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(71) Applicants: **MIDWESTERN UNIVERSITY** [US/US]; 555 31st Street, Downers Grove, IL 60515 (US). **PHARMAZZ, INC.** [US/US]; 50 West 75th Street, Suite 106, Willowbrook, IL 60527 (US).

(72) Inventors: **GULATI, Anil**; 608 Fawell Ct, Naperville, IL 60565 (US). **LAVHALE, Manish**; C-366 Paramount Golf Foreste, Sector Zeta, Upsidc Site C, Greater Noida, Uttar Pradesh-201307 (IN). **KATIA, Bhawna**; House No. 32570, Main Road, Paras Ram Nagar, Near Narotam Furniture House, Bathinda-151001 (IN). **SINGH, Abhishek, Kumar**; H. No.-26, Chandradeep Colony, Near T.V Tower, Post-Umarsia, Bareilly-243001 (IN).

(74) Agent: **ALLIKIAN, Michael, J.** et al.; Marshall, Gerstein & Borun LLP, 233 S. Wacker Drive, 6300 Willis Tower, Chicago, IL 60606-6357 (US).

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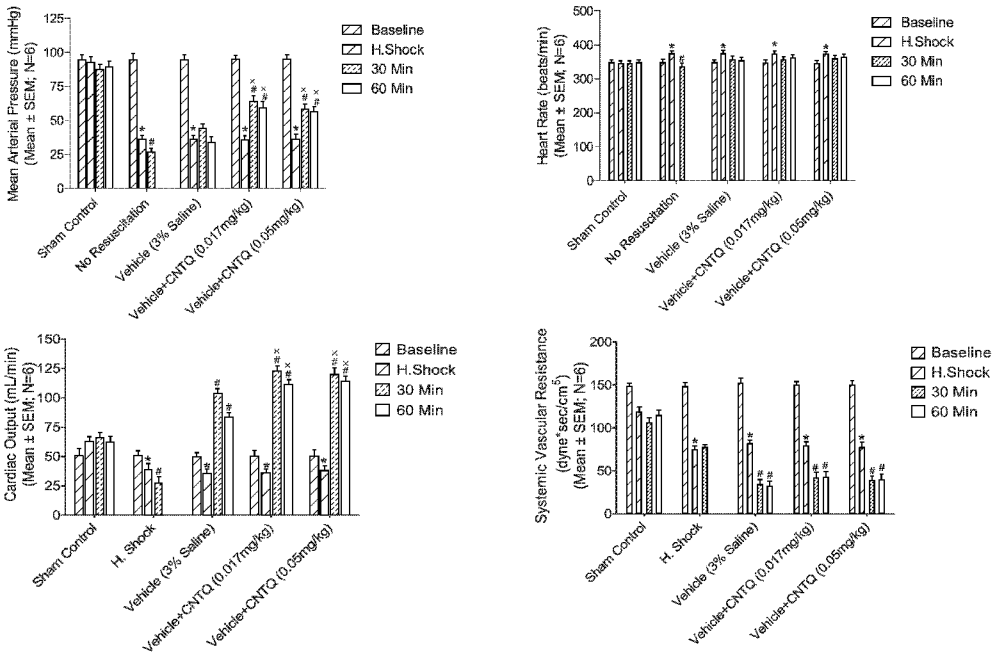


Figure 1

(57) Abstract: The present disclosure is related to methods and compositions for treating or preventing kidney injury or failure, comprising administering an endothelin B (ET_B) receptor agonist and/or an α_2 adrenergic agent.

[Continued on next page]

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ALTERATIONS IN ENDOTHELIN RECEPTORS FOLLOWING HEMORRHAGE AND RESUSCITATION BY CENTHAQUIN

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the priority benefit under 35 U.S.C. § 119(e) of U.S. Provisional Patent Application No. 62/666,675, filed May 3, 2018, and Indian Application No. 201841019588, filed May 25, 2018, the disclosures of which are incorporated herein by reference in their entirety.

FIELD OF THE INVENTION

[0002] The present disclosure is related to methods and compositions for treating or preventing kidney injury or failure, comprising administering an endothelin B (ET_B) receptor agonist and/or an α_2 adrenergic agent.

INCORPORATION BY REFERENCE OF MATERIALS SUBMITTED ELECTRONICALLY

[0003] This application contains, as a separate part of the disclosure, a Sequence Listing in computer readable form (Filename: 50000A_Seqlisting.txt; Size: 673 bytes; Created: May 2, 2019), which is incorporated by reference in its entirety.

BACKGROUND

[0004] Hemorrhagic shock often leads to multiple organ failure due to inadequate blood circulation, perfusion and oxygenation as a result of rapid and excessive blood loss (Wu *et al.* 2009). Multiple compensatory mechanisms to preserve oxygenation and tissue blood flow are initiated with the onset of hemorrhage. Despite resuscitation with intravenous fluids to restore circulation and oxygen delivery, patients may still undergo irreversible loss of blood perfusion, coagulopathy, hypothermia, acidosis, immune suppression, systemic inflammation, oxidative stress, multiple organ failure, and death (Acosta *et al.* 1998; Jacob and Kumar 2014). Deaths from hemorrhagic shock typically occur very early, mostly within the first 6 hours of admission (Shackford *et al.* 1993).

[0005] Endothelin (ET), an endogenous 21 amino acid peptide, was first isolated from porcine aortic endothelial cells nearly 3 decades ago (Yanagisawa *et al.* 1988). There are 3 distinct isopeptides: ET-1, ET-2, and ET-3, which are present in various mammalian tissues performing a myriad of physiological and pathological roles such as regulation of blood pressure and perfusion, apoptosis and cellular proliferation and migration (Ehrenreich *et al.* 2000; Inoue *et al.* 1989; Vidovic *et al.* 2008; Yanagisawa *et al.* 1988). The ET peptides produce their biological effects through activation of G-protein-coupled receptors: ET_A and ET_B (Arai *et al.* 1990). However, ET-1 and its receptors are not limited to the vascular system.

SUMMARY OF THE INVENTION

[0006] In some aspects, the present disclosure is directed to use of the ET_B receptor agonist, IRL-1620, and an adrenergic agent, centhaquin, in the treatment of acute kidney failure. In particular, it has unexpectedly been found that overexpression or stimulation of ET_B receptors significantly increases renal blood perfusion.

[0007] In further aspects, the disclosure is directed to administration of a specific agonist for ET_B receptors alone or in combination with centhaquin to an individual in need thereof. In some embodiments, administration of an ET_B receptor agonist alone or in combination with centhaquin prevents or treats acute kidney injury.

[0008] In some aspects, the disclosure provides a method of preventing or treating kidney injury or failure by administering a therapeutically effective amount of an endothelin B (ET_B) receptor agonist to an individual in need thereof. In some embodiments, the ET_B receptor agonist is selected from the group consisting of N-Succinyl-[Glu^9, $Ala^{11,15}$] endothelin 1 (IRL-1620), BQ-3020, [$Ala^{1,3,11,15}$]-endothelin, sarafotoxin S6c, and endothelin 3. In further embodiments, the ET_B receptor agonist is administered at a dose ranging from about 0.0001 mg/kg to about 0.5 mg/kg. In some embodiments, the method comprises administering multiple doses of the ET_B receptor agonist. In still further embodiments, the administering comprises a single dose of the ET_B receptor agonist. In some embodiments, the method further comprises administering a therapeutically effective amount of centhaquin or a salt thereof to the individual.

[0009] In some embodiments, the kidney injury or failure is acute. In further embodiments, the kidney failure results from exposure to radiocontrast media, a non-steroidal anti-inflammatory drug (NSAID), an antibiotic, a chemotherapeutic agent, nivolumab-induced acute granulomatous tubulointerstitial nephritis or a nephrotoxic drug. In some embodiments, the acute kidney failure is caused by or is associated with critical illness, reduced cardiac output, trauma, reduced blood oxygenation, systemic toxicity caused by reaction to injury in another organ, systemic hypotension resulting from cardiorenal syndrome, cardiac surgery or acute decompensated heart failure, a reduction in circulating volume due to hemorrhage, septic shock, hypovolemic shock, severe dengue, a surgical procedure, rhabdomyolysis or a reduction in local renal blood flow resulting from hepatorenal syndrome or liver transplant, or dehydration caused by diarrhea, vomiting, diuretics or excessive sweating.

[0010] In some embodiments, kidney glomerular filtration rate of the individual is improved. In further embodiments, serum creatinine level of the individual is reduced.

[0011] In some aspects, the disclosure provides a composition comprising (a) an endothelin-B (ET_B) receptor agonist, (b) centhaquin or a salt thereof, and optionally (c) an excipient.

[0012] In further aspects, the disclosure provides an article of manufacture comprising: (a) a packaged composition comprising an endothelin-B (ET_B) receptor agonist and centhaquin or a salt thereof; (b) an insert providing instructions for a simultaneous or sequential administration of the ET_B receptor agonist and the centhaquin or salt thereof to treat a patient; and (c) a container for (a) and (b). In some embodiments, the endothelin-B (ET_B) receptor agonist is N-Succinyl-[Glu^9, $Ala^{11,15}$] endothelin 1 (IRL-1620).

[0013] In some aspects, the disclosure provides a method of treating an individual suffering acute kidney function decline comprising administering to the individual a therapeutically effective amount of a composition comprising centhaquin or a salt thereof. In some embodiments, the acute kidney function decline is associated with acute kidney failure. In some embodiments, the causes of acute decline in kidney functions are prerenal due to decreased blood flow to the kidney, intrinsic where the tissues within the kidneys are directly damaged, and postrenal where the urine flow is blocked. In some embodiments, the acute kidney failure is caused by or is associated with critical illness, reduced cardiac output, trauma, reduced blood oxygenation, systemic toxicity caused by reaction to injury in another organ, systemic hypotension resulting from cardiorenal syndrome, cardiac surgery or acute decompensated heart failure, a reduction in circulating volume due to hemorrhage, septic shock, hypovolemic shock, severe dengue, a surgical procedure, rhabdomyolysis or a reduction in local renal blood flow resulting from hepatorenal syndrome or liver transplant, nephrotoxicity resulting from drugs, radiocontrast media, a non-steroidal anti-inflammatory drug (NSAID), an antibiotic, or a chemotherapeutic agent, or dehydration caused by diarrhea, vomiting, diuretics or excessive sweating.

[0014] In some aspects, the disclosure provides a method of treating an individual suffering from kidney injury comprising administering an effective amount of a composition comprising centhaquin or a salt thereof. In some embodiments, the kidney injury results from an ischemic event or an ischemic reperfusion event. In further embodiments, the salt is citrate, pyruvate, or lactate. In some embodiments, centhaquin or salt thereof is administered at a dose of about 0.0001 mg/kg to about 1.0 mg/kg. In further embodiments, centhaquin or salt thereof is administered in single or multiple doses.

BRIEF DESCRIPTION OF THE FIGURES

[0015] **Figure 1** shows the effect of hemorrhage on mean arterial pressure, heart rate, cardiac output and systemic vascular resistance in sham and hemorrhaged rats. Hemorrhaged rats were resuscitated with hypertonic saline or centhaquin. The values are expressed as mean ± S.E.M. (n=5). *p<0.05 compared to baseline, #p<0.05 compared to hemorrhage, xp<0.05 compared to vehicle treated group.

[0016] **Figure 2** depicts the effect of hemorrhage on the expression of ET_A receptors in sham and hemorrhaged rats. Hemorrhaged rats were resuscitated with hypertonic saline or centhaquin. Lane 1- Sham; Lane 2 – Hemorrhagic shock; Lane 3 – Hypertonic saline (vehicle); Lane 4 – Vehicle + centhaquin (0.017 mg/kg); Lane 5 – Vehicle + centhaquin (0.05 mg/kg). The values are expressed as mean ± S.E.M. (n=4). *p<0.05 compared to sham, #p<0.05 compared to hemorrhage or hypertonic saline.

[0017] **Figure 3** shows the effect of hemorrhage on the expression of ET_B receptors in sham and hemorrhaged rats. Hemorrhaged rats were resuscitated with hypertonic saline or centhaquin. Lane 1- Sham; Lane 2 – Hemorrhagic shock; Lane 3 – Hypertonic saline (vehicle); Lane 4 – Vehicle + centhaquin (0.017 mg/kg); Lane 5 – Vehicle + centhaquin (0.05 mg/kg). The values are expressed as mean ± S.E.M. (n=4). *p<0.05 compared to sham, #p<0.05 compared to hemorrhage or hypertonic saline.

[0018] **Figure 4** shows the effect of hemorrhage on plasma TNF-α, IL-6 and IL-10 in sham and hemorrhaged rats. Hemorrhaged rats were resuscitated with hypertonic saline or centhaquin. The values are expressed as mean ± S.E.M. (n=5). *p<0.05 compared to sham, #p<0.05 compared to hemorrhage, @p<0.05 compared to hypertonic saline.

[0019] **Figure 5** shows the effect of hemorrhage on renal blood perfusion in male rats with massive blood loss. Hemorrhaged rats were resuscitated with vehicle (saline) or centhaquin low dose (0.01 mg/kg) or high dose (0.1 mg/kg). The values are expressed as mean ± S.E.M. (n=5). *p<0.05 compared to hemorrhage, #p<0.05 compared to vehicle (saline).

[0020] **Figure 6** shows the effect of hemorrhage on renal blood perfusion in female rats with massive blood loss. Hemorrhaged rats were resuscitated with vehicle (saline) or centhaquin low dose (0.01 mg/kg) or high dose (0.1 mg/kg). The values are expressed as mean ± S.E.M. (n=5). *p<0.05 compared to hemorrhage, #p<0.05 compared to vehicle (saline).

[0021] **Figure 7** shows results of experiments in which male rats were anaesthetized with urethane, the femoral vein was cannulated for drug administration, the femoral artery was cannulated for measuring mean arterial pressure (MAP), and a laser Doppler flow probe was placed in the renal medulla to measure blood perfusion. Induction of hemorrhagic shock was

initiated by withdrawing blood to maintain the MAP at 35 mmHg for 30 minutes. Norepinephrine infusion was carried out to bring and maintain the MAP to 70 mmHg. The effect of centhaquin on cardiovascular functions were measured before the induction of shock, 30 minutes after shock (hemorrhage) and 15, 30, 45, 60, 75 and 90 minutes after resuscitation. Centhaquin improved renal blood perfusion of hemorrhaged male rats compared to vehicle control following resuscitation. The adverse effects of norepinephrine induced vasoconstriction can be attenuated by centhaquin.

[0022] **Figure 8** shows results of experiments in which female rats were anaesthetized with urethane, the femoral vein was cannulated for drug administration, the femoral artery was cannulated for measuring mean arterial pressure (MAP), and a laser Doppler flow probe was placed in the renal medulla to measure blood perfusion. Induction of hemorrhagic shock was initiated by withdrawing blood to maintain the MAP at 35 mmHg for 30 minutes. Norepinephrine infusion was carried out to bring and maintain the MAP to 70 mmHg. The effect of centhaquin on cardiovascular functions were measured before the induction of shock, 30 minutes after shock (hemorrhage) and 15, 30, 45, 60, 75 and 90 minutes after resuscitation. Centhaquin improved renal blood perfusion of hemorrhaged female rats compared to vehicle control following resuscitation and an improved blood perfusion was observed till the end of experiment. The adverse effects of norepinephrine induced vasoconstriction can be attenuated by centhaquin.

[0023] **Figure 9** shows results of the phase II study of centhaquin as a resuscitative agent for hypovolemic shock due to excessive blood loss in which systolic and diastolic blood pressure were determined when the patient was inducted in the study (baseline) and at the time of discharge from hospital (end of the study). An interim analysis showed that blood pressure increase in centhaquin treated patients was more than that observed in control cohort. These results indicated that centhaquin is an effective resuscitative agent and improved the outcome of patients of hypovolemic shock.

[0024] **Figure 10** shows results of the phase II study of centhaquin as a resuscitative agent for hypovolemic shock due to excessive blood loss in which all subjects received standard of care along with standard shock treatment. Patients were then randomly assigned to either control cohort that received standard treatment along with normal saline or centhaquin cohort that received standard treatment along with centhaquin. Serum creatinine levels were determined when the patient was inducted in the study (baseline) and at the time of discharge from hospital (end of the study). An interim analysis as per approved protocol showed that serum creatinine level decreased by 25.35% in control cohort and by 42.61% in centhaquin

treated patients. The data indicated that reduction of serum creatinine levels by centhaquin is 17.26% more compared to standard treatment.

[0025] **Figure 11** shows results of the phase II study of centhaquin as a resuscitative agent for hypovolemic shock due to excessive blood loss in which all subjects received standard of care along with standard shock treatment. Patients were then randomly assigned to either control cohort that received standard treatment along with normal saline or centhaquin cohort that received standard treatment along with centhaquin. Blood urea nitrogen was determined when the patient was inducted in the study (baseline) and at the time of discharge from hospital (end of the study). An interim analysis as per approved protocol showed that blood urea nitrogen was similar in control cohort and centhaquin treated patients.

[0026] **Figure 12** shows results of the phase II study of centhaquin as a resuscitative agent for hypovolemic shock due to excessive blood loss in which all subjects received standard of care along with standard shock treatment. Patients were then randomly assigned to either control cohort that received standard treatment along with normal saline or centhaquin cohort that received standard treatment along with centhaquin. Glomerular filtration rate was determined when the patient was inducted in the study (baseline) and at the time of discharge from hospital (end of the study). An interim analysis as per approved protocol showed that glomerular filtration rate increased by 40.00% in control cohort and by 81.52% in centhaquin treated patients. The data indicated that an increase in glomerular filtration rate by centhaquin is 41.52% more compared to standard treatment.

DETAILED DESCRIPTION

[0027] It has been established that low doses of centhaquin (2-[2-(4-(3-methyphenyl)-1-piperazinyl)]ethyl-quinoline) citrate, significantly decreased blood lactate, and increased mean arterial pressure (MAP), pulse pressure (PP) and cardiac output (CO) in hemorrhagic shock (Gulati *et al.* 2012; Gulati *et al.* 2013; Lavhale *et al.* 2013; Papapanagiotou *et al.* 2016). Comparative studies (see Example 2) were performed between centhaquin and status quo resuscitative agents grouped into 3 different categories: (a) fluids such as Lactated Ringer's, hypertonic saline; (b) adrenergic agents such as norepinephrine, and (c) fresh blood. Results using (i) a rat model of fixed pressure blood loss, (ii) a rabbit model of uncontrolled bleeding with trauma, and (iii) a pig model of massive blood loss indicate that centhaquin is highly effective in reducing the mortality following hypovolemic shock (Gulati *et al.* 2012; Gulati *et al.* 2013; Lavhale *et al.* 2013; Papapanagiotou *et al.* 2016). Unlike other resuscitative agents (vasopressors), centhaquin increased MAP by increasing stroke volume (SV) and CO; and decreased heart rate and systemic vascular resistance (SVR).

[0028] Elevated plasma ET-1 levels during hemorrhagic shock along with a decrease in blood flow to the kidneys and the lungs have been previously reported (Chang *et al.* 1993; Edwards *et al.* 1994). A decrease in pulmonary and renal blood flow following hemorrhagic shock, causing reduced clearance of ET-1, may be responsible for an increase in circulating plasma ET-1 which plays an important role in maintaining vascular tone and tissue blood perfusion (Chang *et al.* 1993). Circulating ET-1 may regulate cardiovascular system following hemorrhagic shock by acting on ET_A receptors, as a vasoconstrictor and on ET_B receptors as a vasodilator to maintain vascular tone (Bourque *et al.* 2011; Cardillo *et al.* 2000; Helmy *et al.* 2001; Sandoo *et al.* 2010).

[0029] Centhaquin is currently in clinical development as a resuscitative agent for hemorrhagic shock. Without wishing to be bound by theory, the proposed mechanism is that centhaquin acts on venous α_{2B} adrenergic receptors to produce constriction and increase venous return to the heart and stimulate central α_{2A} adrenergic receptors to produce a decrease in SVR. However, adrenergic receptors have been shown to be modulated by endothelin (ET) receptors (Gulati 1992; Gulati and Srimal 1993; Lavhale *et al.* 2010; Sanchez *et al.* 2014) therefore, it is possible that ET receptors may be involved in the mechanism of action of centhaquin in hemorrhagic shock.

Kidney Injury/Failure

[0030] Acute renal failure is the sudden loss of the kidney's ability to filter wastes without losing electrolytes. Most often, acute renal failure (also termed acute kidney injury or AKI) is caused by reduced blood flow to the kidneys (prerenal acute renal failure), though about 20% of the cases are due to infections or toxins affecting the kidneys directly (intrinsic ARF), and about 10% are due to blockages downstream of the kidneys (postrenal obstruction). Acute kidney injury (AKI) has a high prevalence in critical care patients. Early detection might prevent patients from developing chronic kidney disease and requirement for renal replacement therapy. In the majority of hospitalized patients, acute renal failure is caused by acute tubular necrosis, which results from ischemic and/or nephrotoxic insults. Renal hypoperfusion is caused by hypovolemic, cardiogenic and septic shock, by administration of vasoconstrictive drugs or renovascular injury. Nephrotoxins include exogenous toxins such as contrast media and aminoglycosides as well as endogenous toxin such as myoglobin. Studies indicate that apoptosis in renal tissue is prominent in most human cases of acute renal failure, with the principal site of apoptotic cell death being the distal nephron. During the initial phase of ischemic injury, loss of integrity of the actin cytoskeleton leads to flattening of the epithelium, with loss of the brush border, loss of focal cell contacts, and subsequent disengagement of the cell from the underlying substratum. It has been suggested that apoptotic tubule cell death may

be more predictive of functional changes than necrotic cell death (Komarov et al. 1999; Supavekin et al. 2003). An individual at risk for developing ischemic acute renal failure includes individuals with diabetes, underlying renal insufficiency, nephritic syndrome, elderly, atherosclerotic disease, nephrotoxic agent recipients, sepsis, hypotensive individuals, hypoxic individuals, pre-surgery, myoglobinuria-hematuria, pregnancy associated acute renal failure, and liver disease.

[0031] The acute kidney injury is characterized by at least one condition selected from the group consisting of an increase in serum creatinine by at least 50% over baseline, an absolute increase in serum creatinine of at least 0.3 mg/dL over baseline, a reduction in glomerular filtration rate of at least 25% compared to baseline, and a decrease in urine output to 0.5 ml per kilogram of body weight or less per hour persisting for at least 6 hours.

[0032] The incidence of community acquired acute renal failure (ARF) is only about 100 cases per million population with a mortality rate of 7%. The published incidence of ARF ranges from 1 to 13% of all hospital admissions (34×10^6/year in the US) and 20 to 30% of all ICU admissions (4.4×10^6/year in the US). Most cases of ARF are acquired in the hospital as a result of complications from other illnesses or interventions. The most common causes are sepsis, hypovolemia, surgery, imaging contrast agents, chemotherapy drugs, NSAIDS, and some antibiotics.

