The Dissertation Committee for Ernesto Lopez Certifies that this is the approved version of the following dissertation:

The role of arginine vasopressin receptor 2 in microvascular hyperpermeability during severe sepsis and septic shock

Committee:

Perenlei Enkhbaatar, M.D., Ph.D.
Supervisor or Mentor, Chair

Jose M. Barral M.D., Ph.D.

Donald S. Prough, M.D.

Robert A. Cox, Ph.D.

Jae-Woo Lee, M.D.

David W. Niesel, PhD.

Dean, Graduate School

The role of arginine vasopressin receptor 2 in microvascular hyperpermeability during severe sepsis and septic shock

by

Ernesto Lopez, M.D.

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Dedication

I would like to dedicate this work to my parents, Ernesto and Beatriz and my brothers Edgardo, Oscar and German for their unconditional support through all these years.

The role of arginine vasopressin receptor 2 in microvascular hyperpermeability during severe sepsis and septic shock

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Supervisor: Perenlei Enkhbaatar, M.D., Ph.D.

Sepsis compromises more than 18 million lives worldwide every year. Moreover, methicillin-resistant *Staphylococcus aureus* (MRSA) sepsis is one of the most severe types. Despite efforts to provide novel therapies for sepsis, the mortality has not decreased in several decades. Sepsis often leads to vascular leakage, and although the hemodynamic management of this condition targets hypotension, currently no specific treatment is available for vascular leakage. Thus, the overall goal of the current study was to investigate the most important mechanisms of vascular leakage during severe sepsis and propose a novel therapeutic strategy for this condition.

Arginine vasopressin (AVP), a hypothalamic hormone, has been used exogenously in sepsis to promote vasoconstriction by activating AVP receptor 1 (V_1R). However, recent evidence suggests that an increased fluid retention may be associated when AVP receptor 2 (V_2R) is simultaneously activated. Hence, we hypothesized that *V_2R activation induces vascular hyperpermeability*. The hypothesis was tested using a well-characterized ovine model of MRSA sepsis and various *in vitro* cell-based assays with human lung microvascular endothelial cells (HMVECs).

Results demonstrated that the treatment of septic sheep with tolvaptan, an FDA-approved V_2R antagonist, significantly attenuated the sepsis-induced fluid retention and markedly reduced the lung water content. These pathological changes were not affected or augmented by the treatment with V_2R agonist desmopressin (DDAVP). Furthermore, the incubation of cultured HMVECs with DDAVP significantly increased the paracellular permeability. Moreover, endothelial cells subjected to MRSA also augmented the endothelial permeability. Finally, both the DDAVP and MRSA induced elevated hyperpermeability were significantly attenuated by the V_2R antagonist tolvaptan (TLVP). Subsequent protein and gene assays determined that the V_2R-induce increase in permeability is mediated by phospholipase C beta 4 (PLCβ-4) and the potent permeability factor angiopoietin-2.

In conclusion, the results of the present work show that endothelial V_2R activation increases vascular permeability during sepsis and perhaps its closer modulation could improve the outcome of these critically ill patients. The results from this clinically relevant animal study might be translated into clinical practice in the near future.

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List of Abbreviations

ACT	Activated clotting time
ADH	Antidiuretic hormone
AJ	Adherent junctions
ALKP	Alkaline phosphatase
AST	Amino-transferase
AP-1	Activator protein 1
BNP	Brain natriuretic peptide
CCD	Charge-coupled device
CI	Cardiac index
CVP	Central venous pressure
DAG	Diacylglycerol
EC	Endothelial cells
ERK1	Extracellular signal-regulated kinases 1
ET	Expiratory time
FBS	Fetal bovine serum
GPCR	G protein-coupled receptors
HF	Heart failure
HMVEC	Human lung microvascular endothelial cell
HR	Heart rate
ICU	Intensive care unit
IP3	Inositol 1,4,5-trisphosphate
JNK	C-Jun N-terminal kinase
LAP	Left atrium pressure
LVSWI	Left ventricular stroke work index
MAP	Mean arterial pressure
MOI	Multiplicity of infection
MRSA	Methicillin-resistant *Staphylococcus aureus*
NCI	Normalized cell index
NF-κB	Nuclear factor kappa B
OD	Optical density
OI	Oxygenation index
PAMP	Pathogen-associated molecular patterns
PAP	Pulmonary artery pressure
PBS	Phosphate-buffered saline
PCOP	Pulmonary capillary occlusion pressure
PEEP	Positive end-expiratory pressure
PIP	Peak inspiratory airway pressure
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKD	Polycystic kidney disease
PLCβ	Phospholipase C beta
PLCB4	Phospholipase C beta 4
PRR	Pattern recognition receptors
p38 MAPK	P38 mitogen-activated protein kinase

PVRI	Pulmonary vascular resistance index
RIPA	Radio immunoprecipitation Assay
RVSWI	Right ventricular stroke work index
SEM	Standard error mean
SIRS	Systemic inflammatory respond syndrome
SSC	Surviving sepsis campaign
SVI	Stroke volume index
SVRI	Systemic vascular resistance index
TF	Tissue factor
TICU	Translational intensive care unit
TJ	Tight junctions
TLR	Toll-like receptors
TPA	Tissue plasminogen activator
VEGF	Vascular endothelial growth factor
WD	Wet-to-dry
WPB	Weibel-Palade bodies

1. INTRODUCTION

1.1. SEPSIS

1.1.1. HISTORY AND EPIDEMIOLOGY

The concept of sepsis is just as old as the medicine itself, although it has had many different names over the years. In the 19th century, with the observations and conclusions of Pasteur, Koch and Lister, the basis of sepsis was elaborated, and the signs and symptoms of sepsis were attributed to infectious agents (1). The criteria and definitions have been constantly changed over the years (1-4).

Sepsis is a global public health problem. Annually, it is estimated to affect more than 18 million people worldwide (5), with more than 700,000 cases in the United States (U.S.) (6). The high incidence of sepsis is frightening, given the fact that the mortality in sepsis is around 30% (40 to 80% in septic shock) (5, 7-10). Although the understanding of the pathophysiology of sepsis has advanced in the last decades, the mortality rate has not decreased, and its incidence is still rising every year (8, 10, 11).

With regard to etiologic agents, Gram-positive sepsis has increased in incidence in the U.S. compared to Gram-negative bacteria with an estimated of 200,000 vs. 150,000 cases every year, respectively (10, 12, 13). As an infection site, the respiratory system is the most common location; half of the cases are associated with a respiratory infection (10).

1.1.2. DEFINITION AND CRITERIA

The guidelines of the most recent surviving sepsis campaign (SSC) describe three stages of sepsis (sepsis, severe sepsis and septic shock) and the systemic inflammatory response syndrome (SIRS) (3, 4, 14).

SIRS is defined as two or more signs of inflammation. The signs of inflammation described by Levy *et al*. in 2003 are hyperthermia, hypothermia, tachycardia, tachypnea, arterial hypotension, increase of venous oxygen saturation, increase of cardiac index, altered mental status, positive fluid balance, hyperglycemia, leukocytosis, leukopenia, and increase of procalcitonin and C-reactive protein (4). Furthermore, indicative signs of organ dysfunction may also be used to diagnose SIRS. The markers of organ dysfunction include arterial hypoxemia, oliguria, dysregulated coagulation, ileus, thrombocytopenia, hyperbilirubinemia, hypercreatinemia, decrease of capillary refill and hyperlactatemia (4).

The critical care guidelines indicate that a patient fulfills the criteria for sepsis when SIRS is present with evidence of an infectious insult. Furthermore, if the criteria for sepsis are coupled to at least one of the above-mentioned signs of organ dysfunction, the diagnosis is severe sepsis (3, 15).

Patients with sepsis receive a generous amount of fluid to avoid a drop in blood pressure and inappropriate organ perfusion. However, when the blood pressure cannot be maintained despite an aggressive fluid resuscitation or if the patient presents

hyperlactatemia, then this patient meets the clinical characteristics of a septic shock (3, 15).

1.1.3. PATHOPHYSIOLOGY

The onset of sepsis begins when an infectious agent interacts with the host. This interaction is mediated by pattern recognition receptors (PRRs), found on the surface of innate immune cells such as macrophages and dendritic cells or other non-immune cells such as endothelial cells involved in initiating an immune response. Various classes of PRRs exist, some of these include toll-like receptors (TLRs), nucleotide-binding oligomerization domain-like receptors, retinoic-acid-inducible gene 1-like receptors and C-type lectin receptors. PRRs on host cells recognize and interact with pathogen-associated molecular patterns (PAMPs) found on microbial pathogens. PAMPs may include lipopolysaccharide derived from Gram-negative bacteria and the peptidoglycan of Gram-positive bacteria (16, 17).

In the host, the activation of the PPR's leads to an inflammatory response characterized by an upregulation of proinflamatory cytokines, type I interferons, chemokines and antimicrobial proteins. Taken together, this represents the activation of the innate immune system, where inflammatory cells recruitment leads to cellular injury and vascular hyperpermeability (16, 18, 19).

A characteristic of severe sepsis is circulatory dysfunction. The massive release of vasoactive substances, especially potent vasodilators such as nitric oxide (NO) and prostacyclin, causes systemic vasodilation leading to hemodynamic instability (20). The excessive production of NO also diminishes the reactivity of the vascular smooth muscle to vasoconstrictors, reducing the capability of the body to modulate the vascular tone (21, 22). In this scenario, the heart takes a crucial compensatory role by increasing cardiac output to maintain blood pressure. However, that extraordinary effort by the heart is frequently insufficient (23).

Another player in the pathophysiology of sepsis is the damaged endothelium. During sepsis, the endothelium releases multiple inflammatory mediators that eventually cause disruption of the endothelial barrier, which leads to vascular hyperpermeability (24).

Parallel to the complications described above, the hemostatic balance is consistently compromised in sepsis which often leads to disseminated intravascular coagulation (DIC) (15). The homeostasis can be affected by abnormal or dysregulated tissue factor activation (TF), anticoagulant mechanisms, or the capability of removing fibrin deposition (fibrinolysis). In sepsis, these three mechanisms are impaired to some degree, and the endothelium is crucially involved in each of these pathogenic pathways (24).

1.1.4. MANAGEMENT

The current management of sepsis in the intensive care unit (ICU) is based on the elimination of the infectious agent and hemodynamic support to preserve an adequate organ perfusion. To approach the infectious agent, it is indicated to promptly use antibiotic therapy in an empiric manner. At the same time, hemodynamic support has to start with a large amount of intravenous fluid in order to maintain adequate urinary output and mean arterial pressure (MAP) above 65 mmHg. These interventions are sufficient for many of the patients discharged from the ICU with minimal morbidities (3, 25).

Nevertheless, in numerous patients, hypotension persists despite resuscitation due to hyperpermeability and reduced arterial resistance. In these refractory patients the only strategy to follow is to continue administering fluid vigorously and to start using adrenergic vasoconstrictors, such as norepinephrine and epinephrine (14, 26).

At this point, the management may aggravate the pathophysiology. The large amount of fluid received accumulates in the interstitial and the transcellular space, leading to tissue edema and subsequent ischemia. Moreover, the adrenergic vasoconstrictors used may augment tissue ischemia (26-28).

Multiple vasoconstrictors can be used to treat septic shock. The SSC recommends norepinephrine, based on its ionotropic-vasopressor nature, and epinephrine as an adjuvant therapy, although ischemia occurs as a side effect in both applications (3, 14). Arginine vasopressin (AVP), a non-adrenergic vasoconstrictor, is also used as adjuvant

therapy. AVP provides a considerable advantage over other vasoconstrictors in terms of tissue ischemia, although it causes a different set of adverse effects, such as cardiac arrest and hyponatremia (28, 29).

Taken together, the management recommended by the SSC in patients with severe sepsis is often insufficient, resulting in multi-organ failure and death. Despite a large number of studies that seek to identify novel therapies targeting specific aspects in the pathophysiology of sepsis, only activated protein C has been approved in clinical settings. However, it was rapidly withdrawn because of its ineffectiveness (3, 14, 28, 30).

In agreement with various publications, the endothelium is an important target to be approached (24, 31). Evidence suggests that the limited effectiveness of AVP may be associated with its adverse effects on the endothelium. Therefore, derivatives of AVP that may selectively block or activate different subtypes of AVP receptors may overcome this adverse effect (31-35). The different AVP receptors and some of the developed compounds targeting these receptors will be discussed in the following sections.

1.2. ARGININE VASOPRESSIN (AVP)

1.2.1. NEUROREGULATION BY ARGININE VASOPRESSIN

Arginine vasopressin (AVP) is a small neuropeptide first isolated in 1952. AVP is a nonapeptide (9 amino acids) with a molecular weight of 1084 Daltons; its biological structure is identical in most mammalians (36). This neurohormone, also called the antidiuretic hormone (ADH), is synthesized by the magnocellular neurons localized in the paraventricular and the supraoptic nuclei of the hypothalamus. The freshly synthesized AVP drains into the supraoptic-hypophyseal tract, which merges into the presynaptic terminals of the posterior pituitary gland. In less than two hours after its synthesis, it arrives in the pituitary gland, where it internalizes into secretory vesicles for storage (37).

The most important physiological function of AVP is the regulation of plasma osmolarity. An increase in plasma osmolarity activates the osmoreceptors (localized in the portal vein and the third cerebral ventricle) and via the vagus nerve the magnocellular neurons are depolarized promoting the release of AVP (38).

Less important in physiology, but highly relevant in cardiovascular shock, is the modulation of AVP-mediated by the baroreceptors. Baroreceptors (localized in the left heart, the aortic arch, and the carotid sinus) are stimulated when the blood pressure drops substantially, signaling for the release of AVP through the same route as the osmoreceptors (38).

AVP is highly involved in the regulation of adrenocorticotrophic hormone (ACTH), and it has been recently shown to be linked to the neurophysiology of complex brain phenomena, such as memory formation, depression and anxiety (36, 37).

1.2.2. ARGININE VASOPRESSIN RECEPTORS

In order to study the effect of either endogenous or exogenous AVP, it is necessary to consider the main physiological processed mediated by its multiple receptors. AVP binds and activates four different G protein-coupled receptors (GPCRs) (38, 39).

The arginine vasopressin receptor 1 (V_1R) is mainly localized in the vascular smooth muscle, where its activation promotes vasoconstriction. However, V_1R has also been shown to be expressed in platelets and liver cells where it is associated with platelet aggregation and glycogenolysis, respectively (9, 38, 39).

Another natural target of AVP is the arginine vasopressin receptor 2 (V_2R). This receptor is localized in both the kidney collecting duct cells and the endothelial cells. In the kidney, V_2R plays an important role in regulating water and electrolyte balance in the organism (38, 39). In contrast, the role of endothelial-V_2R in physiology has not been completely elucidated. It has been speculated that endothelial-V_2R is involved in the modulation of coagulation and perhaps in the endothelial barrier maintenance (35, 39, 40). The understanding of extrarenal V_2R in physiology as well as in certain pathologic

scenarios needs to be extended. We will review more details of V_2R in sections 1.2.3. and 1.4.

As mentioned above, AVP has a regulatory role over ACTH. This regulation is accomplished trough arginine vasopressin receptor 3 (V_3R), localized in the anterior pituitary gland. The activation of V_3R promotes the release of ACTH (9, 38, 39).

Finally, the oxytocin receptor (OTR) is localized in the myometrium and endometrium of the uterus. Similar to V_1R, its activation triggers smooth muscle constriction, although, only in uterine smooth muscle (9, 38, 39).

1.2.3. ARGININE VASOPRESSIN IN SEPSIS

AVP has brought attention to the field of critical care, not only by its wide use as a vasoconstrictor drug, but also because of the important role of endogenous AVP in the pathophysiology of sepsis (42, 43). At the onset of septic shock, it has been reported that AVP increases 64 fold from basal levels (6 fold from maximal physiologic levels), and in late sepsis, is depleted often below the basal levels (42, 44-46).

The documented deficiency of AVP in late sepsis provided a rationale to use exogenous AVP as a replacement therapy, leading to its introduction to clinical trials in 1997 (43, 47, 48). The results of these first clinical trials led to the implementation of AVP as a vasopressor that may reduce the side effects associated with the use of adrenergic vasoconstrictors (45, 49). In 2008, a large clinical trial (the VASST trial) was

conducted to compare the effects of AVP vs. norepinephrine on the overall mortality rate of septic patients (45). Results from the study indicated that AVP was unable to improve the mortality of septic patients compared to norepinephrine-treated patients. The only positive outcome of the VASST study was that a subgroup of patients with less severe septic shock – that received a low or moderate dose of AVP – showed an improvement in survival, suggesting that the undesirable effects of AVP may be dose and possibly receptor dependent (45). To sum it up, beside some minor beneficial results of the VASST study, the recommendation of vasopressin as therapy for septic shock was tuned-down by the SSC, and currently the use of low dose AVP is recommended only as an adjuvant therapy (14, 50).

Considering the mode of action of AVP, its application for the treatment of septic shock is related to its stimulatory effect on V_1R to promote vasoconstriction. However, its activator effect over V_2R is often underestimated (29, 51-53). It has been shown in experimental animal models that treatment with a V_1R selective agonist provides further therapeutic benefit than AVP, which, as mentioned above, also activates V_2R. However from these experimental models it is unclear whether this positive effect in the management of sepsis is given by the activation of V_1R or by the lack of activation of V_2R (32, 34).

There are several lines of evidence suggesting that activation of V_2R during sepsis is detrimental. Inflammatory mediators, such as NO, von Willebrand factor (vWF) and P-selectin, are secreted upon V_2R activation and cause deleterious effects in septic patients,

such as hypotension, coagulation disorders and immune cell extravasation (40, 51, 52, 54).

The link between V_2R activation and vascular leakage has been also speculated in several publications (Figure 1.1.2), although it has not been fully confirmed (32-35, 52). As mentioned in section 1.1.4., there is a great need to identify novel therapies for vascular leakage. Therefore, V_2R-mediated vascular leakage may be a critical element in the development of a potential therapeutic approach.

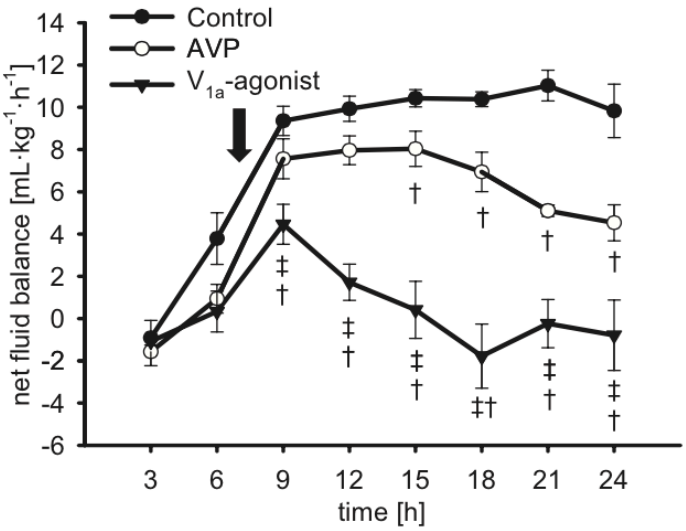


Figure 1.1.2. Fluid net balance reported in septic sheep treated with a V_1R selective agonist or AVP.

Septic sheep were treated with either saline (Control), nonselective V_1R/V_2R agonist AVP or with a V_1R selective agonist (V_{1a}-agonist). Fluid balance was determined by the difference between fluid input and urinary output. ‡p >0.05 vs. AVP; †p >0.05 vs. Control. Study done by Rehberg *et al.* 2012 (33).

1.3. THE ENDOTHELIAL BARRIER AND VASCULAR LEAKAGE

1.3.1. VASCULAR LEAKAGE (INCREASED VASCULAR PERMEABILITY OR VASCULAR HYPERPERMEABILITY)

Vascular leakage is one of the main challenges in the management of sepsis because it has a high correlation with the severity of sepsis. However, understanding of the pathophysiology of vascular leakage remains incomplete (28, 55). Currently, a specific treatment for preventing or reducing vascular leakage is not available in clinical settings, and as described in section 1.1.4., the current management often deteriorates the actual septic condition (33, 56, 57). Hence, it is quite urgent for the critical care field to better understand the pathophysiology of vascular leakage during sepsis and to find a novel therapeutic strategy to approach this complication (55, 58).

