Alterations in Endothelin Receptors Following Hemorrhage and Resuscitation by Centhaquin

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Summary

Endothelin-1 (ET-1) acts on ET_A and ET_B receptors and has been implicated in hemorrhagic shock (shock). We determined effect of shock and resuscitation by hypertonic saline (saline) or centhaquin on ET_A and ET_B receptor expression. Rats were anesthetized, a pressure catheter was placed in the left femoral artery; blood was withdrawn from the right femoral artery to bring mean arterial pressure (MAP) to 35 mm Hg for 30 min, resuscitation was performed and 90 min later sacrificed to collect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.

Key words

Hemorrhagic shock • Centhaquin • Resuscitation • Endothelin • Cytokines

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Introduction

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 h of admission (Shackford *et al.* 1993).

We 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). We further carried out comparative studies 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.*

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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. 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.

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).

The present study was conducted to examine 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.

Methods

Animals

Male Sprague-Dawley rats (340-380 g) (Envigo, Indianapolis, IN, USA) were housed for at least 4 days in a room with controlled temperature (23±1 °C), 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.

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, USA), IL-10 ELISA kit (Catalog No. KRC0101, Invitrogen Corporation, Carlsbad, CA, USA) and TNF-α ELISA kit (Catalog No. ER3TNFA, Thermo Scientific, Rockford, IL, USA) were used for various estimations.

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 h) 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, USA). The right carotid artery was exposed to measure the left ventricular performance. Surgical suture (Deknatel, Research Triangle Park, NC, USA) 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, USA) 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, USA) and secured for resuscitation.

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 min after induction of shock, and 30 and 60 min 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, USA) and analyzed using a pHOx Ultra analyzer (Nova Biomedical Corporation, Waltham, MA, USA). The base deficit was calculated using the formula (Davis *et al.* 1998, Paladino *et al.* 2008):

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

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 mm Hg 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 ~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 min.

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, USA). The blood samples, for biochemical estimations, were collected at 30 min of resuscitation and cardiovascular parameters were monitored till 60 min 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.

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 min 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 min 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 h of incubation and horseradish peroxidase (HRP) labeled monoclonal antibody was then added. After 30 min 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, USA). The measured optical density is directly proportional to the concentration of ET-1 (Lavhale *et al.* 2010).

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, USA). 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, USA), 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, USA). The membranes were washed three times with 1x TBS-Tween (TBST) and incubated with rabbit polyclonal anti-ET_A receptor (ab85163, Abcam, Cambridge, MA, USA, 1:1000) or anti-ET_B receptor (ab117529, Abcam, Cambridge, MA, USA, 1:1000) or mouse monoclonal anti-β-actin (a1978, Sigma-Aldrich, St. Louis, MO, USA) antibodies, followed by horseradish peroxidase (HRP)-conjugated secondary antibodies goat anti-rabbit (sc2004, Santa Cruz Biotechnology, Dallas, TX, USA, 1:2000) or goat anti-mouse (ab98693, Abcam, Cambridge, MA, USA, 1:10,000) and visualized by SuperSignal® West Pico Chemiluminescent Substrate (ThermoFisher Scientific, Hanover Park, IL, USA) using the ChemiDoc™ MP Imaging System (Bio-Rad Laboratories, Hercules, CA, USA) and then analyzed using ImageJ (NIH) software.

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.

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 ± SEM. 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

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).

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.

Table 1. Effect of centhaquin on hematocrit, arterial blood pH, pCO_2, pO_2, lactate and base deficit levels in hemorrhaged rats.

	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	Baseline	32.5 ± 2.1	33.6 ± 1.1	33.3 ± 1.2	33.8 ± 1.2	34.5 ± 2.1
(mm Hg)	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	Baseline	122.2 ± 4.3	125.9 ± 5.7	120.3 ± 1.2	125.5 ± 1.9	120.2 ± 1.2
(mm Hg)	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	Baseline	1.9 ± 0.2	1.8 ± 0.1	1.9 ± 0.1	1.5 ± 0.2	1.8 ± 0.1
(mmol/l)	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	Baseline	-2.7 ± 0.4	-2.4 ± 0.3	-2.4 ± 0.4	-2.9 ± 0.3	-2.8 ± 0.3
(mEq/l)	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$^{*\#\Delta}$

The values are expressed as mean ± SEM. $^{*}p<0.05$ compared to baseline; $^{\#}p<0.05$ compared to hemorrhage; $^{\Delta}p<0.05$ compared to vehicle treated group.

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.