[0033] An acute kidney injury may involve a pre-renal kidney injury caused by or associated with a reduced cardiac output leading to reduced overall blood flow to the kidneys, trauma, reduced blood oxygenation, systemic toxicity caused by reaction to injury in another organ, systemic hypotension resulting from cardiorenal syndrome or acute decompensated heart failure, a reduction in circulating volume due to hemorrhage, a surgical procedure, or a reduction in local renal blood flow resulting from hepatorenal syndrome.

[0034] In a study, acute kidney injury developed in 52.6% (n = 8270) of the entire cohort and in 39.8% of the young adult age group (16-25 years). For the young adult age group, diabetes, surgical reason for admission, severity of illness, hypotension, and certain medications (vancomycin and calcineurin inhibitors) were all independently associated with acute kidney injury. Acute kidney injury was a significant predictor for longer length of stay, intensive care unit mortality, and mortality after discharge. Therefore, acute kidney injury is a common event and is associated with an increased length of stay and a high risk of mortality (Fuhrman et al. 2018).

[0035] Using the 2001 National Hospital Discharge Survey, Liangos et al. (2006) found that 1.9% of all hospital discharges showed a code for ARF, which corresponds to a U.S. incidence of 646,000. The mortality rate was 21.3%. The authors validated the study by examining all the 13,237 patients discharged from St. Elizabeth's (Boston) during 2001. It was noted that 2.6% of the patients were coded for ARF, but lab values showed that 12% of the patients had experienced ARF. Thus, ARF is coded on only about 20% of occurrences (presumably the most serious cases) (Liangos et al. 2006).

[0036] The treatment of acute renal failure is to give fluids to reverse hypovolemia and flush toxins while waiting for the kidneys to recover. In some instances, the patients retain too much water or their electrolyte balance suffers to such an extent that they require dialysis. The most common causes of death in acute renal failure patients are heart failure, sepsis, and respiratory failure. Patients who recover from acute renal failure show increased odds of death and chronic kidney disease over the following 5 and 10 years. Dozens of new treatments and drugs that showed promise in animals have been tested clinically in acute renal failure patients, but none have demonstrated benefits in randomized clinical trials. Some of the treatments tested include diuretics to increase urine flow, dopamine and atrial natriuretic peptide (ANP) to increase blood flow to the kidneys, many cytoprotective agents to preserve tubule epithelial cells such as free radical scavengers, heat shock proteins, hemeoxygenase, xanthine oxidase inhibitors, prostaglandins, and calcium channel blockers and several growth factors to speed the recovery of the proximal tubules (Allgren et al. 1997; Hirschberg et al. 1999).

[0037] Acute kidney injury has been reported after cardiac surgery; liver transplant; severe dengue; septic shock; hypovolemic shock; nephrotoxic drugs such as intravenous radiographic contrast, vancomycin, piperacillin-tazobactam combined with vancomycin, colistin, nivolumab-induced acute granulomatous tubulointerstitial nephritis, anti-cancer drugs, cisplatin-induced acute kidney injury; critically ill patients with solid tumors; Lithiasis-induced acute kidney injury; rhabdomyolysis.

[0038] Studies have been conducted to determine the effect of centhaquin on renal medullary blood flow and the results showed that severe hemorrhage produced a decrease in renal medullary blood flow and worsening renal perfusion leading to ischemia and renal failure (Gulati et al. 2016a; Gulati et al. 2017). It is known that the outer renal medulla has a higher metabolic demand and where about 33% of the filtered sodium chloride is reabsorbed by the thick ascending limb of loop of Henle (Cowley 2008). Hence this region is highly prone to hypoxic or ischemic injury following excessive hemorrhage.

Endothelin

[0039] Endothelin (ET) is an endogenous peptide which acts on two distinct G-protein-coupled receptors, ET_A and ET_B, and performs numerous functions throughout the body (Arai *et al.* 1990; Goto *et al.* 1989). The elevated plasma ET-1 levels during hemorrhagic shock along with a decrease in blood flow to the kidneys and the lungs have been previously reported (Chang *et al.* 1993; Edwards *et al.* 1994). A decrease in pulmonary and renal blood flow following hemorrhagic shock, causing reduced clearance of ET-1, may be responsible for an increase in circulating plasma ET-1 which plays an important role in maintaining vascular tone and tissue blood perfusion (Chang *et al.* 1993). Circulating ET-1 may regulate cardiovascular system following hemorrhagic shock by acting on ET_A receptors, as a vasoconstrictor and on ET_B receptors as a vasodilator to maintain vascular tone (Bourque *et al.* 2011; Cardillo *et al.* 2000; Helmy *et al.* 2001; Sandoo *et al.* 2010). It is therefore of interest to investigate the effect of hemorrhagic shock and resuscitation with centhaquin on changes in ET_A and ET_B receptors in various tissues. In addition, it is known that hemorrhagic shock and resuscitation contribute towards an increased risk of systemic inflammatory response (Chaudry *et al.* 1990) and ET-1 plays a pivotal role in inflammation following sepsis and hemorrhagic shock (Kowalczyk *et al.* 2015).

Endothelin B (ET_B) receptor agonist

[0040] The disclosure contemplates the use of an endothelin B (ET_B) receptor agonist in the compositions and methods disclosed herein. In various embodiments, the ET_B receptor agonist is selected from the group consisting of N-Succinyl-[Glu^9, $Ala^{11,15}$] endothelin 1 (IRL-1620), BQ-3020, [$Ala^{1,3,11,15}$]-endothelin, sarafotoxin S6c, and endothelin 3.

[0041] IRL-1620 [N-Succinyl-[Glu^9, $Ala^{11,15}$] endothelin 1] is an analog of ET-1 with amino acid sequence Suc-Asp-Glu-Glu-Ala-Val-Tyr-Phe-Ala-His-Leu-Asp-Ile-Ile-Trp (SEQ ID NO: 1), molecular weight of 1821.9. IRL-1620 is a potent and specific agonist for the ET_B receptors, with Ki values for ET_A and ET_B receptors of 1.9 µM and 16 pM, respectively, making it approximately 120,000 times more selective for ET_B over ET_A receptors. IRL-1620 has been used in numerous studies to determine the biological actions of ET_B receptors in the pulmonary, hepatic, renal, gastrointestinal, dermatological and endocrine systems (Bauer *et al.* 2000; Fellner and Arendshorst 2007; Khan *et al.* 2006; Lawrence *et al.* 1995; Mathison and Israel 1998; Mazzoni *et al.* 1999).

[0042] According to the disclosure, the ET_B receptor agonist (*e.g.*, IRL-1620) may be administered at a dose ranging from 0.0001 to 0.5 mg/kg. In further embodiments, the

endothelin-B receptor agonist is administered at a dose ranging from about 0.0001 to about 0.5 mg/kg, or from about 0.0001 to about 0.4 mg/kg, or from about 0.0001 to about 0.3 mg/kg, or from about 0.0001 to about 0.2 mg/kg, or from about 0.0001 to about 0.1 mg/kg, or from about 0.001 to about 0.5 mg/kg, or from about 0.001 to about 0.4 mg/kg, or from about 0.001 to about 0.3 mg/kg, or from about 0.001 to about 0.2 mg/kg, or from about 0.001 to about 0.1 mg/kg, or from about 0.01 to about 0.5 mg/kg, or from about 0.01 to about 0.4 mg/kg, or from about 0.01 to about 0.3 mg/kg, or from about 0.01 to about 0.2 mg/kg, or from about 0.01 to about 0.1 mg/kg, or from about 0.0005 to about 0.5 mg/kg, or from about 0.0005 to about 0.4 mg/kg, or from about 0.0005 to about 0.3 mg/kg, or from about 0.0005 to about 0.2 mg/kg, or from about 0.0005 to about 0.1 mg/kg. In additional embodiments, the ET_B receptor agonist (*e.g.*, IRL-1620) is administered at a dose of at least about 0.0001 mg/kg, or at least about 0.0002 mg/kg, or at least about 0.0005 mg/kg, or at least about 0.001 mg/kg, or at least about 0.002 mg/kg, or at least about 0.005 mg/kg, or at least about 0.007 mg/kg, or at least about 0.01 mg/kg, or at least about 0.02 mg/kg, or at least about 0.03 mg/kg, or at least about 0.04 mg/kg, or at least about 0.05 mg/kg, or at least about 0.06 mg/kg, or at least about 0.07 mg/kg, or at least about 0.08 mg/kg, or at least about 0.09 mg/kg, or at least about 0.1 mg/kg, or at least about 0.2 mg/kg, or at least about 0.3 mg/kg, or at least about 0.4 mg/kg. In still further embodiments, the ET_B receptor agonist (*e.g.*, IRL-1620) is administered at a dose of less than about 0.0001 mg/kg, or less than about 0.0002 mg/kg, or less than about 0.0005 mg/kg, or less than about 0.001 mg/kg, or less than about 0.002 mg/kg, or less than about 0.005 mg/kg, or less than about 0.007 mg/kg, or less than about 0.01 mg/kg, or less than about 0.02 mg/kg, or less than about 0.03 mg/kg, or less than about 0.04 mg/kg, or less than about 0.05 mg/kg, or less than about 0.06 mg/kg, or less than about 0.07 mg/kg, or less than about 0.08 mg/kg, or less than about 0.09 mg/kg, or less than about 0.1 mg/kg, or less than about 0.2 mg/kg, or less than about 0.3 mg/kg, or less than about 0.4 mg/kg, or less than about 0.5 mg/kg. In some embodiments, the ET_B receptor agonist (*e.g.*, IRL-1620) is administered at a dose of about 0.1 to about 0.6 µg/kg, or about 0.1 to about 0.5 µg/kg, or about 0.1 to about 0.4 µg/kg, or about 0.1 to about 0.3 µg/kg. In further embodiments, the ET_B receptor agonist (*e.g.*, IRL-1620) is administered at a dose of about 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, or 1.0 µg/kg.

[0043] The ET_B receptor agonist (*e.g.*, IRL-1620), in various embodiments, is administered to a patient repeatedly at intervals of 1 to 6 hours. In some embodiments, the ET_B receptor agonist (*e.g.*, IRL-1620) is administered to the patient every 1 to 5 hours, or every 1 to 4 hours, or every 1 to 2 hours, or every hour, or every 2 hours, or every 3 hours, or every 4 hours, or every 5 hours, or every 6 hours. In further embodiments, the ET_B receptor agonist (*e.g.*, IRL-

1620) is administered to the patient every two to five days, or every three to five days, or every two days, or every three days, or every four days, or every five days. In some embodiments, ET_B receptor agonist (*e.g.*, IRL-1620) is administered one, two, three, four, or five times per day.

Centhaquin

[0044] Centhaquin, an alpha-2 receptor agonist and cardiovascular active agent, has been shown to produce a positive inotropic effect and increased ventricular contractions of the isolated, perfused rabbit heart (Bhatnagar *et al.* 1985). Direct or indirect positive inotropic effect of centhaquin may lead to an increase in cardiac output (CO). In a study conducted in a swine model of hemorrhagic shock it was found that centhaquin (0.015 mg/kg), when administered in the fluid resuscitation phase, augments CO, reduces systemic vascular resistance (SVR), requires smaller volume of fluids for resuscitation, and results in significantly better survival compared to Lactated Ringer's (LR) (Papalexopoulou *et al.* 2017; Papapanagiotou *et al.* 2016). In another study, centhaquin administered in one-fifth the volume of blood loss, maintained mean arterial pressure (MAP) of hemorrhaged rats for a longer length of time and improved the survival (Gulati *et al.* 2013). In a rat model of hemorrhagic shock, centhaquin significantly improved CO, decreased blood lactate and improved survival (Gulati *et al.* 2012; Lavhale *et al.* 2013). Centhaquin has a half-life of about one hour and a high volume of distribution (Gulati *et al.* 2016; O'Donnell *et al.* 2016a; O'Donnell *et al.* 2016b). Successful chemistry, manufacturing and control along with mice, rat and dog toxicological studies led to successful completion of a human Phase I study of experimental centhaquin. The study was conducted in 24 human subjects and centhaquin was found to be safe and was well tolerated (Gulati *et al.* 2016). At present multi-centric Phase II studies are ongoing (CTRI/2017/03/008184) in humans with hemorrhagic shock.

[0045] It is contemplated that, in addition to centhaquin, other α_2 adrenergic agents are useful in the methods of the disclosure. For example and without limitation, the disclosure contemplates the use of an α_2 adrenergic agent selected from the group consisting of centhaquin, clonidine, guanfacine, guanabenz, guanoxabenz, guanethidine, xylazine, tizanidine, methyldopa, fadolmidine, amidephrine, amitraz, anisodamine, apraclonidine, brimonidine, cirazoline, detomidine, dexmedetomidine, epinephrine, ergotamine, etilefrine, indanidine, lofexidine, medetomidine, mephentermine, metaraminol, methoxamine, mivazerol, naphazoline, norepinephrine, norfenefrine, octopamine, oxymetazoline, phenylpropanolamine, rilmenidine, romifidine, synephrine, talipexole, salts thereof, and a combination thereof.

[0046] Salts of the α_2 adrenergic agents also can be used in the present compositions and methods. Examples of suitable salts include, but are not limited to, acid addition salts formed with inorganic acids such as nitric, boric, hydrochloric, hydrobromic, sulfuric, and phosphoric, and organic acids such as oxalic, maleic, succinic, tartaric, and citric. Nonlimiting examples of salts of α_2 adrenergic agents include, but are not limited to, the hydrochloride, hydrobromide, hydroiodide, sulfate, bisulfate, 2-hydroxyethansulfonate, phosphate, hydrogen phosphate, acetate, adipate, alginate, aspartate, benzoate, bisulfate, butyrate, camphorate, camphorsulfonate, digluconate, glycerolphosphate, hemisulfate, heptanoate, hexanoate, formate, succinate, fumarate, maleate, ascorbate, isethionate, salicylate, methanesulfonate, mesitylenesulfonate, naphthylenesulfonate, nicotinate, 2-naphthalenesulfonate, oxalate, pamoate, pectinate, persulfate, 3-phenylproprionate, picrate, pivalate, propionate, trichloroacetate, trifluoroacetate, phosphate, glutamate, bicarbonate, undecanoate, lactate, citrate, tartate, gluconate, methanesulfonate, ethanedisulfonate, benzene sulfonate, and p-toluenesulfonate salts.

[0047] Preferred salts are salts of organic acids, such as citrate, tartrate, malate, succinate, oxalate, fumarate, maleate, ascorbate, lactate, gluconate, diglyconate, and aspartate, for example. A more preferred salt is a citrate salt, a lactate salt, or a tartrate salt.

[0048] Centhaquin, as the free base, may be administered in an amount of 0.001 to less than 0.05 mg per kg of weight of the individual being treated (mg/kg), preferably about 0.003 to about 0.04 mg/kg, and more preferably about 0.005 to about 0.03 mg/kg. More particularly, centhaquin, as the free base, is administered at mg/kg doses of 0.001, 0.002, 0.003, 0.004, 0.005, 0.006, 0.007, 0.008, 0.009, 0.010, 0.011, 0.012, 0.013, 0.014, 0.015, 0.016, 0.017, 0.018, 0.019, 0.020, 0.021, 0.022, 0.023, 0.024, 0.025, 0.026, 0.027, 0.028, 0.029, 0.030, 0.031, 0.032, 0.033, 0.034, 0.035, 0.036, 0.037, 0.038, 0.039, 0.040, 0.041, 0.042, 0.043, 0.044, 0.045, 0.046, 0.047, 0.048, or 0.049, and all ranges and subranges therein.

[0049] Centhaquin also can be administered in the form of salt, *e.g.*, centhaquin citrate, to achieve the benefits of the present methods. Centhaquin citrate is administered in an amount of about 0.0001 to about 1.5 mg/kg, preferably about 0.0002 to about 0.8 mg/kg, and more preferably about 0.0004 to about 0.5 mg/kg. In some embodiments, centhaquin citrate is administered in an amount of about 0.0001 to about 1.0 mg/kg. More particularly, centhaquin citrate can be administered at mg/kg doses (as centhaquin citrate) of about or at least about any of the following doses: 0.0001, 0.0002, 0.0003, 0.0004, 0.0005, 0.0006, 0.0007, 0.0008, 0.0009, 0.001, 0.002, 0.003, 0.004, 0.005, 0.006, 0.007, 0.008, 0.009, 0.010, 0.011, 0.012, 0.013, 0.014, 0.015, 0.016, 0.017, 0.018, 0.019, 0.020, 0.021, 0.022, 0.023, 0.024, 0.025, 0.026,

0.027, 0.028, 0.029, 0.030, 0.031, 0.032, 0.033, 0.034, 0.035, 0.036, 0.037, 0.038, 0.039, 0.040, 0.041, 0.042, 0.043, 0.044, 0.045, 0.046, 0.047, 0.048, 0.049, 0.05, 0.051, 0.052, 0.053, 0.054, 0.055, 0.06, 0.065, 0.07, 0.075, 0.08, 0.085, 0.09, 0.095, 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, 0.45, 0.5, 0.55, 0.6, 0.65, 0.7, 0.75, 0.8, 0.85, 0.9, 0.95, 1.0, 1.1, 1.2, 1.3, 1.4, or 1.5, and all ranges and subranges therein.

[0050] In some embodiments, the α_2 adrenergic agent (*e.g.*, centhaquin or a salt thereof) is coadministered with a resuscitation fluid. The resuscitation fluid can be a colloid solution, a crystalloid solution, blood, a blood component or a blood substitute. Nonlimiting examples of colloid solutions and crystalloid solutions are Ringer's Lactate, saline, hypertonic saline, an albumin solution, a dextran solution, a hydroxyethyl starch solution, a gelatin solution, and a starch solution. Examples of a blood component are plasma, red blood cells, white blood cells, platelets, clotting factors, proteins, and hormones. The blood substitute can be a hemoglobin-based blood substitute or a perflourocarbon-based substitute.

[0051] The resuscitation fluid can administered in a volume amount of up to three times the volume amount of fluid, *e.g.*, blood, plasma, water, lost by an individual. In some embodiments, the resuscitation fluid is administered in a volume amount less than and up to the volume amount of fluid lost by the individual, *e.g.*, a volume amount of 5%, preferably 10% or 20%, and up to 100% of the volume amount of lost fluid.

Compositions/Administration

[0052] In some aspects, the disclosure provides a composition for treating kidney injury or kidney failure comprising a therapeutically effective amount of an ET_B receptor agonist (*e.g.*, IRL-1620), wherein the therapeutically effective amount is about from about 0.0001 mg/kg to about 0.5 mg/kg. In some embodiments, the composition further comprises a therapeutically effective amount of centhaquin or salt thereof, wherein the therapeutically effective amount of centhaquin or salt thereof is administered at a dose of about 0.0001 mg/kg to about 1.0 mg/kg. In some embodiments, the centhaquin salt is centhaquin citrate.

[0053] In some aspects, the disclosure provides a composition for treating acute kidney function decline or kidney injury comprising a therapeutically effective amount of centhaquin or a salt thereof, wherein the therapeutically effective amount of centhaquin or salt thereof is administered at a dose of about 0.0001 mg/kg to about 1.0 mg/kg. In some embodiments, the centhaquin salt is centhaquin citrate.

[0054] The pharmaceutical compositions of the disclosure include those wherein the active ingredients are administered in an effective amount to achieve their intended purpose. More

specifically, a "therapeutically effective amount" means an amount effective to treat or prevent kidney injury or failure, or to treat acute kidney function decline. Determination of a therapeutically effective amount is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein.

[0055] A "therapeutically effective dose" refers to the amount of the active agents that results in achieving the desired effect. Toxicity and therapeutic efficacy of such active agents can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index, which is expressed as the ratio between LD_{50} and ED_{50}. A high therapeutic index is preferred. The data obtained from such data can be used in formulating a range of dosage for use in humans. The dosage of the active agents preferably lies within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage can vary within this range depending upon the dosage form employed, and the route of administration utilized.

[0056] For administration to a human in the methods disclosed herein, oral dosages of centhaquin are about 0.01 to about 200 mg daily for an average adult patient (70 kg), typically divided into two to three doses per day. Thus, for a typical adult patient, individual tablets or capsules contain about 0.1 to about 200 mg centhaquin in a suitable pharmaceutically acceptable vehicle or carrier, for administration in single or multiple doses, once or several times per day. Dosages for intravenous, buccal, or sublingual administration typically are about 0.1 to about 10 mg/kg per single dose as required. In practice, the physician determines the actual dosing regimen that is most suitable for an individual patient, and the dosage varies with the age, weight, and response of the particular patient. The above dosages are exemplary of the average case, but there can be individual instances in which higher or lower dosages are merited, and such are within the scope of this disclosure.

[0057] As discussed in further detail herein above, dosages of an ET_B receptor agonist (*e.g.*, IRL-1620) are about 0.1 to about 0.6 µg/kg for an average adult patient, typically administered one, two, or three times per day. In some embodiments, the dose of the ET_B receptor agonist (*e.g.*, IRL-1620) is about 0.3 µg/kg, administered one, two, or three times per day. Dosages of the ET_B receptor agonist (*e.g.*, IRL-1620) are in a suitable pharmaceutically acceptable vehicle or carrier. In practice, the physician determines the actual dosing regimen that is most suitable for an individual patient, and the dosage varies with the age, weight, and response of the particular patient. The above dosages are exemplary of the average case, but there can be

individual instances in which higher or lower dosages are merited, and such are within the scope of this disclosure.

[0058] The active agents of the present disclosure can be administered alone, or in admixture with a pharmaceutical carrier selected with regard to the intended route of administration and standard pharmaceutical practice. Pharmaceutical compositions for use in accordance with the present disclosure thus can be formulated in a conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries that facilitate processing of the active agents into preparations that can be used pharmaceutically.

[0059] These pharmaceutical compositions can be manufactured in a conventional manner, *e.g.*, by conventional mixing, dissolving, granulating, dragee-making, emulsifying, encapsulating, entrapping, or lyophilizing processes. Proper formulation is dependent upon the route of administration chosen. When a therapeutically effective amount of the active agents are administered orally, the composition typically is in the form of a tablet, capsule, powder, solution, or elixir. When administered in tablet form, the composition can additionally contain a solid carrier, such as a gelatin or an adjuvant. The tablet, capsule, and powder contain about 5% to about 95% of an active agent of the present invention, and preferably from about 25% to about 90% of an active agent of the present invention. When administered in liquid form, a liquid carrier, such as water, petroleum, or oils of animal or plant origin, can be added. The liquid form of the composition can further contain physiological saline solution, dextrose or other saccharide solutions, or glycols. When administered in liquid form, the composition contains about 0.5% to about 90% by weight of active agents, and preferably about 1% to about 50% of an active agents.