1.3.2. PATHOPHYSIOLOGY OF VASCULAR LEAKAGE

In vascular leakage, fluid, proteins and inflammatory cells escape from the intravascular space into the interstitial space and/or to the transcellular space (third space). The formation of this fluid is known as exudate. When it accumulates excessively in the extravascular space, it leads to edema formation (27).

Multiple factors are involved in the migration of molecules in and out of the blood vessels. This concept is better explained by the starling's principle, which denotes that the homeostasis of the vascular fluid depends on the balance between hydrostatic and

oncotic pressures in both the capillaries (intravascular space) and the surrounding interstitium (interstitial space) (59, 60). The endothelial cells (ECs) act as an active barrier, to separate the intravascular space from the interstitial areas, thus allowing the pressures in both compartments to be in balance during physiologic conditions (59, 61).

In sepsis, the endothelial barrier is impaired, which allows water, electrolytes and proteins to move from the capillaries to the interstitium. This flux decreases the oncotic pressure in the capillaries and increases it in the interstitium. In the mean time, an excessive vasodilation slows blood flow, causing stasis. The stasis is associated with an increase in the vascular hydrostatic pressure, which mobilizes more water and protein to be pumped out of the vessels (27, 59, 60). All together, this creates substantial leakage of exudate with deleterious effects in critically ill patients (15, 28).

1.3.3. THE VASCULAR ENDOTHELIAL BARRIER

The vascular endothelial barrier includes endothelial cells, intercellular junctions, and non-cellular components such as glycocalyx and extracellular matrix (62). The integrity of this barrier can be affected either by direct cell damage, by increase in transcellular transport (transcellular leak), or by impairment of the intercellular junctions (paracellular leak). The latter has been thoroughly investigated and described as a major player in vascular hyperpermeability. Therefore, most studies have investigated the pathophysiology of intercellular junctions or cell-to-cell junctions in sepsis (63, 64).

The endothelial cell-to-cell junctions consist of the adherent junctions (AJs) and tight junctions (TJs). Ocludin, junctional adhesion molecules, and claudins are the most important TJs, whereas vascular endothelial cadherin (VE-Cadherin) is considered the predominant component of AJs (62).

1.3.4. MECHANISMS OF ENDOTHELIAL DISRUPTION

It is well-known that the endothelial layer has a key role in the maintenance of circulatory homeostasis and that its disruption is a hallmark in the pathophysiology of sepsis. However, various endothelial alterations leading to impaired vascular permeability need further investigation (24, 58, 65). Here, we will describe some well-elucidated pathways to preserve the intact endothelial barrier during homeostatic balance. We will also detail how these pathways can be disrupted during sepsis and what kind of therapeutic strategy might be proposed to improve endothelial dysfunction.

In endothelial junctions, VE-Cadherin is a particularly important cell-to-cell junction molecule. Under physiological conditions, VE-Cadherin interacts directly with the catenins (ß-catenin and p120-catenin). Catenins are connected with the actin cytoskeleton. Therefore, the association of VE-Cadherin, catenins and actin networks provide a fundamental structure to preserve the endothelial barrier (63, 65, 66).

The internalization of VE-cadherin (by ß-arresting) and its loss of proper junction network occurs by serine phosphorylation, which is a common action of multiple

inflammatory mediators. Both vascular endothelial growth factor (VEGF), a well-characterized permeability factor, and thrombin can signal the serine phosphorylation of VE-cadherin through Src kinase, Vav2 and Rac (63, 66).

The role of the Rho superfamily of GTPases such as, Rac, cell division control protein 42 homolog (Cdc42), and Ras homolog gene family member A (RhoA) in the pathogenesis of endothelial barrier disruption have also been characterized. Rac and Cdc42 have a prominent role in the stabilization of endothelial actin networks. By contrast, signaling of RhoA destabilizes the actin cytoskeleton via cofilin affecting VE-cadherin and the endothelial barrier. Inflammatory players including, thrombin, VEGF and interleukin-1 (IL-1) may also be involved in RhoA signaling and subsequent impairment of endothelial barrier (58).

Promising studies have shown that Simvastatin, an HMG-coA-reductase inhibitor, can inhibit RhoA and activate Rac. Thus, Simvastatin is considered as a promising endothelial barrier promoter (58, 64). Another candidate, sphingosine 1 phosphate (SP1) can also activate Rac preserving cell-to-cell junctions. In the same context, activated protein C has shown to have an antileak effect in an SP1-dependent manner (24, 58, 64).

Angiopoietin-1 and angiopoietin-2 are competitor ligands of the tyrosine kinase receptor 2 (Tie2R) expressed in the endothelial cells. However, they completely differ from one another in origin and function. Angiopoietin-1 is synthesized constitutively in the pericytes and smooth muscle cells. The binding of angiopoietin-1 to Tie2R fosters

cell survival via PI3 kinase/Akt, and it also suppresses the expression and activation of nuclear factor kappa B (NF-κB), a transcription factor that regulates the expression of pro-inflammatory mediators in sepsis (24, 58, 67, 68). Angiopoietin-1 is under investigation as an endothelium-stabilizing agent (58, 69).

In contrast to angiopoietin-1, angiopoietin-2 is produced in the endothelial cells, where it is stored in specialized vesicles termed Weibel-Palade bodies (WPBs). From those vesicles, angiopoietin-2 is released upon endothelial activation. When angiopoietin-2 binds Tie2R, it prevents the physiological function of angiopoietin-1. Therefore, it promotes the upregulation and activation of NF-κB, and halts its cell survival signal. In addition, angiopoietin-2, through RhoA signaling pathways, destabilizes VE-cadherin, and in the presence of VEGF, VE-cadherin endocytosis also occurs (24, 58, 65). Clinically, plasma angiopoietin-2 level correlates well with the prognosis and severity of vascular leakage and sepsis. Therefore, the ratio of angiopoietin-1/angiopoietin-2 has been proposed as an indicator of prognosis in the ICU (52, 65, 70, 71).

1.4 . ARGININE VASOPRESSOR RECEPTOR 2

1.4.1. BIOCHEMISTRY AND FUNCTION

The vasopressin receptor 2 (V_2R) belongs to the GPCRs famlily, and consists of 371 amino acids. It has seven transmembrane domains, including an extracellular N-terminal and an intracellular C-terminal parts (39). The V_2R intracellular domains interact with the G_s protein to stimulate adenylyl cyclase and generate cAMP (72).

Previous studies focused on the V_2R expression in the renal collecting duct cells. The downstream effect of V_2R activation is the elevation of the intracellular cAMP level, which subsequently promotes the phosphorylation of protein kinase A (PKA) and aquaporin 2 (AQP2) to increase the reabsorption of sodium-free water across the nephron (29, 39, 73).

In endothelial cells, V_2R activation has been linked to an increased release of vWF and NO, as well as an elevated activity of tissue plasminogen activator (TPA) and P-selectin. Based on its effect on these molecules, V_2R activation modulates the coagulation cascade, and promotes vasodilation and leucocyte rolling (40, 74-78).

The localization of V_2R in extrarenal locations has received limited attention. Some reports in the literature have described the presence of V_2R in heart, brain, skeletal muscle, and lung. However, its physiological function in those organs remains unclear.

Moreover, recent studies have also demonstrated that V_2R is highly expressed in lung endothelial and epithelial cells (78, 79).

1.4.2. PHARMACOLOGICAL MODULATORS OF V_2R

The endogenous agonist of V_2R is the neurohormone AVP, which activates all three vasopressin receptors mentioned in section 1.2.2. Next to exogenous administration of AVP analogs, which obviously exert the same pharmacological effects as the endogenous hormone, selective vasopressin receptor compounds have also been introduced over the years. Compounds that selectively activate V_2R are 4-valine-8-D-arginine vasopressin (VDAVP), 1-deamino [4-valine-8-D-arginine]-vasopressin (dVDAVP) and, 1-deamino-8-D-arginine vasopressin (DDAVP or desmopressin). Among those agents, DDAVP is better known because it is commonly used for the treatment of diabetes insipidus and von Willebrand's disease (VWD) (77). DDAVP has 2.7 times higher affinity to V_2R vs. V_1R, and its half-life is only 1 – 2 hours vs. 10 to 15 minutes of AVP half-life (39, 80).

More recently, scientists have developed non-peptide analog compounds to block V_2R. This new class of medications are called "vaptans". A list of vaptans is currently in various stages of investigation, including large clinical trials (73, 81). The vaptans were first used to treat hyponatremia, and later utilized for the treatment of heart failure (HF) and polycystic kidney disease (PKD). Two compounds are currently approved by the FDA, conivaptan and tolvaptan (41).

Conivaptan is a V_1R/V_2R antagonist approved for the treatment of hyponatremia, and increasing evidence suggests its high efficacy to treat more complex disorders such as stroke associated edema (81). In contrast, tolvaptan (TLVP) is a selective V_2R antagonist with 1.8 times greater affinity to V_2R than AVP, and 29-fold greater to V_2R than V_1R (82, 83). TLVP is currently used in liver cirrhosis, congestive heart failure, polycystic kidney disease and in other conditions associated with hyponatremia (82, 83). Furthermore, TLVP has not only shown to treat hyponatremia efficiently, but it proved to be beneficial for the improvement of various hemodynamics parameters, such as heart function and arterial pressures (84). Recent evidence also suggests the application of TLVP to treat acute heart failure and to preserve renal function (85). The clinical applications for TLVP are increasing, as it is also clinically approved by the European Union (European Medicine Agency) and Japan (Pharmaceuticals and Medical Devices Agency) (81).

1.5. AIMS AND HYPOTHESES

The overall goal of the current study was to investigate the mechanisms of vascular leakage during severe sepsis and propose a novel therapeutic approach for this condition. This study was mainly focused on the role of V_2R receptor activation in vascular hyperpermeability in a methicillin-resistant *Staphylococcus aureus* (MRSA) sepsis model.

Our overall hypothesis was that, in sepsis, *V_2R activation induces vascular hyperpermeability*. To investigate this hypothesis, we conducted *in vivo* experiments using a highly translational ovine model of MRSA pneumonia/sepsis and *in vitro* assays conducted in human lung microvascular endothelial cell (HMVEC) cultures. To test our hypothesis we used both V_2R agonist (desmopressin) and antagonist (tolvaptan).

Finally, we aimed to elucidate the downstream mechanisms of V_2R activation during vascular leakage. Two sub-hypotheses emerged. 1) *Activation of V_2R promotes hyperpermeability by the release of angiopoietin-2* and, 2) *MRSA-induced hyperpermeability is mediated by V_2R stimulation*. These sub-hypotheses were tested in various *in vitro* assays using HMVECs.

This present study is the first that describes the beneficial role of V_2R inhibition in MRSA sepsis. The data generated by both *in vivo* and *in vitro* models offers highly relevant approaches for sepsis management.

2. Materials and Methods

2.1. Materials

2.1.1. Animals

We used adult female merino sheep between 30 and 40 kg (mean, 33.7 ± 3.1 kg). The use of animals and procedures were carefully approved and supervised by the Institutional Animal Care and Use Committee of the University of Texas Medical Branch and were in compliance with the guidelines established by the U.S. Department of Agriculture Animal Welfare Act and the National Institutes of Health/ Office of the Laboratory Animal Welfare.

2.1.2. Human lung microvascular endothelial cells (HMVECs)

Commercially available HMVECs (Cat# 540-05a, Cell Applications, Inc., San Diego, CA) were subcultured using microvascular endothelial growth media (Cat# 111-500, Cell Applications, Inc., San Diego, CA) and according to vendor's instructions. In brief, cryopreserved cells were revived from passage 3 and were expanded to obtain enough number of cells to use. Various publications have described changes in morphology and lack of expression of endothelial markers in later passages (86), therefore experiments were performed with HMVECs between passage number 5 and 7. The expression of PECAM-1/CD31, VEGF and von Willebrand factor was assessed by the vendor and confirmed by our laboratory (Figure 3.2.1.).

2.1.3. DRUGS

In vivo experiments: Desmopressin (DDAVP) (Ferring Pharmaceuticals, Saint Prex, Switzerland); Up to 32 μg of DDAVP was diluted in 1 L 0.9% sodium chloride solution (saline). Tolvaptan (TLVP) (Biorbyt Ltd; Cambridge, UK); Up to 400 mg of TLVP was dissolved in 1 L of saline solution containing 5% polysorbate 80 and 0.1% dimethyl sulfoxide (DMSO). The formulations were prepared freshly on the day of the experiment and was adjusted to the body weight.

In vitro assays: DDAVP (Cat#SC-391876, Santa Cruz Biotechnology, Dallas, Texas) was re-suspended in water to reach a concentration of 3000 nM, and the aliquots were frozen at -20 ºC and stored until the day of the experiment. Using the same preparation as in the *in vivo* experiments, 1 M TLVP was prepared on the day of the study and then, diluted in culture media to have a 10 nM final concentration in the assay. The concentration of DDAVP and TLVP were selected following a dose-response test and in agreement with previous work (73, 78, 87). Recombinant Human VEGF (Cat#100-20, Peprotech, Rocky Hill, NJ) was re-suspended in culture media with 0.1% fetal bovine serum (FBS) (Cat#F2442, Sigma-Aldrich, St. Louis, NO) as the vehicle. Aliquots of 1 μg/mL were frozen at -20 ºC and stored until the day of the experiment.

2.1.4. METHICILIN-RESISTANT STAPHILOCOCUS AUREUS (MRSA)

Commercially available MRSA bacteria, (Strain USA300 / TCH1516, ATCC, Manassas, VA) were grown in an orbital shaker incubator at 37 ºC with agar media. A standard curve was performed to correlate the optical density (OD) with the bacteria

growth determined as colony forming units per mL (CFU/mL). The day of the experiment, the OD of bacterial suspension was measured, and the concentration of bacteria determined based on the OD vs. CFU/mL standard curve obtained previously. Dilutions were prepared according to the number of bacteria required. Finally, the bacterial suspension was washed two times; centrifuge at 4,000 RPM (Centrifuge 5810R, Eppendorf, Hamburg, Germany) for 10 minutes and re-suspended in phosphate-buffered saline (PBS). The final concentration for the *in vivo* experiments was $3.5x10^{11}$ CFU suspended in 30 mL of PBS. For the *in vitro* assays, a solution containing $2x10^{9}$ CFU suspended in 5 mL of PBS was used as stock. The proportion of the stock solution was mixed with culture media minutes before the bacteria were exposed to the endothelial cells.

2.2. IN VIVO MODEL

2.2.1. OVINE MRSA SEPSIS MODEL

2.2.1.1. Surgical preparation

The animals were surgically prepared according to our well-characterized *in vivo* model (88). Under deep anesthesia (3 to 5 % isoflurane) and analgesia (1.7 mg buprenorphine) and rigorous aseptic conditions, the animals were surgically prepared to place catheters in the pulmonary artery (Swan-Ganz) (79F; Edwards Lifesciences LLC, Irvine, CA), the femoral artery (16-GA, 24 inches; Becton Dickinson, Franklin Lakes, NJ) and the left atrium for continuous hemodynamic monitoring, blood sampling and administration of drugs and fluid.

2.2.1.2. Injury: Smoke inhalation plus bronchoscope directed MRSA instillation

After the surgery, the animals were allowed to recover for five days under analgesia and with *ad libitum* access to water and food in our translational intensive care unit (TICU) facility. The day of the experiment, baseline measurements were taken and then the sheep were randomly allocated to be used as sham or subjected to sepsis-injury. Following the insertion of a cuffed tracheostomy tube (10 mm diameter, Shiley, Irvine, CA, USA) and a urinary bladder catheter (Foley Catheter, 14Fr; BARDEX®, Covington, GA), 48 breaths of cooled cotton smoke were insufflated followed by instillation of MRSA ($3.5X10^{11}$ CFU) into the lungs by bronchoscope via the tracheostomy tube (89). The procedure was conducted under deep anesthesia and analgesia. If within 24 hours of the study an animal met the standardized criteria of severe organ failure (an hour minimum of: PaO_2 <50 mmHg, MAP < 50mmHg or CO_2 < 90 mmHg) (90), then, the animal was euthanized and counted as a fatality. Otherwise, the animals were euthanized after 24 hours of study and the organs processed immediately as previously described (91, 92).

2.2.1.3. Grouping and treatments

Twenty-five animals were randomly divided into one of the four study groups. Sham group (n=6) was not subjected to an injury and was treated 35 mL/hour of saline. The Control group (n=7) received the injury previously described and the same treatment as Sham. DDAVP (n=6) and TLVP (n=6) groups were injured and received 38 ng/kg/hour DDAVP and 435 µg/kg/hour TLVP respectively. The treatments were

administered an hour after the injury as continuous i.v. infusion over 23 hours. The dose of DDAVP was chosen based on previous studies from our laboratory, showing a V_2R stimulation comparable to 0.03 U/minute AVP (34, 51). The dose of TLVP was adapted from published studies (93, 94).

2.2.1.4. Resuscitation and ventilatory management

After the injury, sheep were awakened and placed on mechanical ventilation (AVEA™ Ventilator; Carefusion, Yorba Linda, CA). The cardiopulmonary variables were monitored for 24 hours in a conscious state. A volume/pressure control mode was used with a positive end-expiratory pressure (PEEP) of 5 cmH_2O and a tidal volume (V_t) of 12 mL/kg. During the first 3 hours after the injury the FiO_2 was maintained at 100% in all animals, and then titrated to maintain the arterial PaO_2 close to 100 mmHg. The respiratory rate started at 20 breaths/minute in all animals and was further adjusted to ensure a $PaCO_2$ between 25 and 35 mmHg. The animals were resuscitated with Ringer's lactate starting with 2 mL/kg and then titrated based on a standard protocol to maintain the hematocrit ± 3% from baseline values (89). Administration of resuscitation fluid was provided with an i.v. infusion pump (MicroMacro XL3 Plum XL3, Abbot laboratories, Chicago, IL).

2.2.2. NORMAL RANGE OF PHYSIOLOGIC PARAMETERS

The physiology in sheep closely relates to humans and even though most of the physiologic parameters of our interest have been described in the veterinary literature, we considered informative to determine the normal range of the parameters of our interest

(95-97). The normal ranges described in Table 2.1.1. were determined from the baseline measurements of 162 sheep prior to being studied at the TICU. In agreement with the surviving sepsis campaign guidelines (3, 14), the normal range was defined as ± 2 standard deviations above and below the mean in normally distributed samples.

Parameter	Range		Unit	Parameter	Range		Unit
Blood Temp.	38.4	39.9	°C	PaCO2	28.5	50.7	mmHg
CO	3.1	7.1	L/minute	apH	6.9	7.9	pH
HR	53.6	119.3	bpm	$aHCO_3$	23.5	33.0	mEq/L
MAP	79.5	115.9	mmHg	SaO_2	88.0	94.3	%
PAP	14.2	24.9	mmHg	aCOHb	2.8	8.9	%
PCOP	5.2	16.3	mmHg	PvO_2	28.5	59.3	mmHg
LAP	3.0	12.4	mmHg	$PvCO_2$	30.3	59.5	mmHg
CVP	2.0	10.8	mmHg	SvO_2	42.9	74.4	%
HCT	19.9	34.5	%	Plasma Na	140.6	150.8	mEq/L
HGB	6.9	12.4	g/dL	Plasma K	3.0	4.4	mEq/L
Plasma Prot.	5.0	7.9	g/dL	Plasma Ca	1.0	1.3	mEq/L
PaO_2	88.2	119.8	mmHg				

Table 2.1.1. Normal range in sheep at baseline.

2.2.3. PULMONARY MECHANICS AND GAS EXCHANGE

As previously described, the pulmonary gas exchange was evaluated by calculating the PaO_2/FiO_2 ratio, the pulmonary shunt fraction (Q_s/Q_t) and the pulmonary oxygenation index (OI) (98-100). OI= (FiO_2 X P_{aw} X 100) / (PaO_2). The pulmonary mechanics were evaluated with the mean airway pressure (P_{aw}) (101), determined with the peak inspiratory airway pressure (PIP), the pulmonary end-expiratory pressure (PEEP), the inspiratory time (IT) and the expiratory time (ET). P_{aw}= PEEP + ((PIP - PEEP) x (IT)) / (IT + ET).

