



B. Quantitative analysis of phospho-NF-κB p65 staining



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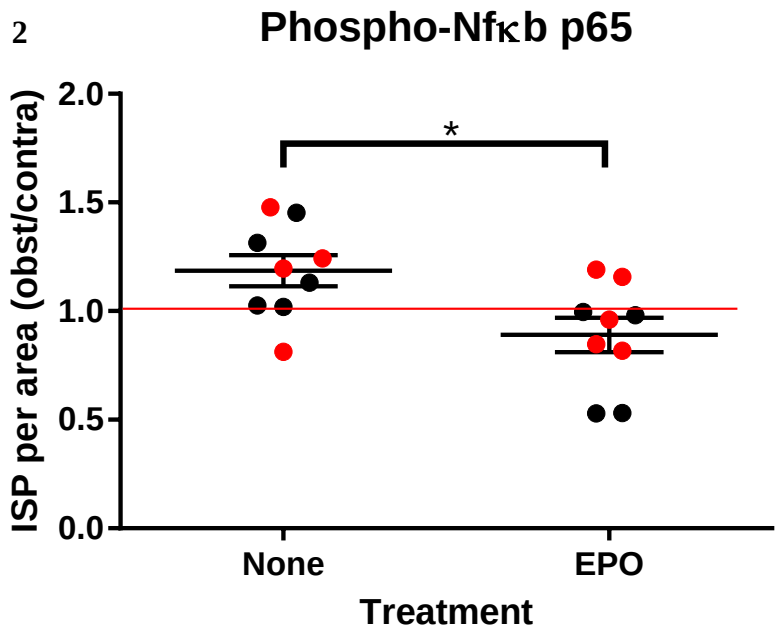


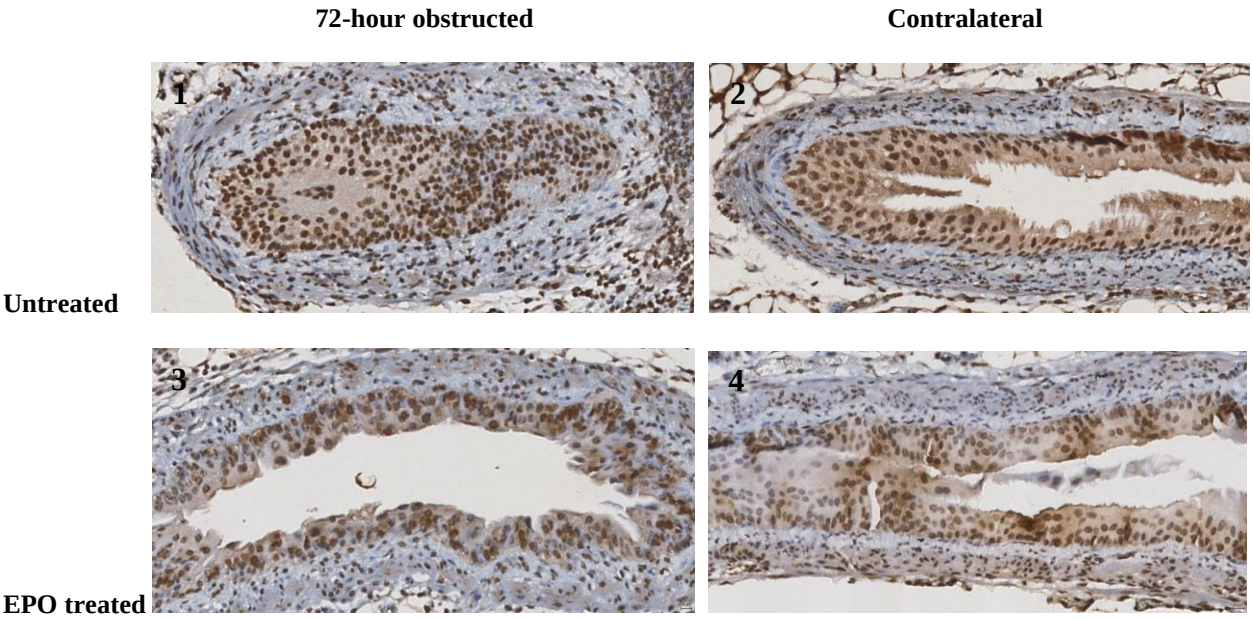
Figure 3-5 Effect on phospho-NF-κB p65 expression in 72-hour obstructed ureters after prophylactic EPO treatment

A. Representative pictures showing immunohistochemistry with antibody for phospho-NF-κB p65 in the 72-hour obstructed ureters with or without prophylactic EPO treatment. Cells with brown staining represent positive staining and cells with blue staining represent negative staining. **B.** Quantitative analysis of phospho-NF-κB p65 expression in uroepithelium in 72-hour obstructed ureters with or without prophylactic EPO treatment. Intense strong positive staining counts per area was counted in three random high power fields of the obstructed or non-obstructed contralateral ureters of 72-hour unilateral ureteral obstruction. Data are presented as mean ± SEM. Asterisk indicates $p < 0.05$. **B1.** Histogram of ISP per area in obstructed ureters and unobstructed contralateral ureters with or without prophylactic EPO treatment. **B2.** ISP per area in obstructed ureters was divided into that in unobstructed contralateral ureters in the individual animal. Each dot represents the ratio of staining in obstructed ureters compared to contralateral ureters of each animal. Different color of dots indicates independent experiment sets (black and red). Red line represents contralateral ureter in each animal. *Obst*, obstruction. *Contra*, contralateral.

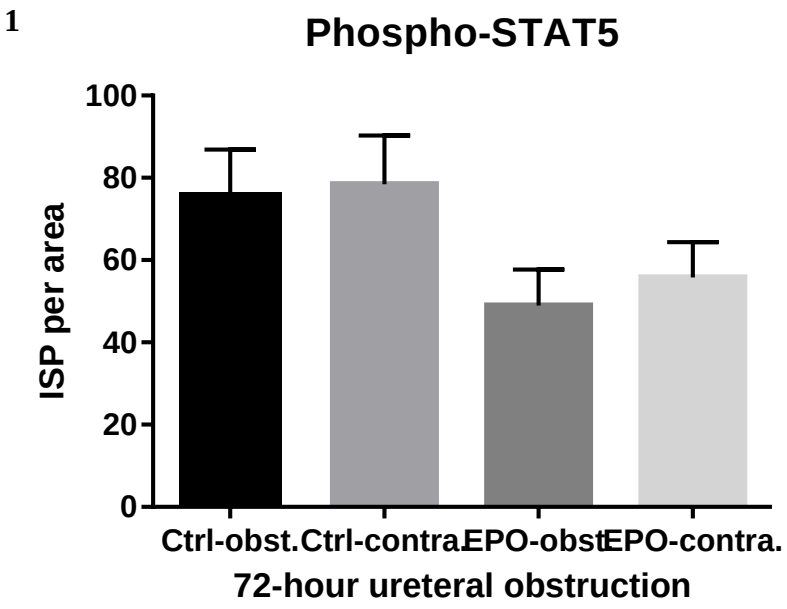
3.3.2.3 Effect of prophylactic EPO on phospho-STAT5 staining in 72-hour obstructed ureters

Our immunohistochemistry work showed that ureteral obstruction does not activate STAT5 (Figure 2-10). As EPO activate STAT5 via JAK2, phospho-STAT5 in 72-hour obstructed ureters treated with EPO was compared to phospho-STAT5 in 72-hour obstructed ureters without treatment. We could still observe STAT5 activation on EPO-treated obstructed ureters, as well as contralateral ureters (Figure 3-6A), however we could not distinguish the difference in staining with or without prophylactic EPO treatment. Immunoreactivity of phospho-STAT5 in the 72-hour obstructed ureters and the contralateral ureters with prophylactic EPO treatment could not be distinguished from one another (1.10-fold, $p = 0.8603$). Immunoreactivity of phospho-STAT5 staining in the ratio of 72-hour obstructed ureters to the contralateral ureters with or without the prophylactic EPO treatment could not be distinguished from one another (1.19-fold, $p = 0.7363$) (Figure 3-6B). As STAT5 activation responds rapidly following the activation, the activated STAT5 by prophylactic EPO treatment would be already disappeared by the end of 72 hours.

A. Paraffin-embedded embedded ureteral tissues immunohistochemically stained for phospho-STAT5



C. Quantitative analysis of phorpho-STAT5 staining



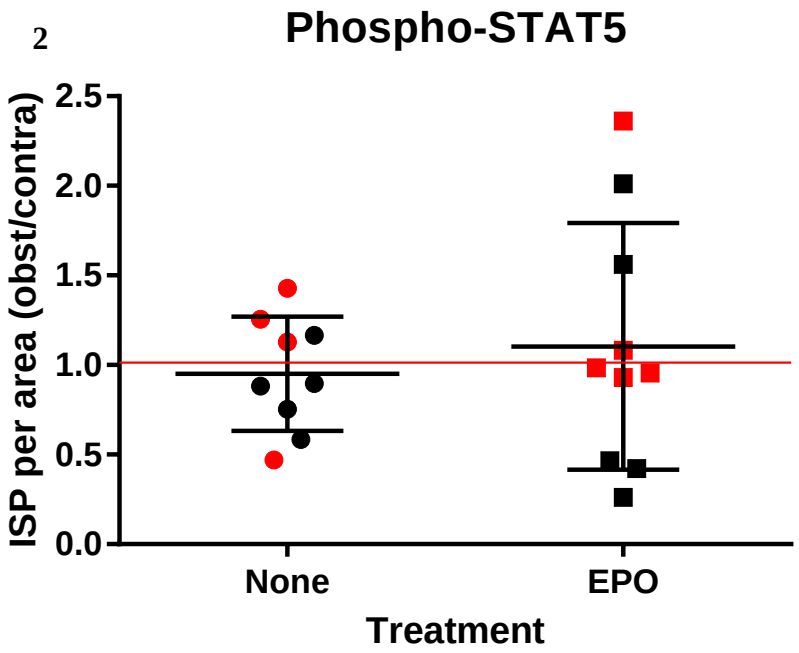


Figure 3-6 Effect on phospho-STAT5 expression in 72-hour obstructed uroepithelium with prophylactic EPO treatment

A. Representative pictures showing immunohistochemistry with antibody for phospho-STAT5 in the 72-hour obstructed ureters with or without prophylactic EPO treatment. Cells with brown staining represent positive staining and cells with blue staining represent negative staining. **B.** Quantitative analysis of phospho-STAT5 expression in uroepithelium in 72-hour obstructed ureters with or without prophylactic EPO treatment. Intense strong positive (ISP) staining counts per area was counted in three random high power fields of the obstructed or non-obstructed contralateral ureters of 72-hour unilateral ureteral obstruction. Data are presented as mean ± SEM. Asterisk indicates $p < 0.05$. **B1.** Histogram of ISP per area in obstructed ureters and unobstructed contralateral ureters with or without prophylactic EPO treatment. **B2.** ISP per area in obstructed ureters was divided into that in unobstructed contralateral ureters in the individual animal. Each dot represents the ratio of staining in obstructed ureters compared to contralateral ureters of each animal. Different color of dots indicates independent experiment sets (black and red). Red line represents contralateral ureter in each animal. *Obst*, obstruction. *Contra*, contralateral.