[0060] When a therapeutically effective amount of the active agents is administered by intravenous, cutaneous, or subcutaneous injection, the composition is in the form of a pyrogen-free, parenterally acceptable aqueous solution. The preparation of such parenterally acceptable solutions, having due regard to pH, isotonicity, stability, and the like, is within the skill in the art. A preferred composition for intravenous, cutaneous, or subcutaneous injection typically contains, in addition to a compound of the present disclosure, an isotonic vehicle.

[0061] Suitable active agents can be readily combined with pharmaceutically acceptable carriers well-known in the art. Such carriers enable the active agents to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a patient to be treated. Pharmaceutical preparations for oral use can be obtained by adding the active agents with a solid excipient, optionally grinding the resulting mixture, and

processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients include, for example, fillers and cellulose preparations. If desired, disintegrating agents can be added.

[0062] The active agents can be formulated for parenteral administration by injection, *e.g.*, by bolus injection or continuous infusion. Formulations for injection can be presented in unit dosage form, *e.g.*, in ampules or in multidose containers, with an added preservative. The compositions can take such forms as suspensions, solutions, or emulsions in oily or aqueous vehicles, and can contain formulatory agents, such as suspending, stabilizing, and/or dispersing agents.

[0063] Pharmaceutical compositions for parenteral administration include aqueous solutions of the active agent in water-soluble form. Additionally, suspensions of the active agents can be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils or synthetic fatty acid esters. Aqueous injection suspensions can contain substances which increase the viscosity of the suspension. Optionally, the suspension also can contain suitable stabilizers or agents that increase the solubility of the compounds and allow for the preparation of highly concentrated solutions. Alternatively, a composition of the disclosure can be in powder form for constitution with a suitable vehicle, *e.g.*, sterile pyrogen-free water, before use.

[0064] The active agents also can be formulated in rectal compositions, such as suppositories or retention enemas, *e.g.*, containing conventional suppository bases. In addition to the formulations described previously, the active agents also can be formulated as a depot preparation. Such long-acting formulations can be administered by implantation (for example, subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the active agents can be formulated with suitable polymeric or hydrophobic materials (for example, as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

[0065] In particular, the active agents can be administered orally, buccally, or sublingually in the form of tablets containing excipients, such as starch or lactose, or in capsules or ovules, either alone or in admixture with excipients, or in the form of elixirs or suspensions containing flavoring or coloring agents. Such liquid preparations can be prepared with pharmaceutically acceptable additives, such as suspending agents. An active agent also can be injected parenterally, for example, intravenously, intramuscularly, subcutaneously, intrathecally, intracisternally, or intracoronarily. For parenteral administration, the active agent is best used in

the form of a sterile aqueous solution which can contain other substances, for example, salts, or monosaccharides, such as mannitol or glucose, to make the solution isotonic with blood.

[0066] For veterinary use, the active agents are administered as a suitably acceptable formulation in accordance with normal veterinary practice. The veterinarian can readily determine the dosing regimen and route of administration that is most appropriate for a particular animal.

EXAMPLES

[0067] The following examples investigated the effect of hemorrhagic shock and resuscitation with centhaquin on endothelin (ET_A and ET_B) receptors in various tissues along with concentration of plasma ET-1 and inflammatory makers in a rat model of hemorrhagic shock.

[0068] Endothelin-1 (ET-1) acts on ET_A and ET_B receptors and has been implicated in hemorrhagic shock (shock). The effect of shock and resuscitation on ET_A and ET_B receptor expression was studied utilizing hypertonic saline (saline) or centhaquin. Rats were anesthetized, a pressure catheter was placed in the left femoral artery; blood was withdrawn from the right femoral artery to bring mean arterial pressure (MAP) to 35 mmHg for 30 minutes, resuscitation was performed and 90 minutes later sacrificed to collect samples for biochemical estimations. Resuscitation with centhaquin decreased blood lactate and increased MAP. Protein levels of ET_A or ET_B receptor were unaltered in the brain, heart, lung and liver following shock or resuscitation. In the abdominal aorta, shock produced an increase (140%) in ET_A expression which was attenuated by saline and centhaquin; ET_B expression was unaltered following shock but was increased (79%) by centhaquin. In renal medulla, ET_A expression was unaltered following shock, but was decreased (-61%) by centhaquin; shock produced a decrease (-34%) in ET_B expression which was completely attenuated by centhaquin and not saline. Shock induced changes in ET_A and ET_B receptors in the aorta and renal medulla are reversed by centhaquin and may be contributing to its efficacy.

Example 1

Methods

[0069] **Animals**. Male Sprague-Dawley rats (340 to 380 g) (Envigo, Indianapolis, IN) were housed for at least 4 days in a room with controlled temperature (23 ± 1 ºC), humidity (50 ± 10%) and light (6:00 A.M. to 6:00 P.M.) before being used. Food and water were made available continuously. Animal care and use for experimental procedures were approved by the Institutional Animal Care and Use Committee of the Midwestern University. All anesthetic and

surgical procedures were in compliance with the guidelines established by the Animal Care Committee.

[0070] Drugs and Chemicals. Centhaquin citrate (PMZ-2010) was synthesized at Pharmazz India Private Limited, Greater Noida, India. Urethane (ethyl carbamate) (Sigma-Aldrich St Louis, MO, USA), Hypertonic Saline Injection, USP (Hospira, Inc, Lake forest IL, USA) and Heparin Sodium Injection, USP (APP Pharmaceuticals, LLC, Schaumburg, IL, USA) were used. Endothelin-1 Enzyme Immunometric Assay Kit (Catalog No. 900-020A, Assay Designs, Inc., Ann Arbor, MI, USA), IL-6 ELISA kit (Catalog No. KRC0061, Invitrogen Corporation, Carlsbad, CA), IL-10 ELISA kit (Catalog No. KRC0101, Invitrogen Corporation, Carlsbad, CA) and TNFα ELISA kit (Catalog No. ER3TNFA, Thermo Scientific, Rockford, IL) were used for various estimations.

[0071] Determination of cardiovascular response. The animals were anesthetized with urethane dissolved in isotonic saline. Urethane was administered in a dose of 1.5 g per kg body weight via intraperitoneal injection. Urethane was selected as an anesthetic agent, because it produces long lasting (8-10 hours) anesthesia with minimal cardiovascular and respiratory system depression. It produces a level of surgical anesthesia characterized by preservation of cardiovascular reflexes (Maggi and Meli 1986). Briefly, anaesthetized rats were immobilized on a surgical board equipped with controlled heating pad. Blood Po_2, Pco_2 and pH, were maintained using a tracheotomy cannula connected to a rodent ventilator (Model 683, Harvard Apparatus Inc., Holliston, MA). The right carotid artery was exposed to measure the left ventricular performance. Surgical suture (Deknatel, Research Triangle Park, NC) was secured around the proximal end of the carotid artery and an ultra-miniature pressure-volume (P-V) catheter SPR-869 (Millar Instruments, Houston, TX) was inserted through a tiny incision made near the proximal end of the artery. The P-V terminal of the catheter was connected to MPVS-300 P-V unit through PEC-4D and CEC-4B cables and advanced into the left ventricle to obtain the P-V signals. The signals were continuously aquired (1000 S^{-1}) using the MPVS-300 P-V unit (AD Instruments, Mountain View, CA, USA) and PowerLab 16/30 data acquisition system (AD Instruments). MAP and HR were measured by cannulating the left femoral artery with pressure catheter SPR-320 (Millar Instruments), connected to the ML221 bridge amplifier (AD Instruments) through AEC-10C connector and the signals were acquired (1000 S^{-1}) using PowerLab 16/30 data acquisition system (Gulati *et al.* 2012; Pacher *et al.* 2008). The left femoral vein was cannulated using PE 50 tubing (Clay Adams, Parsipanny, NJ) and secured for resuscitation.

[0072] Determination of arterial blood gases and base deficit. Baseline arterial blood pH, Po_2, Pco_2, Na^+, K^+ and lactate were monitored prior to induction of shock, 30 minutes after induction of shock, and 30 and 60 minutes following vehicle or centhaquin resuscitation. Blood samples (0.15 ml) were drawn from the arterial cannula using blood gas sampling syringes (Innovative Medical Technologies, Inc. Leawood, KS) and analyzed using a pHOx Ultra analyzer (Nova Biomedical Corporation, Waltham, MA). The base deficit was calculated using the formula (Davis *et al.* 1998; Paladino *et al.* 2008):

$SBD=0.9287 \times [HCO_3^- - 24.4 + 14.83 \times (pH-7.4)]$

[0073] Induction of Hemorrhagic Shock. Hemorrhage was induced by withdrawing blood from the femoral artery at a rate of approximately 0.5 to 1 mL/min until a MAP of 35 mmHg was reached. This MAP was maintained for 30 min by further withdrawal of blood, if necessary. The hemorrhagic shock model used in the present study is a well-established rodent model of manageable pressure hemorrhage (Buehler *et al.* 2000; Gulati *et al.* 1997a; Gulati and Sen 1998). The volume of blood loss was about approximately 8.0 ml in each rat and was similar in various groups, amounting to approximately 40 % of the total blood. Measured hematocrit levels were similar in various groups. The duration of blood withdrawal was approximately 15 minutes.

[0074] Experimental design. To determine the resuscitative effect of centhaquin on cardiovascular system and plasma cytokines in hemorrhagic shock, rats were randomly divided into five groups, *Group 1*: Sham control (Non-hemorrhaged) (n=5), *Group 2*: Hemorrhage with no resuscitation (n=5); *Group 3*: Hemorrhage followed by resuscitation with 3% hypertonic saline (vehicle) (n=5); *Group 4*: Hemorrhage followed by resuscitation with vehicle plus centhaquin (0.017 mg/kg) (n=5); and *Group 5*: Hemorrhage followed by resuscitation with vehicle plus centhaquin (0.05 mg/kg) (n=5). Resuscitation was started 30 min after induction of hemorrhagic shock as an intravenous infusion (1 mL/min) through femoral vein using an infusion pump (Harvard Apparatus Infusion/Withdrawal Pump, Millis, MA). The blood samples, for biochemical estimations, were collected at 30 minutes of resuscitation and cardiovascular parameters were monitored till 60 minutes after which the animal was sacrificed. The volume of resuscitative solution was kept equal to the volume of blood loss. Although, this does not represent a typical human resuscitation, but this volume was selected to minimize confounding factors and allow a more accurate determination of resuscitative effect of centhaquin.

[0075] Determination of ET-1 level in the blood plasma. In order to analyze the change in plasma ET-1 level after hemorrhage followed by centhaquin resuscitation, blood samples were collected from rats of various groups 30 minutes after resuscitation and were collected into

chilled EDTA tubes (1 mg.ml^{-1} of blood) containing aprotinin (500 KIU.mL^{-1} of blood). The blood samples were centrifuged at 1,600 x g for 15 minutes at 0°C and plasma ET-1 level was estimated using enzyme immunoassay. Briefly, plasma samples and standards were added to wells coated with a monoclonal antibody specific for ET-1. The plate was then washed after 24 hours of incubation and horseradish peroxidase (HRP) labeled monoclonal antibody was then added. After 30 minutes incubation the plate was washed and a solution of 3,3',5,5' tetramethylbenzidine substrate was added which generates a blue color. Hydrochloric acid (1N) was added to stop the substrate reaction and the resulting yellow color was read at 450 nm using DTX 800 Multimode detector and the data was analyzed with Multimode Detection Software (Beckman Coulter, Inc., Harbor Boulevard, Fullerton, CA). The measured optical density is directly proportional to the concentration of ET-1 (Lavhale et al. 2010).

[0076] Estimation of ET_A and ET_B receptor expression. Expression of ET_A and ET_B receptors was determined using the western blotting technique (Briyal *et al.* 2015; Leonard and Gulati 2013) with some modifications. After completion of cardiovascular experiments animals were sacrificed and the organs (brain, heart, liver, lung, kidney and abdominal aorta) were immediately dissected out, flash frozen on dry ice, and stored at -80 °C for further analysis. The tissue was homogenized with 10x (w/v) RIPA lysis buffer (20 mM Tris-HCl pH 7.5, 120 mM NaCl, 1.0% TritonX-100, 1.0% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 10% glycerol, 1 mM disodium ethylene diamine tetraacetic acid (EDTA), 1 mM ethylene glycol-bis(β–aminoethyl ether)-N,N,N',N'-tetraacetic acid tetrasodium salt (EGTA), phosphatase inhibitors and Complete Mini Protease inhibitor cocktail tablet (Roche Diagnostics, Indianapolis, IN). Proteins were isolated in solubilized form and concentrations were measured by Folin-Ciocalteu's phenol reagent (Lowry *et al.* 1951). Solubilized protein (60 µg) was denatured in Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA), resolved on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto the nitrocellulose membrane followed by blocking the membrane with SuperBlock® Blocking Buffer in tris-buffered saline (TBS) (ThermoFisher Scientific, Hanover Park, IL). The membranes were washed three times with 1x TBS-Tween (TBST) and incubated with rabbit polyclonal anti-ET_A receptor (ab85163, Abcam, Cambridge, MA, 1:1000) or anti-ET_B receptor (ab117529, Abcam, Cambridge, MA, 1:1000) or mouse monoclonal anti-β-actin (a1978, Sigma-Aldrich, St. Louis, MO) antibodies, followed by horseradish peroxidase (HRP)-conjugated secondary antibodies goat anti-rabbit (sc2004, Santa Cruz Biotechnology, Dallas, TX, 1:2000) or goat anti-mouse (ab98693, Abcam, Cambridge, MA, 1:10,000) and visualized by SuperSignal® West Pico Chemiluminescent Substrate (ThermoFisher Scientific, Hanover Park, IL) using the

ChemiDoc™ MP Imaging System (Bio-Rad Laboratories, Hercules, CA) and then analyzed using ImageJ (NIH) software.

[0077] Determination of IL-6, IL-10 and TNF-α levels in the blood plasma. Plasma levels of IL-6, IL-10 and TNF-α were estimated using commercially available rat enzyme-linked immunosorbent assay kits: IL-6 (Invitrogen Corporation, with a lower detection limit of 5 pg/ml; highly specific for rat IL-6 with no cross-reactivity with other cytokines), IL-10 (Invitrogen Corporation, with a lower detection limit of 5 pg/ml; highly specific for rat IL-10 with no cross-reactivity with other cytokines) and TNF-α ELISA kit (Thermo Scientific, with a lower detection limit of 15 pg/ml; highly specific for rat TNF-α with no cross-reactivity with other cytokines) were used for various estimations. All assays were performed using plasma samples that have not been thawed previously according to the protocols provided by the manufacturers.

[0078] Statistical Analysis. A Power Analysis was conducted using GraphPad Instat-2.00. The power was set to 80% (beta = 0.8) and the level of significance (alpha) used was 0.05. Power Analysis indicated that a sample size of 5 for cardiovascular and 4 for biochemical estimation per group was sufficient to achieve a power of 80%, when level of significance alpha=0.05. Data are presented as mean ± S.E.M. The significance of differences was estimated by one-way analysis of variance followed by a post hoc test (Bonferroni's method). A P value of less than 0.05 was considered to be significant. The statistical analysis was processed with GraphPad Prism 7.00 (GraphPad, San Diego, CA, USA).

Results

[0079] Effect of centhaquin on arterial blood pH, pO_2, pCO_2, hematocrit, blood lactate and base-deficit of hemorrhaged rats. A significant reduction in blood pH was observed in rats following hemorrhage, which was further decreased following administration of hypertonic saline. Centhaquin administration (0.017 and 0.05 mg/kg) significantly prevented the reduction of pH in hemorrhaged rats. Hemorrhage produced a significant decrease in pCO_2 and increase in pO_2 which was not affected by resuscitation with hypertonic saline or centhaquin (Table 1).

[0080] There was no change in percent hematocrit in control rats throughout the experimental period, while hematocrit lowered significantly ($p<0.001$) after hemorrhage. Hemorrhaged rats, when resuscitated with hypertonic saline or with 0.017 and 0.05 mg/kg doses of centhaquin showed no change in hematocrit after treatment.

[0081] There was no change in blood lactate levels in control rats throughout the experimental period, while lactate levels were significantly increased ($p<0.001$) following hemorrhage. Hemorrhaged rats, when resuscitated with 0.017 and 0.05 mg/kg doses of

centhaquin showed a significant decrease ($p<0.001$) in blood lactate levels compared to the hypertonic saline group (Table 1). There was no change in base deficit of control rats during the experimental period. Base deficit significantly ($p<0.001$) increased after induction of hemorrhage, which was not affected by resuscitation with hypertonic saline. Rats resuscitated with 0.017 and 0.05 mg/kg doses of centhaquin, on the other hand, showed a significant decrease ($p<0.001$) in base deficit (-12.0 ± 0.2 and -11.6 ± 0.5, respectively) compared to hypertonic saline (-16.3 ± 1.3) (Table 1). Hemorrhaged rats that were not resuscitated could not survive till 60 min and hence no data could be obtained at that time point.

Table 1. Effect of centhaquin on hematocrit, arterial blood pH, pCO_2, pO_2, lactate and base deficit levels in hemorrhaged rats. The values are expressed as mean ± S.E.M. $^{*}p < 0.05$ compared to baseline; $^{\#}p < 0.05$ compared to hemorrhage; $^{\Delta}p < 0.05$ compared to vehicle treated group.

	Time	Sham Control	Hemorrhage (No resuscitation)	Hemorrhage (3% saline)	Hemorrhage (centhaquin; 0.017 mg/kg)	Hemorrhage (centhaquin; 0.05 mg/kg)
Hematocrit (%)	Baseline	36.0 ± 2.4	38.3 ± 0.8	38.3 ± 0.8	36.8 ± 1.3	39.3 ± 1.6
	H. Shock	38.3 ± 0.8	24.7 ± 2.4*	22.2 ± 1.5*	24.8 ± 1.5*	22.2 ± 0.7*
	60 min	36.5 ± 1.5		19.5 ± 1.8	21.5 ± 1.8	21.3 ± 1.3
pH	Baseline	7.40 ± 0.01	7.36 ± 0.01	7.37 ± 0.01	7.38 ± 0.01	7.38 ± 0.01
	H. Shock	7.39 ± 0.01	7.22 ± 0.02*	7.17 ± 0.03*	7.23 ± 0.02*	7.25 ± 0.03*
	60 min	7.38 ± 0.01		7.13 ± 0.02	7.24 ± 0.02 $^{\Delta}$	7.28 ± 0.01 $^{\Delta}$
pCO_2 (mmHg)	Baseline	32.5 ± 2.1	33.6 ± 1.1	33.3 ± 1.2	33.8 ± 1.2	34.5 ± 2.1
	H. Shock	29.3 ± 2.1	15.8 ± 0.7*	15.9 ± 1.1*	15.9 ± 0.9*	16.3 ± 1.5*
	60 min	30.8 ± 2.2		27.8 ± 2.8$^{\#}$	26.0 ± 1.6$^{\#}$	27.3 ± 1.9$^{\#}$
pO_2 (mmHg)	Baseline	122.2 ± 4.3	125.9 ± 5.7	120.3 ± 1.2	125.5 ± 1.9	120.2 ± 1.2
	H. Shock	121.7 ± 3.9	142.6 ± 1.3*	140.3 ± 5.6*	145.6 ± 1.9*	142.8 ± 1.8*
	60 min	112.3 ±3.9		128.3 ± 4.8	131.3 ± 5.4$^{\#}$	121.3 ± 4.6$^{\#}$
Lactate (mmol/L)	Baseline	1.9 ± 0.2	1.8 ± 0.1	1.9 ± 0.1	1.5 ± 0.2	1.8 ± 0.1
	H. Shock	1.7 ± 0.1	7.4 ± 0.2*	7.3 ± 0.2*	7.6 ± 0.3*	7.4 ± 0.4*
	60 min	1.2 ± 0.1		3.7 ± 0.2$^{\#}$	1.9 ± 0.1$^{\#\Delta}$	1.7 ± 0.2$^{\#\Delta}$
Base-deficit (mEq/L)	Baseline	-2.7 ± 0.4	-2.4 ± 0.3	-2.4 ± 0.4	-2.9 ± 0.3	-2.8 ± 0.3
	H. Shock	-2.9 ± 0.5	-15.7 ± 1.5*	-16.1 ± 0.6*	-15.3 ± 0.4*	-15.4 ± 0.9*
	60 min	-3.9 ± 0.4		-16.3 ± 1.3	-12.0 ± 0.2$^{\#\Delta}$	-11.6 ± 0.5$^{\#\Delta}$
ET-1	60 min	13.6 ± 0.9	21.8 ± 0.9*	25.3 ± 1.35*	37.9 ± 3.0$^{*\#\Delta}$	38.3 ± 2.7$^{*\#\Delta}$

[0082] Effect of centhaquin on mean arterial pressure and heart rate of hemorrhaged rats. Control rats did not show any change in MAP during the experimental period. MAP

significantly decreased ($p<0.001$) in all the treatment groups after induction of hemorrhage. Hemorrhaged rats, resuscitated with hypertonic saline, did not show any improvement in MAP at either 30 or 60 minutes post resuscitation. Rats resuscitated with centhaquin (0.017 and 0.05 mg/kg) showed a significant increase ($p<0.01$) in MAP for at least 60 min post resuscitation (Figure 1). Prior to hemorrhage, the baseline HR was approximately 345 beats/min in all groups. Hemorrhage produced a slight increase in HR (approximately 372 beats/min). In rats resuscitated with hypertonic saline HR dropped to 353 ± 11 beats/min at 60 minutes, while in rats resuscitated with centhaquin in the doses of 0.017 and 0.05 mg/kg HR was 361 ± 10 and 363 ± 10, respectively (Figure 1). No significant difference was observed in HR following resuscitation with hypertonic saline or centhaquin.

[0083] Effect of centhaquin on cardiac output and systemic vascular resistance of hemorrhaged rats. CO significantly decreased following hemorrhage in all the groups. Hemorrhaged rats resuscitated with hypertonic saline and centhaquin both produced a significant increase in CO (Figure 1). Centhaquin resuscitation significantly increased CO at 30 and 60 min post resuscitation as compared to hypertonic saline alone. SVR decreased from 148 ± 5 to 77 ± 3 dyne*sec/cm^5 following hemorrhage, and it further decreased at 30 and 60 minutes of resuscitation with both hypertonic saline or centhaquin treatments (Figure 1).