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 min 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 (Fig. 1). Prior to hemorrhage, the baseline HR was approximately 345 beats/min in all groups. Hemorrhage produced a slight increase in HR (~372 beats/min). In rats resuscitated with hypertonic saline HR dropped to 353±11 beats/min at 60 min, 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 (Fig. 1). No significant difference was observed in HR following resuscitation with hypertonic saline or centhaquin.

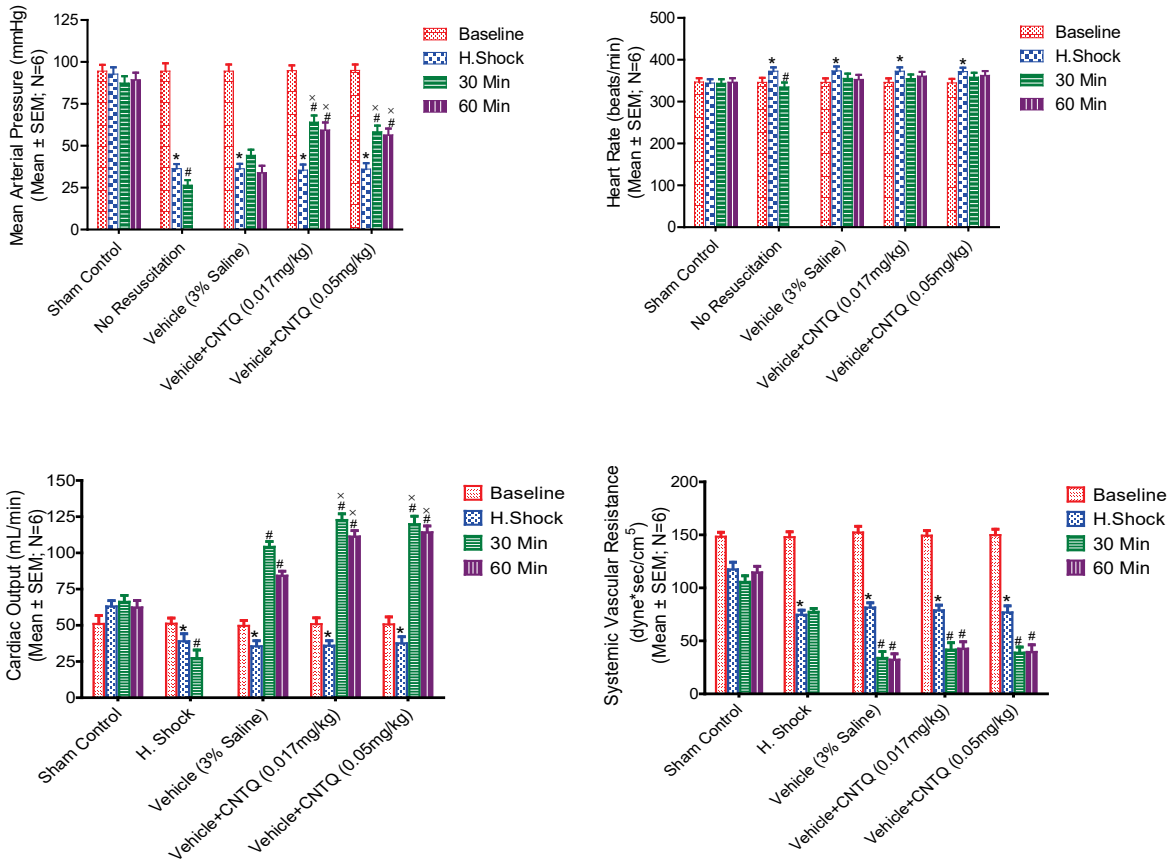

Fig. 1. Effect of hemorrhage on mean arterial pressure, heart rate, cardiac output and systemic vascular resistance in sham and hemorrhaged rats. Hemorrhaged rats were resuscitated with hypertonic saline or centhaquin. The values are expressed as mean ± SEM. (n=5). $^{*}p<0.05$ compared to baseline, $^{\#}p<0.05$ compared to hemorrhage, $^{x}p<0.05$ compared to vehicle treated group.

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. 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 min of resuscitation with both hypertonic saline or centhaquin treatments (Fig. 1).

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 (Table 1).

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 (Fig. 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. 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 (Fig. 2).