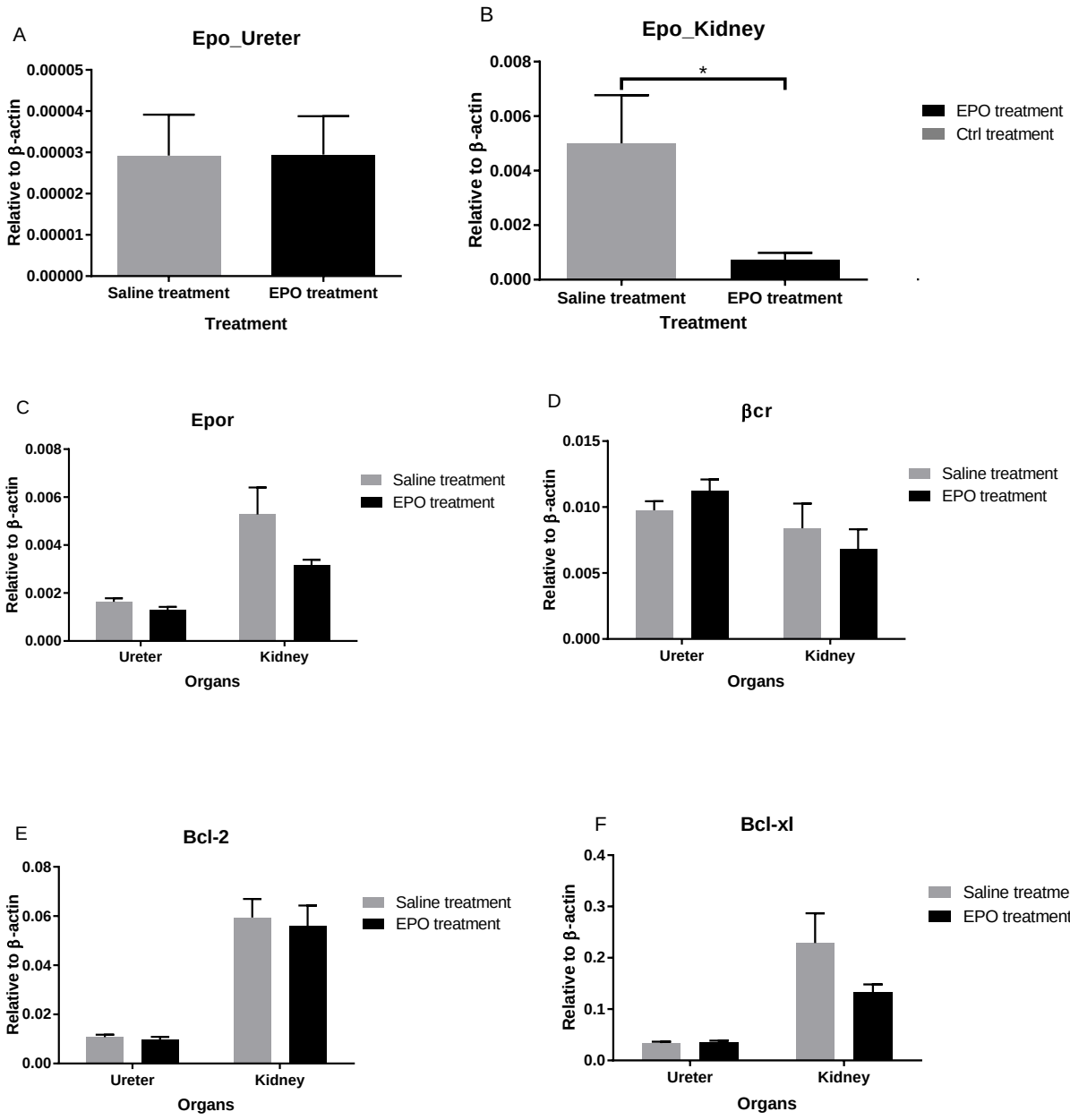
3.3.3 Effect of prophylactic EPO treatment on RNA expression

3.3.3.1 Effect of prophylactic EPO treatment on RNA expression of EPOR signaling in normal ureters and kidneys

We first examined if EPO treatment can induce pre-conditioning of EPOR-βCR signaling genes without injury which are downregulated in obstructed ureters. RNA expression of EPOR signaling in the mice treated with either EPO treatment or saline treatment without obstruction was compared in the ureters and the kidneys. Other than decreased EPO expression on EPO treated kidneys, we could not observe distinguishable difference between two groups. EPO expression was decreased in kidneys in the EPO treatment group compared to the saline treatment group ($p < 0.05$) (Figure 3-7B). This suggests a negative feedback loop in the body to sustain homeostasis in EPO level. EPO gene is shown to have own negative regulatory element in its sequence with GATA motif[164,165]. As the organ that produces EPO hormone, the kidney may be more susceptible to the changes in EPO level compared to the ureter. Similarly, there was a slight decrease observed in EPOR expression after EPO treated kidneys, similar to EPO expression. (Figure 3-7C) In contrast, EPO and EPOR expression in the ureters between the EPO treatment group and the saline treatment group could not be distinguished from one another (Figure 3-7A). βCR expression between the EPO treatment group and the saline treatment group in the ureters and the kidneys could not be distinguished from one another (Figure 3-7D). In terms of downstream genes, BCL-2 expression in the ureters and the kidneys between the EPO treatment group and the saline treatment group could not be distinguished from one another (Figure 3-7E). There was a slight decrease observed in BCL-XL expression in EPO treated kidneys, which could be resulted from decreased EPO and EPOR expression (Figure 3-7F). BAX expression in the ureters and the kidneys between the EPO treatment group and the saline

treatment group could not be distinguished from one another (Figure 3-7G). NF-κB p105 expression in the ureters and the kidneys between the EPO treatment group and the saline treatment group could not be distinguished from one another (Figure 3-7H). BAX expression in the ureters and the kidneys between the EPO treatment group and the saline treatment group could not be distinguished from one another (Figure 3-7I). Taken together, we found decreased EPO expression in the kidneys after EPO treatment. It is suggested to be a negative regulatory mechanism of EPO related to hematopoiesis. However, EPO's pre-conditioning effects accompanies increased BCL-2 and BCL-XL[80,166]. Decreased EPO expression in the kidneys could be resulted from our treatment schedule, where 4 doses over 4 days were provided before the sacrifice compared to a single dose provided in other studies on pre-conditioning. Therefore, our experiment provided enough time for EPO to induce negative feedback loop. Our results suggest that EPO treatment does not induce anti-apoptotic pre-conditioning effect in the kidneys and the ureters. Chang et al. showed that EPO treated rat kidneys subjected to sham operation had the same expression profile as sham operated kidneys with vehicle treatment[107]. However, there is conflicting evidence surrounding conditioning of prophylactic EPO, as Yang et al. showed increased BCL-2 expression after prophylactic EPO treatment without injury in a rat kidney ischemia-reperfusion model[80]. Our study is the first study to examine the effect of prophylactic EPO treatment in the ureters. This suggests that changes in the expression of EPOR signaling occur under the condition of obstructive injury, but not under conditions of prophylactic EPO treatment without injury. Effect of EPO treatment derives from remaining EPO in the body from prophylactic treatment might protect tissues once obstructive injury occurs. More importantly, our results imply that EPOR signaling on contralateral unobstructed

ureters and kidneys is not affected by prophylactic EPO treatment, which would serve as a proper control for obstructed ureters with EPO treatment.



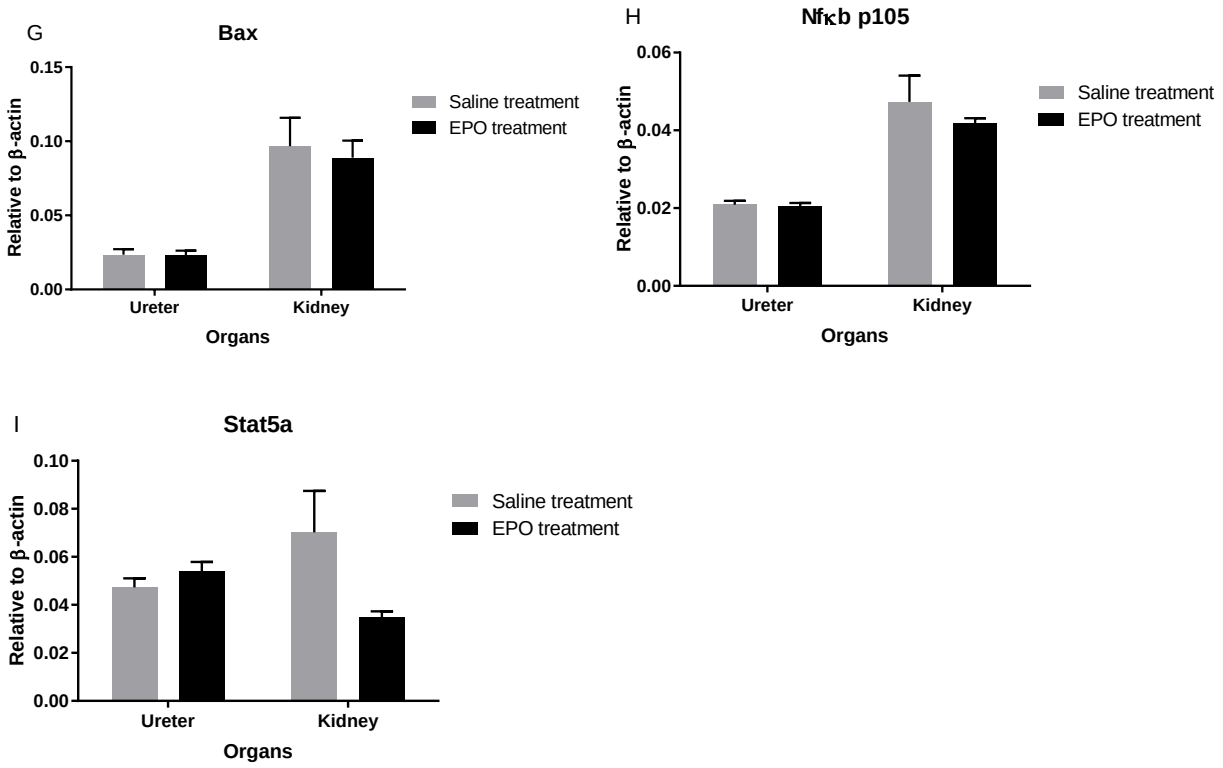


Figure 3-7 Effect of EPO treatment on RNA expression of EPOR signaling

Quantitative real-time PCR analysis in ureters and kidneys from mice treated with either EPO or saline. RNA expression of EPO, EPOR, βCR, BCL-2, BCL-XL (BCL2L1), BAX, NF-κB p105 and STAT5A in ureters and kidneys after each treatment was analyzed by qRT-PCR (A to H). Grey bars represent the gene expression of mice with the saline treatment. Black bars represent the gene expression of mice with the EPO treatment. Data are presented as mean ± SEM.