[0084] Effect of centhaquin on plasma ET-1 level of hemorrhaged rats. The baseline plasma ET-1 levels were 13.6 ± 0.87 pg.ml^{-1}. After hemorrhage, ET-1 levels were significantly increased to 21.8 ± 0.87 pg.ml^{-1} ($p<0.001$). In rats treated with hypertonic saline, ET-1 levels were 25.3 ± 1.35 pg.ml^{-1}, with no significant change compared to the untreated hemorrhagic shock group. However, in rats treated with centhaquin (0.017 and 0.05 mg/kg), the ET-1 levels were significantly increased (37.9 ± 3.03 and 38.3 ± 2.7 pg.ml^{-1}, respectively) compared to hemorrhaged rats resuscitated with hypertonic saline.

[0085] Effect of centhaquin on the expression of ET_A receptors in hemorrhaged rats. There was no change in the expression of ET_A receptors in the brain, heart, liver, lungs and kidney cortex (Figure 2). A significant ($p<0.0001$) increase in the expression of ET_A receptors was observed following hemorrhagic shock in the abdominal aorta. The expression of ET_A receptors in the abdominal aorta of hemorrhaged rats increased by 140% compared to sham group. In hemorrhaged rats treated with hypertonic saline and hypertonic saline + centhaquin (0.017 and 0.05 mg/kg), a significant decrease (-48.8, -47.6 and -49.2 %, respectively) in ET_A expression was observed in the abdominal aorta compared to hemorrhaged rats with no treatment (Figure 2). No change in ET_A expression was observed in the renal medulla following hemorrhagic shock in rats. However, rats treated with centhaquin (0.017 and 0.05 mg/kg)

presented with a significant decrease (-61.3 % and -70.5 %, respectively) in the expression of ET_A receptors compared to hemorrhagic shock (Figure 2).

[0086] Effect of centhaquin on the expression of ET_B receptors in hemorrhaged rats. There was no change in the expression of ET_B receptors in brain, heart, liver, lungs and kidney cortex (Figure 3). No change in ET_B expression was observed in the abdominal aorta following hemorrhagic shock in rats. However, the expression of ET_B receptors in abdominal aorta of rats treated with centhaquin (0.017 and 0.05 mg/kg) significantly increased (79.7 % and 57.4 %, respectively) compared untreated hemorrhaged rats (Figure 3). A significant ($p<0.0001$) decrease (-34 %) in the expression of ET_B receptors was observed following hemorrhagic shock in kidney medulla compared to the sham group. In hemorrhaged rats treated with centhaquin (0.017 and 0.05 mg/kg), a significant increase (76.6 % and 69.4 %, respectively) in ET_B expression was observed in the kidney medulla compared to untreated hemorrhaged rats (Figure 3).

[0087] Effect of centhaquin on plasma IL-6, IL-10 and TNF-α levels of hemorrhaged rats. To further evaluate whether centhaquin treatment affected the inflammatory response, we measured a select panel of cytokines in rat plasma. Overall, the levels of plasma IL-6, IL-10 and TNF-α were increased in all hemorrhaged rats with or without resuscitation with hypertonic saline and centhaquin. TNF-α and IL-6 levels were higher after hemorrhagic shock and resuscitation with hypertonic saline compared with sham control. Centhaquin further increased ($p<0.01$) the levels of TNF-α and IL-6 as compared to hypertonic saline alone. There was no statistically significant difference in plasma IL-10 between rats after hemorrhagic shock and resuscitation with hypertonic saline or centhaquin (Figure 4).

Discussion

[0088] Centhaquin significantly decreased blood lactate and restored MAP and enhanced the resuscitative effect of hypertonic saline, confirming previous findings (Gulati *et al.* 2012; Gulati *et al.* 2013; Lavhale *et al.* 2013; Papapanagiotou *et al.* 2016). The effect of hemorrhagic shock and resuscitation using hypertonic saline alone or with centhaquin on ET_A and ET_B receptors expression in different tissues, plasma ET-1 levels and inflammatory markers were determined. It was found that ET_A and ET_B receptors in the abdominal aorta and renal medulla are involved in its resuscitative action. No change in ET_A or ET_B receptor levels were observed in the brain, heart, lung and liver following hemorrhagic shock or resuscitation with either hypertonic saline or centhaquin.

[0089] Vascular ET_A receptors have been well established to have a strong vasoconstrictor effect (Schneider *et al.* 2007). Hemorrhage produced an increase in the expression of ET_A receptors in the abdominal aorta. Resuscitation with hypertonic saline and centhaquin significantly reversed the hemorrhage-induced increase in ET_A receptor expression in the abdominal aorta. However, ET_B receptors were unaltered following hemorrhagic shock, but were increased by centhaquin treatment. It is possible that following hemorrhagic shock an increase in the expression of vasoconstrictor ET_A receptors in the blood vessels occurs to maintain vascular tone and MAP. However, an increase in circulating ET-1 along with increased vascular ET_A receptors may produce undesired vasoconstriction and reduce tissue perfusion.

[0090] On the other hand, an increase in plasma ET-1 levels following hemorrhagic shock has been reported to be acting as a compensatory mechanism to maintain blood pressure (Chang *et al.* 1993; Edwards *et al.* 1994; Gulati *et al.* 1997b; Sharma *et al.* 2002). It was also found that a precursor of ET-1 improved the resuscitative effect of hemoglobin based blood-substitute diaspirin cross-linked hemoglobin in severely hemorrhaged rats (Gulati *et al.* 1995). In normal rats ET-1 produces a biphasic response: an initial transient decrease followed by a sustained increase in blood pressure (Gardiner *et al.* 1994; Yanagisawa *et al.* 1988), however, in hemorrhaged rats, ET-1 produced a monophasic effect where only an increase in blood pressure was observed along with improved survival (Jochem *et al.* 2003). The resuscitative effect of ET-1 in hemorrhaged rats was mediated through ET_A receptors since it was blocked by BQ123, a specific ET_A receptor antagonist (Jochem *et al.* 2003). Without wishing to be bound by theory, vascular ET_A receptors are increased following hemorrhagic shock as part of compensatory mechanism which is reversed upon resuscitation with either hypertonic saline or centhaquin. ET_B receptors in the abdominal aorta were unaltered following hemorrhagic shock, but increased by centhaquin and not by hypertonic saline resuscitation. Since vascular ET_B receptors produce vasodilation (Arai *et al.* 1990; Cardillo *et al.* 2000; Yanagisawa *et al.* 1988) therefore centhaquin induced increase in the expression of vascular ET_B receptors may contribute towards an increase in tissue blood perfusion thereby decreasing blood lactate levels of hemorrhaged rats.

[0091] ET receptors crosstalk with each other and with adrenergic. The effect of ET_B receptor desensitization is revealed in the presence of ET_A receptor blockade (Mickley *et al.* 1997). On the other hand, ET_B receptors are capable of altering the pharmacology of ET_A receptors. It has been shown that venous ET_A receptor blockade inhibited ET-1 induced contraction to a larger degree when ET_B receptors were blocked (Thakali *et al.* 2008). Crosstalk

between ET_A and ET_B receptors has been shown to take place in several different blood vessels in rodents (Lodge *et al.* 1995; Thakali *et al.* 2008) and all of these vessels possess contractile ET_A and ET_B receptors and suggest that pharmacological ET_A and ET_B receptor interaction require the presence of contractile ET_B receptors (Thakali *et al.* 2008). It has been shown that ET_A receptors modulate the cardiovascular responses of adrenergic agent such as clonidine (Gulati 1992; Gulati and Srimal 1993; Lavhale *et al.* 2013). Since centhaquin acts on adrenergic receptors, it is possible that changes in expression of ET_A receptors is responsible for some of the resuscitative effects of centhaquin.

[0092] It is demonstrated herein that in the renal medulla, ET_A receptor levels were unaltered following hemorrhagic shock, but were decreased by centhaquin, whereas ET_B receptor expression decreased following hemorrhagic shock, which was completely attenuated by centhaquin and not with hypertonic saline. In the kidney, ET-1 produces vasoconstriction and decreases glomerular filtration rate which is mediated through ET_A receptors (Harris *et al.* 1991; Kon *et al.* 1989). A decrease in the expression of ET_A receptors induced by centhaquin could reduce the vasoconstrictor effect of ET-1 in the renal medulla. The outer renal medulla is the site where extensive reabsorption of sodium chloride takes place by the thick ascending limb of loop of Henle making outer renal medulla a site for high metabolic activity and demand for better blood perfusion (Cowley 2008). Hence this region is highly prone to hypoxic or ischemic injury following excessive hemorrhage. It is possible that centhaquin, by decreasing the concentration of ET_A receptors, prevents the renal medullary region from ischemic injury following hemorrhagic shock. On the other hand, ET_B receptor stimulation has been found to increase renal medullary blood flow mediated through vasodilators such as NO, cyclo-oxygenase and cytochrome p-450 metabolites (Hercule and Oyekan 2000; Vassileva *et al.* 2003). As shown herein, hemorrhagic shock decreased renal medullary ET_B receptor expression which was not affected by resuscitation with saline but was attenuated by centhaquin. The results provided herein show that severe hemorrhage produces a decrease in the expression of ET_B receptors in the renal medulla which may contribute towards a decrease in blood flow to the renal medulla causing ischemia and renal failure. Since resuscitation with centhaquin did not produce any decrease in renal medullary ET_B receptor expression, it is possible that hemorrhage induced renal medullary ischemic effects could be attenuated by centhaquin. Therefore, centhaquin induced changes in ET_A and ET_B receptors both may be contributing to prevent the renal medulla from ischemic injury following hemorrhagic shock.

[0093] The renal medullary ET_B receptors also play a role in the control of sodium and water excretion (Kohan *et al.* 2011; Schneider *et al.* 2007). ET_B receptors in the epithelium of the

renal medullary collecting ducts, are mainly responsible for inhibition of ET-1 action on sodium and water reabsorption (Kitamura *et al.* 1989; Kohan *et al.* 2011). The diuretic and natriuretic response to ET-1 was found to be attenuated by an ET_B receptor antagonist (Hoffman *et al.* 2000). Patients with excessive blood loss presenting with anuria or oliguria warrant emergency medical attention because acute kidney failure is the main cause of death in such patients (Rossaint *et al.* 2006). It is possible that a decrease in the expression of renal medullary ET_B receptors may contribute towards oliguria which could be attenuated by centhaquin. It may be speculated that centhaquin could be a novel pharmacological intervention to reduce renal injury mediated by hemorrhagic shock. These findings are preliminary and only suggestive, they need to be extensively investigated in animal studies. Studies are needed to investigate the effect of centhaquin on renal blood flow and whether those changes can be antagonized by specific ET_A and ET_B receptor antagonists.

[0094] In the present study it was found that hemorrhage increased the plasma concentration of ET-1, TNF-α, IL-6 and IL-10. Resuscitation with hypertonic saline did not alter plasma ET-1, TNF-α, IL-6 or IL-10; however, centhaquin significantly increased plasma ET-1, TNF-α and IL-6 without affecting plasma IL-10 concentration. In a study conducted in mongrel dogs an increase in plasma ET-1 levels was observed following hemorrhage which co-related with the amount of blood loss (Chang *et al.* 1993). ET-1 increases superoxide anion production and cytokine secretion (Kowalczyk *et al.* 2015; Virdis and Schiffrin 2003), along with activation of transcription factors such as NF-κB and expression of pro-inflammatory cytokines such as TNF-α, IL-1, and IL-6. Cytokines, reciprocally, have been shown to modulate the secretion of ET-1 (Breuiller-Fouche *et al.* 2005; Yeager *et al.* 2012).

[0095] Hemorrhagic shock compromises the metabolic, cellular and inflammatory responses which can lead to multiple organ failure (Bonanno 2011; Gutierrez *et al.* 2004; Marik and Flemmer 2012). The response is typically characterized by release of pro-inflammatory cytokines such as IL-6 or TNF-α appearing immediately following hemorrhagic shock (Mees *et al.* 2009). This is followed by a sustained release of anti-inflammatory cytokines such as IL-10 which may contribute towards immune depression (Oberholzer *et al.* 2000). The overall impact of excessive IL-6 and TNF-α production in hemorrhage is still controversial. The present findings confirm increases in plasma levels of TNF-α, IL-6 and IL-10 after hemorrhage. Resuscitation with hypertonic saline did not alter plasma TNF-α, IL-6 or IL-10; however, centhaquin significantly increased plasma TNF-α and IL-6 without affecting plasma IL-10 concentration. Previous investigations have shown that an increased expression of ET_B receptors may correlate with an increase in certain pro-inflammatory cytokines (Breuiller-Fouche

et al. 2005; Pernow *et al.* 2000; White *et al.* 2000). It is demonstrated herein that centhaquin resuscitation increased ET_B receptor expression in the abdominal aorta and renal medulla along with elevating plasma TNF-α and IL-6 concentration. Studies have suggested that a robust early TNF-α response is associated with survival in trauma victims and early elevation of plasma TNF-α serves either to limit organ damage or to induce reparative processes (Namas *et al.* 2009). Cytokine IL-6 seems to play a significant role in the systemic response to inflammation. Although several studies have shown beneficial effect of blockage of IL-6 in arthritis (Peake *et al.* 2006), multiple myeloma (Gado *et al.* 2000) and Crohn's disease (Atreya *et al.* 2000), inhibition of IL-6 has not been found to be beneficial in hemorrhagic shock (Mees *et al.* 2009). IL-6 plays a dual role in the inflammatory response to injury, often classified as pro-inflammatory locally and anti-inflammatory systemically. Studies have shown the beneficial effects of IL-6 deficiency in experimental paradigms of thermal injury, sepsis, and hemorrhage (Fontanilla *et al.* 2000; Mommsen *et al.* 2011; Yang *et al.* 2007). In contrast, other studies demonstrate that IL-6 administration prevents epithelial cell and cardio-myocyte apoptosis induced by hemorrhage (Alten *et al.* 2008; Moran *et al.* 2009). Systemic infusion of IL-6 following hemorrhagic shock reduces inflammation and injury in the liver and lung (Meng *et al.* 2000). Studies have also shown the beneficial effects of TNF-α. Mice lacking TNF receptors have larger infarcts in ischemic brain injury (Bruce *et al.* 1996). TNF-α release in the hippocampus may promote neuroprotection and activate repair processes of the cerebral microvasculature as well as mediate neuronal plasticity (Kim *et al.* 2014; Sriram and O'Callaghan 2007). Several experimental studies suggest that both cytokines display protective actions in the brain (Bruce *et al.* 1996; Gadient *et al.* 1990; Hama *et al.* 1989; Kossmann *et al.* 1996). While centhaquin did increase IL-6 and TNF-α in the present study, more markers for inflammation are being examined in order to fully understand the influence of centhaquin on inflammation, both in normal and hemorrhaged animals, as these cytokines have the capacity to perform both pro- and anti-inflammatory functions.

[0096] Endothelin-1 (ET-1) acts on ET_A and ET_B receptors and has been implicated in hemorrhagic shock (shock). The effect of shock and resuscitation on ET_A and ET_B receptor expression was studied herein utilizing hypertonic saline (saline) or centhaquin. Rats were anesthetized, a pressure catheter was placed in the left femoral artery; blood was withdrawn from the right femoral artery to bring mean arterial pressure (MAP) to 35 mmHg for 30 minutes, resuscitation was performed and 90 minutes later sacrificed to collect samples for biochemical estimations. Resuscitation with centhaquin decreased blood lactate and increased MAP. Protein levels of ET_A or ET_B receptor were unaltered in the brain, heart, lung and liver following

shock or resuscitation. In the abdominal aorta, shock produced an increase (140%) in ET_A expression which was attenuated by saline and centhaquin; ET_B expression was unaltered following shock but was increased (79%) by centhaquin. In renal medulla, ET_A expression was unaltered following shock, but was decreased (-61%) by centhaquin; shock produced a decrease (-34%) in ET_B expression which was completely attenuated by centhaquin and not saline. Shock induced changes in ET_A and ET_B receptors in the aorta and renal medulla are reversed by centhaquin and may be contributing to its efficacy.

[0097] In summary, centhaquin significantly improved resuscitation following hemorrhagic shock (HS) in rats. The administration of centhaquin following HS resulted in a decrease in the expression of vasoconstrictor ET_A receptor and an increase in the expression of vasodilator ET_B receptors, the mechanism for these alterations remains to be determined. Similarly, the significance of elevation in cytokines following hemorrhagic shock and resuscitation with centhaquin is contemplated that these changes improve tissue blood perfusion.

[0098] Centhaquin has been shown to have significant resuscitative effect following extensive hemorrhage in rat, rabbits and swine models. Hemorrhage decreases the expression of ET_B receptors and it is contemplated that resuscitation with centhaquin attenuates this effect through the increased expression of ET_B receptors. Specifically, centhaquin-induced increase in ET_B receptor expression in the renal medulla could lead to a vasodilatory effect and promote diuresis and natriuresis, preventing injury to the renal medulla.

Example 2

[0099] The following example establishes that low doses of centhaquin (2-[2-(4-(3-methyphenyl)-1-piperazinyl)]ethyl-quinoline) citrate, significantly decreased blood lactate, and increased mean arterial pressure (MAP), pulse pressure (PP) and cardiac output (CO) in hemorrhagic shock (Gulati et al. 2012; Gulati et al. 2013; Lavhale et al. 2013; Papapanagiotou et al. 2016). Comparative studies were performed between centhaquin and status quo resuscitative agents grouped into 3 different categories: (a) fluids such as Lactated Ringer's, hypertonic saline; (b) adrenergic agents such as norepinephrine, and (c) fresh blood. Our results using (i) a rat model of fixed pressure blood loss, (ii) rabbit model of uncontrolled bleeding with trauma, and (iii) a pig model of massive blood loss indicate that centhaquin is highly effective in reducing the mortality following hypovolemic shock (Gulati et al. 2012; Gulati et al. 2013; Lavhale et al. 2013; Papapanagiotou et al. 2016). Unlike other resuscitative agents (vasopressors), centhaquin increased MAP by increasing stroke volume (SV) and CO; and

decreased heart rate and systemic vascular resistance (SVR). Centhaquin is currently in clinical development as a resuscitative agent for hemorrhagic shock.

[0100] Hemorrhagic shock is a major cause of morbidity and mortality following trauma, particularly during the early stages of injury (Wu *et al.* 2009). Most of the deaths due to hemorrhagic shock occur in the first 6 hours after trauma (Shackford *et al.* 1993) and many of these deaths can be prevented (Acosta *et al.* 1998). Shock is accompanied by circulatory failure, which is mainly responsible for mortality and morbidity. The current recommended fluid therapy of using large volumes of lactated Ringer (LR) solution is effective in restoring hemodynamic parameters but presents logistic and physiological limitations (Vincenzi *et al.* 2009). Resuscitation with a large volume of crystalloids has been associated with secondary abdominal compartment syndrome, pulmonary edema, cardiac dysfunction, and paralytic ileus (Balogh *et al.* 2003). A secondary sequelae of circulatory failure in hemorrhagic shock is renal failure, which may be exacerbated by large volume fluid resuscitation. The incidence of acute kidney injury and renal failure following hemorrhagic shock is extremely high, with many requiring renal replacement therapy. The pathophysiology of renal failure in hemorrhagic shock is a result of splanchnic and renal vasoconstriction that directs blood flow to the heart and brain but may lead to ischemic injury of the kidney. Injury of tubular cells is most prominent in the straight portion of the proximal tubules and in the thick ascending limb of the loop of Henle, especially as it dips into the relatively hypoxic medulla. Changes in the proximal tubular cells are apical blebs and loss of the brush border membrane followed by a loss of polarity and integrity of the tight junctions. On a cellular level, ischemia causes depletion of adenosine triphosphate (ATP), an increase in cytosolic calcium, free radical formation, metabolism of membrane phospholipids, and abnormalities in cell volume regulation.

Methods

[0101] **Animal Studies**. Male Sprague-Dawley rats (340 to 380 g) (Envigo, Indianapolis, IN) were housed for at least 4 days in a room with controlled temperature (23 ± 1 ºC), humidity (50 ± 10%) and light (6:00 A.M. to 6:00 P.M.) before being used. Food and water were made available continuously. Animal care and use for experimental procedures were approved by the Institutional Animal Care and Use Committee of the Midwestern University. All anesthetic and surgical procedures were in compliance with the guidelines established by the Animal Care Committee.

[0102] **Drugs and Chemicals**. Centhaquin citrate (PMZ-2010) was synthesized at Pharmazz India Private Limited, Greater Noida, India. Urethane (ethyl carbamate) (Sigma-Aldrich St Louis,

MO, USA), Saline Injection, USP (Hospira, Inc, Lake forest IL, USA) and Heparin Sodium Injection, USP (APP Pharmaceuticals, LLC, Schaumburg, IL, USA) were used.

[0103] Determination of cardiovascular response. The animals were anesthetized with urethane dissolved in isotonic saline. Urethane was administered in a dose of 1.5 g per kg body weight via intraperitoneal injection. Urethane was selected as an anesthetic agent, because it produces long lasting (8-10 hours) anesthesia with minimal cardiovascular and respiratory system depression. It produces a level of surgical anesthesia characterized by preservation of cardiovascular reflexes (Maggi and Meli 1986). Briefly, anaesthetized rats were immobilized on a surgical board equipped with controlled heating pad. Blood Po_2, Pco_2 and pH, were maintained using a tracheotomy cannula connected to a rodent ventilator (Model 683, Harvard Apparatus Inc., Holliston, MA). MAP and HR were measured by cannulating the left femoral artery with pressure catheter SPR-320 (Millar Instruments), connected to the ML221 bridge amplifier (AD Instruments) through AEC-10C connector and the signals were acquired (1000 S^{-1}) using PowerLab 16/30 data acquisition system (Gulati *et al.* 2012; Pacher *et al.* 2008). A Perimed laser Doppler flow probe was placed in the renal medulla to measure blood perfusion and data was captured on PowerLab 16/30 data acquisition system. The left femoral vein was cannulated using PE 50 tubing (Clay Adams, Parsipanny, NJ) and secured for resuscitation.

[0104] Determination of arterial blood gases and base deficit. Baseline arterial blood pH, Po_2, Pco_2, Na^+, K^+ and lactate were monitored prior to induction of shock, 30 minutes after induction of shock, and 30 and 60 minutes following vehicle or centhaquin resuscitation. Blood samples (0.15 ml) were drawn from the arterial cannula using blood gas sampling syringes (Innovative Medical Technologies, Inc. Leawood, KS) and analyzed using a pHOx Ultra analyzer (Nova Biomedical Corporation, Waltham, MA). The base deficit was calculated using the formula (Davis *et al.* 1998; Paladino *et al.* 2008):

$$SBD=0.9287 \times [HCO_3^- - 24.4 + 14.83 \times (pH-7.4)]$$

[0105] Induction of Hemorrhagic Shock. Hemorrhage was induced by withdrawing blood from the femoral artery at a rate of approximately 0.5 to 1 mL/min until a MAP of 35 mmHg was reached. This MAP was maintained for 30 minutes by further withdrawal of blood, if necessary. The hemorrhagic shock model used in the present study is a well-established rodent model of manageable pressure hemorrhage (Buehler et al. 2000; Gulati et al. 1997; Gulati and Sen 1998). The volume of blood loss was about approximately 8.0 ml in each rat and was similar in various groups, amounting to approximately 40 % of the total blood. Measured hematocrit

levels were similar in various groups. The duration of blood withdrawal was approximately 15 minutes.