2.2.4. ASSESSMENT OF HEMODYNAMICS

Central venous pressure (CVP), pulmonary artery pressure (PAP), pulmonary capillary occlusion pressure (PCOP), left atrium pressure (LAP), mean arterial pressure (MAP) and heart rate (HR) were recorded by hemodynamic monitors (IntelliVue MP50, Philips Medizin Systeme Boeblingen, Boeblingen, Germany) via pressure transducers connected to the vascular catheters. The lung hydrostatic pressure (P_c) was calculated with a standard equation: P_c= (0.6 X PCOP) + (0.4 X PAP). The thermodilution technique was used for determination of cardiac output (PX1800, Edwards Lifesciences LLC, Irvine, CA) and core blood temperature. Cardiac index (CI), left ventricular stroke work index (LVSWI), right ventricular stroke work index (RVSWI), systemic vascular resistance index (SVRI), pulmonary vascular resistance index (PVRI) and stroke volume index (SVI) were calculated using a standard equations previously used (102).

2.2.5. BLOOD PARAMETERS AND BIOMARKERS

Blood samples were taken every three hours, processed as fresh heparinized blood or as plasma or serum and stored at -20 °C until termination of the study. Fresh heparinized blood was used to determine PO_2, CO_2, pH, HCO_3, carboxyhemoglobin (COHb), SO_2, hemoglobin, glucose, lactate and electrolytes using a blood gas analyzer (GEM Premier 3000; Instrumentation Laboratories, Lexington, MA), hematocrit using microhematocrit capillary tubes (Fisher Brand, Pittsburgh, Pa), activated clotting time (ACT) using a whole blood coagulation system (HEMOCHRON 801; International Technidyne Corporation, Piscataway, NJ) and complete cell count using a hematology system (Hemavet 850™ ; Drew Scientific, Inc.,Oxford, CT). Thawed plasma samples were used to measure total protein (Refractometer. National Instrument, Baltimore, MD), nitric

oxide (NO) (Nitrates/nitrites, Colorimetric Assay Kit, Cayman Chemicals Company, Ann Arbor, MI, USA) and arginine vasopressin (AVP) (AVP Chemiluminescent Immunoassay kit; Arbor assays, Ann Arbor, MI). Thawed serum samples were used to measure creatinine (Spectrophotometric Two-point Rate Reaction), urea nitrogen (Colorimetric assay), unconjugated bilirubin (Colorimetric assay), total bilirubin (Colorimetric assay), alanine aminotransferase (ALT) (Enzymatic, Multi-point Rate), alkaline phosphatase (ALKP) (Enzymatic, Multi-point Rate), aspartate amino-transferase (AST) (Enzymatic, Multi-point Rate), brain natriuretic peptide (BNP) (N-terminal pro-BNP Chemiluminescence assay) and albumin (Colorimetric assay).

2.2.5. URINE ANALYSIS

Urine output was continuously collected with the urinary bladder catheter (Foley Catheter, 14Fr; BARDEX®, Covington, GA) both for an accurate assessment of the fluid balance and to collect urine samples for further analysis. Urine was collected and quantified every 3 hours and a sample taken and frozen immediately. After termination of the study, the urine samples were thawed and screened for urine osmolality (Freezing Point Depression), urine sodium and potassium (Potentiometric) and urine creatinine (Spectrophotometric Two-point Rate Reaction).

2.2.6. WESTERN IMMUNOBLOTTING OF LUNG AND HEART TISSUES

Sheep heart and lung tissues were homogenized in tissue protein extraction reagent (T-PER™) (Cat#78510, Thermo Fisher Scientific Inc., Waltham, MA). Both assay buffers were supplemented with proteinase inhibitor cocktail (Cat# P8340, Sigma Aldrich, St. Louis, MO), and phosphatase inhibitors (Phenylmethanesulfonyl fluoride;

Sigma-Aldrich, St. Louis, MO). Lysates were diluted in Laemmeli denaturing loading buffer (Biorad, Hercules, CA), sonicated, boiled and resolved on 4–12% Criterion XT Bis-Tris acrylamide gels (Biorad, Hercules, CA) and transferred to PVDF membranes to immobilize the proteins. Then, membranes were blocked with Starting Block™ T20 (Thermo Fisher Scientific Inc., Waltham, MA) and were probed overnight with anti-arginine-vasopressin-receptor-2 antibodies (1:1000, Cat#ab109326, Abcam, Cambridge, MA). Anti-rabbit-horseradish peroxidase conjugate (HRP, 1:3000, Abcam, Cambridge, MA) and enhanced chemiluminescent substrate (Pierce ECL) (Thermo Fisher Scientific Inc., Waltham, MA) were used to detect the chemiluminescent signal on a charge-coupled device (CCD) camera–based detection system (GBox;Syngene USA, Frederick, MD). To normalize the signal, membranes were re-probed with anti-beta-actin antibody overnight. The anti-arginine vasopressin receptor 2 and beta-actin at 40 kDa were detected. The intensity of Western blot signals was quantified by densitometry using ImageJ 1.45s software (U.S. National Institutes of Health, Bethesda, MD).

2.2.7. SIMPLE WESTERN IMMUNOBLOTTING

Lung and heart tissues were homogenated and the protein denaturated with the same methodology from section 2.2.6. The samples were analyzed with a Simple Western instrument for protein quantification (Simon™ , ProteinSimple, Santa Clara, CA) using a protein quantification kit (Rabbit 12-180 kDa master kit for Simon, Cat#Simon-01-01, ProteinSimple, Santa Clara, CA). In short, we loaded a 384-well plate according to the manufacturer's instructions using 5 μg of protein diluted in Laemmeli denaturing loading buffer (Biorad, Hercules, CA) mixed with fluorescent standards and primary anti-angiopoietin-2 antibody (Cat# ab8452, Abcam, Cambridge, MA) diluted in antibody

diluent buffer at a 1:20 ratio and mixed with anti-actin (Cat#A2066, Sigma-Aldrich, St. Louis, NO) diluted at the same ratio. The chemiluminescence signal representing the amount of protein that was integrated and analyzed with Compass software Version 2.7.1 (ProteinSimple, Santa Clara, CA) (103).

2.2.8. LUNG WET-TO-DRY WEIGHT RATIO AND BRONCHOALVEOLAR LAVAGE FLUID

To determine lung water content or lung edema by the wet-to-dry ratio (W/D), 960 ± 182 milligrams of lung tissue were weighted before and after the water evaporated at 60 °C in a vacuum oven (91). In regards to the bronchoalveolar lavage fluid (BALF), during the autopsy, the left lung was separated and using a urinary catheter, 100 mL of saline were infused into the lung with a 60 mL syringe. The lung was then gently shacked for 10 seconds and finally 40 to 60 mL of the solution was withdrawn with the same syringe. The solution was finally centrifuge at 4,000 RPM (Centrifuge 5810R, Eppendorf, Hamburg, Germany) for 10 minutes and the supernatant frozen at -80 °C. Protein concentration in BALF was determined with a BCA protein assay kit (Cat#23225, Thermo Fisher Scientific Inc., Waltham, MA).

2.3. IN VITRO ASSAYS

2.3.1. IN VITRO VASCULAR PERMEABILITY ASSAY

An *in vitro* vascular permeability assay was established and optimized using a previously described method with minor modifications (86). Three μm pore size transwell inserts (Cat# 354575, Thermo Fisher Scientific Inc., Waltham, MA) were coated with sterile extracellular matrix (Matrigel™, Cat# 354234, Thermo Fisher

Scientific Inc., Waltham, MA) and incubated at 37 °C. Then, 200,000 of passage 5 HMVECs were seeded during two consecutive days. The cells were then allowed to form a tight monolayer during 48 hours. In this model, the extracellular matrix over the multipore membrane represents the basal membrane and the microvascular endothelial cells seeded at a high concentration form a tight endothelial monolayer. The system together represents a microvascular vessel. These *in vitro* microvascular vessels were challenged with different pharmacological agents, MRSA or the combination of both according to Table 2.3.1.

On the day of the study, the culture media was aspirated from the wells and the pretreatment (TLVP or starvation media) was added and 30 minutes later it was replaced by the indicated treatment. Fluorescent dextran (FITC-Dextran, Thermo Fisher Scientific Inc., Cat# D-3306, Waltham, MA) at a concentration of 10 μg/mL was added to the lower chamber in combination with the treatment. Every 30 minutes after the treatment was initiated, 20 μL of supernatant media were collected from the upper chamber and placed in a black plate (Costar, Cat#06-443-2, Thermo Fisher Scientific Inc., Waltham, MA) containing 90 μL of water per well (Figure 2.3.1.). The amount of fluorescent dextran in the media was quantified with fluorometer reader (Powerwave HT, Biotek, Winooski, VT) with excitation of 485 nm and emission of 535 nm.

The concentrations of DDAVP and TLVP were adapted from the work previously done (73, 78, 87) and confirmed in our laboratory with a dose response test (data not shown). Treatments or pretreatments were conducted in starvation media using a volume of 1 mL for the lower chamber and 300 μL for the upper chamber. For starvation media, we used basal media (Cell Applications Inc., Cat#100-500, San Diego, CA) with 1% of

FBS (Cat#F2442, Sigma-Aldrich, St. Louis, NO) and as the vehicle we used starvation media with 0.001% DMS0 and 0.01% polysorbate 80. All treatments and pretreatments were incubated at 37 °C and 5% CO_2 prior to cell exposure. For the wells exposed to MRSA, we targeted a multiplicity of infection (MOI) of 15, therefore we applied $3x10^7$ CFU MRSA suspended in 50 µL of starvation media. The smaller volume used over the insert facilitates the interaction of the cells with the bacteria. Cells and bacteria were allowed to interact during 6 hours before the permeability was evaluated with the fluorescent dextran polymers.

Group	Pre-treatment	Treatment
Control	Vehicle	Vehicle
DDAVP	Vehicle	10 nM TLVP + 300 nM DDAVP
TLVP	TLVP 10 nM	10 nM TLVP
TLVP + DDAVP	TLVP 10 nM	10 nM TLVP + 300 nM DDAVP
MRSA	Vehicle	3 X 10^7 CFU MRSA
TLVP + MRSA	TLVP 10 nM	10 nM TLVP + 3 X 10^7 CFU MRSA
MRSA + DDAVP	Vehicle	3 X 10^7 CFU MRSA + 300 nM DDAVP
TLVP + MRSA + DDAVP	TLVP 10 nM	10 nM TLVP + 3 X 10^7 CFU MRSA + 300 nM DDAVP

Table 2.3.1. Treatment groups used with the *in vitro* vascular permeability assay.

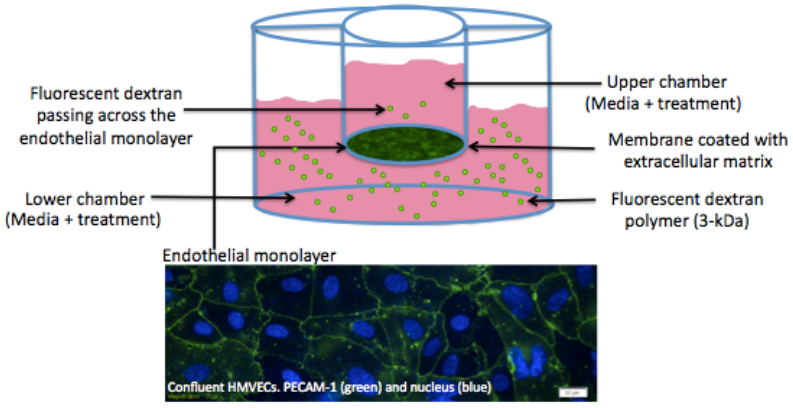


Figure 2.3.1. 3D cell culture model of the *in vitro* endothelial vascular permeability assay.

2.3.2. ELECTRICAL IMPEDANCE ASSAY

Based on previous work (104, 105), HMVECs were seeded in a 96-well cell culture plate equipped with gold electrodes (E-plate, Cat#05232376001, ACEA BIO, San Diego, CA) at a concentration of 15,000 cells per well. The electrical impedance was measured every 15 minutes by cell-microelectronic sensing (XCELLigence™ RTCA, ACEA BIO, San Diego, CA). When cells became confluent (indicated by a plateau of electrical impedance), the cells were incubated with starvation media for 2 hours followed by replacement with media containing DDAVP, VEGF or only starvation media. The electrical impedance continued to be recorded every 15 minutes during 12 hours.

2.3.3. LACTATE DEHYDROGENASE (LDH) ACTIVITY ASSAY

As previously described (106), 30 μL of culture media were collected at each time point (1, 3 and 6 hours for this study) and stored at 4 ºC until the last sample was collected. The culture media sample was mixed with 100 µl of LDH assay reagent constituted with 85 mM lactic acid, 1040 mM nicotinamide adenine dinucleotide, 224 mM N-methylphenazonium methyl sulfate, 528 mM 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride and 200 mM Tris-pH 8.2. Immediately after the culture media was mixed with the LDH reagent, the optical density was read with a monochromator-based reader (Powerwave HT, Biotek, Winooski, VT) at 492 nm during 15 minutes of incubation at 37 ºC. Using the Michaelis-Menten formula, the LDH activity was determined by calculating the maximum velocity achieved by the system

(V_{max}). The higher the $\%V_{max}$ indicated by the assay, the higher the LDH activity was in the cells investigated.

2.3.4. PROTEIN DETERMINATION IN CELL CULTURE SUPERNATANT.

HMVECs were allowed to grow until confluence in 25 cm^2 cell culture flasks. The day of the experiment, the culture media was replaced with 2.6 mL of starvation media with or without treatment. Then, supernatant was collected, mixed with 1.4 mL of PBS and 40 μL of proteinase inhibitor cocktail (Cat# P8340, Sigma Aldrich, St. Louis, MO), added into a 4 mL ultra filter for protein concentration (Amicon Cat# UFC8-003-24, EMD Millipore, Darmstadt Germany) and centrifuge for 60 minutes at 4,000 RPM (Centrifuge 5810R, Eppendorf, Hamburg, Germany). Approximately 150 μl of concentrated supernatant was achieved with this method, which represents a 27-fold concentration increase. The treatment tested was 300 nM DDAVP and was administered during 1, 3 or 6 hours. Culture media without exposure to cells was also concentrated under the same conditions and was used as additional control (negative control). This negative control is important because the protein of interest, angiopoietin-2, is often found in the culture media. The protein concentration in the concentrated samples was determined with a BCA protein assay kit (Thermo Fisher Scientific Inc., Cat#23225, Waltham, MA) and then diluted in PBS to a concentration of 1.4 μg/μL. Finally, the concentration of angiopoietin-2 was determined with simple western immunoblotting as described in 2.2.7. In this method our protein of interest (angiopoietin-2) was undetected in the negative control samples.

2.3.5. QUANTITATIVE REAL TIME POLYMERASE CHAIN REACTION

Following the different experimental treatments, RNA was isolated using an RNA isolation kit (RNeasy® Mini Kit, Cat# 74106, Qiagen, Valencia, CA). Briefly, culture media was aspirated, and lysis buffer was applied to the culture plates at a concentration of 79 μL/cm^2. The cell lysate was collected and placed into a centrifuge tube, mixed with 70% ethanol (1:1 concentration) vortex for 2 minutes and then transfered into a spin column and processed as indicated by the manufacturer. The RNA was diluted in RNase-free H_2O, 0.075 μL of H_2O for each μL of cell lysate used. The RNA concentration and quality was determined by measuring the OD with a nanodrop spectrophotometer (Biotek, Winooski, VT).

To convert RNA into cDNA, 500 ng of RNA diluted in 16 μL of RNase-free H_2O were mixed with 4 μL of iScript™ RT supermix (Cat# 170-8841, Bio-Rad, Hercules, CA). The reaction mixture was incubated using a thermal cycler (T100, Bio-Rad, Hercules, CA) according to the specification provided by the manufacturer (5 minutes at 25ºC, 30 minutes at 42ºC and 5 minutes at 85ºC)

To quantify the expression of the genes of interest we performed quantitative real-time polymerase chain reaction (RT-qPCR) using PrimePCR™ assays previously validated (Cat# 10025636, Bio-Rad, Hercules, CA) and optimized based on previous studies (107). Each sample was measured in duplicate using a 10 μL reaction containing 4 μL cDNA [2.5 ng/μL], 5 μL SsoAdvanced™ Universal SYBR® Green Supermix (Cat# 172-5270, Bio-Rad, Hercules, CA) and 1 μL PrimePCR™ assay [250 nM]. The 96-well plates were then run in a CFX96 RT-qPCR detection system (Cat# 10025636, Bio-Rad, Hercules, CA) with 30 seconds at 95°C for activation and 45 amplification cycles for 15

seconds at 95°C and 60 seconds at 60°C. Under these conditions, we measured the gene expression using PrimePCR™ assays for beta actin (ACTB) (UniqueAssayID: qHsaCID0017615), angiopoietin-2 (ANGPT2) (UniqueAssayID: qHsaCED003626) and phospholipase beta 4 (PLCB4) (UniqueAssayID: qHsaCID0014933).

Relative normalized expression of genes of interest was determined by relative quantification using the threshold cycle (C_T) of interest gene vs. reference gene with the equation $C_T(\Delta\Delta C_T)$. The calculations were done using CFX Manager™ Software (Bio-Rad, Hercules, CA). ACTB was the reference gene, which showed to be unchanged after the treatments. TATA box binding protein (TBP) and hypoxanthine phosphoribosyltransferase 1 (HRP1) were also evaluated as reference genes, although CFX Manager™ determined higher efficacy for ACTB.

2.3.6. WESTERN BLOT IMMUNOBLOTTING OF CELL LYSATE

HMVEC cells were lysed in Radio-Immunoprecipitation Assay Buffer (RIPA) (Cat# 89900, Thermo Fisher Scientific Inc., Waltham, MA) and process and analyzed by the same method described in 2.2.6.

2.3.7. IMMUNOFLUORESCENCE LABELING

HMVECs were cultivated in the transwell multi-pore membrane in same conditions described in 2.3.1. The method was adapted from a well-characterized assay (86). The media was aspirated and the membranes washed twice with pre-warmed DPBS and fixed with 4% paraformaldehyde for at least 30 minutes at 4 ºC. Then, the membranes were washed in the following order: DPBS: 10 minutes (twice), DPBS + 1 M glycine: 10 minutes, DPBS: 10 minutes (twice) and DPBS + 10% goat serum: 30

minutes. The membranes were then incubated overnight with the desired primary antibody diluted in DPBS + 1% BSA. The next day, the membranes were washed in the following order: DPBS + 1% BSA: 10 minutes (three times), DPBS + 1% BSA + Secondary antibody: 60 minutes and, DPBS + 0.1% BSA: 10 minutes (three times). The membranes were then carefully separated from the insert and mounted on a glass slide with Vectashield antifade mounting medium containing 4', 6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Cat# H-1200, Burlingame, CA) and analyzed with a fluorescent microscope (Olympus America, Center Valley, PA).

2.4. STATISTICAL ANALYSIS

Results were compared by two-way analysis of variance. Scores or measurements taken at a single time point were compared using one-way analysis of variance. To compare the difference among groups we used a "Sidak-Bonferroni post hoc" test. No other adjustments were made for the multiple comparison tests. Scores or measurements taken at a single time point were compared using Student *t* test. All values are expressed as mean ± standard error of mean (SEM). Differences were considered significant when the p-value was smaller than 0.05. The statistical methodology was conducted, accordingly (98).

3. RESULTS

3.1. The effect of a vasopressin receptor 2 selective agonist and antagonist on an ovine model of methicillin-resistant *Staphilococcus Aureus* pneumonia/sepsis

We have thoroughly investigated the role of V_2R activation during sepsis using an ovine model of MRSA pneumonia/sepsis. This translational ovine model has been well-characterized by our research group (89). The principal aim of our study was to elucidate various pathophysiological responses attributed to V_2R activation and inhibition. The sheep that developed sepsis were treated with V_2R agonist or antagonist and were compared to untreated septic sheep or uninjured sheep.

Recent studies in our experimental animal model showed that treatment with a selective V_1R agonist provides further therapeutic benefits than nonselective AVP, a V_1/V_2 R agonists on its own (33, 34, 54). However, based on these results it was unclear whether the beneficial effect of administrating a selective V_1R agonist was attributed to the activation of V_1R or to the lack of V_2R activation. For this reason, it appears to be necessary for the critical care field to define the role of V_2 receptor activation/inhibition in septic conditions. Therefore, in this study we focused on the role of V_2 receptor in vascular leakage during sepsis utilizing an *in vivo* ovine model and several *in vitro* endothelial cell-based assays.