3.3.3.2 Effect of prophylactic EPO treatment on EPO, EPOR and βCR expression in obstructed kidneys and ureters

Since EPO exhibits a protective effect against ureteral dysfunction via binding to EPOR-βCR, we examined the effect of prophylactic EPO on EPOR-βCR signaling in ureteral obstruction. We could observe increasing trend of EPO expression in both the ureters and the kidneys after EPO

treatment compared to without treatment. Increased sample size will be required to validate the treatment effect on EPO expression. EPO expression between 24, 72-hour obstructed ureters and contralateral ureters with the EPO treatment could not be distinguished from one another (Figure 3-8-1A). EPO expression was decreased in 24, 72-hour obstructed kidney with the EPO treatment ($p < 0.01$, $p < 0.001$) (Figure 3-8-2A). EPO expression of ureters and kidneys with or without prophylactic EPO treatment could not be distinguished from one another (Figure 3-8-1A, 3-8-2A). Interesting results was observed on EPOR expression in the EPO treated ureters, where prophylactic EPO treatment increased EPOR expression after 24-hour obstruction and decreased EPOR expression after 72-hour obstruction ($p < 0.05$, $p < 0.05$). As half-life of EPO in rodents is relatively short, EPO may not be enough till the end of 72-hour obstruction, whereas EPO may still be available at 24-hour obstruction. It might explain different patterns of EPOR expression. EPOR expression was decreased in 24, 72-hour obstructed ureters with prophylactic EPO treatment ($p < 0.0001$, $p < 0.0001$) (Figure 3-8-1B). EPOR expression was decreased in 24, 72-hour obstructed kidneys with prophylactic EPO treatment ($p < 0.001$, $p < 0.0001$) (Figure 3-8-2B). Compared to no treatment, βCR expression after 24, 72-hour obstruction with prophylactic EPO and its contralateral kidney could not be distinguished from one another. One of explanation can be reduced inflammation by EPO treatment, as EPO has been shown to decrease cytokine production[67]. βCR is a common receptor used in various cytokines signaling[167]. Prophylactic EPO may suppress cytokine production by obstruction that may decrease βCR expression. Current work focuses on anti-apoptotic function of EPO, and future work focusing on inflammation by obstruction would be required to validate it. βCR expression was decreased in 24-hour obstructed ureter in both EPO treated and untreated group ($p < 0.01$) (Figure 3-8-1C). βCR expression of obstructed kidneys and contralateral kidneys with the EPO treatment could

not be distinguished from one another, whereas βCR expression was increased in untreated 72-hour obstructed kidney (Figure 3-8-2C). βCR expression of the ureters and the kidneys between prophylactic EPO treated obstruction group and the untreated obstruction group could not be distinguished from one another. Overall, though increased EPO expression is observed, EPO and EPOR-βCR expression was not affected by prophylactic EPO treatment. It suggests that increased EPO after the treatment still have enough receptors available to induce its anti-apoptotic effect.

1. **Ureter** 2. **Kidney**

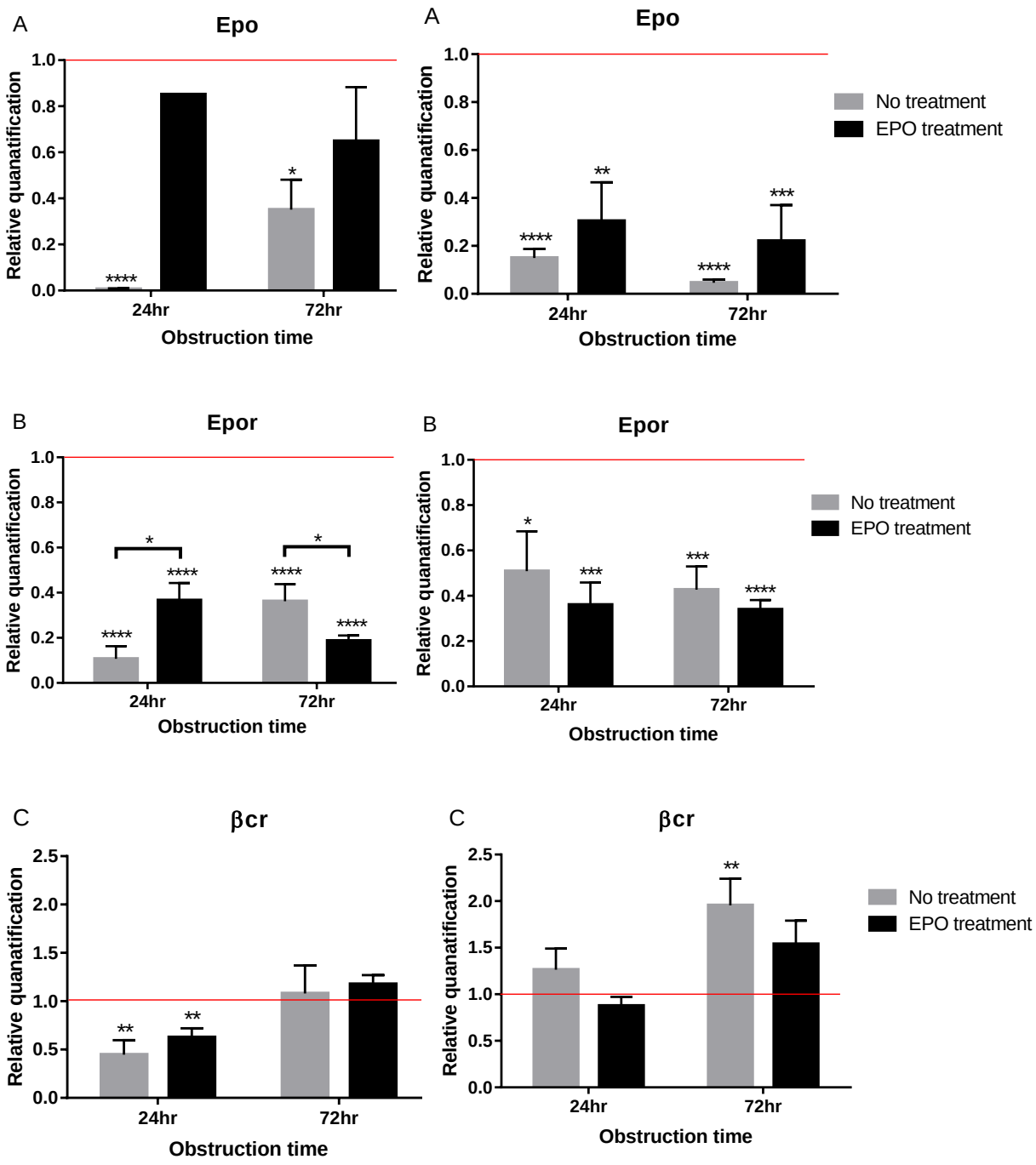


Figure 3-8 Effect of EPO treatment on RNA expression of EPO, EPOR, βCR in ureters and kidneys under unilateral ureteral obstruction

Real-time quantitative PCR analysis in 24, 48, 72-hour obstructed ureters and kidneys after EPO treatment and compared to those in unobstructed contralateral side per each animal. RNA expression of EPO, EPOR, βCR in the ureters and the kidneys after unilateral ureteral obstruction was analyzed by qRT-PCR (A to F). Grey bars represent RNA expression without treatment (Chapter 2). Black bars represent RNA expression with prophylactic EPO treatment. Red line represents contralateral side. Data are presented as mean ± SEM. Asterisk indicates $p < 0.05$. Double asterisks indicate $p < 0.01$. Triple asterisks indicate $p < 0.001$. Quadruple asterisks indicate $p < 0.0001$.

3.3.3.3 Effect of prophylactic EPO treatment on BCL-2, BCL-XL, BAX, NF-κB p105 and STAT5A expression in obstructed kidneys and ureters

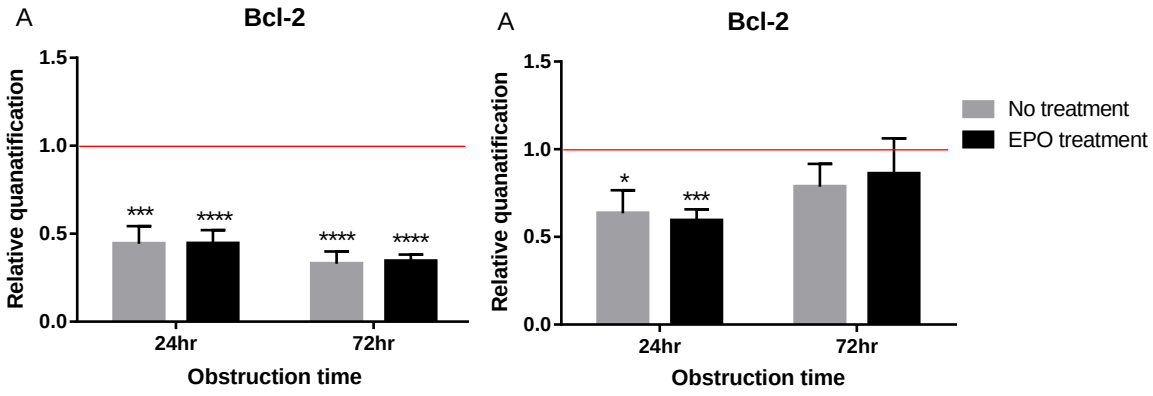
We then examined that downstream genes of EPOR-βCR signaling after EPO treatment, as they are more closely related to regulating apoptosis. We could observe decreased pro-apoptotic BAX expression in obstructed ureters and kidneys after prophylactic EPO treatment, which was not observed in non-treated ureters. Pro-apoptotic BAX expression was decreased in 72-hour obstructed ureters with the EPO treatment, which was increased after ureteral obstruction (Figure 3-9-1C). BAX expression was decreased in 24, 72-hour obstructed kidneys with the EPO treatment ($p < 0.05$, $p < 0.01$), in whereas BAX expression of the obstructed kidneys and contralateral kidneys without treatment could not be distinguished from one another (Figure 3-9-2C). Prophylactic EPO treatment increased BAX expression in 24-hour obstructed ureters ($p < 0.05$). Though BAX expression in the ureters showed decreasing trend after 24-hour obstruction with prophylactic EPO, BAX expression in 24-hour obstructed ureters and contralateral ureters with prophylactic EPO treatment could not be distinguished from one another. Decreased BAX expression following prophylactic EPO treatment could result from biological chance where biological group without treatment was relatively more sensitive to obstructive injury. Decreased

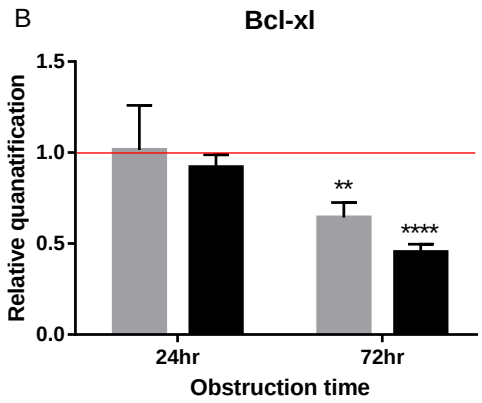
BAX expression shows us anti-apoptotic signaling of EPO to compensate decreased anti-apoptotic genes following obstruction.