[0106] Experimental design. To determine the resuscitative effect of centhaquin on cardiovascular system in hemorrhagic shock, rats were randomly divided into three groups: Hemorrhaged rats were administered 100% shed blood volume of either normal saline (vehicle); 100% shed blood volume of either normal saline plus centhaquin (0.01 mg/kg) or 100% shed blood volume of either normal saline plus centhaquin (0.10 mg/kg) during the first 10 minutes of resuscitation. Additional experiments were also carried out where the amount of norepinephrine (a very commonly used vasopressor during resuscitation) required to maintain MAP at 70 mmHg in normal saline (NS) or centhaquin (0.01 mg/kg) treated rats (volume equal to blood loss) was determined.

[0107] Statistical Analysis. A Power Analysis was conducted using GraphPad Instat-2.00. The power was set to 80% (beta = 0.8) and the level of significance (alpha) used was 0.05. Power Analysis indicated that a sample size of 5 for cardiovascular and 4 for biochemical estimation per group was sufficient to achieve a power of 80%, when level of significance alpha=0.05. Data are presented as mean ± S.E.M. The significance of differences was estimated by one-way analysis of variance followed by a post hoc test (Bonferroni's method). A P value of less than 0.05 was considered to be significant. The statistical analysis was processed with GraphPad Prism 7.00 (GraphPad, San Diego, CA, USA).

Results

[0108] Effect of centhaquin on arterial blood pH, pO_2, pCO_2, hematocrit, blood lactate and base-deficit of hemorrhaged rats. A significant reduction in blood pH was observed in rats following hemorrhage, which was further decreased following administration of saline. Centhaquin administration (0.01 and 0.10 mg/kg) significantly prevented the reduction of pH in hemorrhaged rats. Hemorrhage produced a significant decrease in pCO_2 and increase in pO_2 which was not affected by resuscitation with saline or centhaquin.

[0109] There was no change in percent hematocrit in control rats throughout the experimental period, while hematocrit lowered significantly after hemorrhage. Hemorrhaged rats, when resuscitated with saline or with centhaquin showed no change in hematocrit after treatment.

[0110] There was no change in blood lactate levels in control rats throughout the experimental period, while lactate levels were significantly increased following hemorrhage. Hemorrhaged rats, when resuscitated with centhaquin showed a decrease in blood lactate levels compared to the saline group.

[0111] Effect of centhaquin on mean arterial pressure (MAP) of hemorrhaged rats. Control rats did not show any change in MAP during the experimental period. MAP significantly decreased in all the treatment groups after induction of hemorrhage. Hemorrhaged rats, resuscitated with saline, showed only transient improvement in MAP post resuscitation. Rats resuscitated with centhaquin (0.01 and 0.10 mg/kg) showed a significant increase ($p<0.01$) in MAP post resuscitation.

[0112] Effect of centhaquin on renal blood perfusion of hemorrhaged rats. Hemorrhaged rats were resuscitated with vehicle (saline) or centhaquin low dose (0.01 mg/kg) or high dose (0.1 mg/kg). The effect of centhaquin on renal blood perfusion was measured before the induction of shock, 30 minutes after shock (hemorrhage) and 10, 30, 60, 90 and 120 minutes after resuscitation. Low doses of centhaquin significantly improved renal blood perfusion of hemorrhaged male rats compared to equal volume of saline. This improved renal medullary blood perfusion is indicative of a use of centhaquin to prevent or treat injury to the kidneys (Figure 5). Centhaquin significantly improved renal blood perfusion of hemorrhaged female rats compared to equal volume of saline (Figure 6). A low dose of 0.02 mg/kg of centhaquin was also found to be effective in increasing the renal blood flow of hemorrhaged rats subjected to 20 minutes of renal artery occlusion. A ten times higher dose of 0.2 mg/kg centhaquin did not have the same efficacy in increasing renal blood flow as a low dose of 0.02 mg/kg centhaquin. Hence, a low dose (0.02 mg/kg) of centhaquin was more effective than a high dose (0.2 mg/kg) of centhaquin in improving renal blood flow of hemorrhaged rats subjected to 20 minutes of renal artery occlusion.

[0113] Male rats were anaesthetized with urethane. The femoral vein was cannulated for drug administration, femoral artery was cannulated for measuring mean arterial pressure (MAP) and a laser Doppler flow probe was placed in the renal medulla to measure blood perfusion. Induction of hemorrhagic shock was initiated by withdrawing blood to maintain the MAP at 35 mmHg for 30 minutes. Norepinephrine infusion was carried out to bring and maintain the MAP to 70 mmHg. The effect of centhaquin on cardiovascular functions were measured before the induction of shock, 30 minutes after shock (hemorrhage) and 15, 30, 45, 60, 75 and 90 minutes after resuscitation. Centhaquin improved renal blood perfusion of hemorrhaged male rats compared to vehicle control following resuscitation. The adverse effects of norepinephrine induced vasoconstriction can be attenuated by centhaquin (Figure 7). Similarly, centhaquin improved renal blood perfusion of hemorrhaged female rats compared to vehicle control following resuscitation and an improved blood perfusion was observed till the end of experiment

(Figure 8). The adverse effects of norepinephrine induced vasoconstriction can be attenuated by centhaquin.

Example 3

[0114] A prospective, multi-centric, randomized, double-blind, parallel, saline controlled phase II study of PMZ-2010 (centhaquin) as a resuscitative agent for hypovolemic shock due to excessive blood loss (CTRI/2017/03/008184) is being conducted. All subjects received standard treatment for shock (the type of treatment and care the enrolling institution would provide). Patients were randomly assigned to either control cohort (N=7) that received standard treatment along with normal saline or PMZ-2010 cohort (N=12) that received standard treatment along with PMZ-2010. Interim analysis showed comparable demographics of patients in both cohorts (Table 2).

Table 2. An interim analysis showing comparable demographics of patients in both cohorts of the phase II study.

Patient Demographics (Mean±SEM)						
Group	Gender	Age (Years)	Body Weight (Kg)	Height (Cm)	BMI (Kg/m2)	BSA (m2)
Normal Saline (N=7)	(5M/2F)	37.00±6.41	64.29±5.01	167.86±2.24	22.96±1.24	1.74±0.07
PMZ-2010 (N=12)	(11M/1F)	41.08±3.59	65.42±3.88	166.50±2.57	23.46±0.99	1.73±0.06

[0115] The investigational drug PMZ-2010 met its primary endpoint of safety and no adverse event was reported. The number of doses required in PMZ-2010 treated were less than those required in control cohort (Table 3). Of the total hospital stay, PMZ-2010 treated patients spent only 39.3% time in intensive care unit compared to 57.3% of control. Time spent on ventilator was only 0.85 ± 0.71 days in patients from PMZ-2010 group while it was 5.09 ± 3.14 days in patients from control group. Total fluids needed in the first 48 hours of resuscitation was 19.9% less in PMZ-2010 treated patients; similarly, total blood products administered in the first 48 hours was 23.4% less in PMZ-2010 treated group compared to control. Systolic blood pressure increased by 34.5% from baseline (at the time of inclusion) until 48 hours after resuscitation in the control group while an increase of 45.2% was observed in PMZ-2010 treated group (Figure

9). Similarly, diastolic blood pressure increased by 15.4% in the control group while 34.9% in PMZ-2010 treated group 48 hours after resuscitation (Figure 9). Total amount of vasopressors needed in first 48 hours of resuscitation were 18.4 ± 12.1 mg in the control group, while only 1.3 ± 1.2 mg was needed in PMZ-2010 treated patients. Blood lactate levels decreased by 47% in control and 63% in PMZ-2010 treated patients. Interim analysis of a small number of patients indicated that PMZ-2010 improved numerous parameters that are indicative of its effectiveness as a resuscitative agent.

Table 3. Patients in the phase II study were randomly assigned to either control cohort that received standard treatment along with normal saline or PMZ-2010 cohort that received standard treatment along with PMZ-2010. An interim analysis as per approved protocol showed that number of doses required in PMZ-2010 treated were less than those required in control cohort. Although statistically not significant, there is a trend showing that resuscitation was more effective than standard treatment and required about 25% less doses.

Number of Doses of Study Drug Administered in First 48 hours (Mean±SEM)			
Group	Gender	Total Number of doses administered	Number of doses per patient
Normal Saline (N=7)	(5M/2F)	10 doses in 7 patients	1.43±0.43
PMZ-2010 (N=12)	(11M/1F)	13 doses in 12 patients	1.08±0.08

[0116] Serum creatinine levels were determined when the patient was inducted in the study (baseline) and at the time of discharge from hospital (end of the study). An interim analysis as per approved protocol showed that serum creatinine level decreased by 25.35% in control cohort and by 42.61% in PMZ-2010 treated patients. The data indicated that reduction of serum creatinine levels by PMZ-2010 is 17.26% more compared to standard treatment. See Figure 10.

[0117] Blood urea nitrogen was determined when the patient was inducted in the study (baseline) and at the time of discharge from hospital (end of the study). An interim analysis as per approved protocol showed that blood urea nitrogen was similar in control cohort and PMZ-2010 treated patients. See Figure 11.

[0118] Glomerular filtration rate was determined when the patient was inducted in the study (baseline) and at the time of discharge from hospital (end of the study). An interim analysis as per approved protocol showed that glomerular filtration rate increased by 40.00% in control cohort and by 81.52% in PMZ-2010 treated patients. The data indicated that an increase in glomerular filtration rate by PMZ-2010 is 41.52% more compared to standard treatment. See Figure 12.

Discussion

[0119] The principal feature of acute renal failure is an abrupt decline in glomerular filtration rate (GFR), resulting in the retention of nitrogenous wastes (urea, creatinine). In the general world population 170-200 cases of severe acute renal failure per million population occur annually. To date, there is no specific treatment for acute renal failure. Several drugs have been found to ameliorate toxic and ischemic experimental acute renal failure, as manifested by lower serum creatinine levels, reduced histological damage and faster recovery of renal function in different animal models. These include anti-oxidants, calcium channel blockers, diuretics, vasoactive substances, growth factors, anti-inflammatory agents and more. However, those drugs that have been studied in clinical trials showed no benefit, and their use in acute renal failure has not been approved. The foregoing example demonstrates the use of centhaquin and its salts to prevent and/or treat patients with acute renal failure.

[0120] Centhaquin has been shown to have significant resuscitative effect following extensive hemorrhage in rat, rabbits and swine models. Centhaquin was found to be an effective resuscitative agent and induced an increase in blood flow to the renal medulla and decrease serum creatinine levels in patients with hypovolemic shock. In addition, centhaquin increased glomerular filtration rate in patients with hypovolemic shock. It is contemplated that centhaquin and its salts will lead to a vasodilator effect and promote diuresis and natriuresis, and improve renal functions by increasing the glomerular filtration rate and decrease serum creatinine levels.

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WHAT IS CLAIMED IS:

1. A method of preventing or treating kidney injury or failure by administering a therapeutically effective amount of an endothelin B (ET_B) receptor agonist to an individual in need thereof.

2. The method of claim 1, wherein the ET_B receptor agonist is selected from the group consisting of N-Succinyl-[Glu^9, $Ala^{11,15}$] endothelin 1 (IRL-1620), BQ-3020, [$Ala^{1,3,11,15}$]-endothelin, sarafotoxin S6c, and endothelin 3.

3. The method of claim1 or claim 2, wherein the ET_B receptor agonist is administered at a dose ranging from about 0.0001 mg/kg to about 0.5 mg/kg.

4. The method of any one of claims 1-3, wherein the method comprises administering multiple doses of the ET_B receptor agonist.

5. The method of any one of claims 1-3, wherein the administering comprises a single dose of the ET_B receptor agonist.

6. The method of any one of claims 1-5, further comprising administering a therapeutically effective amount of centhaquin or a salt thereof to the individual.

7. The method of any one of claims 1-6, wherein the kidney injury or failure is acute.

8. The method of any one of claims 1-6, wherein the kidney failure results from exposure to radiocontrast media, a non-steroidal anti-inflammatory drug (NSAID), an antibiotic, a chemotherapeutic agent, nivolumab-induced acute granulomatous tubulointerstitial nephritis or a nephrotoxic drug.

9. The method of claim 7, wherein the acute kidney failure is caused by or is associated with critical illness, reduced cardiac output, trauma, reduced blood oxygenation, systemic toxicity caused by reaction to injury in another organ, systemic hypotension resulting from cardiorenal syndrome, cardiac surgery or acute decompensated heart failure, a reduction in circulating volume due to hemorrhage, septic shock, hypovolemic shock, severe dengue, a surgical procedure, rhabdomyolysis or a reduction in local renal blood flow resulting from hepatorenal syndrome or liver transplant, or dehydration caused by diarrhea, vomiting, diuretics or excessive sweating.

10. The method of any one of claims 6-9, wherein kidney glomerular filtration rate of the individual is improved.

11. The method of any one of claims 6-10, wherein serum creatinine level of the individual is reduced.

12. A composition comprising (a) an endothelin-B (ET_B) receptor agonist, (b) centhaquin or a salt thereof, and optionally (c) an excipient.

13. An article of manufacture comprising:

(a) a packaged composition comprising an endothelin-B (ET_B) receptor agonist and centhaquin or a salt thereof;

(b) an insert providing instructions for a simultaneous or sequential administration of the ET_B receptor agonist and the centhaquin or salt thereof to treat a patient; and

(c) a container for (a) and (b).

14. The article of claim 13, wherein the endothelin-B receptor agonist is N-Succinyl-[Glu^9, $Ala^{11,15}$] endothelin 1 (IRL-1620).

15. A method of treating an individual suffering acute kidney function decline comprising administering to the individual a therapeutically effective amount of a composition comprising centhaquin or a salt thereof.

16. The method of claim 15, wherein the acute kidney function decline is associated with acute kidney failure.

17. The method of claim 16, wherein the acute kidney failure is caused by or is associated with critical illness, reduced cardiac output, trauma, reduced blood oxygenation, systemic toxicity caused by reaction to injury in another organ, systemic hypotension resulting from cardiorenal syndrome, cardiac surgery or acute decompensated heart failure, a reduction in circulating volume due to hemorrhage, septic shock, hypovolemic shock, severe dengue, a surgical procedure, rhabdomyolysis or a reduction in local renal blood flow resulting from hepatorenal syndrome or liver transplant, nephrotoxicity resulting from drugs, radiocontrast media, a non-steroidal anti-inflammatory drug (NSAID), an antibiotic, or a chemotherapeutic agent, or dehydration caused by diarrhea, vomiting, diuretics or excessive sweating.

18. A method of treating an individual suffering from kidney injury comprising administering an effective amount of a composition comprising centhaquin or a salt thereof.

19. The method of claim 18, wherein the kidney injury results from an ischemic event or an ischemic reperfusion event.

20. The method of any one of claims 6-19, wherein the salt is citrate, pyruvate, or lactate.

21. The method of any one of claims 6-20, wherein centhaquin or salt thereof is administered at a dose of about 0.0001 mg/kg to about 1.0 mg/kg.

22. The method of claim 21, wherein centhaquin or salt thereof is administered in single or multiple doses.

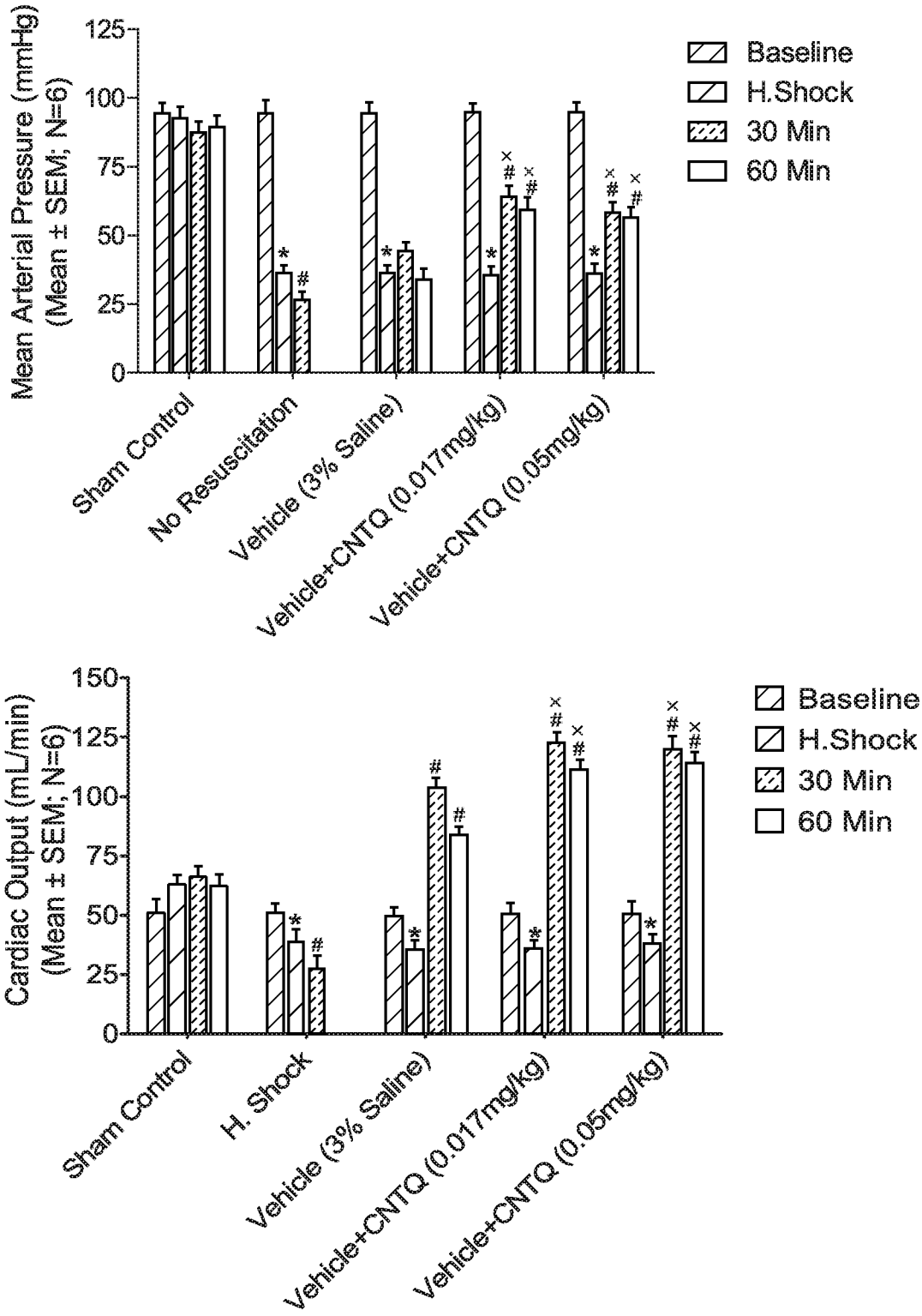


Figure 1

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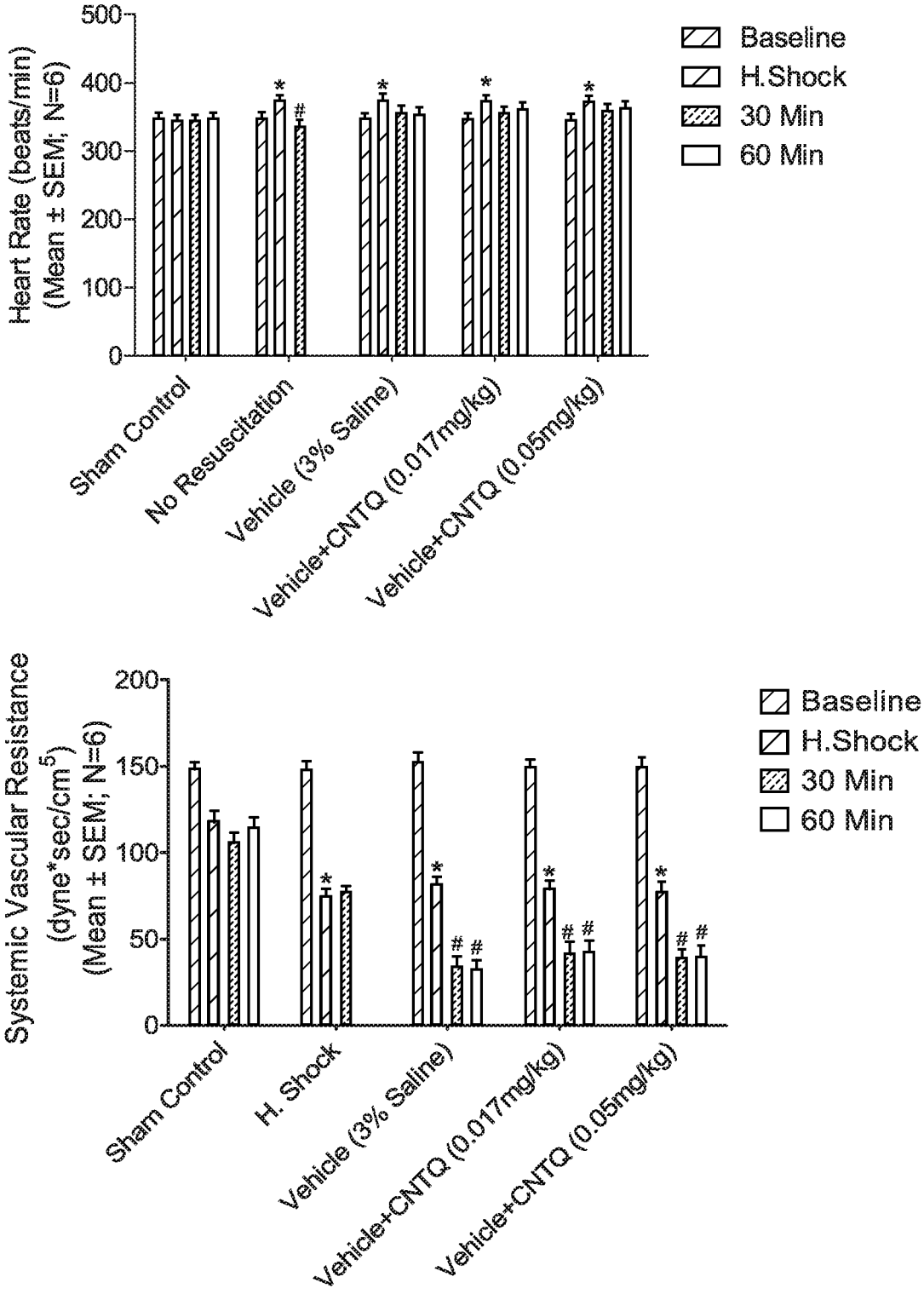