3.1.1. After smoke inhalation injury the arterial carboxyhemoglobin (aCOHb) levels proved to be similar in all injured groups

All injured groups had a similar aCOHb level after smoke inhalation. The mean in the Control group was 71.3 ±7.8 %, compared to 74.2 ± 5.2 % in DDAVP and, 82.8 ± 3.5 % in TLVP groups (p=0.4). Sham group had a mean aCOHb of 5.4 ± 0.3 %, p>0.0001 vs. any group.

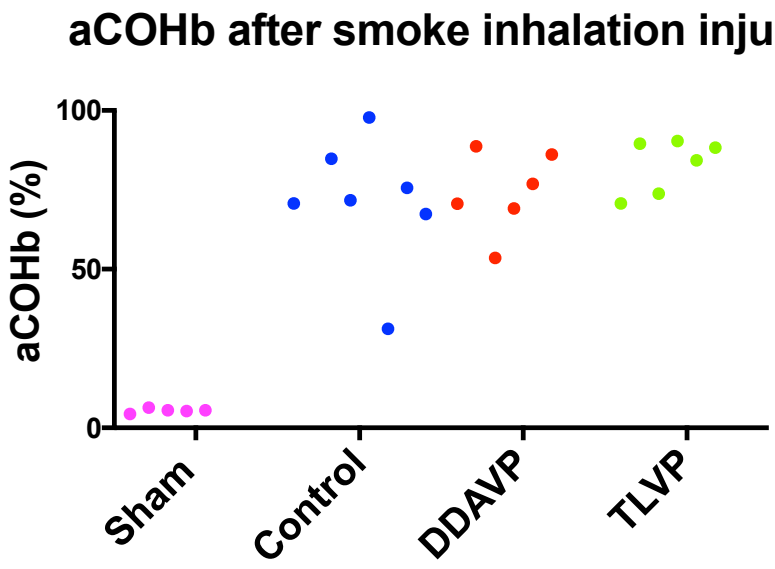


Figure 3.1.1. Arterial COHb level after smoke inhalation.

In the scatter plot, each dot represents one individual value in Control (Blue), DDAVP (Red), TLVP (Green) and, Sham group (Pink).

3.1.2. Mortality rate of the TLVP group was superior vs. Control group

Sheep were subjected to acute MRSA pneumonia/sepsis and monitored for 24 hours. All 25 studied animals survived the first 12 hours (Control 7/7, DDAVP 6/6, TLVP 6/6 and Sham 6/6). By 15 hours, the Control group had two fatalities (Control 5/7,

DDAVP 6/6, TLVP 6/6 and Sham 6/6). By 18 hours, the Control group had one more fatality (Control 4/7, DDAVP 6/6, TLVP 6/6 and Sham 6/6). By 21 to 24 hours, TLVP had one fatality and the Control group had its fourth fatality. Therefore, in the Control group just 3 out of 6 animals survived 24 hours compared to TLVP and DDAVP where 5 out of 6 and 6 out of 6 survived respectively. In the Sham group, 6 out of 6 animals survived 24 hours as well. In other words, by 24 hours the Control group showed the highest mortality rate (57%). Moreover, there was 17% mortality in the TLVP group while no mortality was detected in DDAVP and Sham groups.

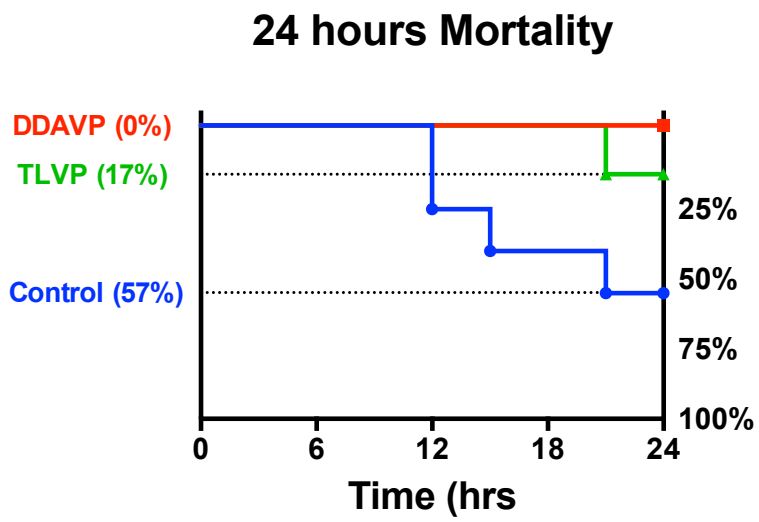


Figure 3.1.2 Time course of mortality rates in control, DDAVP and TLVP sheep subjected to MRSA pneumonia/sepsis.

3.1.3. Injured groups developed sepsis by 3 hours and septic shock by 9 hours after the injury.

Based on the established guidelines for sepsis (3, 4), after 3 hours all injured groups developed sepsis as determined by the increase of blood temperature and heart rate

compared to Sham (Figure 3.1.3). By 9 hours after injury, the animals from the injured groups were receiving a high rate of fluid (513.5, 340.8 and 355.1 mL/h in control, DDAVP and TLVP, respectively). The Sham group had a fluid rate of 94.8 mL/h by 9 hours (fluid rate adjusted to hematocrit). Mean arterial pressure (MAP) had a significant reduction in Control (98.8 ± 3.9 mmHg for baseline vs. 75.7 ± 5.1 mmHg at 9 hours), DDAVP (97 ± 1.8 mmHg for baseline vs. 72.7 ± 2.1 mmHg at 9 hours) and TLVP groups (98.3 ± 3.6 mmHg for baseline vs. 71.3 ± 3.7 mmHg at 12 hours). In the Sham group, MAP was 97.7 ± 3.9 mmHg at baseline without significant change during the study. The reduction of MAP while receiving an aggressive fluid resuscitation indicated that within 12 hours the induced injury led to septic shock in all injured groups. Leucocytes were also statistically alterated by 12 hours in all injured groups compared to the Sham group (data not shown).

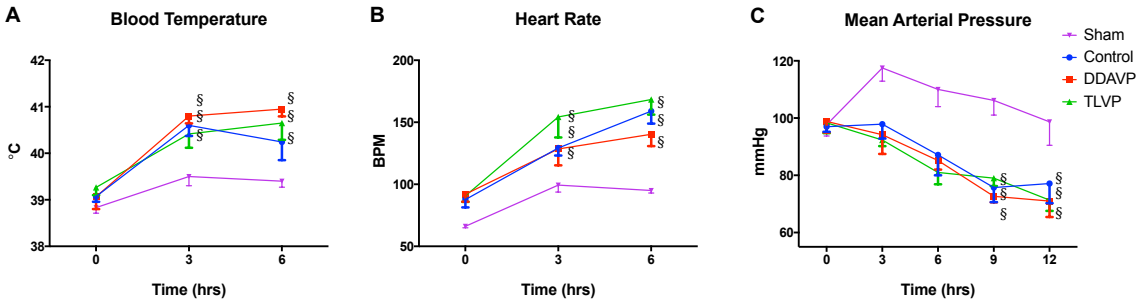


Figure 3.1.3. Signs of sepsis and septic shock during the first 12 hours.

Figures denote blood temperature (A), heart rate (B) and mean arterial pressure (C). All of the injured groups significantly differ from the sham group in all three parameters at early time points of sepsis. Data are expressed as mean ± SEM. §p <0.05 vs. Sham.

3.1.4. Lung and heart tissues express V_2R protein

Heart and lung tissues harvested from Sham, Control, DDAVP and TLVP animal groups had a positive expression of V_2R determined by western blot analysis. The V_2R expression rate was quantified by densitometry analysis, which demonstrated that the V_2R protein level was unchanged by the injury or the treatments.

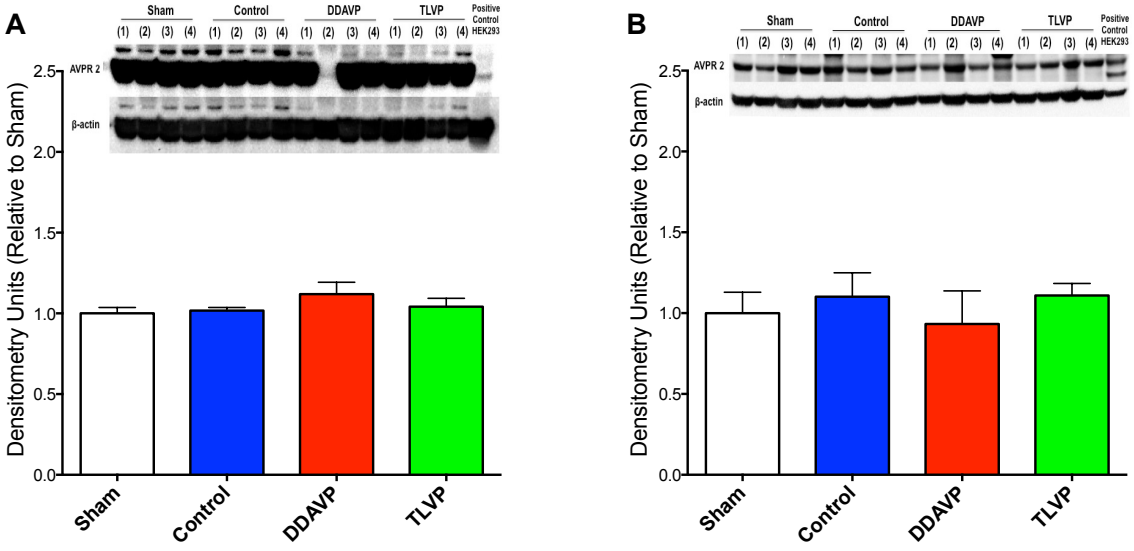


Figure 3.1.4. Arginine vasopressin receptor 2 protein expression in lung and heart tissue

A) Western blot of heart tissue homogenate. B) Western blot of lung tissue homogenate.

3.1.5. TLVP treatment reduces the fluid requirement

The total fluid requirement in the Control (8199 ± 2334 mL) and DDAVP groups (6837 ± 1517 mL) was higher than Sham (2327 ± 814 mL). In contrast, the amount of fluid received by the TLVP group (4606 ± 432) was not statistically different from the fluid requirement of the Sham group. Comparing the injured groups, only the TLVP group

required significantly less fluid than Control (Figure 3.1.5.). As mentioned in section 2.2.1, the fluid input was titrated based on the hematocrit changes, therefore these results indicate that the extravasation of water was attenuated with TLVP treatment -- suggesting its antileak effect.

3.1.6. The urinary output shows similarity among TLVP, Control and Sham groups but being decreased in the DDAVP group.

The accumulated urinary output had no statistical difference in Control (3375 ± 1058 mL), DDAVP (2165 ± 486 mL) or TLVP (3623 ± 788) compared to the Sham group (2366 ± 684 mL). Comparison among the injured groups showed that the urinary output in the DDAVP group was significantly decreased vs. TLVP group (Figure 3.1.5.). This information demonstrates the antidiuretic effect of DDAVP in sepsis.

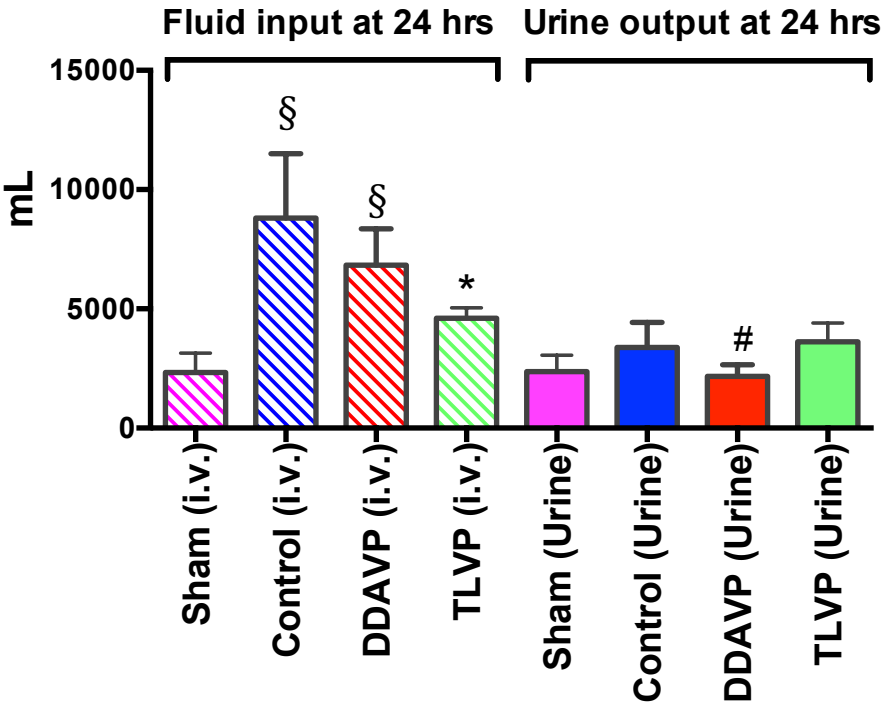


Figure 3.1.5. Accumulated fluid input and urinary output at 24 hours are shown in all groups.

TLVP treatment significantly decreased the fluid requirement during sepsis without having effects on urinary output. Data are expressed as mean ± SEM.

* $p < 0.05$ vs. Control; # $p < 0.05$ vs. TLVP; §$p < 0.05$ vs. Sham.

3.1.7. TLVP treatment prevents the systemic fluid retention

In the Control group (126 ± 52 mL/Kg, at 24 hours), the accumulated fluid was statistically greater than Sham (1 ± 8 mL/Kg, at 24 hours) by 12 to 24 hours. In the DDAVP group (142 ± 42 mL/Kg, at 24 hours), the accumulation was significantly increased vs. Sham between 15 and 24 hours. In TLVP group (30 ± 30 mL/Kg, at 24 hours), the amount of fluids retained was statistically similar to the Sham group, and lower than the DDAVP and Control groups (Figure 3.1.6.). These results imply that in our model, the amount of fluid retention at 24 hours was significantly elevated, correlating well with the characteristics of critically ill septic patients (55). TLVP treatment proved to be efficient in preventing this excessive amount of fluid retention during sepsis.

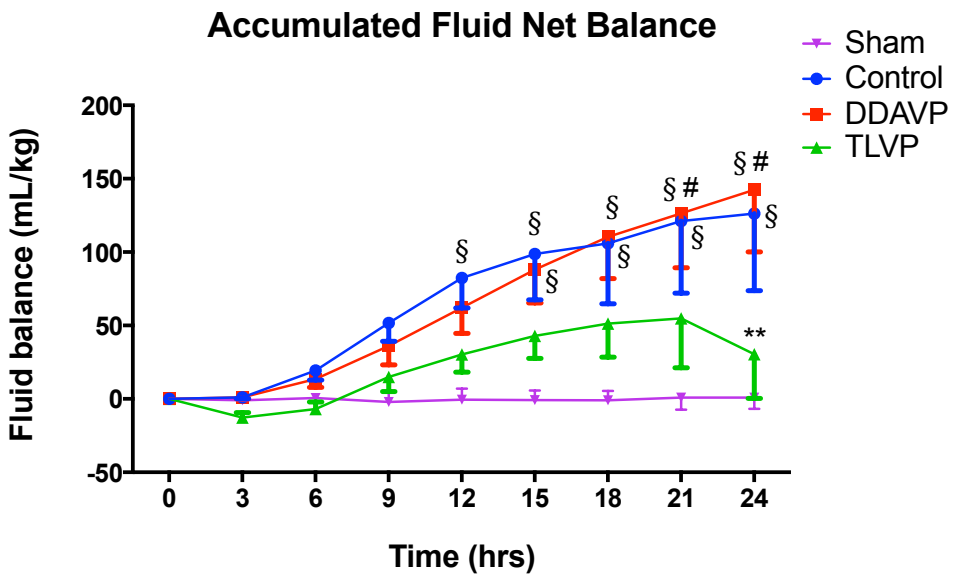


Figure 3.1.6. Accumulated fluid net balance in all groups.

Data are expressed as mean ± SEM. *p <0.05 vs. Control; #p <0.05 vs. DDAVP; §p <0.01 vs. Sham.

3.1.8. The hematocrit and the hemoglobin were similar in all groups at 24 hours

The hematocrit level increased at 3 and 6 hours in the Control and TLVP groups vs. Sham and their baselines. The levels of hemoglobin (data not shown) also increased at 6 hours in Control and TLVP vs. Sham and their baselines. At later time points, both hemoglobin (data not shown) and hematocrit were statistically similar in all groups (Figure 3.1.7). The comparable levels of hemoglobin and hematocrit in all groups support the fact that, the fluid retention was not a product of the increased plasma volume, but more to the appearance of capillary leak induced by severe sepsis.

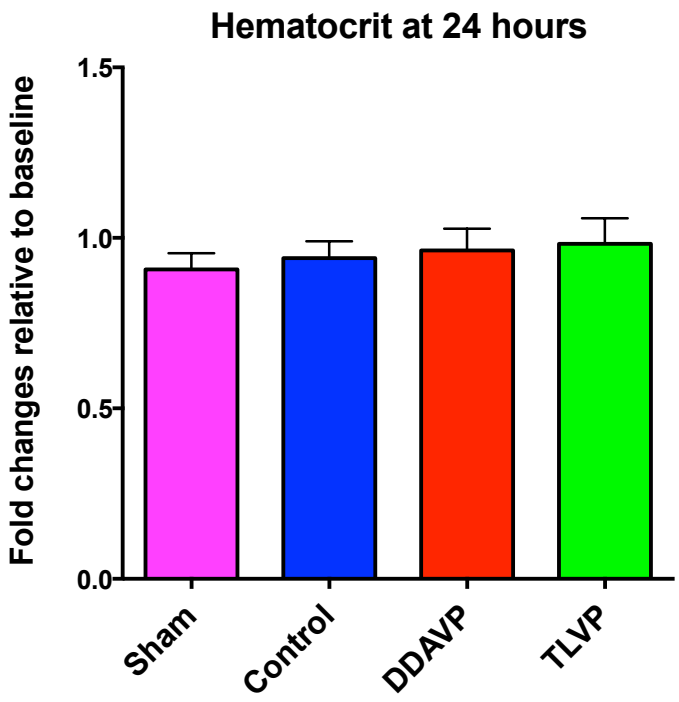


Figure 3.1.7. Hematocrit levels at 24 hours are not altered among the groups.

Data are expressed as mean ± SEM.

3.1.9. TLVP treatment has no effect over the injury-induced loss of plasma proteins.

The total plasma protein concentration among all the injured groups (Control, DDAVP and TLVP) was decreased vs. Sham between 9 and 24 hours (Figure 3.1.8.A). The oncotic pressure also dropped in injured animals vs. Sham between 6 and 24 hours (Figure 3.1.8.B). Serum albumin was decreased in all three injured groups vs. Sham between 12 and 24 hours (Figure 3.1.8.C). However, no difference in plasma protein, oncotic pressure or albumin was observed among the injured groups.

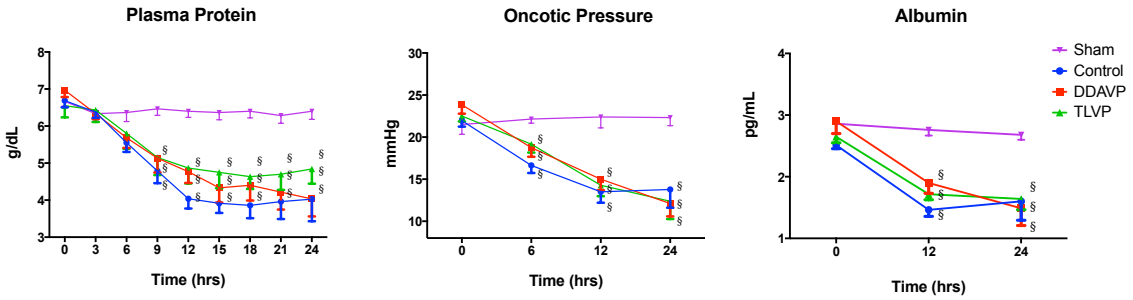


Figure 3.1.8. Total plasma protein concentration (A), oncotic pressure (B) and, plasma albumin concentration (C) are shown.

All injured group significantly differ from Sham. Data are expressed as mean ± SEM. §p <0.05 vs. Sham.