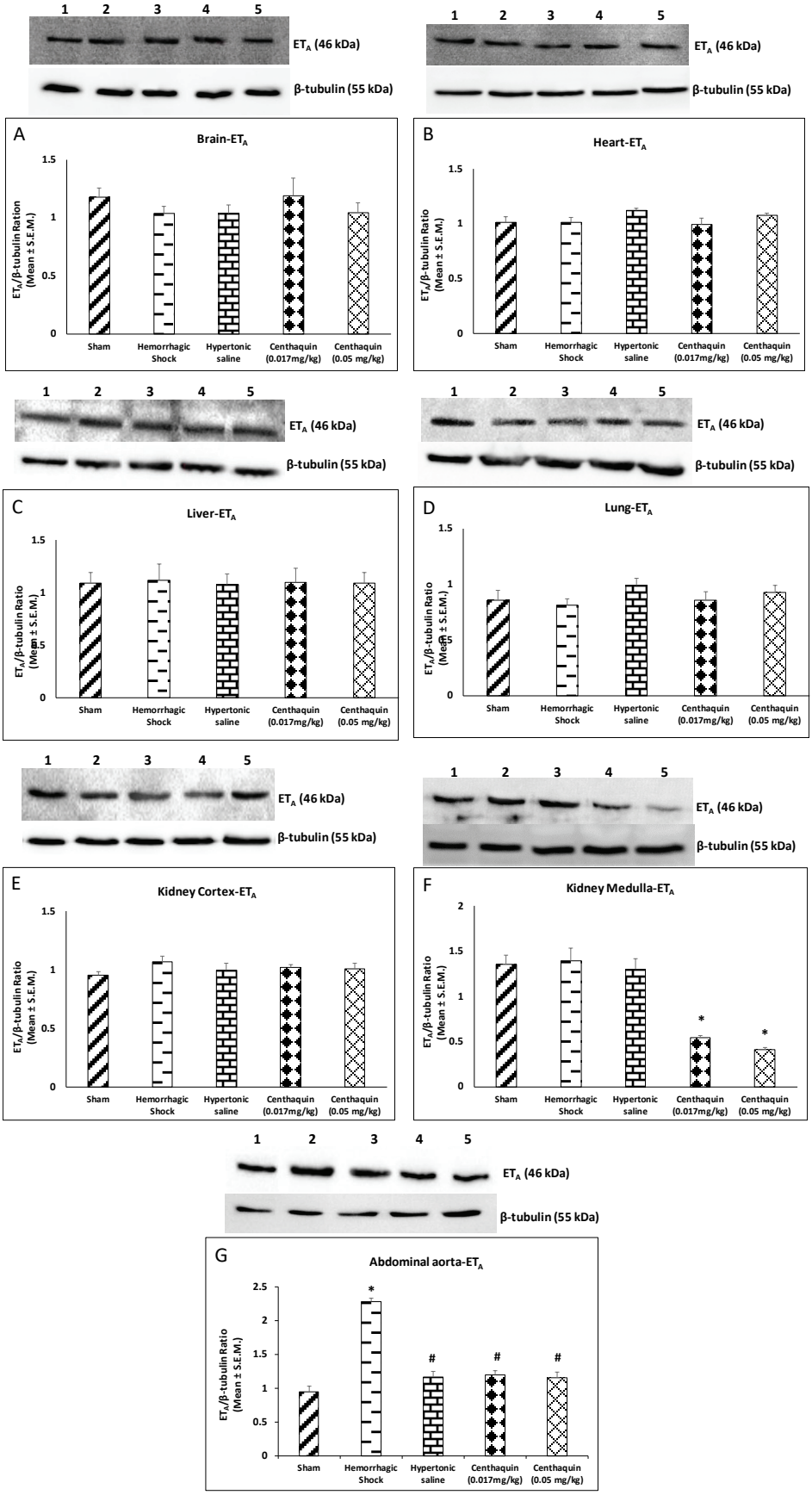

Fig. 2. Effect of hemorrhage on the expression of ET_A receptors in sham and hemorrhaged rats. Hemorrhaged rats were resuscitated with hypertonic saline or centhaquin. Lane 1 – Sham, Lane 2 – Hemorrhagic shock, Lane 3 – Hypertonic saline (vehicle), Lane 4 – Vehicle + centhaquin (0.017 mg/kg), Lane 5 – Vehicle + centhaquin (0.05mg/kg). The values are expressed as mean ± SEM. (n=4). $^{*}p<0.05$ compared to sham, $^{\#}p<0.05$ compared to hemorrhage or hypertonic saline.