In anti-apoptotic genes, we could not find distinguishable change after prophylactic EPO treatment. Expression of BCL-2 after prophylactic EPO was similar to no treatment following obstruction. BCL-2 expression was decreased in 24, 72-hour obstructed ureters with the EPO treatment ($p < 0.0001$, $p < 0.0001$) (Figure 3-9-1A). BCL-2 expression was decreased in 24-hour obstructed kidneys with the EPO treatment ($p < 0.001$) (Figure 3-9-2A). BCL-2 expression in 72-hour obstructed kidneys and contralateral kidneys with the prophylactic EPO treatment could not be distinguished from one another. BCL-2 expression of the ureters and the kidneys with or without prophylactic EPO could not be distinguished from one another. Similar to what we observed at BCL-2, expression of BCL-XL after prophylactic EPO was similar to no treatment following obstruction. BCL-XL expression in 24-hour obstructed ureters and contralateral ureters with the prophylactic EPO treatment could not be distinguished from one another. BCL-XL expression was decreased in 72-hour obstructed ureters with the prophylactic EPO treatment ($p < 0.0001$) (Figure 3-9-1B). BCL-XL expression was decreased in 24-hour obstructed kidneys with the prophylactic EPO treatment ($p < 0.05$) BCL-XL expression in 72-hour obstructed kidneys and contralateral kidneys with the prophylactic EPO treatment could not be distinguished from one another (Figure 3-9-2B). BCL-XL expression of the ureters and the kidneys with or without prophylactic EPO could not be distinguished from one another. Expression of NF-κB p105 after prophylactic EPO was similar to no treatment following obstruction. NF-κB p105 expression was decreased in 24, 72-hour obstructed ureters with the prophylactic EPO treatment ($p < 0.001$, $p < 0.001$). Also, there was an increase in NF-κB p105 expression in 24-hour obstructed ureters with the prophylactic EPO treatment compared to the

untreated obstruction group ($p < 0.05$) (Figure 3-9-1D), which may show anti-apoptotic function of prophylactic EPO via p50 homodimers. NF-κB p105 expression in 24, 72-hour obstructed kidneys and contralateral kidneys with the prophylactic EPO treatment could not be distinguished from one another, similarly to the untreated obstructed kidneys (Figure 3-9-2D). Expression of STAT5A after prophylactic EPO was similar to no treatment following obstruction. STAT5A expression was decreased in 24, 72-hour obstructed ureters with the prophylactic EPO treatment ($p < 0.0001$, $p < 0.0001$) (Figure 3-9-1E). STAT5A expression was decreased in 24, 72-hour obstructed kidneys with the prophylactic EPO treatment ($p < 0.0001$, $p < 0.0001$) (Figure 3-9-2E). STAT5A expression of the obstructed ureters and kidneys with or without the prophylactic EPO treatment could not be distinguished from one another. Taken together, our results showed that prophylactic EPO treatment showed anti-apoptotic signaling via decreased pro-apoptotic BAX expression in the obstructed ureters and kidneys without altering the expression of EPOR-βCR.

1. Ureter **2. Kidney**

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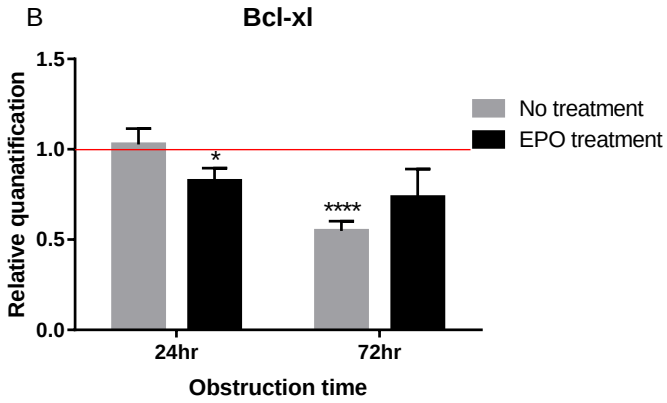
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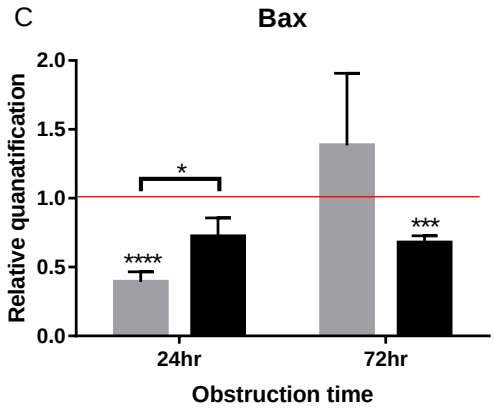
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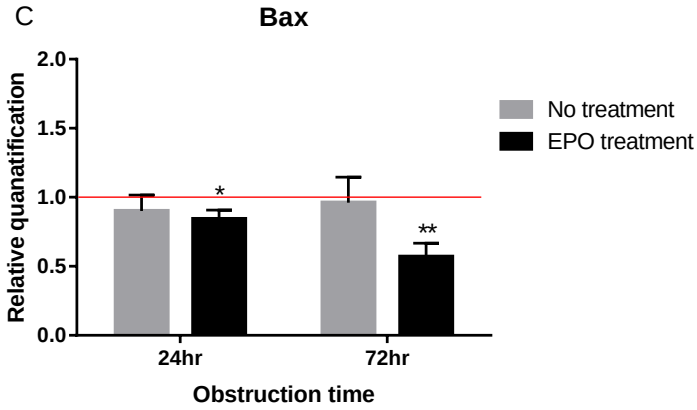
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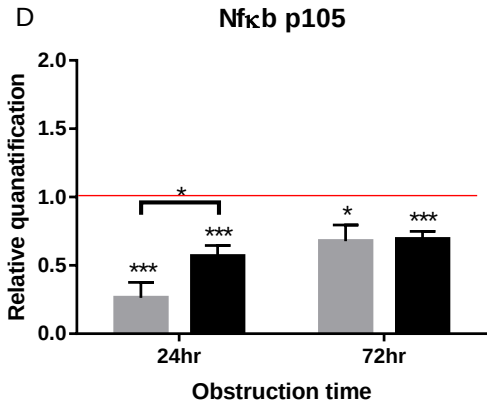
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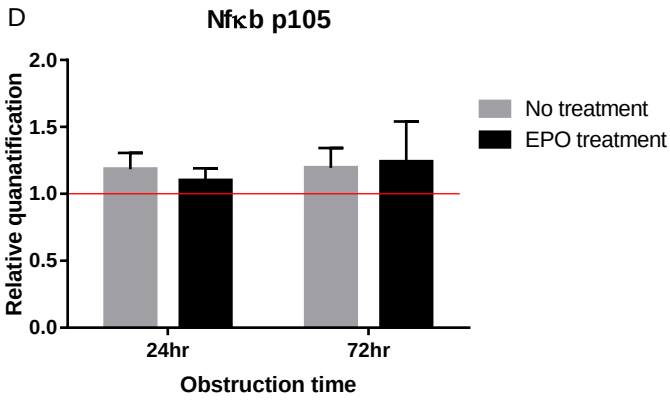
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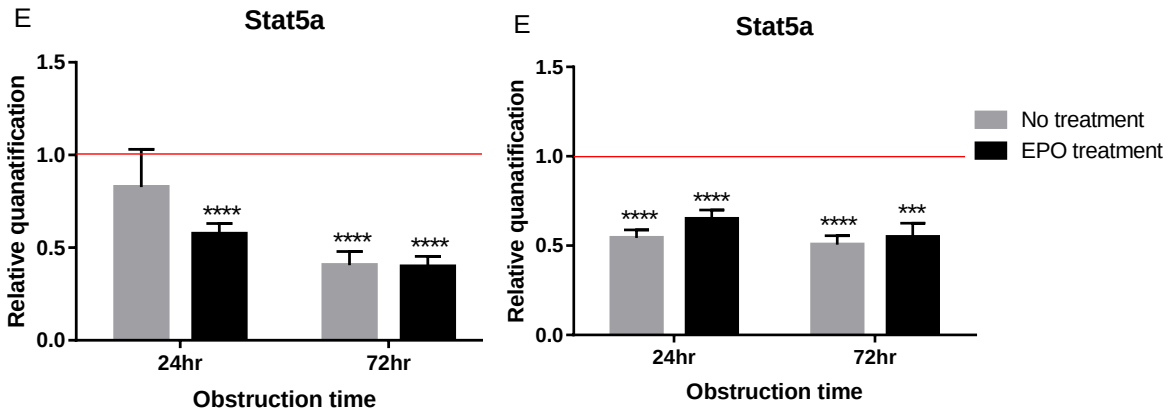

Figure 3-9 Effect of EPO treatment on RNA expression of BCL-2, BCL-XL, BAX, NF-κB p105 and STAT5A in ureters and kidneys under unilateral ureteral obstruction

Real-time quantitative PCR analysis in 24, 48, 72-hour obstructed ureters and kidneys with or without EPO treatment and compared to those in unobstructed contralateral side. RNA expression of BCL-2, BCL-XL, BAX, NF-κB p105 and STAT5A in obstructed ureters and kidneys after EPO treatment was analyzed by qRT-PCR (A to J). Grey bars represent RNA expression without treatment. Black bars represent RNA expression with prophylactic EPO treatment. Red line represents contralateral side. Data are presented as mean ± SEM. Asterisk indicates $p < 0.05$. Double asterisks indicate $p < 0.01$. Triple asterisks indicate $p < 0.001$. Quadruple asterisks indicate $p < 0.0001$.

3.3.4 Effect of prophylactic EPO treatment on hematopoiesis

EPO stimulates red blood cell production in the bone marrow. Some people argued that EPO's protective effect may in part derive from increased blood circulation via increased blood cells. To assess hematopoietic effects of EPO in our experiment, complete blood counts were performed on the blood samples from prophylactic EPO treatment compared to saline treatment without obstruction. Levels of hematocrit, hemoglobin and red blood cell count were selected to evaluate hematopoiesis according to the current clinical guideline. Red blood cell count level after either prophylactic EPO treatment or the saline treatment could not be distinguished from

one another (1.03-fold, 9.280 vs 9.008, p = 0.6824, normal range: 7.0-12.0) (Figure 3-10). Hematocrit level after either prophylactic EPO treatment or the saline treatment could not be distinguished from one another (1.12-fold, 15.920 vs 14.18, p = 0.1616, normal range: 12.2-16.2) (Figure 3-10). Hemoglobin level after either prophylactic EPO treatment or the saline treatment could not be distinguished from one another (1.08-fold, 45.02 vs 41.45, p = 0.2063, normal range: 35.0-45.0) (Figure 3-10). This result showed us that EPO treatment did not induce hematopoiesis in our experimental model. Parfrey et al showed that EPO did not increase hemoglobin levels until 1 month after the treatment[168]. Srisawat et al. showed that hematocrit level started to increase from 14 days after EPO 5000 IU/kg treatment in ureteral obstruction[105], and the protective effect of EPO against tubulointerstitial injury and tubular apoptosis in obstructed kidneys was already found from the earliest timepoint of day 3. Current clinical use of EPO for treating anemia suggests that a significant increase in haemoglobin is usually not observed in less than 2 weeks, and it can take up to 10 weeks after treatment[169]. The hematological assessment on the current experiment was performed 4 days after starting EPO treatment, which suggests that this was a relatively short time to see any change in hematological assessment. Delayed hematopoietic effect of EPO in our study showed that EPO's protective effect is an independent mechanism from increased blood circulation, which increases its potential for clinical use in the future by avoiding cardiovascular side effects. This is also supported by many studies showing derivatives of EPO, which do not induce hematopoiesis, are also protective against injuries through EPOR-βCR[170,171]. Our study is the first study examining EPO's hematopoietic effect on mice ureteral obstruction model.