Figure 1

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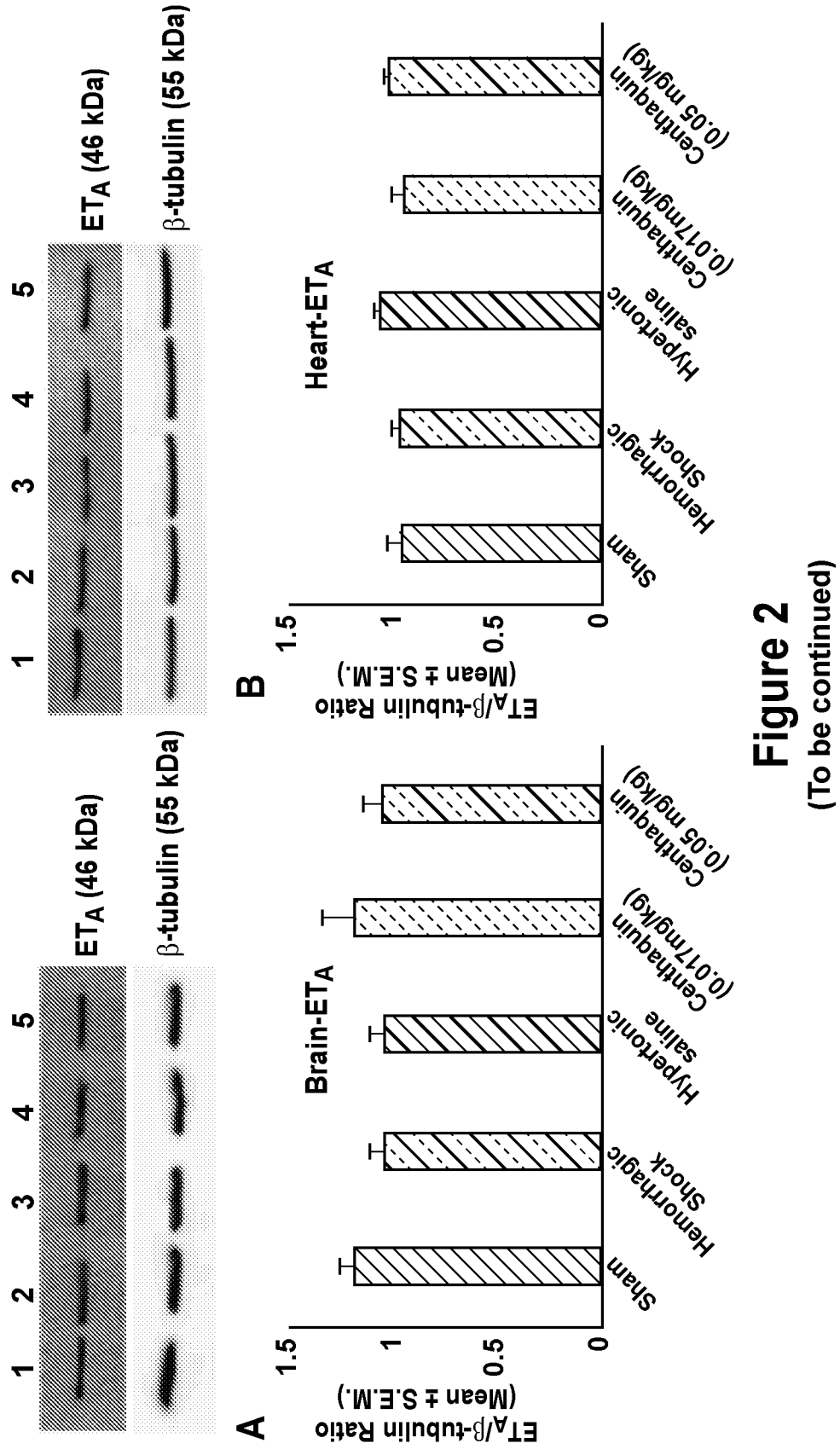


Figure 2
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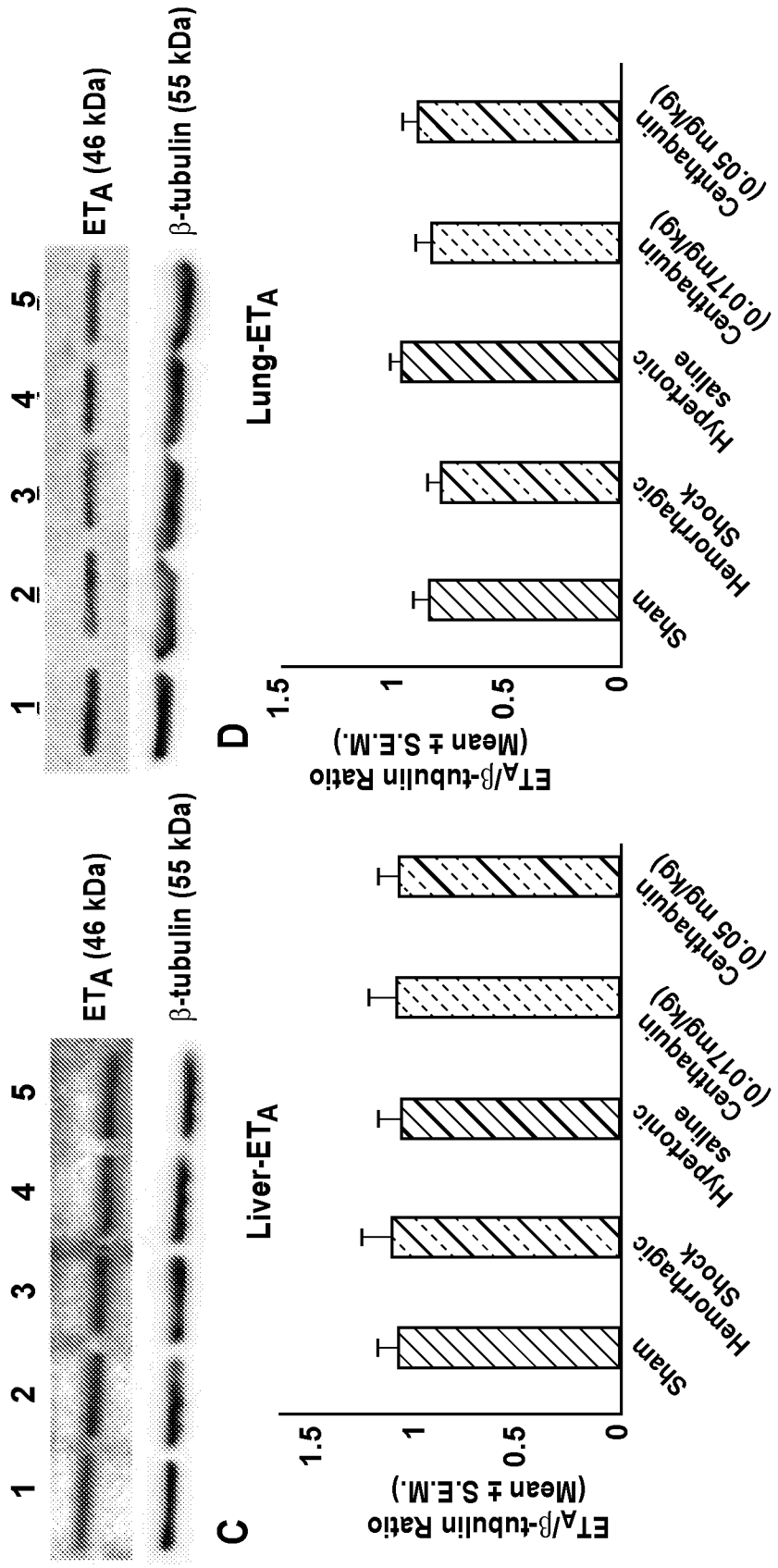


Figure 2
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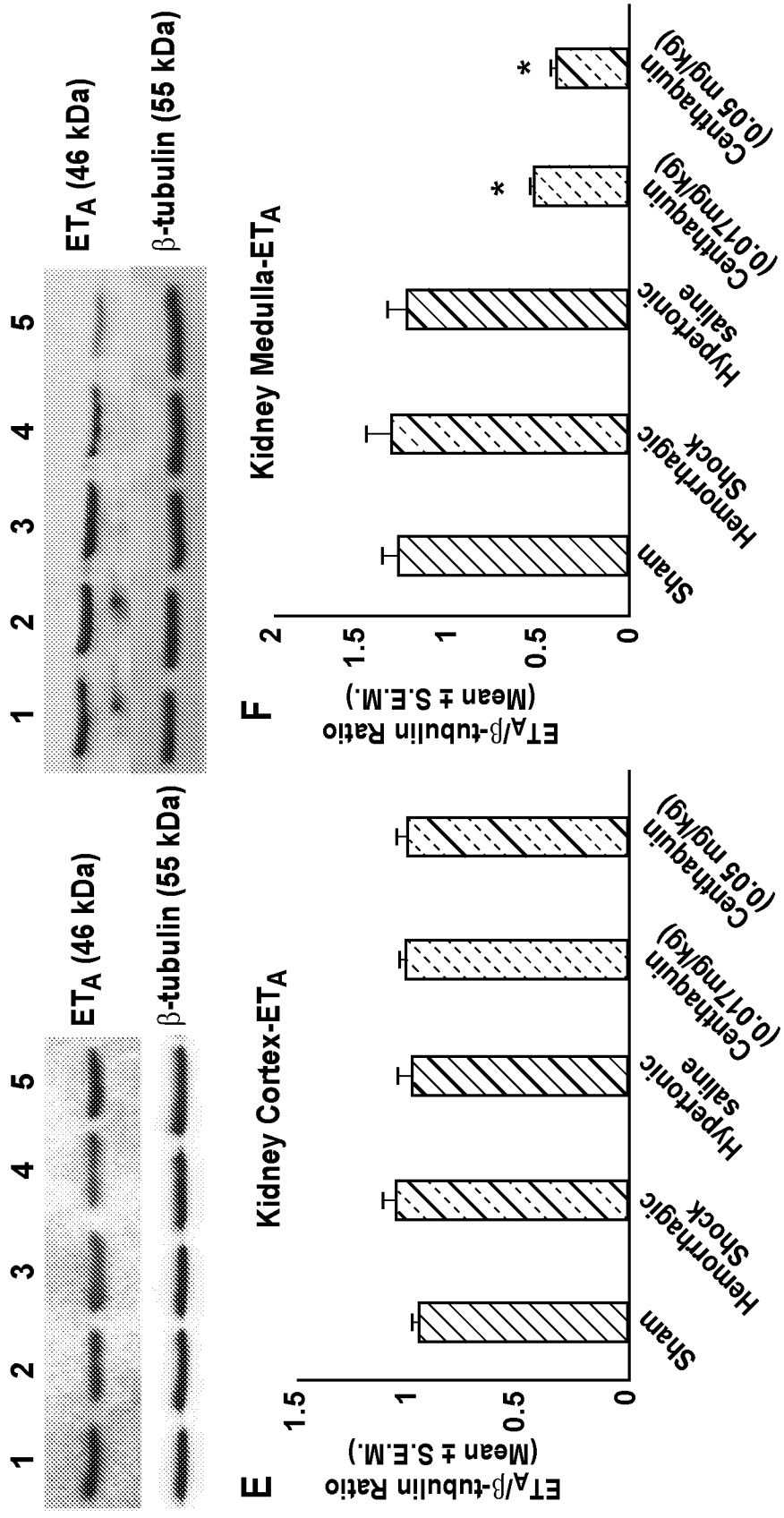


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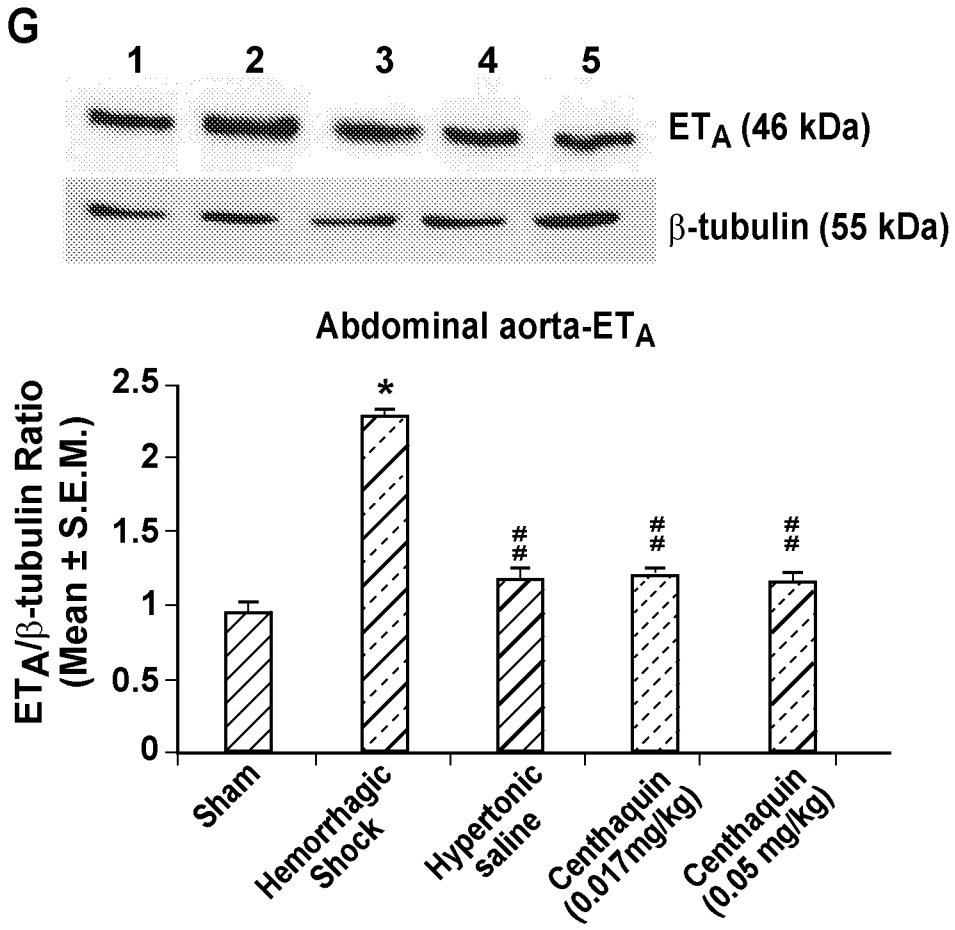


Figure 2
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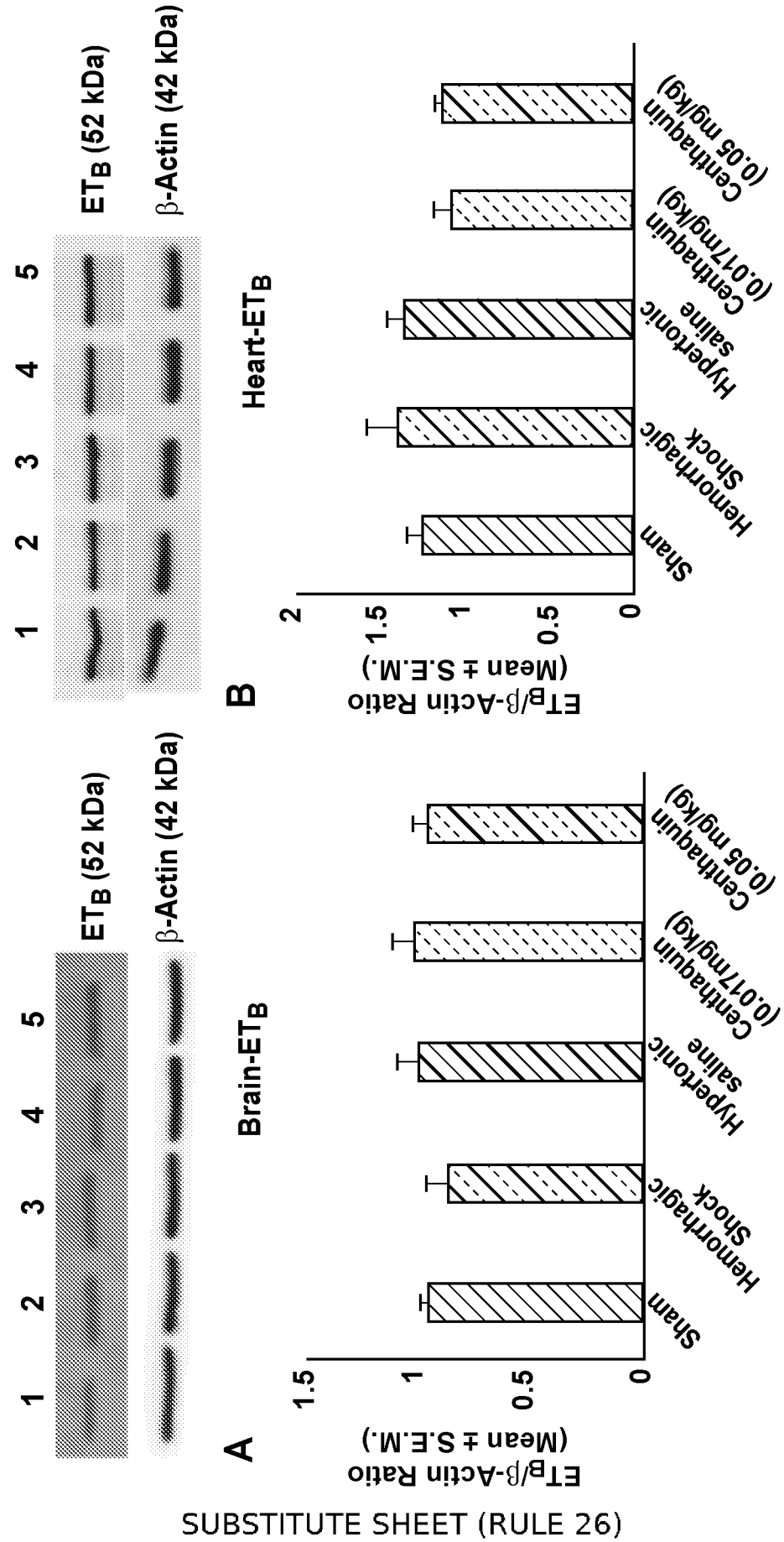


Figure 3
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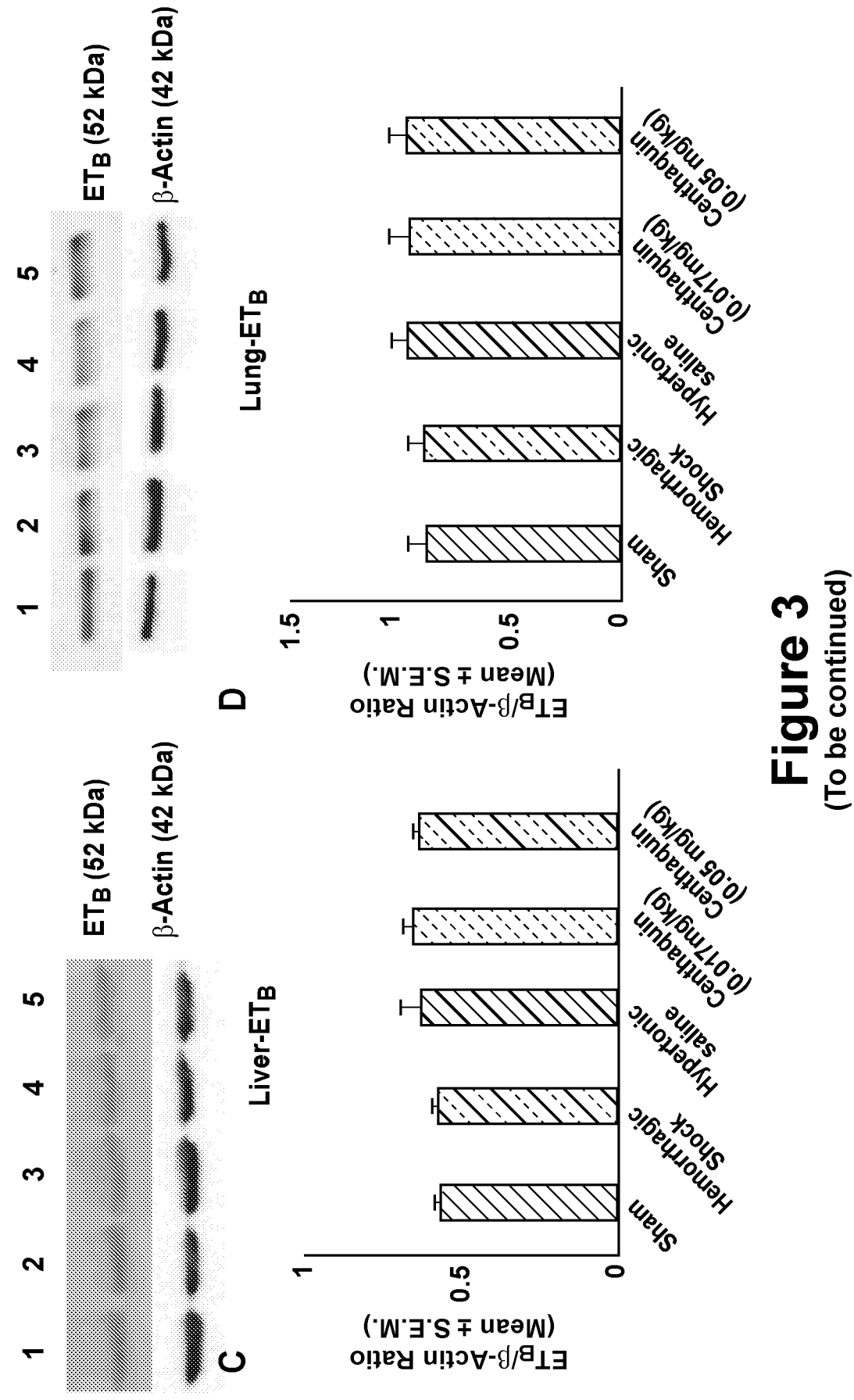