3.1.10. TLVP treatment prevented the increase in left atrium pressure (LAP)

Compared to Sham, LAP increased between 15 and 24 hours in the Control group and between 12 and 24 hours in the DDAVP group. However, in septic sheep TLVP treatment significantly decreased the LAP after 15 hours vs. Control, and had no statistical difference vs. Sham (Figure 3.1.9.A). The PCOP increased vs. Sham at 12 hours in the Control group and between 18 and 24 hours in the DDAVP group. In the TLVP group, PCOP decreased vs. Control at 12 and 24, hours and had no difference vs. Sham (Figure 3.1.9.B). Taken together, TLVP treatment proved to be effective to maintain the pressure in the left heart during the 24 hours of the study.

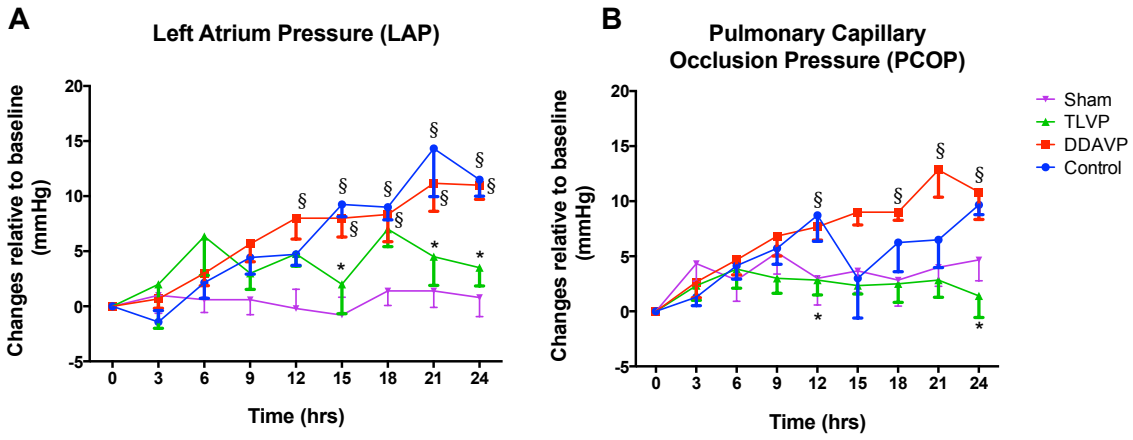


Figure 3.1.9. TLVP remarkably reduced the left atrium pressure (A) and pulmonary capillary occlusion pressure (B) after 15 hours.

Data are expressed as mean ± SEM. *p <0.05 vs. Control; #p <0.05 vs. DDAVP; §p <0.05 vs. Sham.

3.1.11. The increase of brain natriuretic peptide was abolished with TLVP treatment

The level of brain natriuretic peptide (BNP) in plasma was statistically elevated in the Control group vs. Sham. In the DDAVP group, there was an increase in BNP vs. Sham, although with no statistical difference. In the TLVP group, levels of BNP were lower than Control and close to the levels of BNP in the Sham group (Figure 3.1.10.). BNP is a marker of myocardial wall stress (108), thus in conjunction with the section 3.1.15, this finding proves that TLVP improves the heart performance.

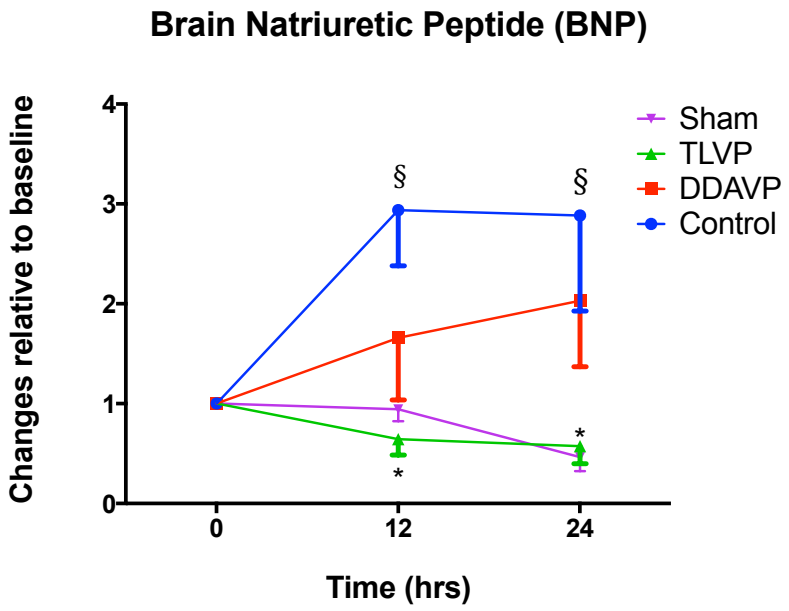


Figure 3.1.10. TLVP treatment prevented the increase in circulating brain natriuretic peptide.

Data are expressed as mean ± SEM. *p <0.05 vs. Control; §p <0.01 vs. Sham.

3.1.12. TLVP treatment attenuated the elevated in pulmonary resistance

Compared to Sham, PAP increased between 12 and 24 hours in the Control group, and between 15 and 24 hours in the DDAVP group. In the TLVP group, PAP decreased between 12 and 24 hours vs. Control, and had no difference vs. Sham (Figure 3.1.11.). The attenuation of pulmonary hypertension by TLVP supports the view that the V_2R blockage is a promising therapeutic approach during severe sepsis.

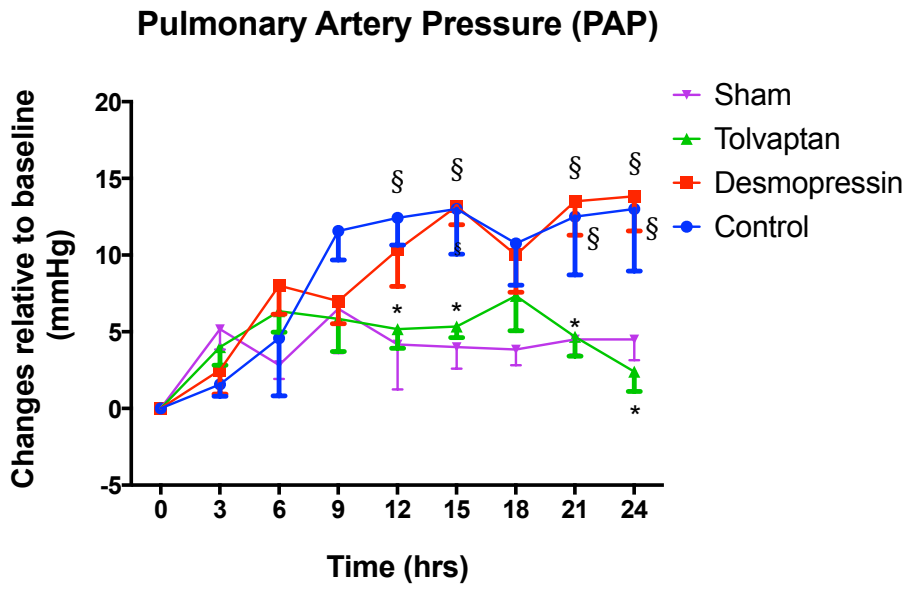


Figure 3.1.11. The pulmonary artery pressure did not increase with TLVP treatment. Data are expressed as mean ± SEM. *p <0.05 vs. Control; §p <0.01 vs. Sham.

3.1.13. TLVP decreased the pulmonary microvascular capillary pressure (P_c)

Compared to Sham, P_c increased at 15 hours in the Control group and between 15 and 24 hours in the DDAVP group. In the TLVP group, the P_c decreased at 12 and 24 hours vs. Control, and had no difference vs. Sham (Figure 3.1.12.). The reduced P_c, an indicator of capillary hydrostatic pressure (98), was a beneficial effect of the TLVP treatment associated with diminished vascular stasis.

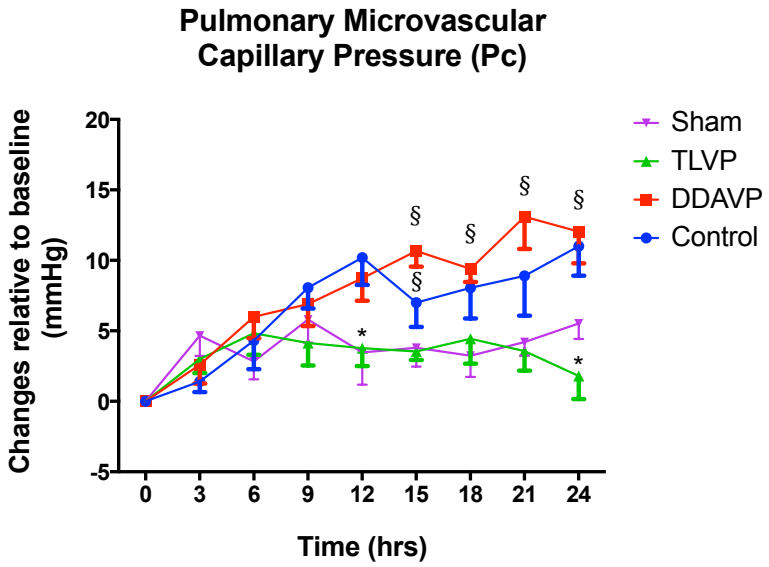


Figure 3.1.12. The capillary hydrostatic pressure indicated by the pulmonary microvascular capillary pressure proved to be reduced in septic sheep treated with TLVP.

Data are expressed as mean ± SEM. *p <0.05 vs. Control; §p <0.05 vs. Sham.

3.1.14. Retention of water in lung was attenuated with TLVP

Lung edema was determined by measuring changes in the tissue wet-to-dry ratio (W/D). The Control and DDAVP groups had a significant accumulation of water in lungs compared to Sham. However, the W/D of the TLVP group had no statistical difference vs. Sham animals (Figure 3.1.13.A), suggesting that TLVP treatment reduced lung edema in septic sheep. The total protein concentration in bronchialveolar lavage (BALF) increased in all injured groups vs. Sham. But, a trend of reduced protein concentration was observed in DDAVP and TLVP groups vs. Control (Figure 3.1.13.B).

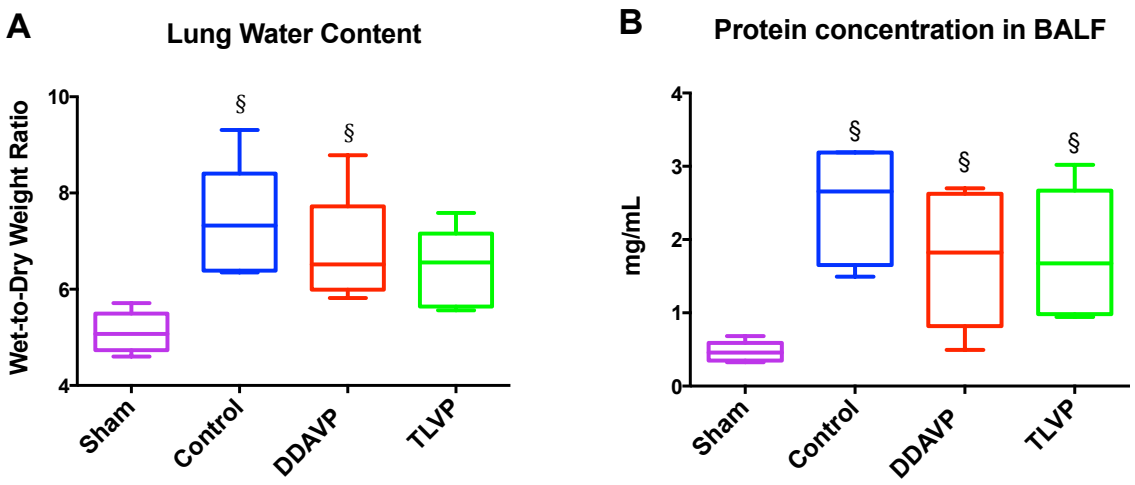


Figure 3.1.13. Lung Wet-to-Dry weight ratio (A) and the total protein in BALF (B) measurements are shown.

Please note the TLVP group proved to mitigate the accumulation of water in lung tissue. Data are expressed as mean ± SEM. §p <0.01 vs. Sham.

3.1.15. TLVP treatment reduced the levels of angiopoietin-2 in heart tissue

The concentration of angiopoietin-2 in lung tissue was not significantly different among groups. However, in heart tissue, the level of angiopoietin-2 significantly reduced in the TLVP treated group vs. the Control and DDAVP groups. DDAVP treatment tended to increase the amount of angiopoietin-2 in heart tissue vs. the Control group and was significantly augmented vs. the TLVP group.

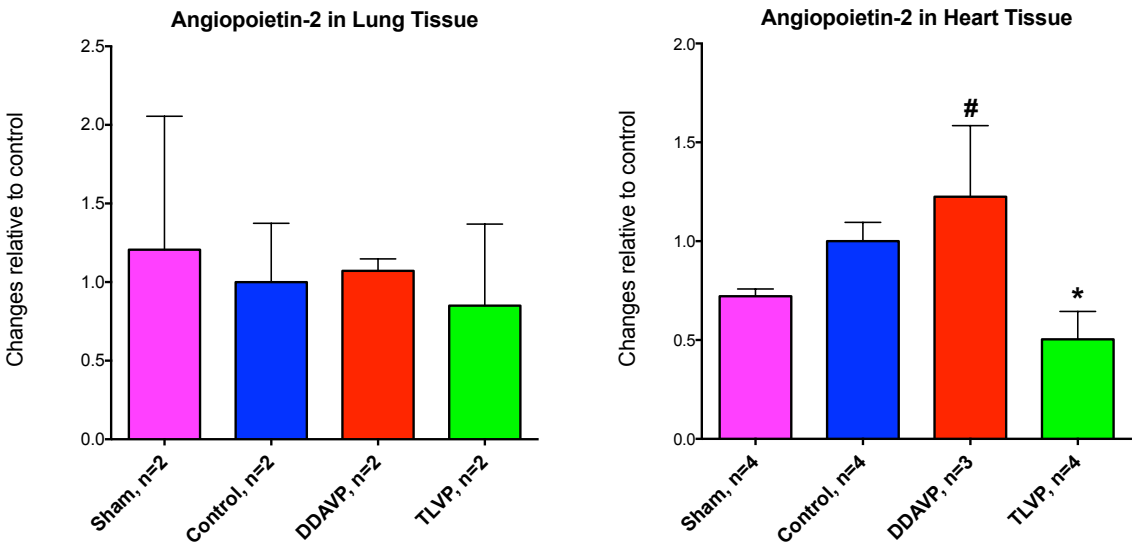


Figure 3.1.14. Angiopoietin-2 protein concentration in lung and heart tissue detected by immunoblotting analysis.

TLVP treatment statistically reduced the angiopoietin-2 expression in heart tissue. Data are expressed as mean ± SEM. *p <0.05 vs. Control; §p <0.05 vs. Sham; #p <0.05 vs. DDAVP.

3.1.16. Endogenous AVP increased in all injured groups

The plasma AVP levels increased in the Control group between 3 hours (138 ± 43 pg/mL) and 12 hours (181.7 ± 70.5 pg/mL) vs. its baselines (29.5 ± 1.6 pg/mL). In the TLVP group, the level of AVP was statistically greater at 6 hours (133 ± 14.9 pg/mL) vs. its baselines (22.8 ± 1.0 pg/mL). In the DDAVP group, there was a slight increase between 3 hours (65.8 ± 24.6 pg/mL) and 12 hours (71.7 ± 30 pg/mL) vs. its baselines (21.7 ± 1.4 pg/mL), but without statistical relevance (Figure 3.1.15.). A consensus of studies concluded that within the pathophysiology of sepsis, there is early increase of plasma AVP above 100 pg/mL which is reflected in the control and TLVP group (42). However, there

may be some feedback mechanism in the DDAVP treated animals that attenuates the release of AVP.

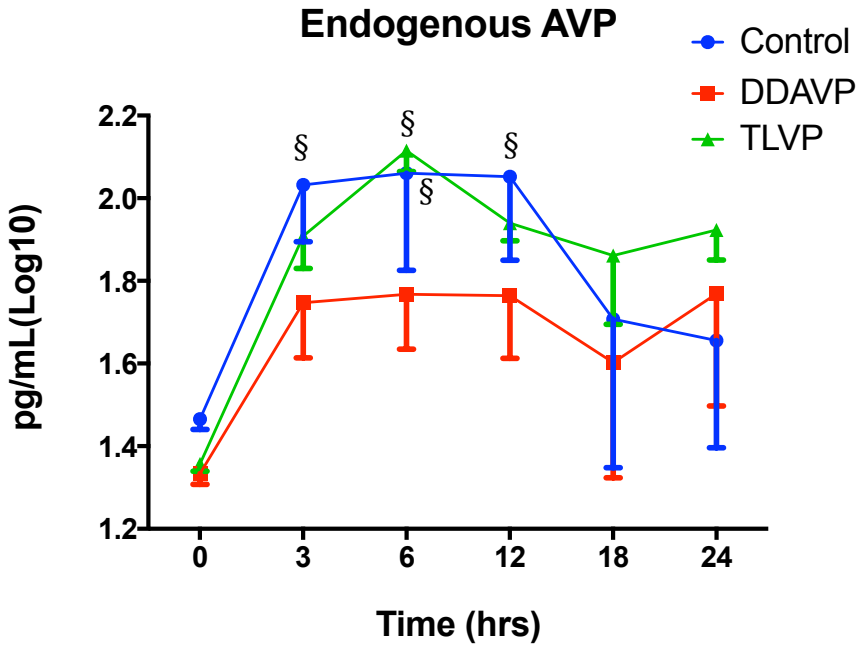


Figure 3.1.15. Endogenous plasma AVP increased above 100 pg/mL in Control and TLVP groups.

Data are expressed as mean ± SEM. §, $p < 0.05$ vs. baseline.

3.1.17. Gas exchange and ventilatory pressures

The fraction of inspired oxygen (PaO_2/FiO_2 ratio) and the shunt fraction (Qs/Qt) increased in all injured animals at 12 and 24 hours vs. Sham. In DDAVP, the Qs/Qt was decreased at 12 hours vs. Control. Compared to Sham, the oxygenation index (OI) increased in the Control group at 12 hours and in Control, DDAVP and TLVP groups at 24 hours. Compared to Control, at 12 hours the OI was lower in the DDAVP and TLVP groups. The mean airway pressure (P_{aw}) increased at 12 and 24 hours in Control and at 24

hours in the DDAVP group vs. Sham. In the TLVP group, P_{aw} was lower at 12 hours vs. Control (Table 3.1.1.). The lack of significant increase of P_{aw} in TLVP vs. Sham suggests a therapeutic effect provided of TLVP in lung. However, the evidence is limited, as no improvement was observed with the PaO_2/FiO_2 ratio and Qs/Qt.

	PaO_2/FiO_2 (mmHg)				Qs/Qt (%)		
Time (H)	0	12	24	Time (H)	0	12	24
Control	512 6	108 § 38	161 § 89	Control	0.18 0.01	0.58 § 0.08	0.42 § 0.1
DDAVP	497 18	227 § 38	195 § 71	DDAVP	0.22 0.01	0.4 § * 0.04	0.47 § 0.08
TLVP	505 15	222 § 37	141 § 37	TLVP	0.18 0.01	0.42 § 0.1	0.53 § 0.08
Sham	510 16	600 32	587 21	Sham	0.25 0.03	0.2 0.01	0.2 0.03
	OI				P_{aw} (cmH_20)		
Time (H)	0	12	24	Time (H)	0	12	24
Control	1.7 0.3	20 § 5	18 § 7.1	Control	8.9 1.5	13.5 § 1.3	15.6 § 2.4
DDAVP	1.8 0.3	5.5 * 0.9	15 § 5.7	DDAVP	8.9 1.1	10.8 0.4	13.7 § 1.6
TLVP	1.6 0.2	5.2 * 1.3	12 § 3.9	TLVP	7.9 1	9.2 * 0.2	11.9 1.6
Sham	1.9 0.1	1.4 0.1	1.7 0.2	Sham	9.8 0.2	8.4 0.4	9.9 0.7

Table 3.1.1. Ventilatory variables and gas exchange. Data are expressed as mean ± SEM. *p <0.05 vs. Control; §p <0.05 vs. Sham; #p <0.05 vs. DDAVP.