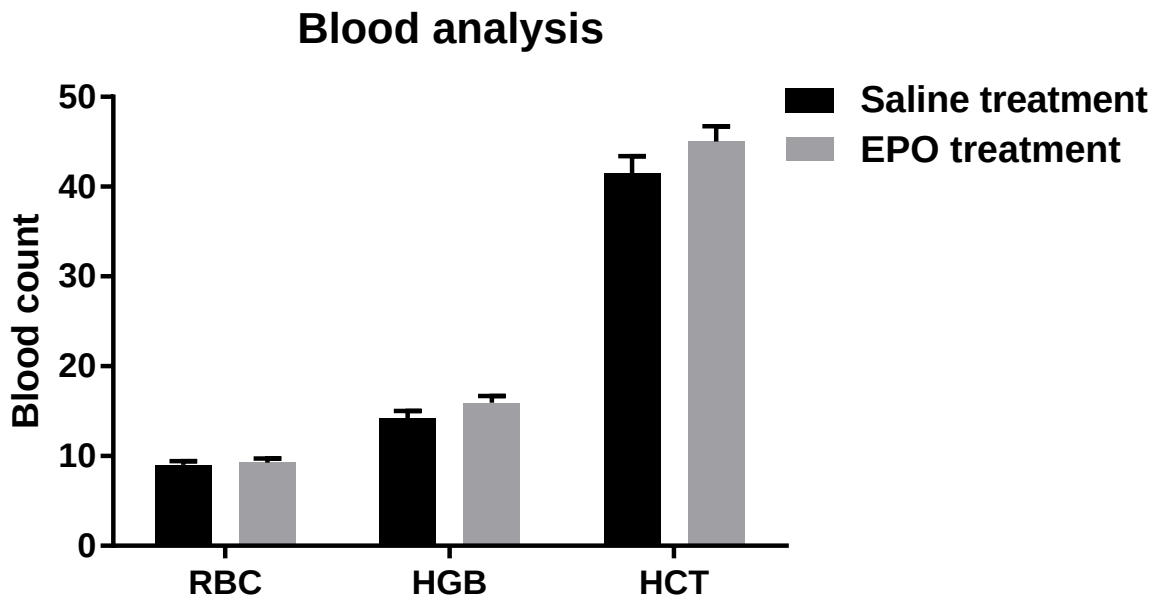

Figure 3-10 Effect of EPO treatment on erythropoiesis

Complete blood cell count was analyzed by blood analyzer on blood sample from the animals treated with either prophylactic EPO or control (saline). Red blood cell count (RBC) and hemoglobin (HGB), hematocrit (HCT) were analyzed. Units of red blood cell (RBC) and hemoglobin (HGB) and hematocrit (HCT) were 10^{12}/L, g/dl and % respectively. Black bars represent the level of each factor on mice receiving saline treatment. Grey bars represent the level of each factor on mice receiving EPO treatment. Data are presented as mean ± SEM.

3.4 Discussion

Here, we demonstrated that prophylactic EPO treatment suppressed ureteral apoptosis after 72-hour obstruction, accompanying improved peristaltic function in 24-hour obstructed ureters. Ozdamar et al. showed that EPO treatment restored intestinal peristaltic activity and bowel damage in hypocontractile gastroschisis[102]. Nagib et al. showed that prophylactic EPO treatment restored the intestinal motility in gentamicin-induced intestinal motility dysfunction ex vivo

without any increase in hematological factors[172]. Previous study in our laboratory, which was the first study showing EPO's effect on accelerating the recovery from peristaltic dysfunction, measured ureteral peristalsis only after recovery time[11]. Our study is the first study to show that prophylactic EPO treatment protected ureteral peristaltic function in the obstructed ureters. Ureteral peristaltic counts showed that EPO treatment improved ureteral dysfunction following obstruction. 80% of mice retained ureteral movement with prophylactic EPO treatment whereas 30% of mice retained ureteral movement after 24-hour obstruction. Interestingly, one mice from the EPO treated 72-hour obstruction group retained ureteral activity. However, most of mice in 72-hour obstruction group did not show any peristaltic activity regardless of the treatment. Though our results on TUNEL assays suggest that EPO treatment decreased ureteral apoptosis after 72-hour obstruction, there might be a delay in the recovery of ureteral peristaltic function even with suppressed apoptosis by EPO treatment. Previous work in our laboratory also showed that full recovery of ureteral function from 72-hour obstruction took 2 weeks after obstruction release even with prophylactic EPO treatment [11].

TUNEL assays showed that increased apoptosis from ureteral obstruction was supressed by prophylactic EPO treatment. This suggests that decreased apoptosis by the EPO treatment is one of the main mechanisms that EPO induce to promote functional recovery from ureteral obstruction. Several studies have shown that EPO suppressed apoptosis by ureteral obstruction in kidneys[105-107]. The results of the current study further support these findings by showing EPO's effects in suppressing apoptosis in ureteral tissues in addition to renal tissues. This is important new finding as the ureters might be more directly affected by the obstruction than the kidneys in terms of proximity. Apoptosis works as a defense mechanism when cells are damaged by various

cellular stress. Disruption of the apoptotic pathway is implicated in pathophysiology of various diseases. Decreased apoptosis is found in different diseases including cancers and autoimmune diseases. On the other hand, some diseases induce hyper-active apoptosis that leads to tissue damage and loss, including in neurodegenerative disorders[173,174] and viral infections[175]. The treatments available for these diseases are targeted toward modulating the disrupted apoptotic pathways[176-178]. Studies have shown that EPO prevented injuries by decreasing apoptosis in various disease models. In addition, mechanical stretch on epithelium is shown to disrupt peristalsis of the airway[179]. Ureteral obstruction induces mechanical stretch on the ureters, which can disrupt ureteral peristalsis. By suppressing the apoptosis of the ureters to preserve its normal functioning in obstructed ureters, EPO treatment can accelerate the recovery of ureteral peristaltic function as well as minimizing the functional injury of obstructed ureters. Our study is the first study to show that EPO treatment suppressed apoptosis in obstructed ureters.

Chaung et al. investigated several therapeutic agents that can prevent ureteral damage from obstruction, including COX-2 inhibitor[180], NF-κB inhibitor[181] and 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor[182]. These inhibitors decreased inflammatory signaling and apoptosis by ureteral obstruction. However, these agents were shown to be only effective in long-term ureteral obstruction starting from 14 days, and it was not effective for shorter obstruction durations. It is interesting to notice that 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor, referred as a statin, has been shown to have pleiotropic effects against renal injuries along with EPO and angiotensin-converting-enzyme inhibitors. Statins have been shown to be protective against injuries mainly by modulating the immune system and improving lipid metabolism. On the other hand, EPO has been shown to be

protective against injuries through decreasing apoptosis and increasing proliferation. (reviewed by Gluhovschi et al.[183]) EPO's primary mechanism on suppressing apoptosis may explain why EPO protected the damage by ureteral obstruction more rapidly than statins.

Immunohistochemistry detecting phospho-STAT5 showed that there was no change in STAT5 expression with prophylactic EPO treatment. Studies using different cell lines showed that STAT5 is activated in 5 to 30 minutes after EPO treatment[38,184,185]. In the current experiment, prophylactic EPO treatment was provided before inducing ureteral obstruction, and ureteral tissues were collected after 72 hours of ureteral obstruction. This suggests that the effect of EPO treatment on STAT5 expression could be already diminished at the time of tissue collection. In addition, exogenous EPO a short half-life when administered to rodents (3.3 hours~), suggesting that external EPO and its effect on STAT5 activation may not last till the end of our experiment. This may explain why we could not find any significant difference in intense positive staining per area in 72-hour obstructed ureters between the EPO treatment group and the no treatment group.

EPO treatment suppressed the activation of phospho-NF-κB following 72-hour ureteral obstruction. Results in this study are supported by outcomes of other studies investigating EPO treatment on ureteral obstruction. Acikgoz et al. showed EPO treatment decreased renal NF-κB expression and renal fibrosis following 14-day ureteral obstruction[109]. Several studies have shown EPO-induced protection against injuries with decreased NF-κB expression in various injury models including myocardial ischemia-reperfusion injuries[186], peritoneal fibrosis[187], hemorrhagic shock[188] and traumatic brain injury[189]. Many studies have shown the dual roles of

NF-κB, which are role as an inflammatory[190] and pro-apoptotic[191] mediator and a role as an anti-apoptotic[192] mediator. As NF-κB is activated by various cellular stress, abrogated NF-κB activation by EPO treatment indicates that EPO treatment suppressed cellular stress by ureteral obstruction. Our results suggest that EPO treatment rescued ureteral tissues from apoptosis by suppressing NF-κB activation. It is supported by another study by Chaung et al. where an NF-κB inhibitor suppressed apoptosis in ureteral smooth muscle cells after 14-day ureteral obstruction[181]. Our result was observed in the ureteral epithelium where most of signaling exists as well as directly affected by dilation from ureteral obstruction. Several studies have shown the mechanisms of EPO in suppressing NF-κB activation to suppress apoptosis. One of proposed mechanisms is via increasing Hsp70 by EPO treatment. EPO treatment was shown to activate Hsp70 through JAK2 and STAT5 in various experimental models[80,88,193]. Hsp70 inhibits apoptosis through blocking oligomerization of Apaf-1 from recruiting pro-caspase-9[194,195]. Heat shock response and heat shock proteins suppressed NF-κB by inhibiting the degradation of I-κBα and therefore stabilizing NF-κB complex[196-200]. Xu et al showed that EPO treatment increased Hsp70 and decreased NF-κB to decrease myocardial infarct size in myocardial ischemia-reperfusion injury[186]. Another proposed mechanism is via inhibiting GSK-3β by EPO treatment. EPO treatment induced phosphorylation of GSK-3β through PI3-K and AKT to inhibit its apoptotic function[201,202]. And GSK-3β inhibition subsequently leads to inhibition of NF-κB[203,204]. Several studies discussed pro-apoptotic and anti-apoptotic function of NF-κB. NF-κB is shown to block apoptosis by interacting with XIAP, c-IAPs, FLIP and BCL-2 related proteins. Several studies have shown that NF-κB has pro-apoptotic functions. Kaltschmidt et al. showed that the nature of apoptotic inducers determines whether NF-κB is pro-apoptotic or anti-apoptotic[205]. Several apoptosis inducers, including serum withdrawal, UV-C radiation, H_2O_2, protein-tyrosine

phosphatase inhibitors, chemotherapeutic agents and non-steroidal anti-inflammatory agents, leads to pro-apoptotic function of NF-κB[206,207]. Stark et al. showed that aspirin induced I-κBα degradation, NF-κB nuclear translocation and apoptosis in colorectal cancer cell lines[208]. Additional studies in this group showed that additional cofactor is recruited in NF-κB complex inside the nucleus to bind and repress anti-apoptotic targets transcription[209]. Campbell et al. showed that NF-κB actively repressed BCL-XL translation[210]. RNA expression of NF-κB p105 was increased after prophylactic EPO treatment in 24-hour obstructed ureters. NF-κB p105 is a precursor of NF-κB p50, which can form a homodimer to exhibit its own function without interacting with p65. p50 homodimer cannot induce transcriptional change by itself because of the lack of transcription domain. However, its binding to BCL-3 can induce transcription that leads to anti-apoptosis[211]. Also, p50 homodimer is shown to suppress NF-κB activation via attenuating the affinity of NF-κB binding site[212]. It can be one of mechanisms in suppressing NF-κB activation of prophylactic EPO in 72-hour obstructed ureters. Therefore, increased NF-κB p50 precursor may show us the activation of anti-apoptotic pathway by prophylactic EPO treatment. Immunohistochemistry with phospho-NF-κB p50 in future work will be needed to verify our finding.