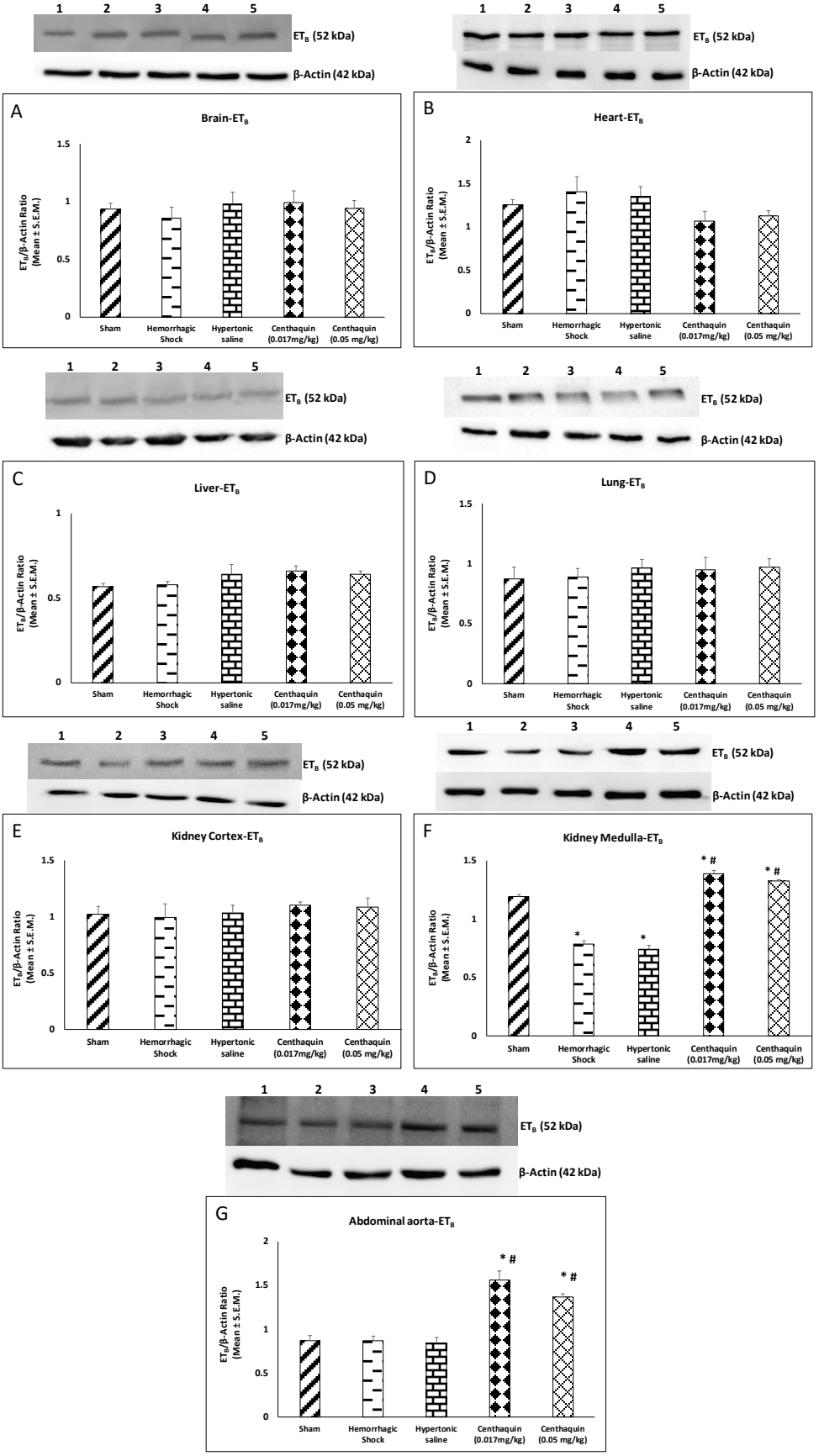

Fig. 3. Effect of hemorrhage on the expression of ET_B receptors in sham and hemorrhaged rats. Hemorrhaged rats were resuscitated with hypertonic saline or centhaquin. Lane 1 – Sham, Lane 2 – Hemorrhagic shock, Lane 3 – Hypertonic saline (vehicle), Lane 4 – Vehicle + centhaquin (0.017 mg/kg), Lane 5 – Vehicle + centhaquin (0.05 mg/kg). The values are expressed as mean ± SEM. (n=4). $^{*}p<0.05$ compared to sham, $^{\#}p<0.05$ compared to hemorrhage or hypertonic saline.