3.1.18. Treatment with TLVP prevented the decrease of potassium in plasma

Compared to the Sham group, the potassium level in plasma (Kpl) was decreased by 18 to 24 hours in the DDAVP group and by 9 hours only in the Control and TLVP groups. Compared to Control, Kpl in TLVP was increased by 15 to 21 hours and DDAVP was decreased at 24 hours. Kpl was also statistically lower than TLVP at 24 hours. At 24 hours, the Kpl in TLVP was 3.9 ± 0.2 mEq/L compared to 4 ± 0.15 mEq/L, 3.3 ± 0.1

mEq/L and 2.9 ± 0.2 mEq/L in Sham, Control and DDAVP groups respectively (Figure 3.1.16.A).

3.1.19. Treatment with TLVP had an undesired increase of sodium in plasma

The levels of sodium in plasma (Napl) had a very modest change in the Control, DDAVP and Sham groups. In the TLVP group Napl had a significant increase vs. Control between 3 and 24 hours and vs. Sham between 6 and 24 hours. At 24 hours the Napl in the TLVP group was 158.4 ± 1.6 mEq/L compared to 144.7 ± 0.7 mEq/L, 144.7 ± 2.3 mEq/L and 142.3 ± 1.7 mEq/L in Sham, Control and DDAVP groups respectively (Figure 3.1.16.B). Similar to the levels of Napl, the plasma osmolarity and osmolality increased in the TLVP group between 3 and 24 hours vs. Control and, between 9 and 24 hours vs. Sham (data not shown).

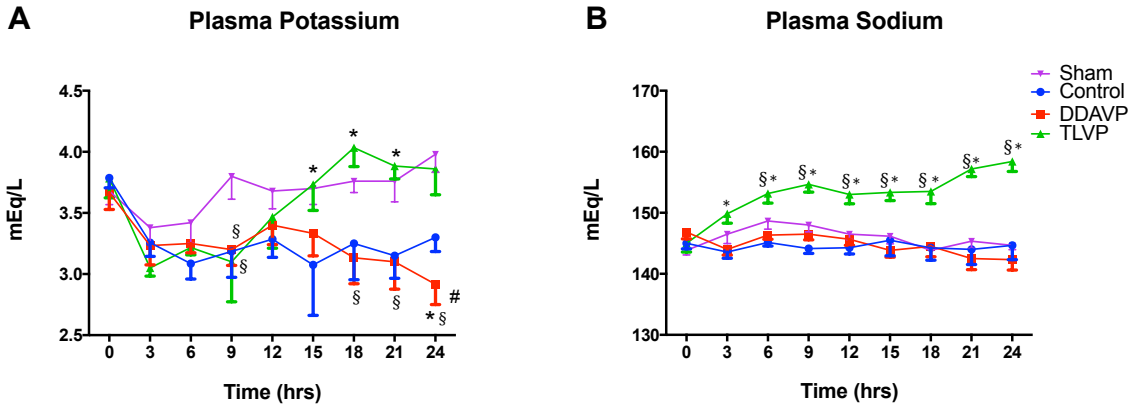


Figure 3.1.16. Plasma potassium (A) and sodium (B) levels are shown. Data are expressed as mean ± SEM. *p <0.05 vs. Control; #p <0.05 vs. DDAVP; §p <0.05 vs. Sham.

3.1.20. TLVP increased the elimination of hypoosmolar urine

The osmolality in urine (OsUr) was decreased vs. Sham in the Control group between 6 and 18 hours, in the DDAVP group at 9 and 12 hours and in the TLVP group between 3 and 24 hours. Compared to Control, OsUr was decreased in TLVP at 3 and 6 hours and increased in DDAVP between 18 and 21 hours (Figure 3.1.17.A). The accumulated urinary sodium (NaUr) was decreased vs. Sham between 9 and 24 hours in TLVP, and 12 and 24 hours in both Control and DDAVP groups (Figure 3.1.17.B). The accumulated urinary potassium (KUr) was decreased in TLVP vs. Sham between 15 and 24 hours, vs. Control at 24 hours, and vs. DDAVP between 18 and 24 hours (Figure 3.1.17.C). The concentration of urine electrolytes indicates that even though the severe sepsis led to a milder elimination of NaUr and KUr (as seen in the Control), the TLVP treatment was related to the reduced elimination of both NaUr and KUr. In addition, these results also clearly define that DDVAP is associated with lower urinary output with high osmolality.

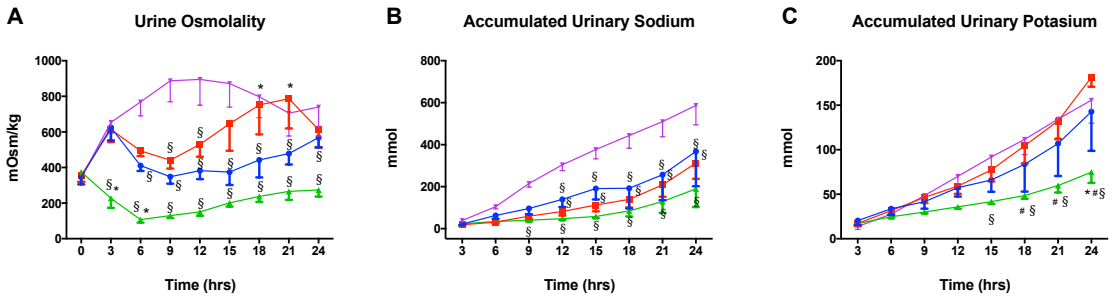


Figure 3.1.17. Urinary osmolality (A), urinary sodium and (B), urinary potassium (C) are shown.

TLVP treatment decreased the urine osmolality by reducing the sodium and potassium urinary excretion. Data are expressed as mean ± SEM. *p <0.05 vs. Control; #p <0.05 vs. DDAVP; §p <0.05 vs. Sham.

3.1.21. TLVP treatment was linked with a decreased clearance of urea

The accumulated urinary urea nitrogen (UnUr) was reduced vs. Sham between 9 and 24 hours in DDAVP and TLVP groups, and between 9 and 21 hours in the Control group (Figure 3.1.18.A). The accumulated excretion of creatinine was similar among the 4 groups (Figure 3.1.18.B).

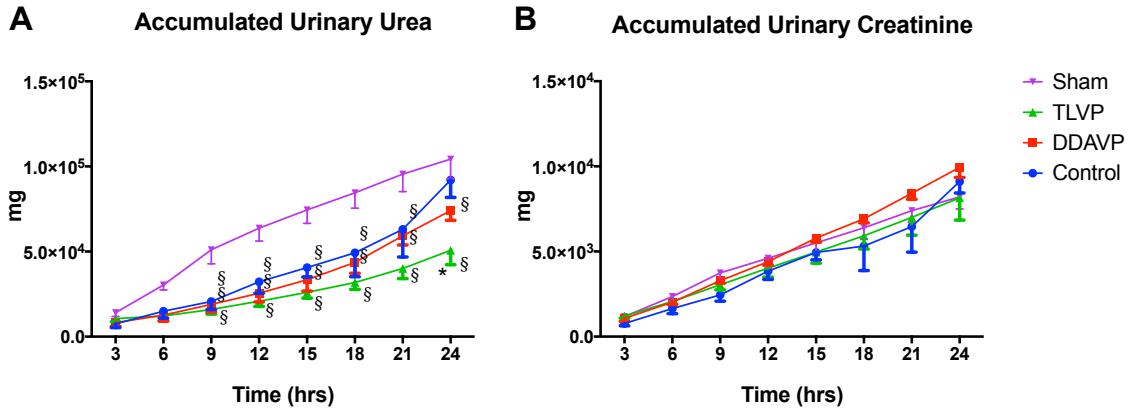


Figure 3.1.18. Figure represents the accumulated urea (A) and creatinine (B).

Data are expressed as mean ± SEM. *p <0.05 vs. Control; #p <0.05 vs. DDAVP; §p <0.05 vs. Sham.

3.1.22. The heart rate (HR), cardiac index (CI), mean arterial pressure (MAP), and central venous pressure (CVP) are not affected by DDAVP or TLVP treatments

The HR increased in all injured groups between 3 and 24 hours vs. Sham. The CI increased vs. Sham at 12 and 24 hours in the Control group, and at 12 hours in the DDAVP group. In the TLVP group, CI was statistically higher vs. Sham and lower vs. Control at 24 hours. MAP decreased in DDAVP vs. Sham at 12 hours and, in TLVP vs. Sham, MAP decreased at 12 and 24 hours. CVP increased in the Control and DDAVP groups at 12 and 24 hours vs. Sham. PVRI was increased in the Control group at 12 hours vs. Sham. SVI was decreased in TLVP at 12 hours vs. Sham. There was no statistical difference comparing the values of LVSWI, RVSWI and SVRI among groups (Table 3.1.2.).

HR	BL	12	24
Control	87.7 ± 6.3	162.4 § ± 7.5	147 § ± 12.4
DDAVP	92 ± 6.0	164 § ± 5.3	126.5 § ± 9.2
TLVP	90.7 ± 4.8	166 § ± 12.3	147.8 § ± 9.8
Sham	66.2 ± 1.2	94.2 ± 3.7	95.8 ± 5.6

CVP	BL	12	24
Control	5.6 ± 1	10.7 § ± 1.3	13.3 § ± 0.9
DDAVP	5.3 ± 1.1	11.2 § ± 1.4	14.3 § ± 1.7
TLVP	5.5 ± 0.8	8.7 ± 1.2	10.2 ± 1.3
Sham	7.7 ± 0.6	7.5 ± 1.1	9.7 ± 0.7

SVRI	BL	12	24
Control	1251.2 ± 69.5	609 § ± 99.0	579.7 § ± 121.0
DDAVP	1237 ± 38.8	505.9 § ± 86.6	826.8 § ± 160.0
TLVP	1219.6 ± 79.6	658.6 § ± 51.5	551 § ± 171.1
Sham	1368.7 ±	1252 ±	1293.9 ± 119.1

CI			
Control	5.9 ± 0.3	9.3 § ± 0.6	10 § ± 1.2
DDAVP	6.1 ± 0.4	10.1 § ± 0.9	7.4 ± 0.8
TLVP	6.2 ± 0.4	7.8 ± 0.5	8.9 § ± 1.1
Sham	5.4 ± 0.4	6 ± 0.4	5.9 ± 0.4

LVSWI			
Control	7.1 ± 0.3	7.3 ± 0.8	8.5 ± 0.2
DDAVP	7.6 ± 0.7	7.3 ± 0.6	6.1 ± 0.9
TLVP	7.4 ± 0.6	6.1 ± 0.6	5.9 ± 0.9
Sham	6.1 ± 0.3	6.7 ± 0.6	6.6 ± 0.5

PVRI			
Control	131.1 ± 11.2	119 § ± 17.6	113.5 ± 22.2
DDAVP	129.2 ± 11.5	101.9 ± 12.2	158.6 ± 41.5
TLVP	113.6 ± 13.0	116.1 ± 14.1	97.9 ± 22.4
Sham	119.6 ± 15.6	165.3 ± 28.4	138.6 ± 16.7

MAP			
Control	97 ± 1.8	77.1 ± 6.9	82.3 ± 7.7
DDAVP	98.8 ± 3.9	71 § ± 5.6	83.2 ± 8.7
TLVP	98.3 ± 3.6	71.3 § ± 3.7	63.8 § ± 9.5
Sham	97.7 ± 3.9	98.7 ± 8.2	103 ± 4.7

RVSWI			
Control	12.1 ± 1.6	15.6 ± 2.0	16.8 ± 3.0
DDAVP	12.3 ± 2.2	14.2 ± 0.9	13.8 ± 1.4
TLVP	12.9 ± 1.5	10 ± 1.0	9.4 ± 1.9
Sham	15.4 ± 3.0	15.1 ± 1.7	13.7 ± 2.7

SVI			
Control	70.3 ± 6.3	57.6 ± 4.1	68.4 ± 7.2
DDAVP	68.7 ± 7.8	61 ± 3.9	58.3 ± 5.5
TLVP	69.4 ± 6.1	48 § ± 4.1	60.1 ± 5.4
Sham	82.5 ± 5.8	64.1 ± 4.1	63 ± 6.1

Table 3.1.2. The present table summarizes the various hemodynamic parameters. Data are expressed as mean ± SEM. *p <0.05 vs. Control; #p <0.05 vs. DDAVP; §p <0.05 vs. Sham.

3.1.23. Blood biomarkers:

Creatinine increased in the DDAVP group vs. Sham at 12 hours. In the TLVP group, creatinine was increased at 12 and 24 hours vs. Sham and Control groups. BUN increased in DDAVP at 12 hours vs. Sham and it was decreased vs. TLVP at 24 hours. BUN in the TLVP group increased at 12 and 24 hours vs. Sham. Mean total bilirubin was slightly increased in the DDAVP group, although the levels between groups were not statistically different. Unconjugated bilirubin was increased in DDAVP vs. Sham and TLVP at 24 hours. The levels of nitrite/nitrate increased in Control, DDAVP and TLVP groups vs. Sham at 12 hours. In the TLVP group, nitrite/nitrate was also increased at 24

hours vs. Sham. Lactate increased in the Control group at 12 hours and in the TLVP group at 24 hours vs. Sham. Lactate was also diminished at 12 hours in DDAVP vs. Control. ALT was increased at 12 hours in TLVP group vs. Control and Sham. AST was increased in TLVP group at 12 hours vs. Sham, Control and DDAVP groups. ACT was elevated in the DDAVP group vs. Sham at 24 hours.

Creatinine (mg/dL)			
Control	0.72	0.80	0.81
	0.06	0.05	0.09
DDAVP	0.74	1.11 §	0.95
	0.07	0.12	0.21
TLVP	0.69	1.21 *§	1.65 *§
	0.02	0.12	0.23
Sham	0.65	0.60	0.60
	0.04	0.03	0.02

Unconjugated Bilirubin (mg/dL)			
Control	0.00	0.00	0.15
	0.00	0.00	0.05
DDAVP	0.00	0.04	0.65 §#
	0.00	0.04	0.52
TLVP	0.00	0.01	0.16
	0.00	0.01	0.08
Sham	0.00	0.00	0.00
	0.00	0.00	0.00

ALT (U/L)			
Control	32.00	28.86	39.00
	2.85	2.06	8.54
DDAVP	37.00	36.33	41.50
	5.27	4.56	4.64
TLVP	40.83	45.50 *§	43.20
	2.97	4.64	4.96
Sham	26.80	28.20	37.80
	2.42	2.73	7.64

BUN (mg/dL)			
Control	14.29	17.14	19.67
	1.73	1.50	1.45
DDAVP	15.17	21.80 §	17.17 #
	2.12	2.65	2.47
TLVP	13.83	23.83 §	28.60 §
	0.75	1.68	3.39
Sham	16.40	13.20	12.80
	1.47	2.03	1.32

Nitrite/Nitrate (uM)			
Control	0.41	6.79 §	2.79
	0.39	0.97	1.10
DDAVP	0.85	7.95 §	3.93
	0.37	2.30	1.11
TLVP	1.33	9.22 §	5.52 §
	0.19	0.39	0.65
Sham	0.69	1.18	0.25
	0.50	1.04	0.32

AST (U/L)			
Control	104.43	94.71	199.33
	10.81	8.57	75.90
DDAVP	103.17	103.17	147.33
	13.17	15.57	34.19
TLVP	149.50	246.67 *§#	159.40
	19.68	66.13	24.40
Sham	103.40	91.80	112.40
	10.75	10.26	16.80

Total Bilirubin (mg/dL)			
Control	0.27	0.53	0.30
	0.03	0.41	0.06
DDAVP	0.27	0.22	0.85
	0.03	0.05	0.53
TLVP	0.27	0.20	0.36
	0.03	0.04	0.11
Sham	0.22	0.14	0.16
	0.04	0.02	0.02

Lactate mmol/L			
Control	0.90	6.81 §	2.53
	0.55	1.52	1.32
DDAVP	1.33	3.05 *	4.20
	0.78	0.39	1.53
TLVP	0.53	4.33	6.70 §
	0.12	1.00	2.95
Sham	0.34	0.78	0.70
	0.07	0.10	0.03

ACT (sec)			
Control	151.83	180.83	186.00
	10.34	15.58	17.52
DDAVP	144.67	165.40	197.33 §
	23.25	15.21	22.31
TLVP	164.83	187.80	168.50
	13.17	18.82	13.28
Sham	159.83	174.17	164.00
	4.03	11.93	13.19

Table 3.3.2. The present table summarizes different blood parameters. Data are expressed as mean ± SEM. *p <0.05 vs. Control; #p <0.05 vs. DDAVP; §p <0.05 vs. Sham.

3.2. *In vitro* assays using human lung microvascular endothelial cells (HMVECs)

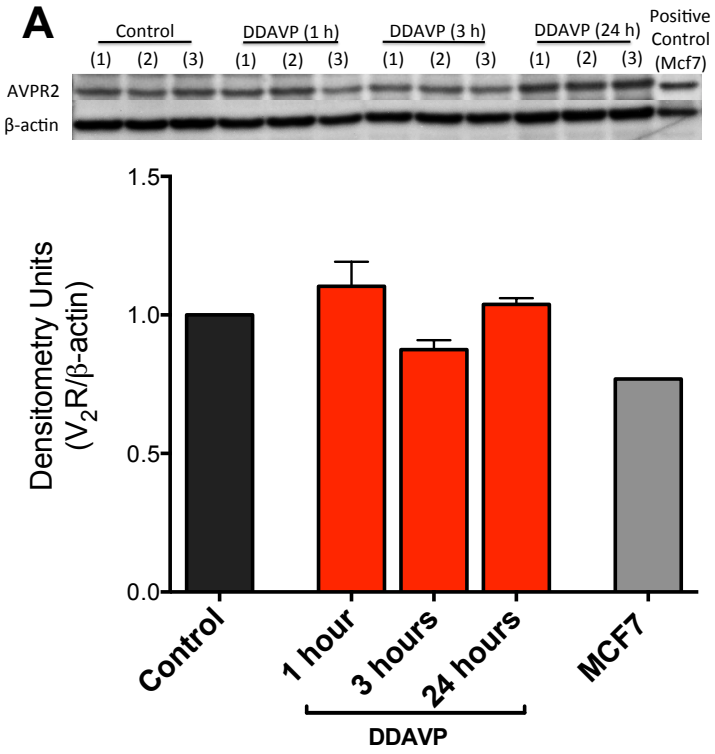
As mentioned in section 1.3, the disruption of the endothelial barrier play a major role in the pathophysiology of vascular leakage. Several investigators have focused their attention on seeking a therapeutic strategy to preserve the integrity of the endothelial barrier (24, 64). To further elucidate the molecular aspects of the beneficial effect of TLVP in our MRSA sepsis model, we conducted several cell-based *in vitro* assays.

It is well-known that to study vascular leakage, it is necessary to specifically investigate the microvascular endothelial cells as they are majorly implicated in this phenomena (21, 61, 86). Therefore, for our *in vitro* studies we selected the widely used HMVECs. This cell line has been demonstrated to express V_2R (and its activation by DDAVP) a selective V_2R agonist, is associated with the release of several deleterious molecules such as vWF and NO (75, 78). Furthermore, as respiratory infections are the most common sepsis etiology, it is necessary to study lung microvascular cells rather than microvascular cells derived from other organs (10).

To study the changes in the endothelium during MRSA or V_2R agonist stimulation, we used an electrical impedance assay and a 3D cell culture based permeability assay. Gene and protein expression levels of key mediators were also investigated to characterize the damage to the endothelial barrier triggered by V_2R activation. In these cell culture assays, we also co-incubated HMVECs with MRSA in order to model the most characteristic phenomena in the development of sepsis.

3.2.1. Arginine vasopressin receptor 2 (V_2R) and platelet endothelial cell adhesion molecule 1 (PECAM-1) expression are confirmed in HMVECs

Western blot analysis of HMVECs without treatment (control) or treated with 300 nM DDAVP for 1, 3 or 24 hours showed positive V_2R protein expression indicated by a consistent band around 40 kDa. Michigan Cancer Foundation-7 (MCF7) cell line served as positive control (87). The protein expression was quantified by densitometry analysis and the different groups were subsequently statistically compared. However, the V2R expression among the groups had no statistical relevance and the DDAVP treatment did not alter the V2R expression level in HMVECs (Figure 3.2.1A). Finally, the expression and cellular localization of V_2R were also confirmed by immunofluorescence. HMVECs were stained green after being exposed to anti-V_2R antibody (Figure 3.2.1B). The expression of the cell junction protein PECAM-1 was identified by immunofluorescence representing a confluent monolayer of HMVECs (Figure 3.2.1C).