EPO treatment decreased BAX expression in obstructed kidneys and ureters. BAX belongs to the BCL-2 family of apoptosis-regulators mediated by p53[213]. The relative proportion of pro- and anti-apoptotic members of the BCL-2 family controls the fate of cells to undergo apoptosis[214]. The activation of BAX leads to an increase in the amount of protein, which forms BAX homodimers that are translocated from the cytoplasm to the mitochondria to further proceed apoptosis[215]. Several studies have shown that EPO decreased apoptosis by decreasing BAX

expression[80,86,89,92,94,216]. Our current study showed that apoptotic BAX was decreased after EPO treatment along with supressed apoptosis. Chaung et al. showed that ureteral obstruction increased BAX expression along with ureteral apoptosis[118]. This suggests the importance of BAX in transmitting apoptotic signaling in ureteral cells in obstructive uropathy. And EPO can block its apoptotic transmission to rescue ureteral tissues from apoptosis. EPO was shown to prevent apoptosis by modulating the BCL-2 family of apoptosis regulators. Though there was no innate change in upper signaling in EPO and EPOR after EPO treatment, EPO treatment decreased apoptosis through decreased BAX and NF-κB p65. This suggests that extraneous EPO was enough to induce EPOR signaling, and further transmit anti-apoptotic signaling. Dzietko et al. also showed that EPO treatment did not affect innate expression of EPOR, but EPO treatment was still effective in preventing against neurotoxicity in the brain[135]. Additionally, EPO treatment could also induce other EPO-induced signaling, such as MAPK, PI3-K that we did not focus on this study. Future work will be needed to investigate the other EPOR downstream signaling as well as additional BCL-2 family members.

3.5 Conclusion

Our work showed that prophylactic EPO treatment protected peristaltic function of obstructed ureters. Prophylactic EPO treatment suppressed apoptosis in 72-hour obstructed ureters via downregulating BAX expression and phospho-NF-κB p65. Anti-apoptotic function of EPO treatment suggests the mechanism of EPO treatment on accelerating the recovery of ureteral peristaltic function after transient obstruction. (Figure 3-12) In addition, hematopoietic effect of EPO was not observed in our experimental scheme, showing that protective effect of EPO in

accelerating ureteral recovery from ureteral dysfunction is an independent effect from increased blood circulation via hematopoiesis.

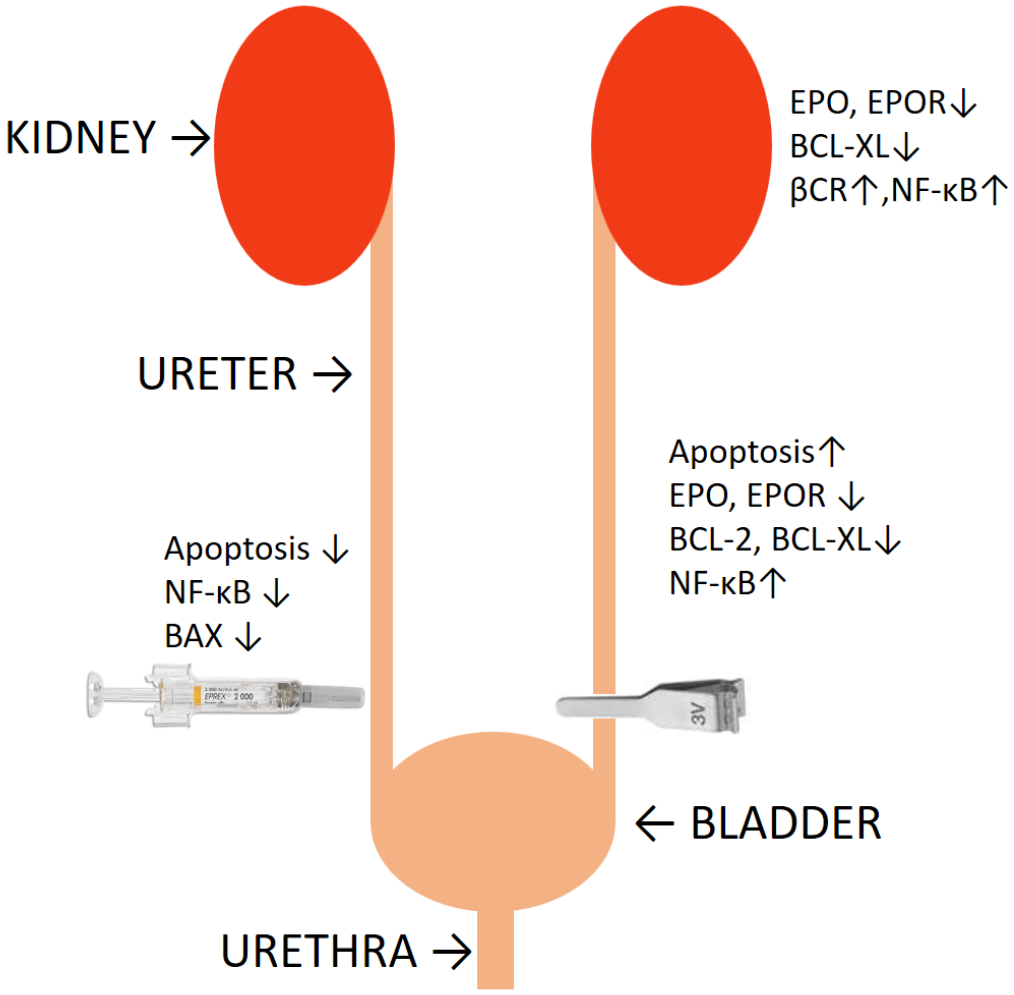

Figure 3-11 Overview on the effect of prophylactic EPO on EPOR-βCR signaling

Representative diagram of EPOR signaling in the ureters and the kidneys after ureteral obstruction in 2 strains of rodents. ↑ represents increased expression or immunoreactivity. ↓ represents decreased expression or immunoreactivity. Clip represents the unilateral ureteral obstruction. Syringe represents prophylactic EPO treatment provided for 4 days prior to the obstruction.

Chapter 4: Concurrent EPO on 72-hour ureteral obstruction showed similar results as prophylactic EPO

4.1 Introduction

In order to test the protective effect of concurrent EPO treatment on ureteral obstruction, we examined EPOR signaling in ureteral obstruction with concurrent EPO treatment and compared it to without EPO treatment (Chapter 2). EPO has relatively a short half-life in rodents (3.3-10 hours)[217], which means EPO concentration is negligible from the bodies as early as 16.5 hours after the last treatment. Results from our study (section 3.3.3.1) suggested that prophylactic EPO treatment does not affect EPOR signaling in advance without injury, and EPO can protect tissues effectively only when applied during or shortly after injury. This suggests that therapeutic effect of prophylactic EPO was mostly from the remaining EPO that was present during ureteral obstruction. Considering that our ureteral obstruction model is performed for 72 hours, we concluded that EPO may not be available for the duration of the injury. Therefore, *we hypothesized that concurrent EPO treatment at the same time of the obstruction may provide more protection over 72-hour ureteral obstruction than prophylactic EPO treatment, by providing consistent EPO supplying on the injury site*.

4.2 Methods

4.2.1 Animals

A total of 10 C57BL/6 were used in this experiment. Animals were treated as detailed in section 2.2.1.

4.2.2 Unilateral ureteral obstruction model

Unilateral ureteral obstruction model was induced as detailed in section 2.2.2.

4.2.3 EPO treatment

20 IU of Human biosynthetic epoetin alfa (Eprex, 200µl) diluted in saline (100 IU/ml) was administered once daily intraperitoneally for 4 consecutive days starting on the day 1 of ureteral obstruction.

4.2.4 Ureteral function evaluation

After ureteral obstruction with EPO treatment for 72 hours, ureteral function was assessed as detailed in section 2.2.3.

4.2.5 Quantitative Real-Time PCR

Quantitative real-time PCR was performed as detailed in section 2.2.4.

4.2.6 Statistical analysis

Statistical analysis was performed as detailed in section 2.2.7.

4.3 Results

4.3.1 Ureteral function evaluation

4.3.1.1 Effect of concurrent EPO treatment on ureteral peristaltic activity in 72-hour obstructed ureters

In order to test the effect of concurrent EPO treatment against ureteral dysfunction following 72-hour obstruction, peristaltic activity in obstructed ureters with concurrent EPO treatment was measured. Similar to what we observed on 72-hour obstructed ureters, peristaltic function was completely lost in 72-hour obstructed ureters with concurrent EPO treatment, from average 14 peristalsis per minute before the obstruction ($p < 0.0001$). Ureteral peristaltic count was decreased in 72-hour obstructed ureters compared to that of contralateral after obstruction with the concurrent EPO treatment ($p < 0.001$). Similar to what we observed in no treatment group, ureteral peristaltic count was also decreased in contralateral ureters after 72-hour obstruction compared to that of before obstruction (0.50-fold, $p < 0.05$) (see section 2.3.1.2). Peristaltic count in 72-hour obstructed ureters with or without the concurrent EPO treatment could not be distinguished from one another ($p = 1$) (Figure 4-1). Our results showed that concurrent EPO

treatment did not affect ureteral peristaltic activity in 72-hour obstructed ureters. EPO is effective in decreasing injury as well as accelerating recovery in obstructed ureters[11] however, it may not be enough to make ureters retain its function after severe damage by longer obstruction.

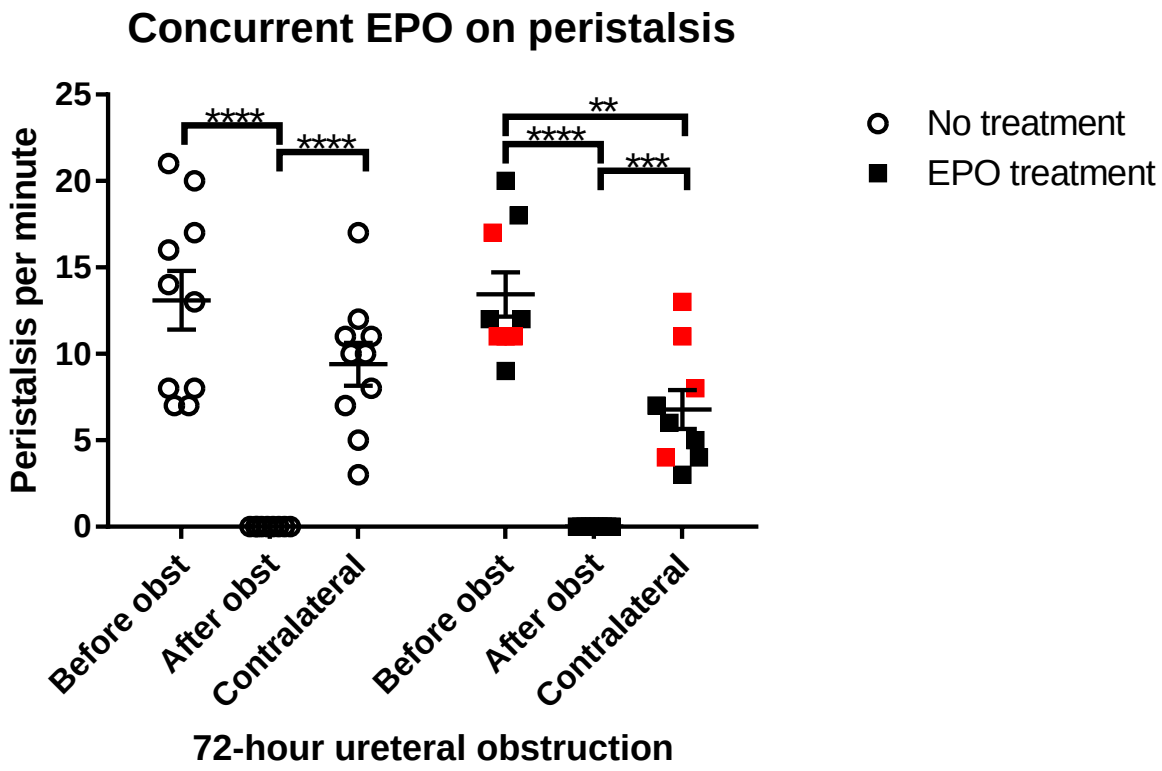

Figure 4-1 Effect of concurrent EPO treatment in ureteral peristaltic activity in 72-hour obstructed ureters

Ureteral peristaltic activity per minute under surgical microscope after 72-hour ureteral obstruction with or without the concurrent EPO treatment. Ureteral peristaltic activity after 72-hour unilateral ureteral obstruction of ureters before and after obstruction and its unobstructed contralateral ureter after obstruction. Different color of dots indicates independent experiments (black and red). Data are presented as mean ± SEM. Asterisk indicates $p < 0.05$. Double asterisks indicate $p < 0.01$. Triple asterisks indicate $p < 0.001$. Quadruple asterisks indicate $p < 0.0001$. *Obst*, obstruction.