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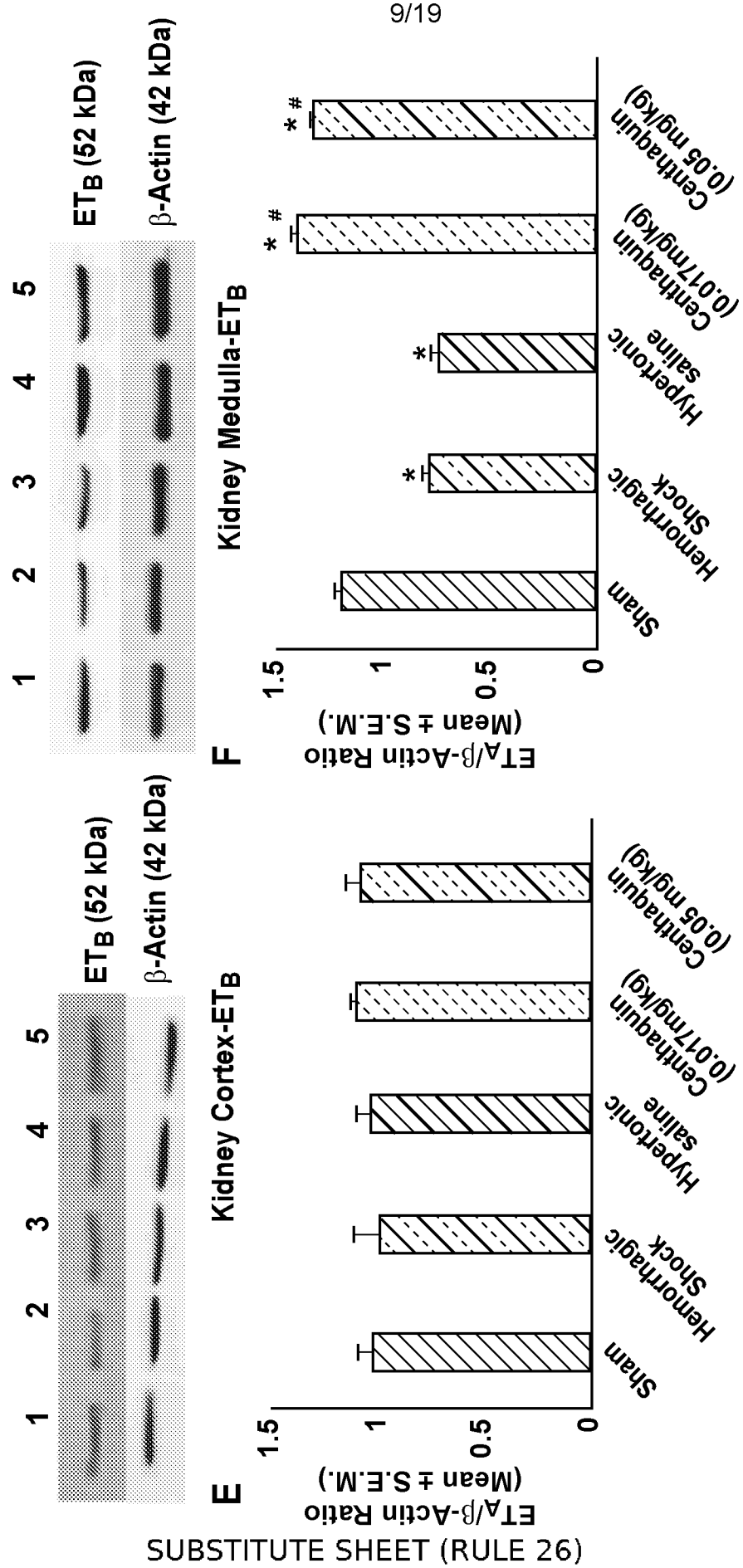


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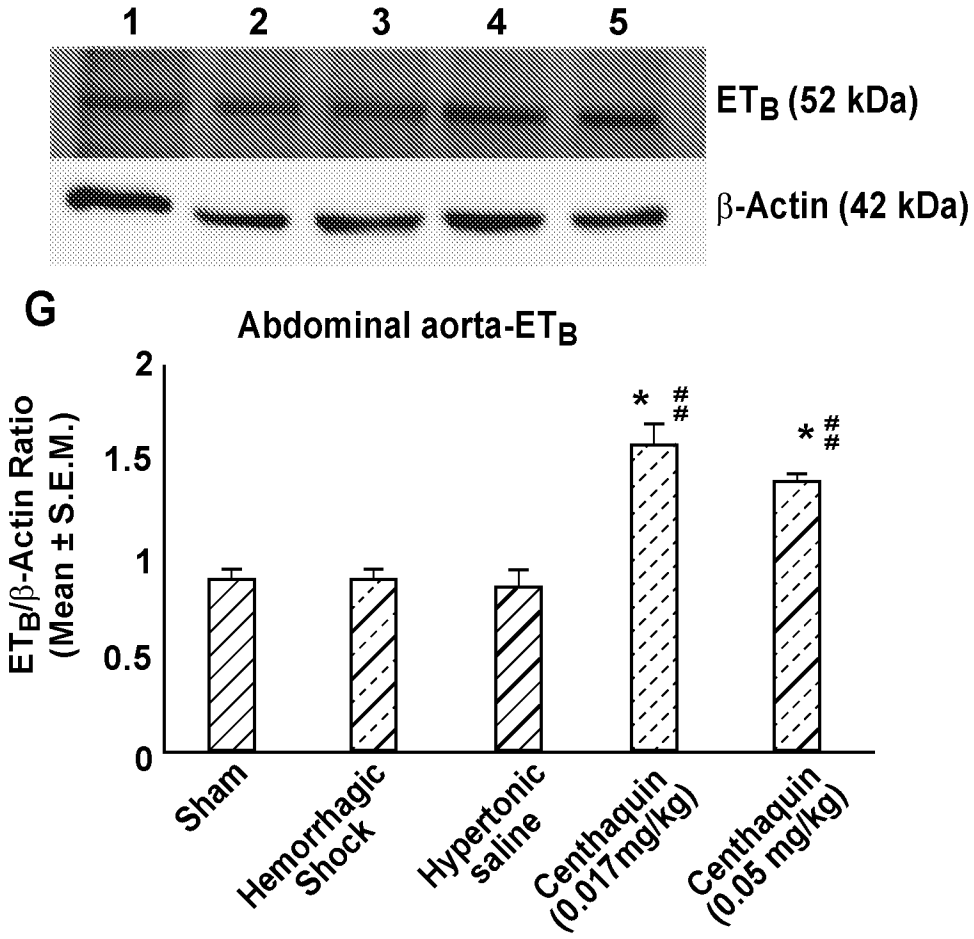


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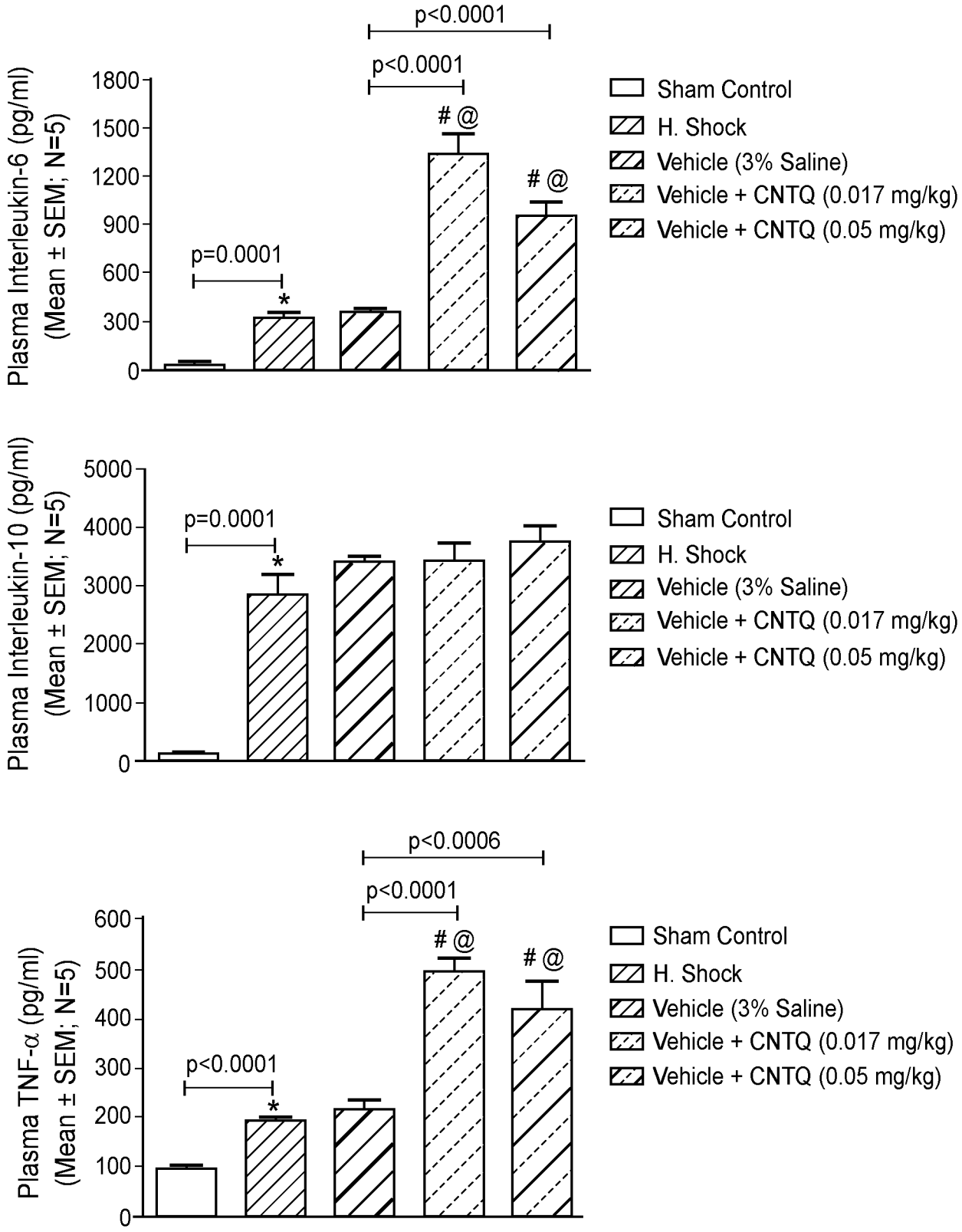

p<0.0001
p<0.0001
#@
#@
p=0.0001
*
1800
1500
1200
900
600
300
0
Plasma Interleukin-6 (pg/ml)
(Mean ± SEM; N=5)
Sham Control
H. Shock
Vehicle (3% Saline)
Vehicle + CNTQ (0.017 mg/kg)
Vehicle + CNTQ (0.05 mg/kg)
5000
4000
3000
2000
1000
0
p=0.0001
*
Plasma Interleukin-10 (pg/ml)
(Mean ± SEM; N=5)
Sham Control
H. Shock
Vehicle (3% Saline)
Vehicle + CNTQ (0.017 mg/kg)
Vehicle + CNTQ (0.05 mg/kg)
p<0.0006
p<0.0001
#@
#@
600
500
400
300
200
100
0
p<0.0001
*
Plasma TNF-α (pg/ml)
(Mean ± SEM; N=5)
Sham Control
H. Shock
Vehicle (3% Saline)
Vehicle + CNTQ (0.017 mg/kg)
Vehicle + CNTQ (0.05 mg/kg)

Figure 4

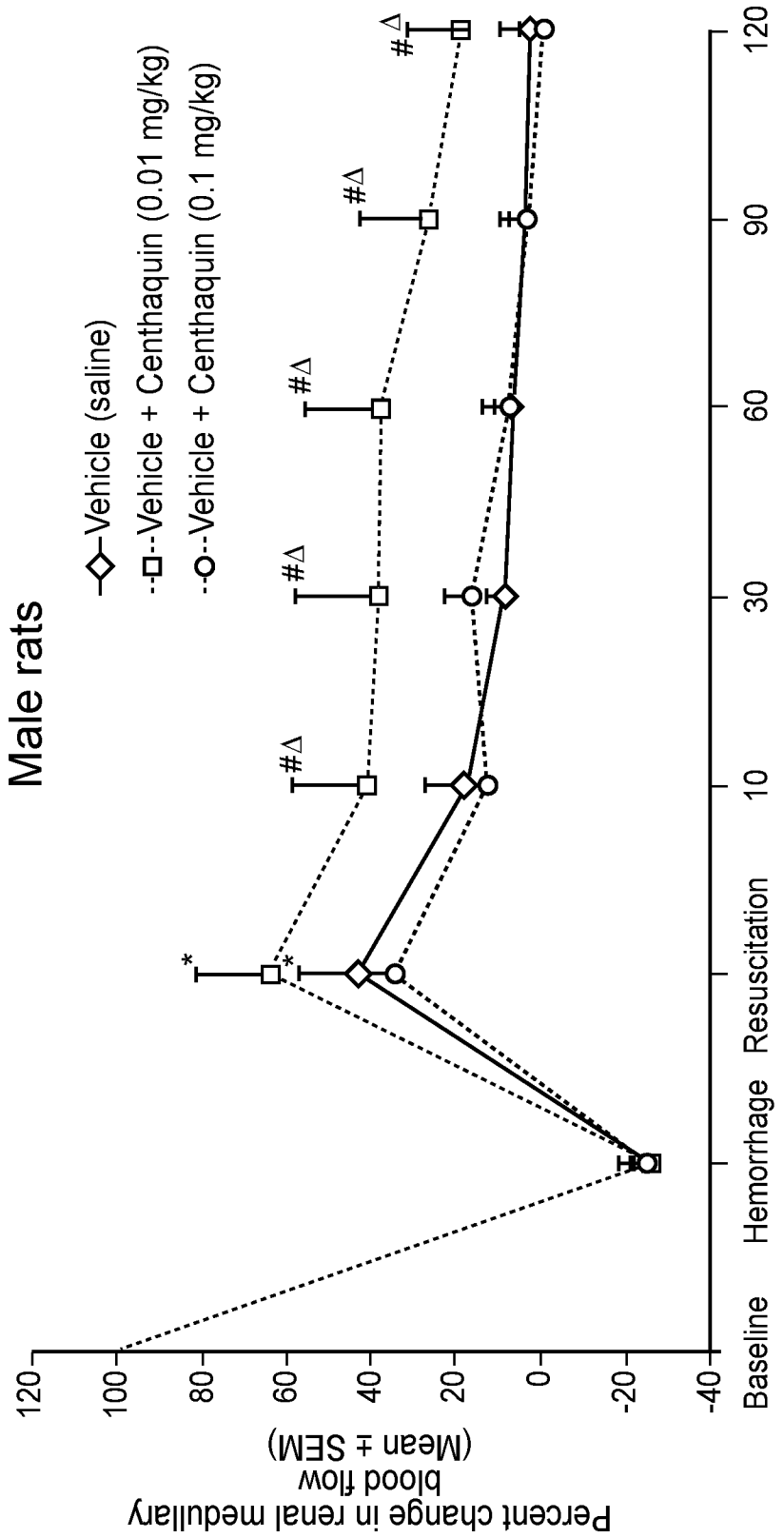

Male rats
Vehicle (saline)
Vehicle + Centhaquin (0.01 mg/kg)
Vehicle + Centhaquin (0.1 mg/kg)
Percent change in renal medullary blood flow (Mean ± SEM)
120
100
80
60
40
20
0
-20
-40
Baseline
Hemorrhage
Resuscitation
10
30
60
90
120
Time (Min)
*
#Δ

Figure 5

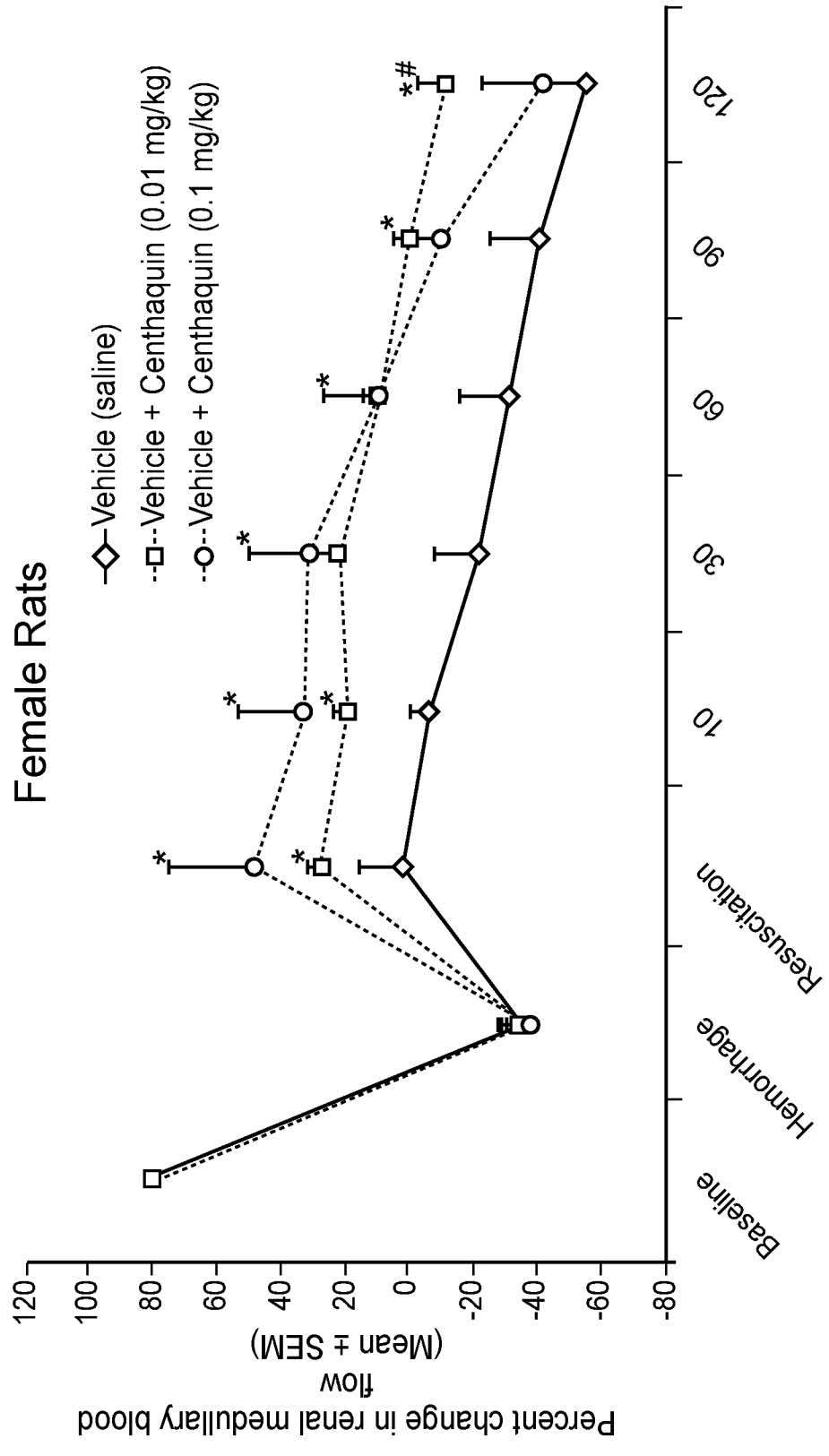

Female Rats
Vehicle (saline)
Vehicle + Centhaquin (0.01 mg/kg)
Vehicle + Centhaquin (0.1 mg/kg)
Percent change in renal medullary blood flow (Mean ± SEM)
120
100
80
60
40
20
0
-20
-40
-60
-80
Baseline
Hemorrhage
Resuscitation
10
30
60
90
120
Time (Min)
*
*
*
*
*
*
*
*#

Figure 6

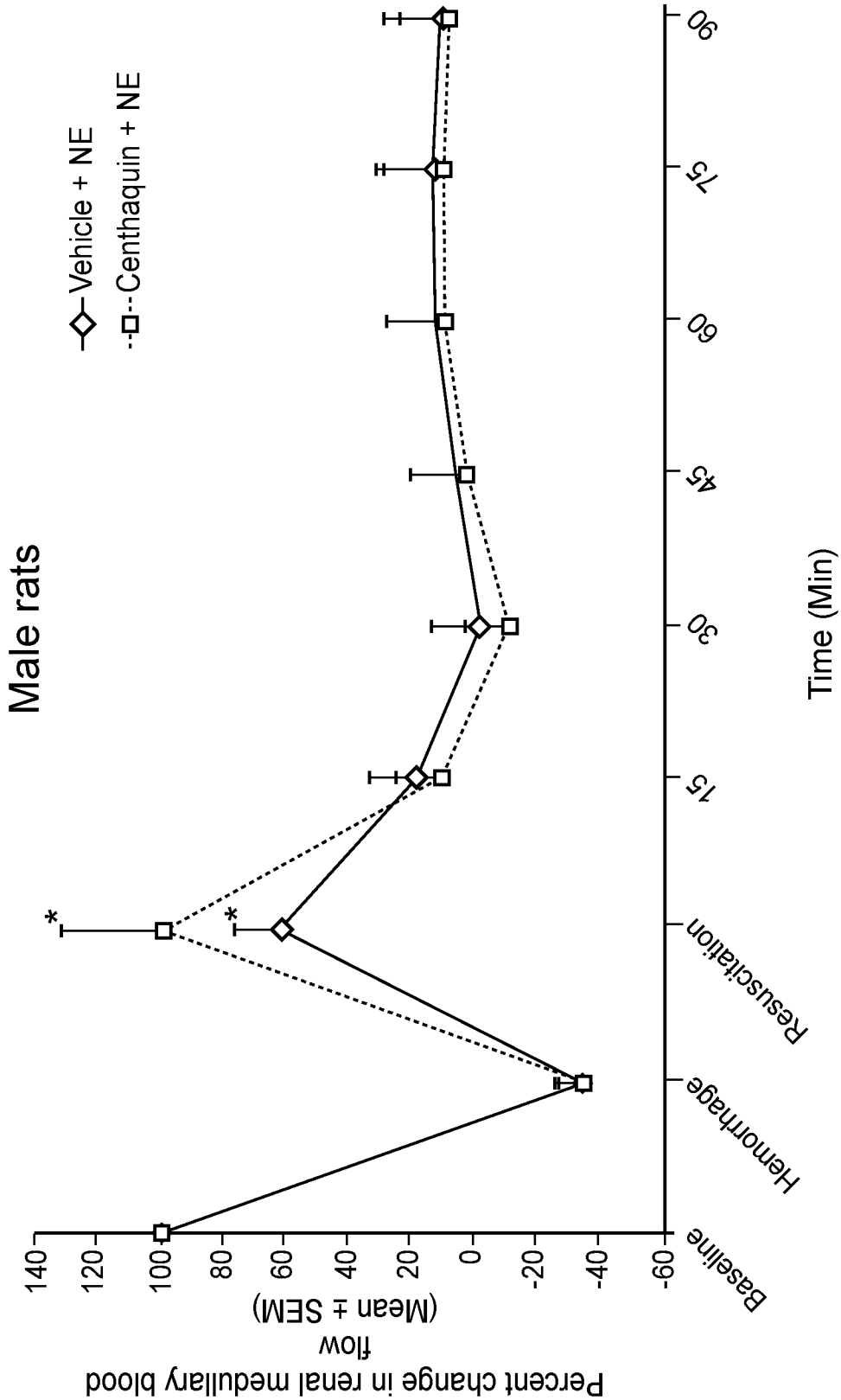

Male rats
Vehicle + NE
Centhaquin + NE
Percent change in renal medullary blood flow (Mean ± SEM)
140
120
100
80
60
40
20
0
-20
-40
-60
Baseline
Hemorrhage
Resuscitation
15
30
45
60
75
90
Time (Min)
*
*