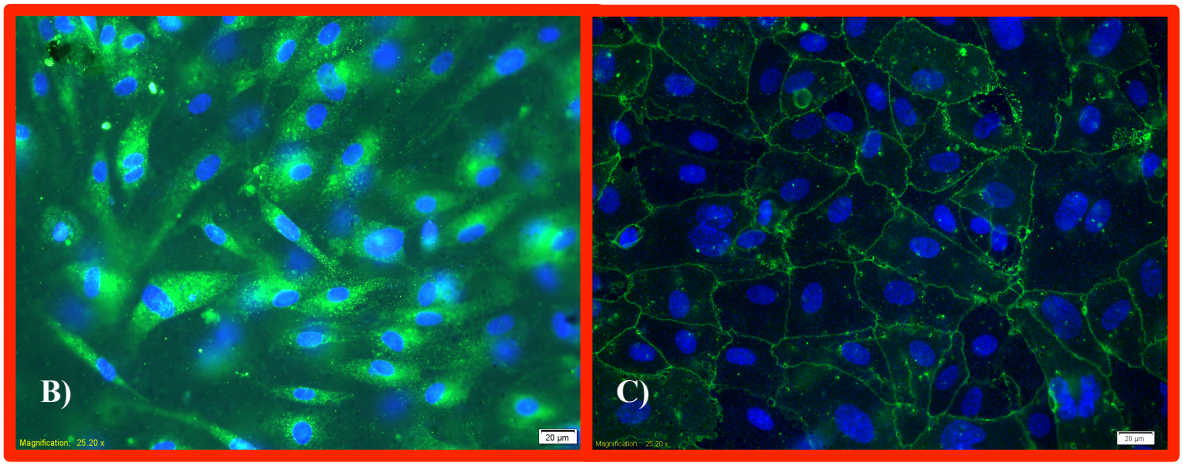


Figure 3.2.1. Protein expression of V_2R and PECAM-1 are determined in HMVECs

A) Western blot analysis of V_2R in HMVECs lysates. B) Immunostaining to determine the localization of V2R (green). The Nuclei was stained with DAPI (blue). C) Immunostaining to determine the localization of PECAM-1 (green). The nuclei was stained with DAPI (blue).

3.2.2. V_2R agonist, DDAVP increases the endothelial barrier permeability.

Treatment of *in vitro* microvascular vessels with DDAVP and VEGF increased the passage of dextran across the endothelial monolayer compared to Control. When the microvessels were pretreated with TLVP the DDAVP-induced increment in endothelial permeability was attenuated. Treatment with TLVP did not cause any significant change in endothelial permeability vs. Control. These results indicate that DDAVP treatment significantly disrupted the endothelial barrier similar to VEGF, a potent permeability factor and positive control of this assay (109). Moreover, TLVP pretreatment significantly prevented the DDAVP-induced increase in endothelial barrier permeability.

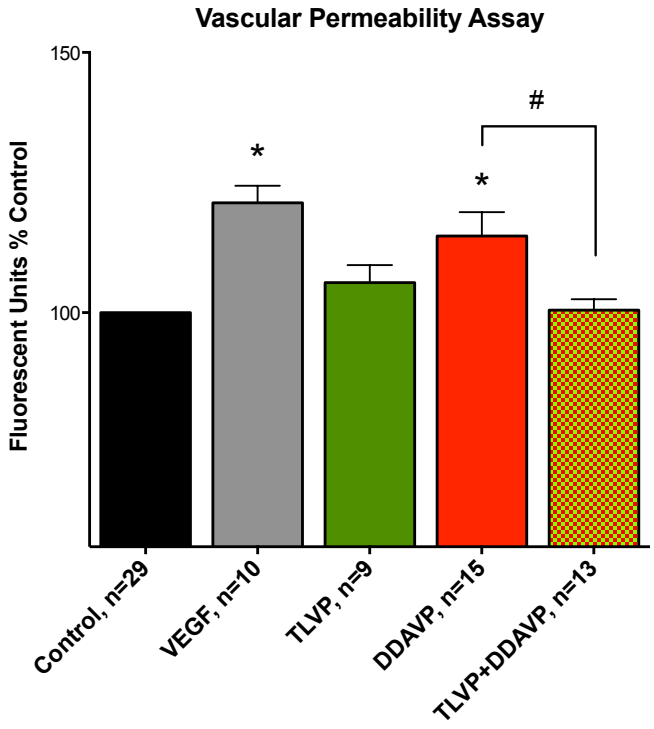


Figure 3.2.2. The changes in endothelial permeability after DDAVP, TLVP and VEGF treatment.

DDAVP treatment significantly disrupted the endothelial barrier similar to VEGF and pretreatment with TLVP attenuated the V_2R agonist-induced hyperpermeability. Data are expressed as mean ± SEM. *p <0.05 compared to Control; #p <0.05

3.2.3. V_2R antagonist attenuates the endothelial permeability induced by MRSA.

Treatment of *in vitro* microvascular vessels with MRSA augmented the passage of dextran through the endothelial monolayer vs. Control. However, when the vessels were pretreated with TVLP prior to the addition of MRSA, the amount of dextran across the monolayer did not differ from the Control group. The co-incubation of MRSA plus DDAVP enhanced barrier permeability as well, but this effect was similar to the

treatment with MRSA on its own. These data suggest that MRSA treatment alone induced a maximal rate of endothelial permeability that did not further increase in combination with DDAVP. Treatment with TLVP and subsequent addition of MRSA plus DDAVP showed a slight decrease in permeability without any statistical relevance. These results support the view that the MRSA-induced increment in endothelial barrier permeability is also mediated by V_2R activation as this effect was significantly attenuated by pretreatment with TLVP.

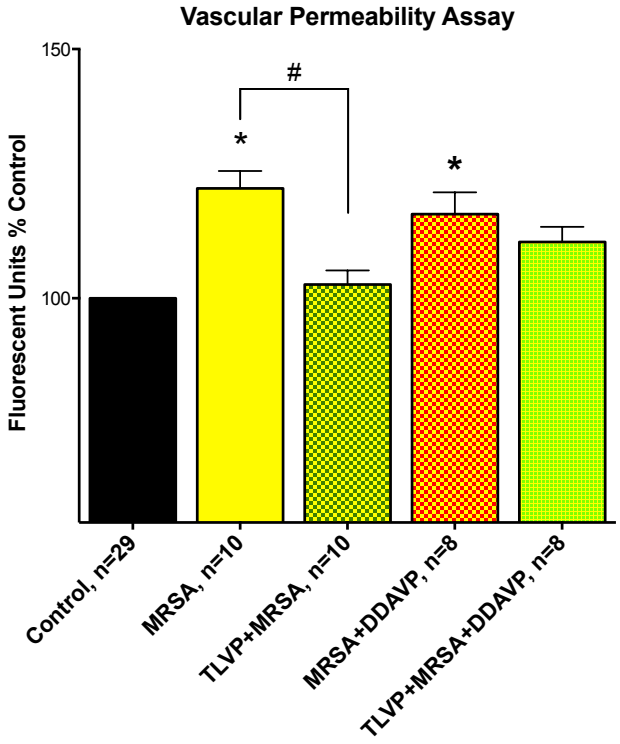


Figure 3.2.3. MRSA-induced increment in endothelial barrier permeability is mediated by V_2R activation.

Data are expressed as mean ± SEM. *p <0.05 compared to Control; #p <0.05.

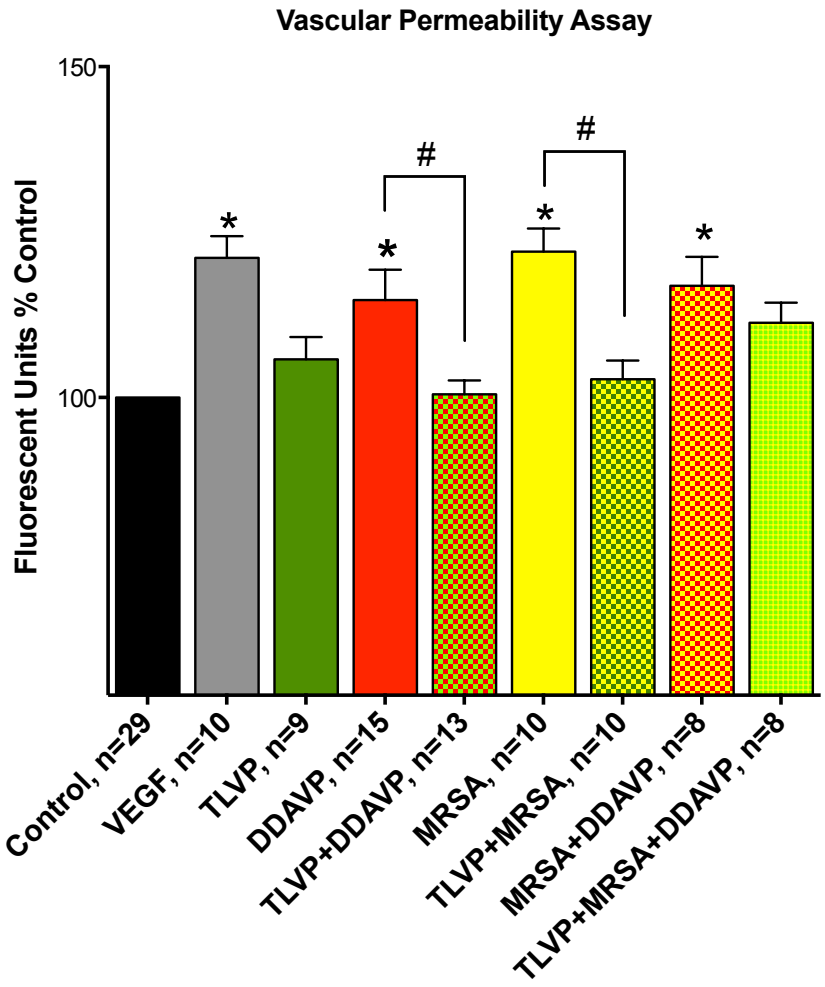


Figure 3.2.4. TLVP significantly reversed the increased endothelial permeability induced by DDAVP or MRSA treatment

Data are expressed as mean ± SEM. *p <0.05 compared to Control; #p <0.05 shows significant inhibitory effect of TLVP on endothelial permeability.

3.3.4. The cell viability is not affected in HMVECs treated with DDAVP and TLVP

The lactate dehydrogenase (LDH) activity in the HMVECs cell culture supernatant was significantly elevated after administrating H_2O_2 for 1, 3 and 6-hour-long periods vs. untreated cells (Control). H_2O_2 challenge, serving as a positive control,

significantly induced cell death and subsequent LDH release in the cell culture supernatant. HMVECs treated with DDAVP at 300 and 1000 nM or TLVP at 10 nM concentration did not affect cell viability, indicating that DDAVP and TLVP are not cytotoxic compounds.

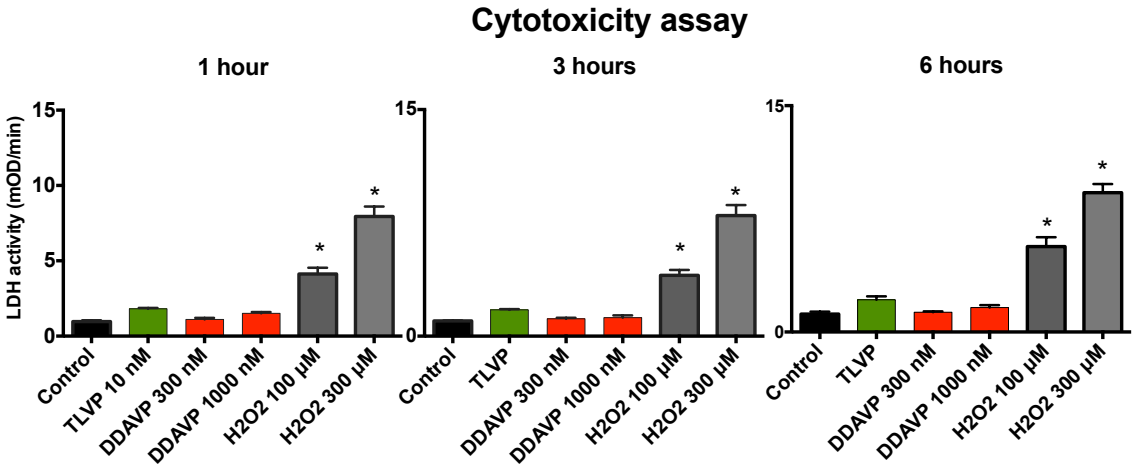


Figure 3.2.5. TLVP or DDAVP in HMVEC cultures have no effect on cytotoxicity.

LDH activity values are shown as Vmax for kinetic assay in mOD/min. H_2O_2 was used as cytotoxic agent (positive control). Data are expressed as mean ± SEM. *p <0.05 vs. Control.

3.3.5. V_2R agonist DDAVP decreases the electrical impedance demonstrating the disruption of cell-to-cell junctions.

In HMVECs treated with 3 and 10 ng/mL VEGF (used as positive control), the electrical impedance decreased 30 minutes after treatment and remained reduced vs. Control (Figure 3.2.4A). In the groups treated with 0.3, 1 and 3 nM DDAVP, electrical impedance was slightly decreased vs. Control. However, electrical impedance in the group treated with 10 and 30 nM DDAVP was statistically decreased in 30 minutes

(Figure 3.2.4A) and continually diminished vs. Control during the following hours as well. Figure 3.2.4B represents that DDAVP treatment decreased the electrical impedance in a dose dependent fashion after a short, only 30-min-long, incubation period. A reduction in electrical impedance correlates well with the decrease in cellular connectivity, which is a relevant indicator of the increased endothelial permeability (63, 110).

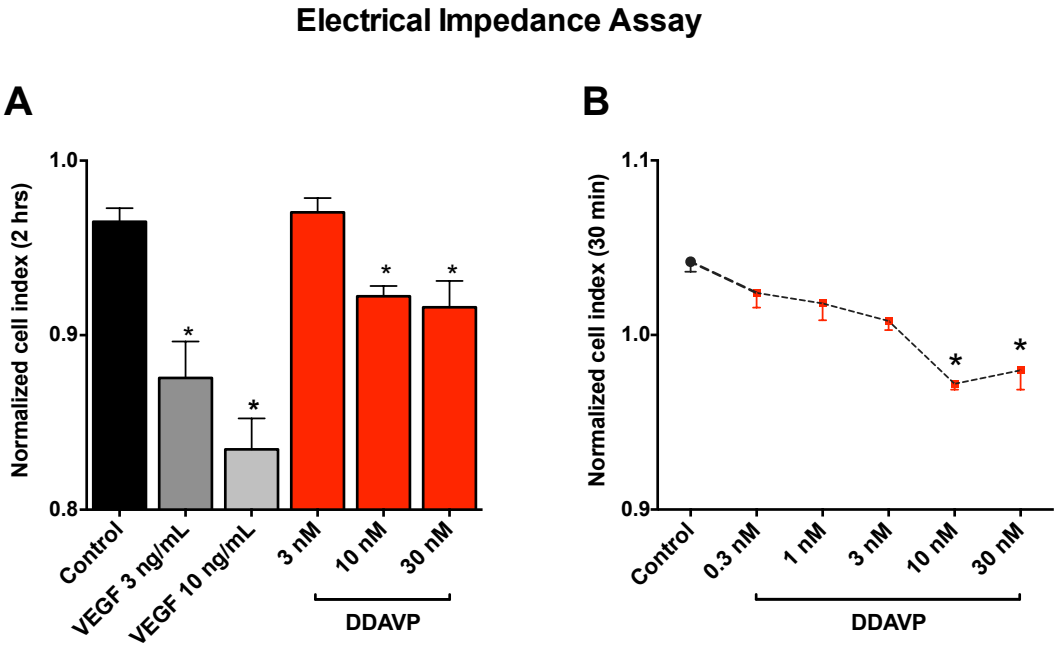


Figure 3.2.6. DDAVP at higher concentration significantly diminishes the electrical impedance in confluent HMVECs.

A) Normalized cell index (NCI) of confluent HMVECs treated for 2 hours with different concentrations of DDAVP or VEGF. B) Dose dependent effect of DDAVP in HMVECs treated with multiple doses of DDAVP in 30 minutes. Data are expressed as mean ± SEM. * $p < 0.01$ vs. Control.

3.3.6. The V_2R agonist, DDAVP increases the mRNA expression of angiopoietin-2 in HMVECs and promotes the secretion of angiopoietin-2 protein in cell culture supernatant

The mRNA expression of angiopoietin-2 in HMVECs subjected to DDAVP (n=9) for 6 hours was significantly elevated by 2.3 ± 0.3 fold vs. Control (n = 9) (Figure 3.2.5B). The level of angiopoietin-2 protein in the cell culture supernatant after a 6-hour-long DDAVP treatment (n = 5, 78596 ± 115) was also significantly increased 1.6 fold vs. control (n=5, 48622 ± 4550) (Figure 3.2.5A). These two experiments provide strong evidence to point out that the production and exocytosis of angiopoietin-2 occurs with V_2R activation.

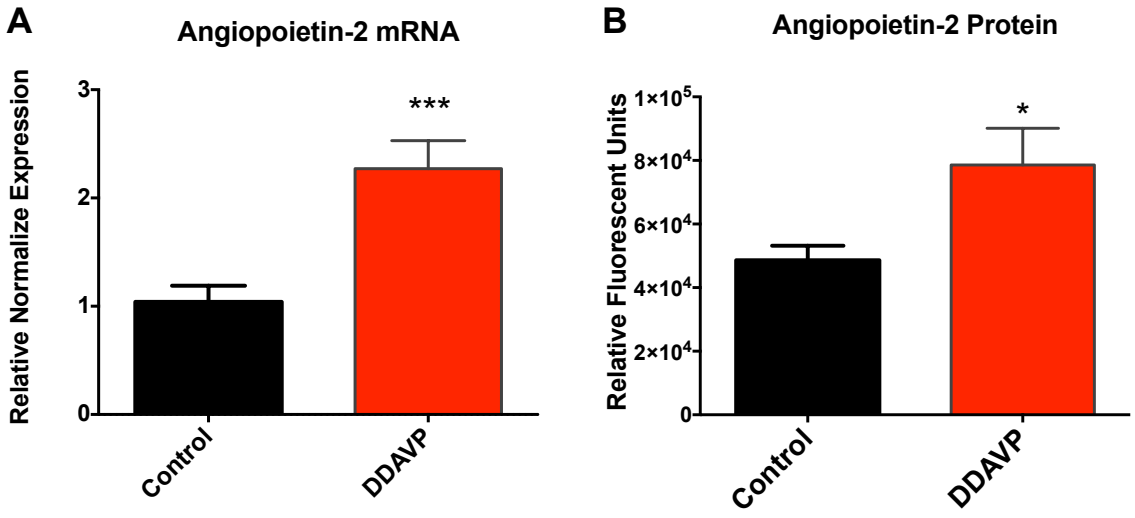


Figure 3.2.7. Angiopoietin-2 mRNA expression (A) and release of angiopoietin-2 protein (B).

Data are expressed as mean ± SEM. Two tail t-test, *p<0.05 vs. Control; ***p<0.001 vs. Control.

3.3.8. TLVP pretreatment attenuates the DDAVP-induced elevated mRNA expression of angiopoietin-2

The mRNA expression of angiopoietin-2 increased by 2.3 fold in HMVECs treated with DDAVP in 6 hours. When the cells were pretreated with TLVP prior to the addition of DDAVP, the increment in angiopoietin-2 mRNA was reduced and had no significant difference vs. the Control group (1.6 folds vs. Control). Cells treated only with TLVP did not significantly alter the mRNA expression level of angiopoietin-2.