4.3.2 Effect of concurrent EPO treatment on RNA expression of EPOR-βCR signaling in obstructed kidneys and ureters

The expression of genes in the obstructed ureters were compared to the contralateral side which served as the control.

4.3.2.1 Effect on EPO, EPOR, βCR expression in 72-hour obstructed ureters and kidneys with concurrent EPO treatment

In order to test the effect of concurrent EPO treatment on EPOR-βCR signaling by obstruction, we first examined RNA expression of EPO and EPOR-βCR. We could not detect distinguishable change of EPO and EPOR-βCR expression after concurrent EPO treatment on 72-hour obstructed ureters and kidneys. EPO expression of obstructed ureters and kidneys and contralateral side with concurrent EPO treatment could not be distinguished from one another. EPO expression was decreased in 72-hour obstructed kidneys with concurrent EPO treatment similar to untreated kidneys ($p < 0.0001$). EPO expression of 72-hour obstructed ureters with or without the concurrent EPO treatment could not be distinguished from one another. (Figure 4-2A). EPOR expression was decreased in 72-hour obstructed ureters with the concurrent EPO treatment similar to no treatment ($p < 0.0001$). EPOR expression was decreased in 72-hour obstructed kidneys with the concurrent EPO treatment similar to no treatment ($p < 0.0001$). EPOR expression of 72-hour obstructed ureters and kidneys with or without the concurrent EPO treatment could not be distinguished from one another. (Figure 4-2B). βCR expression of 72-hour obstructed ureters and contralateral ureters with the concurrent EPO treatment could not be

distinguished from one another. βCR expression was increased in 72-hour obstructed kidneys in both with or without the concurrent EPO treatment ($p < 0.001$). βCR expression of 72-hour obstructed ureters with or without the concurrent EPO treatment could not be distinguished from one another (Figure 4-2C). Overall, there was no distinguishable difference in EPO, EPOR-βCR expression after concurrent EPO treatment. It supports our previous finding on prophylactic EPO treatment where EPO treatment did not necessarily need to increase internal EPOR-βCR expression to induce its protective effects.

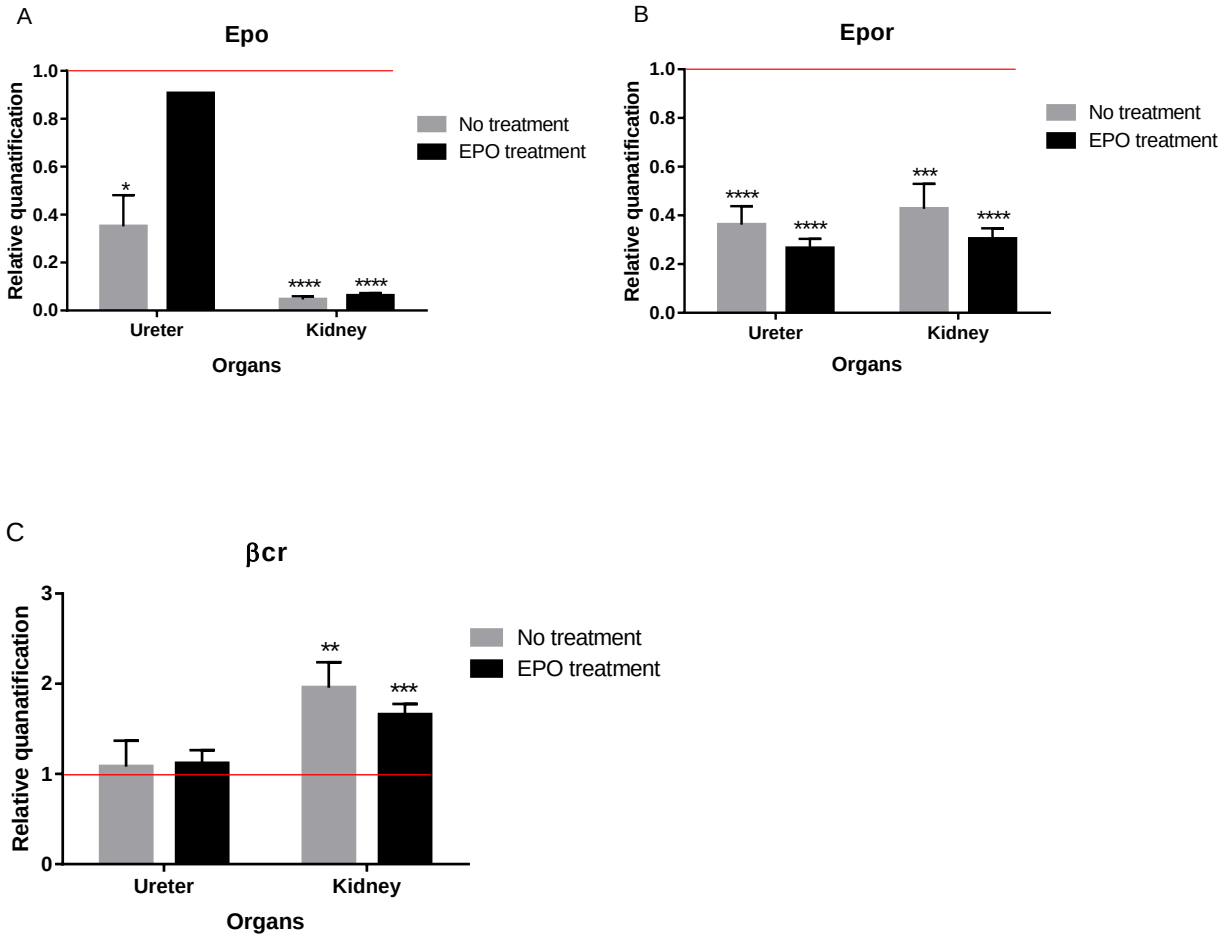

Figure 4-2 Effect of concurrent EPO treatment on RNA expression of EPO, EPOR and βCR in 72-hour obstructed ureters and kidneys

Real-time quantitative PCR analysis in 72-hour obstructed ureters and kidneys and compared to those in unobstructed contralateral side with or without the concurrent EPO treatment. RNA expression of EPO, EPOR, βCR in the ureters and the kidneys after 72-hour unilateral ureteral obstruction was analyzed by qRT-PCR (A to F). Light gray bars represent RNA expression with concurrent EPO treatment. Dark gray bars represent RNA expression without EPO treatment. Red line represents contralateral side. Data are presented as mean ± SEM. Asterisk indicates $p < 0.05$. Double asterisks indicate $p < 0.01$. Triple asterisks indicate $p < 0.001$. Quadruple asterisks indicate $p < 0.0001$.

4.3.2.2 Effect on BCL-2, BCL-XL, BAX, NF-κB p105, STAT5A expression in 72-hour obstructed ureters and kidneys with concurrent EPO treatment

We then examined the expression of downstream genes in EPOR-βCR signaling after concurrent EPO treatment. RNA expression of apoptosis-regulating genes that we examined in the previous experiments was analyzed. Similar to what we observed in obstructed ureters treated with prophylactic EPO, there was no distinguishable change in anti-apoptotic EPOR downstream genes except BAX expression. BAX expression was decreased in both ureters and kidneys, which was shown to be anti-apoptotic mechanism of prophylactic EPO in the previous experiment (Figure 3-9), which supports our previous finding. BAX expression was decreased in 72-hour obstructed kidney with the concurrent EPO treatment ($p < 0.05$). We could also observe decreasing trend of BAX expression in obstructed ureters after concurrent EPO treatment, though it was not statistically significant due to high variance in the untreated group (Figure 4-3C). In anti-apoptotic genes, we could not observe distinguishable difference after concurrent

EPO treatment. BCL-2 expression was decreased in 72-hour obstructed ureters and kidneys with EPO treatment ($p < 0.0001$, $p < 0.0001$). BCL-2 expression after 72-hour obstruction with or without concurrent EPO treatment could not be distinguished from one another (Figure 4-3A). BCL-XL expression was decreased in 72-hour obstructed ureters and kidneys with the concurrent EPO treatment ($p < 0.0001$). BCL-XL expression after 72-hour obstruction with or without concurrent EPO treatment could not be distinguished from one another (Figure 4-3B). NF-κB p105 expression was decreased in 72-hour obstructed ureter with the concurrent EPO treatment ($p < 0.001$). On the other hand, NF-κB p105 expression was increased in 72-hour obstructed kidneys with the concurrent EPO treatment ($p < 0.05$). NF-κB p105 expression after 72-hour obstruction with or without concurrent EPO treatment could not be distinguished from one another (Figure 4-3D). STAT5A expression was decreased in 72-hour obstructed ureter and kidney with the concurrent EPO treatment ($p < 0.0001$, $p < 0.0001$). STAT5A expression after 72-hour obstruction with or without concurrent EPO treatment could not be distinguished from one another (Figure 4-3E).

Also, we could not distinguish the difference in EPOR signaling genes between concurrent and prophylactic EPO treatment in ureteral obstruction (Figure 4-3). In the kidney tissues, decreased expression of BAX, BCL-2 and BCL-XL and increase in βCR and NF-κB p105 after the concurrent EPO treatment, similar to prophylactic EPO treatment (Figure 4-3). In the ureteral tissues, we could find a slight decrease in BAX expression with concurrent EPO treatment, similar to prophylactic EPO treatment (Figure 4-3C).