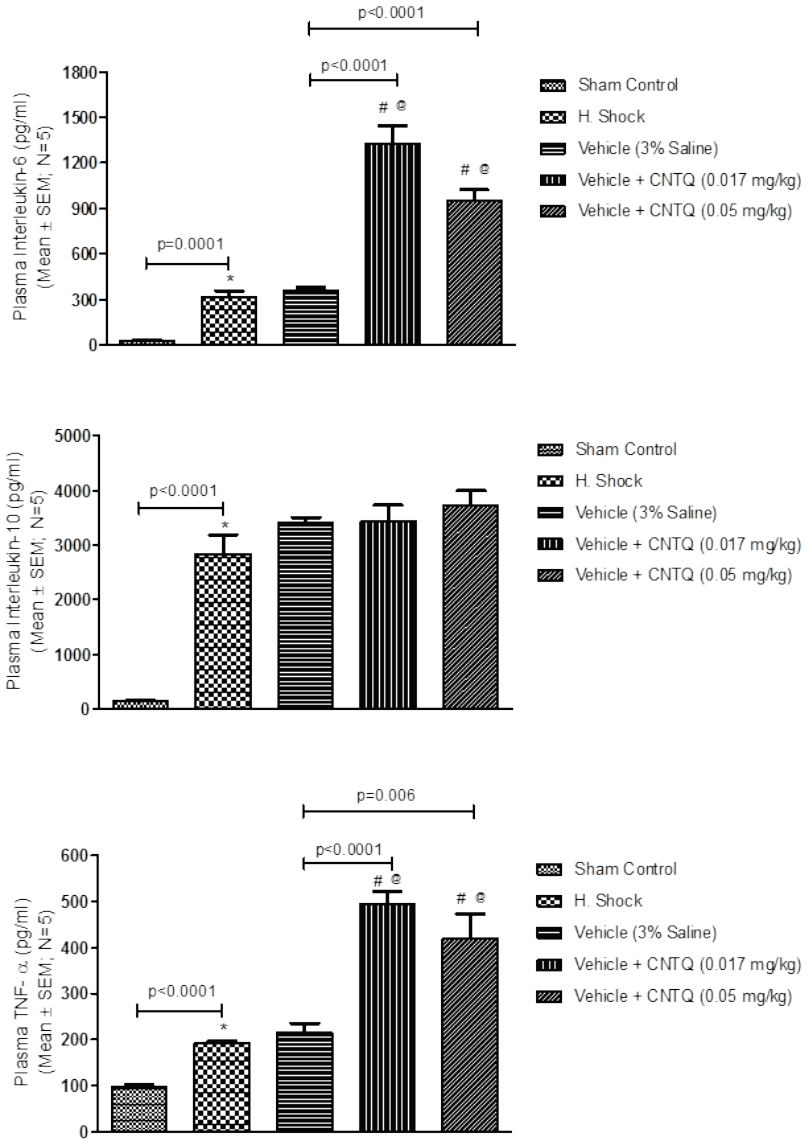

Fig. 4. Effect of hemorrhage on plasma TNF-α, IL-6 and IL-10 in sham and hemorrhaged rats. Hemorrhaged rats were resuscitated with hypertonic saline or centhaquin. The values are expressed as mean ± SEM. (n=5). $^{*}p<0.05$ compared to sham, $^{\#}p<0.05$ compared to hemorrhage, $^{@}p<0.05$ compared to hypertonic saline.

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 (Fig. 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. 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 (Fig. 3).

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 (Fig. 4).

Discussion

Centhaquin significantly decreased blood lactate and restored MAP and enhanced the resuscitative effect of hypertonic saline confirming our 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. We found that ET_A and ET_B receptors in the abdominal aorta and renal medulla appear to be 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.

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. 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). It can be speculated that 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.

ET receptors crosstalk with each other and with adrenergic receptors. 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). We have 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 may be responsible for some of the resuscitative effects of centhaquin.

We found 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 may prevent 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). In the present study, hemorrhagic shock decreased renal medullary ET_B receptor expression which was not affected by resuscitation with saline but was attenuated by centhaquin. Our results suggest 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.

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.

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).

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). We found 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 would need to be 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.

The limitations of this study are the lack of mechanistic detail and causal relationships. Further studies are needed to confirm whether an increase in the expression of ET_B receptors is responsible for an elevation in the concentration of plasma cytokines and how an increase in certain cytokines may contribute towards beneficial effect of centhaquin following hemorrhagic shock. In addition, studies using specific ET receptor antagonists are needed to confirm the involvement of ET receptors and it is possible that these finding are associative.

In summary, centhaquin significantly improves resuscitation following hemorrhagic shock 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 not known and needs to be investigated. It is speculated that these changes may improve tissue blood perfusion.

Conflict of Interest

Dr. Gulati has a pending patent and Dr. Lavhale is presently employed by Pharmazz, Inc. having rights to pending patent.

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