Figure 7

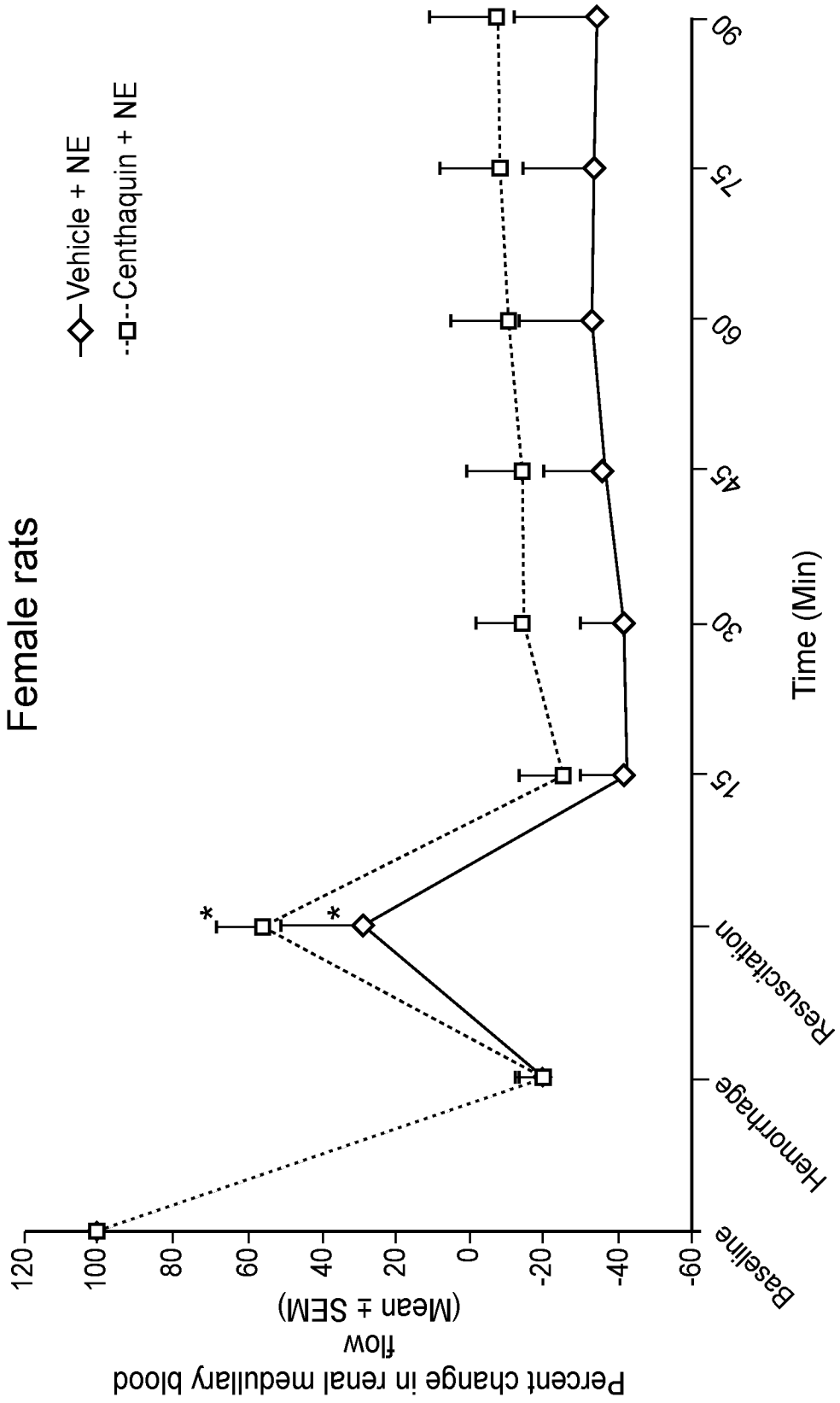

Female rats
Vehicle + NE
Centhaquin + NE
Percent change in renal medullary blood flow (Mean ± SEM)
120
100
80
60
40
20
0
-20
-40
-60
Baseline
Hemorrhage
Resuscitation
15
30
45
60
75
90
Time (Min)
*
*

Figure 8

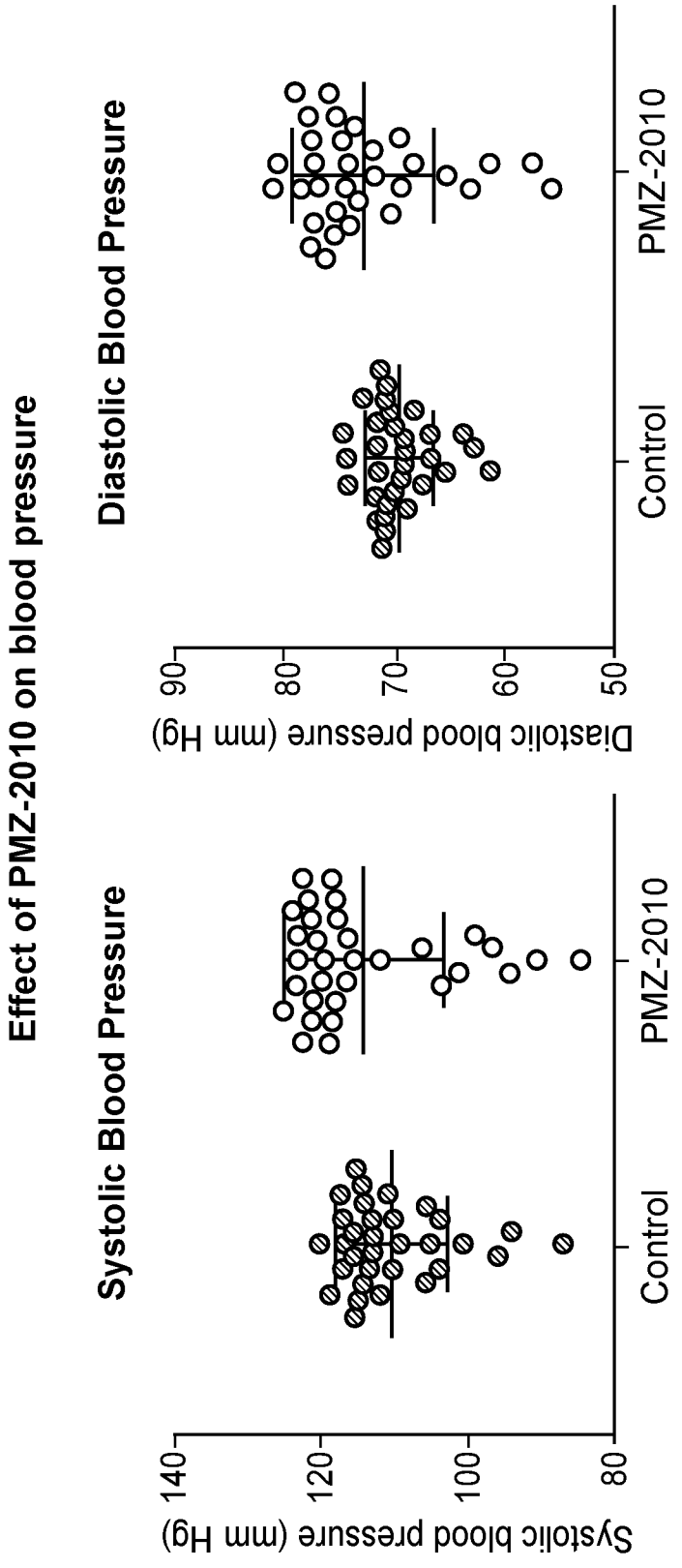

Effect of PMZ-2010 on blood pressure
Systolic Blood Pressure
Systolic blood pressure (mm Hg)
140
120
100
80
Control
PMZ-2010
Diastolic Blood Pressure
Diastolic blood pressure (mm Hg)
90
80
70
60
50
Control
PMZ-2010

Figure 9

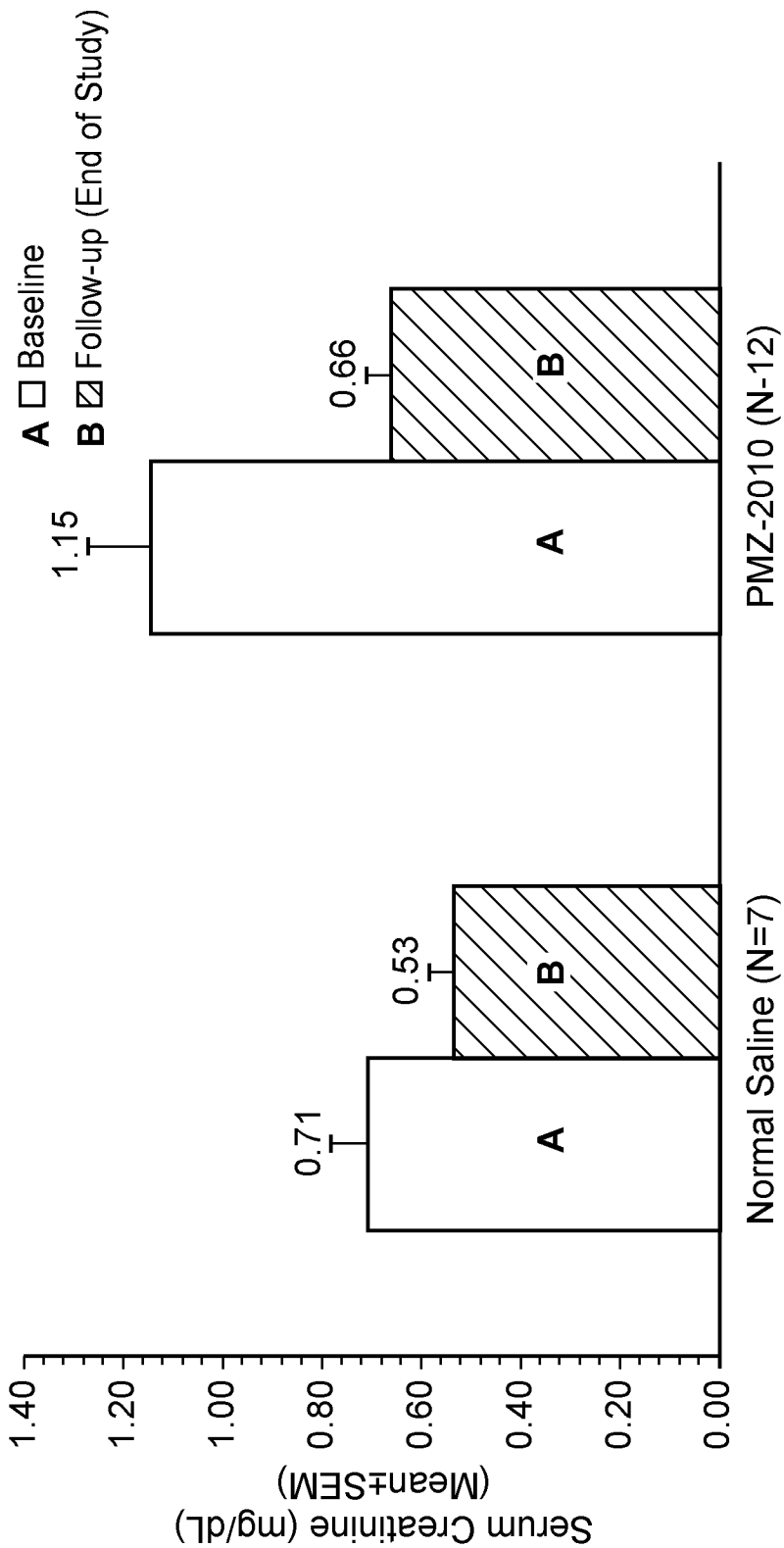

A ☐ Baseline
B ▨ Follow-up (End of Study)
Serum Creatinine (mg/dL)
(Mean±SEM)
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0.71
0.53
1.15
0.66
A
B
A
B
Normal Saline (N=7)
PMZ-2010 (N-12)

Figure 10

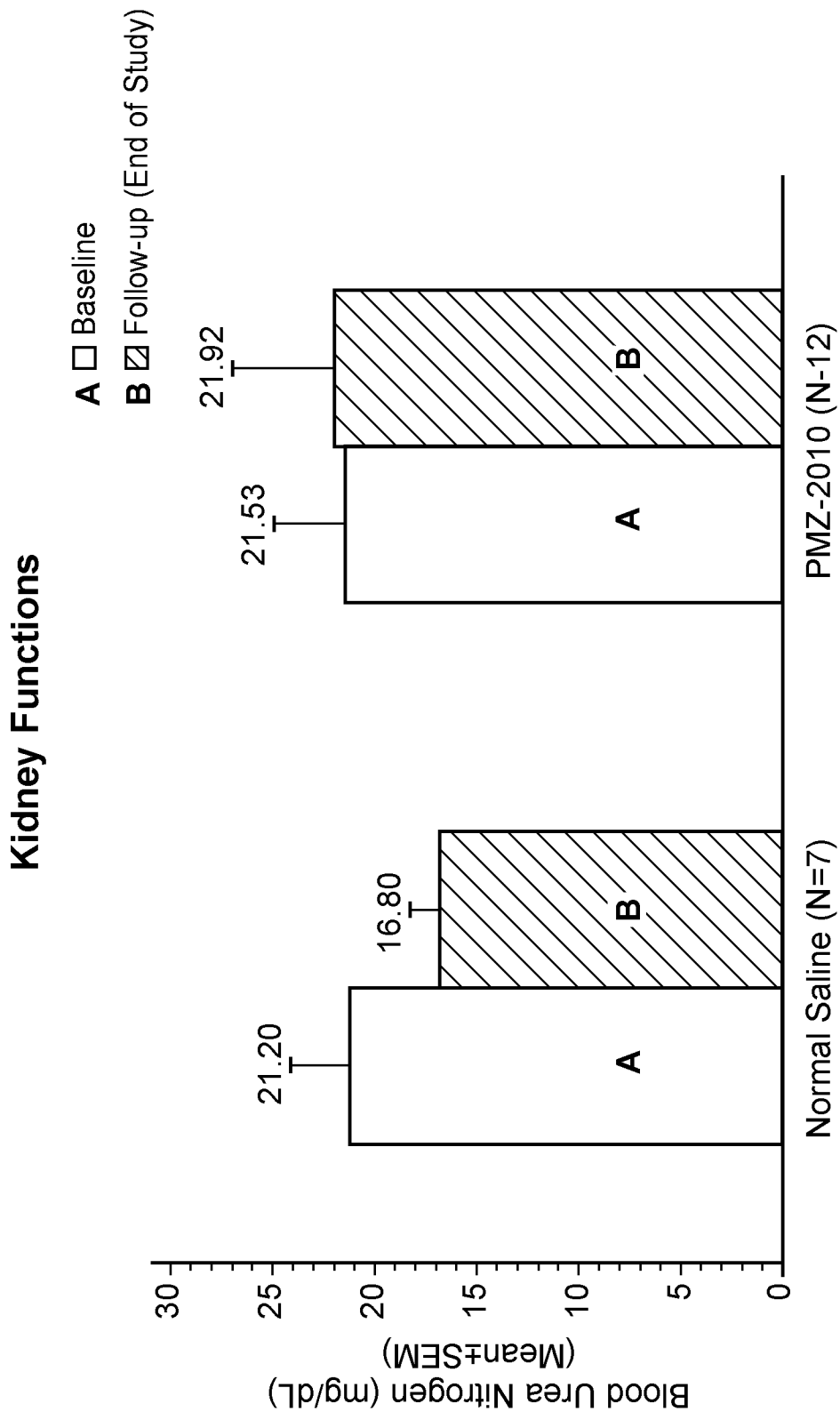

Kidney Functions
A ☐ Baseline
B ☑ Follow-up (End of Study)
Blood Urea Nitrogen (mg/dL)
(Mean±SEM)
0
5
10
15
20
25
30
21.20
16.80
21.53
21.92
A
B
A
B
Normal Saline (N=7)
PMZ-2010 (N-12)

Figure 11

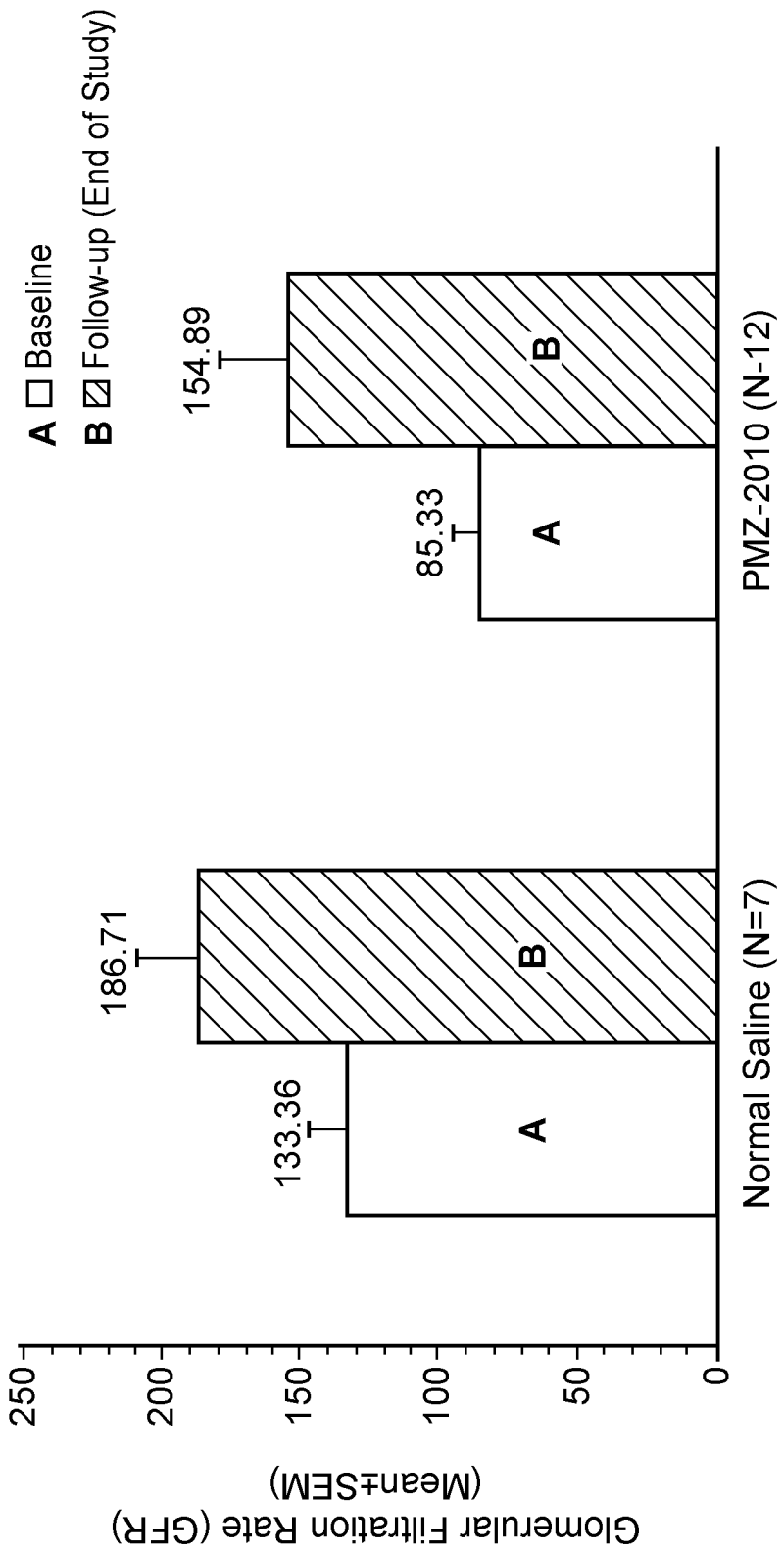

Kidney Functions
A ☐ Baseline
B ☑ Follow-up (End of Study)
Glomerular Filtration Rate (GFR)
(Mean±SEM)
250
200
150
100
50
0
186.71
133.36
154.89
85.33
A
B
A
B
Normal Saline (N=7)
PMZ-2010 (N-12)

Figure 12

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2019/030652

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61P 9/00; A61P 13/12; A61P 25/00; A61P 35/00; A61P 43/00 (2019.01)

CPC - A61K 31/137; A61K 31/155; A61K 31/165; A61K 31/401; A61K 31/4025; A61K 31/415; A61K 31/4168; A61K 31/4174; A61K 31/4178; A61K 31/433; A61K 31/4375; A61K 31/444; A61K 31/4725; A61K 31/496; A61K 31/498; A61K 31/506; A61K 31/54; A61K 31/55; A61K 45/06; A61P 13/12 (2019.05)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

USPC - 424/94.61; 424/133.1 (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	US 2010/0209433 A1 (BERGMANN et al) 19 August 2010 (19.08.2010) entire document	1, 2 ------ 3
X	US 2012/0083447 A1 (GULATI) 05 April 2012 (05.04.2012) entire document	12-14
Y	US 2016/0151450 A1 (MIDWESTERN UNIVERSITY) 02 June 2016 (02.06.2016) entire document	3
Y	US 2007/0066568 A1 (DALTON et al) 22 March 2007 (22.03.2007) entire document	15-17
Y	US 2015/0250782 A1 (PHARMAZZ, INC et al) 10 September 2015 (10.09.2015) entire document	15-19
Y	US 2012/0308644 A1 (BROMLEY et al) 06 December 2012 (06.12.2012) entire document	18, 19
P, X	BRIYAL et al. "Alterations in Endothelin Receptors Following Hemorrhage and Resuscitation by Centhaquin," Physiological Research, 27 June 2018 (27.06.2018), Vol. 67, Suppl. 1, Pgs. 199-214. entire document	1-3, 12-19
A	GULATI et al. "Abstract 20622: Endothelin Receptor Alteration Following Hemorrhagic Shock and Resuscitation by Centhaquin," Circulation, 14 November 2017 (14.11.2017), Vol. 136, No. 1, Pgs. 1-2. entire document	1-3, 12-19
A	EP 2890376 B1 (PHARMAZZ INC et al) 27 September 2017 (27.09.2017) entire document	1-3, 12-19

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 June 2019	25 JUL 2019

Name and mailing address of the ISA/US	Authorized officer
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300	Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2019/030652

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 4-11, 20-22
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.