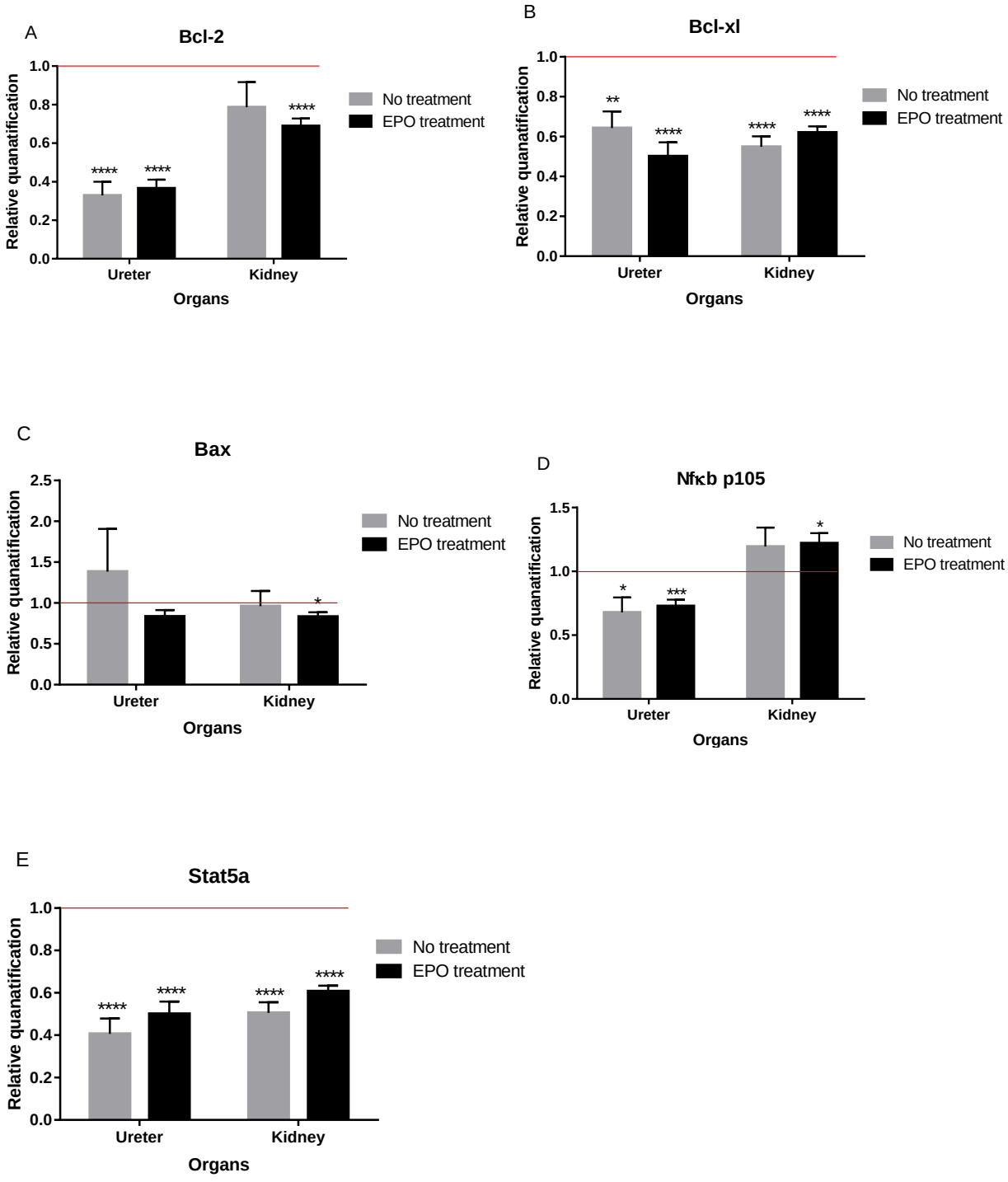

Figure 4-3 Effect of concurrent EPO treatment on RNA expression of BCL-2, BCL-XL, BAX, NF-κB p105, STAT5A in in 72-hour obstructed ureters and kidneys

Real-time quantitative PCR analysis in 72-hour obstructed ureters and kidneys and compared to those in unobstructed contralateral side with or without concurrent the EPO treatment. RNA expression of BCL-2, BCL-XL, BAX, NF-κB p105, STAT5A in the ureters and the kidneys after unilateral ureteral obstruction was analyzed by qRT-PCR (A to E). Light gray bars represent RNA expression with concurrent EPO treatment. Dark gray bars represent RNA expression without EPO treatment. Red line represents contralateral side. Data are presented as mean ± SEM. Asterisk indicates $p < 0.05$. Double asterisks indicate $p < 0.01$. Triple asterisks indicate $p < 0.001$. Quadruple asterisks indicate $p < 0.0001$.

4.4 Discussion

Here, we showed that concurrent EPO treatment provides overall similar effect via similar mechanism as prophylactic EPO treatment in protecting from ureteral obstruction. Decreased expression of EPOR signaling pathway after ureteral obstruction with concurrent EPO treatment was consistent with prophylactic EPO treatment. Results from our study that concurrent EPO has a similar efficacy as prophylactic EPO suggests that EPO provides protection in an on-off switch manner rather than consistent stimulation manner. This is supported by other studies where short-acting EPO analogs (half-life = 5 minutes ~) showed protection against various injuries after a single dose[218-221]. Literatures suggested that the tissue protective effects of EPO can be provided after a short stimulation of higher concentration, in contrast to hematopoiesis by EPO which needs a consistent stimulation of lower concentration[64,65].

Ureteral peristaltic count showed that concurrent EPO treatment has a similar effect as prophylactic EPO treatment. Concurrent EPO-treated ureters did not show any peristaltic activity after 72-hour obstruction. It was shown that the ureters require relatively longer time to recover

its peristaltic function than the kidneys[11]. It is interesting to note that contralateral ureters of 72-hour obstructed ureters decreased peristaltic activity compared to that prior to the obstruction. The decreasing trend of peristaltic count compared to the basal level on the contralateral ureter was noticeable in obstruction group without EPO treatment, though it was not statistically significant. As one side of upper urinary tract cannot function properly because of the obstruction, the contralateral side of ureters and kidneys need to process almost twice the work compared to normal conditions. The high overload on the contralateral ureter under 72-hour obstruction may lead to slight damage in its normal function, which was represented as decreased peristaltic activity of contralateral ureters of the obstructed. Further work with immunohistochemistry including TUNEL assay will be needed to validate the effect of concurrent EPO treatment.

4.5 Conclusion

Our work showed that concurrent EPO treatment induce similar EPOR signaling as prophylactic EPO treatment in 72-hour obstructed ureters and kidneys. It is important finding considering clinical perspectives, where it is difficult to provide prophylactic treatment in most of cases of clinical ureteral obstruction. It further expands the use of EPO as therapeutics to treat ureteral dysfunction following ureteral obstruction.

Chapter 5: Conclusion

5.1 Summary

Ureteral obstruction leads to dysfunction in ureteral peristaltic activity, which further damages kidney function. Prophylactic EPO treatment accelerated functional recovery of the ureters and the kidneys resulting from the obstructive uropathy. Our current study showed that ureteral peristaltic activity was decreased after 24-hour obstruction and abolished its activity after 48 and 72-hour obstruction. Ureteral obstruction decreased the expression of EPO, EPOR and its downstream anti-apoptotic BCL-2, BCL-XL and STAT5A in both CD-1 and C57BL/6 strains. Decreased anti-apoptotic gene expression in the ureters after ureteral obstruction, led us to investigate apoptosis in the obstructed ureters. We found increased apoptosis in 72-hour obstructed ureters compared to contralateral ureters. Furthermore, we found that βCR was upregulated in kidneys by obstruction. It suggests the activation of non-hematopoietic mechanisms in response to ureteral dysfunction in obstruction, which may be impaired due to decreased EPO and EPOR expression. Immunohistochemistry with phospho-NF-κB p65 antibody showed a significant increase in phospho- NF-κB p65 by obstruction compared to its contralateral ureters.

Prophylactic EPO treatment improved ureteral function as shown by increasing the number of animals that retained ureteral peristalsis after 24-hour obstruction. Prophylactic EPO treatment on ureteral obstruction decreased the expression of BAX, which showed anti-apoptotic signaling by EPO treatment to compensate for decreased EPOR signaling by obstruction. Prophylactic

EPO treatment of mice obstructed for 72 hours suppressed apoptosis in the ureters by obstruction, which aligns well with the decreased apoptotic signaling by EPO treatment. And there was no significant difference in apoptosis between 72-hour obstructed ureters and contralateral ureters after EPO treatment. Immunohistochemistry with phospho-NF-κB p65 antibody showed that there is no significant difference in phospho-NF-κB p65 between 72-hour obstructed ureters and contralateral ureters with EPO treatment. This suggests that prophylactic EPO treatment suppressed NF-κB activation to prevent apoptosis in the obstructed ureters.

Concurrent EPO treatment on ureteral obstruction showed similar level of EPOR signaling as were found with prophylactic EPO treatment. It shows that duration of EPO exposure on obstructive injury can be short to activate EPO's protection against injury by ureteral obstruction. It further expands EPO's potential use as a therapeutic for clinical situations, where it would be difficult to provide prophylactic treatment in most of clinical cases of ureteral obstruction.

EPO treatment without obstructive injury did not affect the expression of EPOR signaling. It suggests that EPO treatment does not have any pre-conditioning effect on ureters and kidneys, and prophylactic EPO shows protective effects only when the injury co-exists in the urinary tracts. Additionally, EPO treatment did not increase red blood cells and hematopoietic markers in our experiment of 4 days, which confirms that EPO's protective effect is mediated via a separate mechanism from increased blood circulation through erythropoiesis by EPO treatment. In vitro experiment showed that there was no significant change in EPOR downstream genes after EPO incubation, which supports our previous in vivo experiment.

The results from my thesis study suggest that the EPO treatment accelerated the functional recovery of ureters and kidneys via anti-apoptotic mechanisms. Our study findings may contribute to the development of the therapeutics to treat ureteral dysfunction following ureteral obstruction. Furthermore, we showed that anti-apoptotic EPOR signaling is suppressed by obstruction in two different mice strains. It implies the importance of EPOR signaling in obstructive uropathy that leads to ureteral apoptosis.

5.2 Limitation

We first focused on studying the effects in CD-1 strain for Chapter 2 as that is the mouse strain that the original observations of accelerated ureteral recovery following obstruction were made in. Considering the difference in physiological responses from different mouse strain, C57BL/6 was chosen to verify the finding from CD-1. Given that we noticed C57BL/6 mice to be more sensitive as well as to show the same pattern of expression in EPOR signaling genes in both strains, we pursued further studies in C57BL/6 strain. The effect of EPO treatment on functional recovery of ureteral obstruction could show the difference in different strains.

We selectively investigated the expression of the genes that are involved in anti-apoptotic EPOR signaling. Further analysis on the expression of additional apoptosis regulatory genes will be needed to validate our findings. TUNEL assay was performed to detect apoptosis in 72-hour obstructed ureters, presuming that prolonged obstruction would lead to more severe damage based on the results from ureteral functional assay. That said, waiting for this later time point may minimize the difference in apoptosis between EPO treated and untreated animals, as

apoptosis cells may have been removed by phagocytes as art of the early inflammatory response. The beneficial effect of EPO on decreasing apoptosis may therefore be greater at earlier time points where apoptosis is at its highest.

The effect of concurrent EPO treatment was only validated on RNA expression, which would need further investigation using immunohistochemistry.

Renal pacemaker cells such as ICC-like cells located in renal pelvis have a significant role in peristalsis and the current study has not addressed any potential role of EPO in pacemaker cell activity.

5.3 Future direction

Given the finding that EPO treatment suppressed BAX and NF-κB expression to ameliorate obstruction-induced apoptosis in ureteral tissues, direct inhibition of BAX and NF-κB on ureteral obstruction may provide avenues for therapeutic development to treat ureteral dysfunction following obstruction. Also, further investigation should look at the protein expression of EPOR signaling. Lastly, more apoptosis regulatory targets should be investigated, along with their protein activation on mitochondria.

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