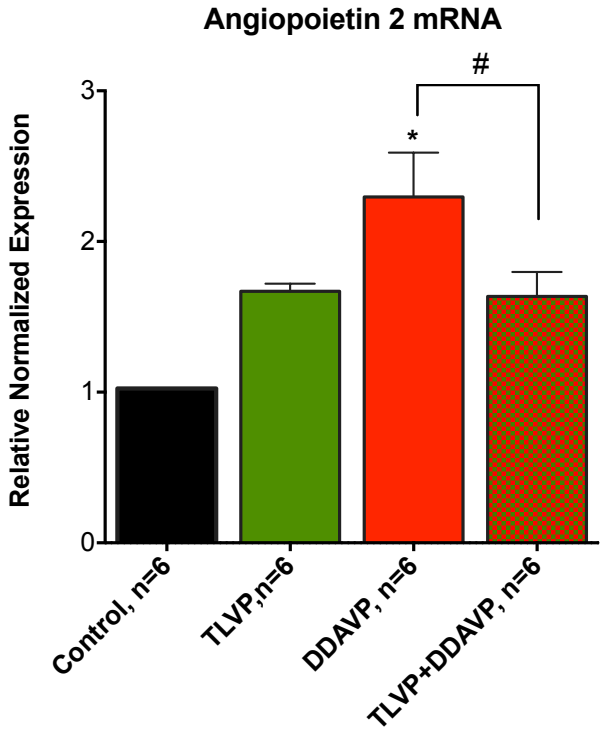


Figure 3.2.8. The DDAVP-induced elevated angiopoietin-2 mRNA expression is reduced by TLVP pretreatment.

Data are expressed as mean ± SEM. *p <0.05 vs. Control; #p <0.05 shows significant difference between DDAVP and TLVP + DDAVP group.

3.3.9. Co-incubation of HMVECs with different concentrations of MRSA upregulates angiopoietin-2 mRNA expression in a dose dependent manner

The expression of angiopoietin-2 mRNA tended to increase in cells exposed to $3x10^6$ CFU (1.2 folds vs. Control) and $6x10^6$ CFU (1.5 folds vs. Control). The increment in angiopoietin-2 mRNA reached statistical significance in the cells subjected to $1.2x10^7$ CFU (1.8 folds vs. Control). The highest concentration of MRSA significantly upregulated the angiopoietin-2 mRNA level and, in agreement, the milder increase in angiopoietin-2 mRNA in the presence of lower concentrations of MRSA provides evidence towards a MRSA-induced dose-dependent effect on angiopoietin-2 gene expression level. This elevation of angiopoietin-2 after MRSA challenge is consistent with previous work (111).

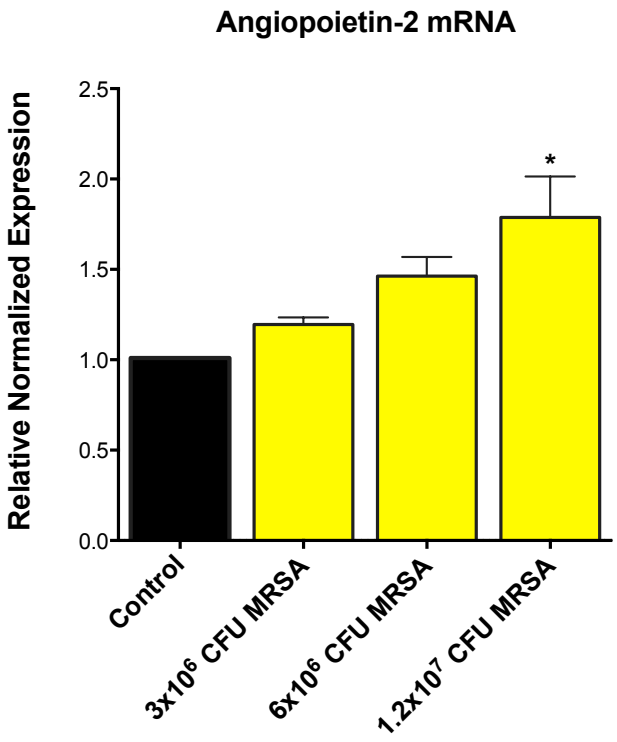


Figure 3.2.9. Increased angiopoietin-2 mRNA expression is detected in MRSA challenged HMVECs.

Data are expressed as mean ± SEM. *p <0.05 vs. Control.

3.3.10. MRSA-induced increase in angiopoietin-2 is attenuated by TLVP and potentiated by DDAVP

The expression of angiopoietin-2 mRNA significantly increased in cells exposed to $1.2x10^7$ CFU (2.4 folds vs. Control). Cells exposed to the same concentration of MRSA but pretreated with TLVP exhibited a decreased upregulation of angiopoietin-2 mRNA (2 folds vs. Control). Remarkably, co-incubation of MRSA and DDAVP induced an increase in mRNA expression of angiopoietin-2 higher than Control (3.3 folds vs. Control) and also higher than DDAVP or MRSA on its own (Figure 3.2.11.). These results demonstrate that the angiopoietin-2 upregulation induced by MRSA is mediated

by V_2R activation as TLVP pretreatment slightly inhibited the angiopoietin-2 mRNA level. The results also indicate that the selective V_2R agonist, DDAVP, potentiates the MRSA-induced upregulation of angiopoietin-2.

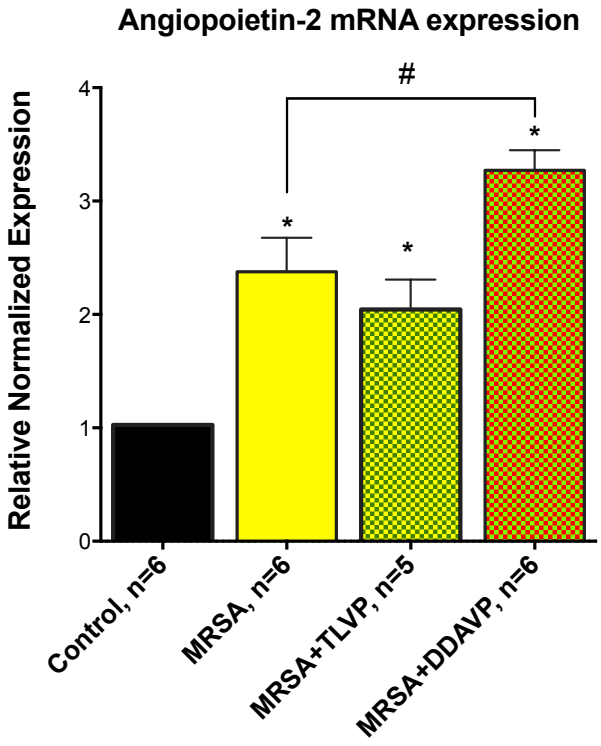


Figure 3.2.10. Changes in angiopoietin-2 mRNA expression in MRSA challenged HMVECs with and without TLVP and DDAVP treatment.

Data are expressed as mean ± SEM. *p <0.05 vs. Control; #p <0.05

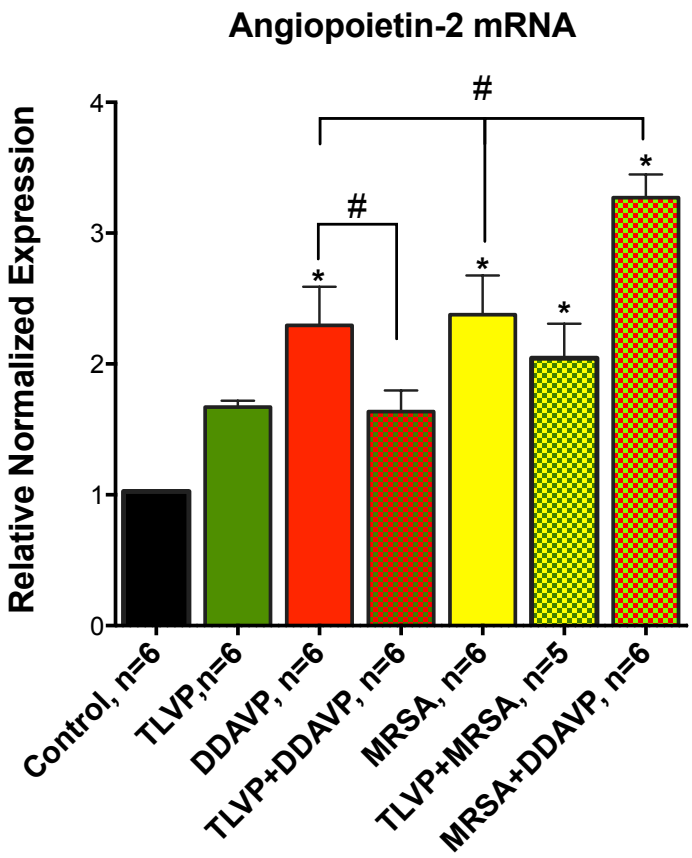


Figure 3.2.11. Angiopoietin-2 mRNA expression in HMVECs. Integrated figure 3.2.6. and 3.2.8.

Data are expressed as mean ± SEM. *p <0.05 vs. Control; #p <0.05.

3.3.11. Phospholipase C beta 4 (PLCB4) is significantly upregulated after DDAVP treatment.

A G proteins receptor p38 and JNK regulation multigene screening was conducted focusing on 36 different genes known to be mediators in this type of receptor. The name of the genes and their mRNA levels expressed in relative to Control are listed in table 3.3.1. Among all these data the PLCB4 gene expression was significantly increased by 2.7 folds vs. Control. The majority of the remaining genes showed a slight degree of upregulation relative to Control (31 out of 35). It is important to note that a

significant upregulation was also detected in the GNBI gene next to PLCB4. A trend of decreased expression of RAC1, PAK1, BTK and VAV1 vs. Control was present as well. These results suggest that PLCB4-mediated signalling pathways might have a role in the downstream pathways of V_2R activation.

GENE	RNE	SEM	GENE	RNE	SEM	GENE	RNE	SEM
PLCB4	2.72*	0.58	CDC42	1.5	0.22	AC013461.1	1.39	0.29
GNB1	1.79*	0.2	GNA12	1.5	0.27	MAPK13	1.39	0.28
GNAQ	1.77	0.4	MAP2K6	1.48	0.24	MAP2K4	1.33	0.17
PKN1	1.73	0.43	MAP3K1	1.48	0.29	MAP2K3	1.31	0.3
MAP2K7	1.72	0.26	MAPK9	1.48	0.21	PIK3CG	1.29	0.16
MEF2C	1.68	0.36	GNA13	1.48	0.28	RHOA	1.23	0.2
PRKCE	1.65	0.42	ATF2	1.44	0.31	GNB3	1.21	0.38
JUN	1.65	0.32	ARHGEF12	1.43	0.27	MAPK12	1.1	0.13
PLCB1	1.58	0.28	MAP3K4	1.43	0.29	RAC1	0.93	0.12
PTK2B	1.57	0.36	SRC	1.42	0.25	PAK1	0.91	0.19
MAPK8	1.57	0.3	MAPK14	1.42	0.25	BTK	0.82	0.23
GNAI2	1.55	0.3	ROCK1	1.41	0.32	VAV1	0.48	0.11

Table 3.3.1. Multi-gene screening. Relative normalized expression (RNE), standard error mean (SEM). *p <0.05 vs. Control.

3.3.12. mRNA level of PLCB4 gene proves to be upregulated in a dose dependent fashion in HMVECs co-incubated with different concentrations of MRSA

The expression of PLCB4 mRNA tended to increase in cells exposed to $3x10^6$ CFU (1.1 folds vs. Control) and $6x10^6$ CFU (1.4 folds vs. Control). The increase in PLCB4 mRNA reached statistical significance in the cells exposed to $1.2x10^7$ CFU (2.8 folds vs. Control). These findings provide evidence that, in endothelial cells, PLCB4 mediated pathways might have a role in MRSA induced endothelial cell injury. Furthermore, the same amount of MRSA is required to upregulate both angiopoietin-2 and PLCB4 mRNA levels, suggesting a common signaling pathway.

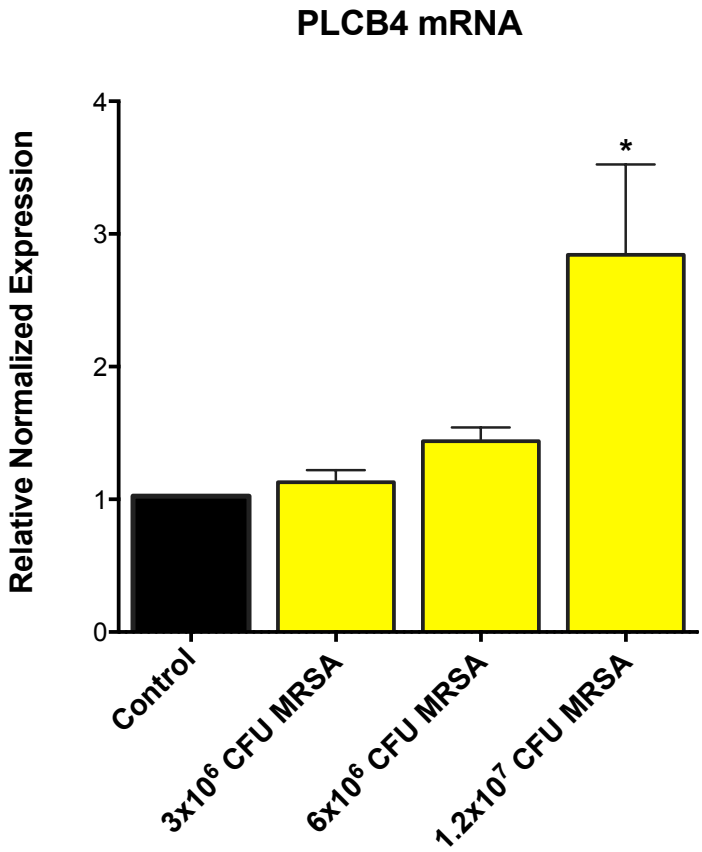


Figure 3.2.13. PLCB4 mRNA expression in MRSA treated HMVECs

Data are expressed as mean ± SEM. *p <0.05 vs. Control.

3.3.13. PLCB4 gene is upregulated by MRSA and DDAVP challenges.

The PLCB4 expression increased after a 6-hour-long treatment of DDAVP at 300 nM concentration (1.8 folds vs. Control) and $1.2x10^7$ CFU MRSA (2.2 folds vs. Control). This comparison suggests that PLCB4 might be a mediator in the cellular responses induced by both DDAVP and MRSA mediated stimuli.

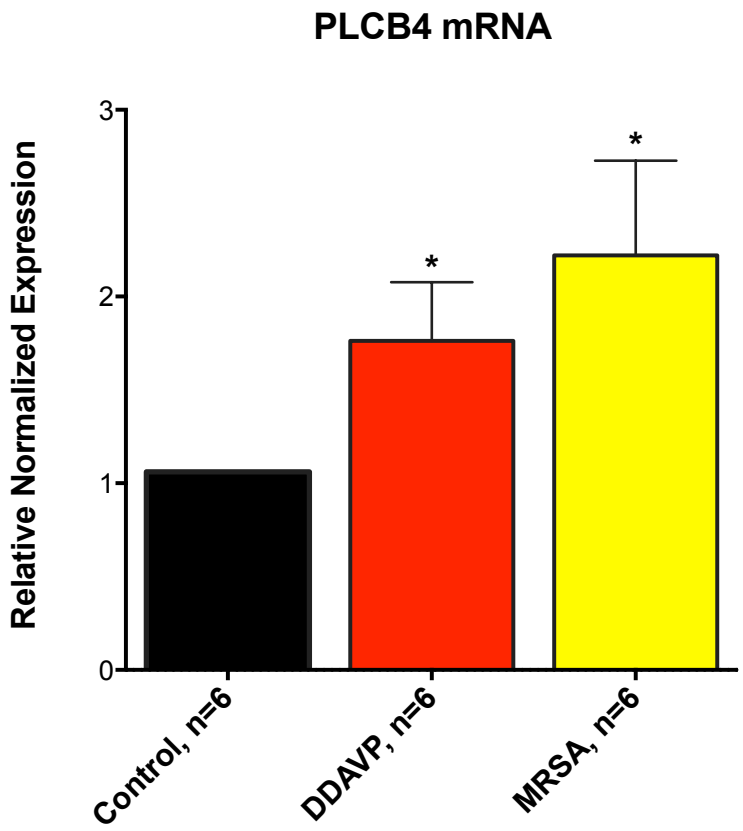


Figure 3.2.14. PLCB4 mRNA expression shows increment in MRSA treated and DDAVP treated HMVECs.

Data are expressed as mean ± SEM. *p <0.05 vs. Control.

3.3. Pilot study using AVP and lower doses of TLVP

In section 3.1., we showed the efficacy of the V_2R antagonist TLVP in attenuating vascular leakage. The therapeutic effect of TLVP was primarily proven by the reduction of retained fluid, but also by the improvement of several hemodynamic parameters.

However, the results of the study were not able to completely prove the safety of TLVP. We had a special concern about the undesired increase of Napl level in the TLVP-treated group. In addition, the level of creatinine in both urine and plasma was increased with comparison to the Control group. The alterations of Napl and creatinine are well documented as transitory effects of the drug (112, 113). However, given the fact that the dosage used is around 6 fold higher than the maximal dose approved by the FDA (83), we decided to conduct a pilot study using lower doses of TLVP.

Our goals were to; 1) determine whether low doses of TLVP could provide a protective effect against vascular leakage and 2) whether TLVP has also a superior therapeutic effect compare to AVP treatment. For this purpose, we compared the treatment with TLVP at lower concentrations vs. AVP in our MRSA sepsis model. The parameters of each studied group are described in table 3.3.1.

Group	Injury	Treatment	N
Sham	No injury	Saline	6
Control	SII + MRSA	Saline	5
TLVP-M	SII + MRSA	TLVP 114 mg/day	3
TLVP-L	SII + MRSA	TLVP 60 mg/day	2
AVP	SII + MRSA	0.01 – 0.1 U	1

Table 3.3.1. The present table summarizes the different animal groups to test lower doses of TLVP and also AVP as an additional treatment in our ovine model. Smoke inhalation injury (SII).

3.3.1. Lower doses of TLVP were effective in preventing the retention of fluid

The Control had a positive net fluid balance of (125 ± 18.9 mL at 24 hours) compared to the close to neutral fluid balance in Sham group (0.84 ± 7.6 mL at 24 hours). In TLVP-M (60.4 ± 54.9 mL at 24 hours), the fluid balance was lower than Control between 12 and 24 and statistically higher than Sham at 24 hours. In TLVP-L (35 ± 62 mL at 24 hours), the fluid balance was lower than Control between 18 and 24 hours and statistically unchanged vs. Sham. The levels of hematocrit at 24 hours, were comparable among the 4 groups (data not shown). These results indicate that both intermediate and lower doses of TLVP may be efficacious in attenuating sepsis-induced accumulation of fluid.

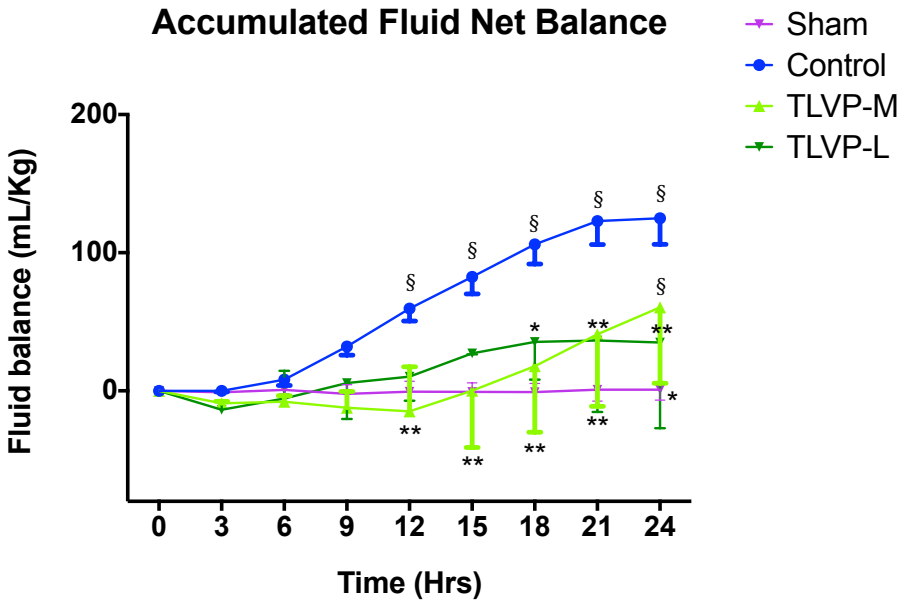


Figure 3.3.1. Fluid retention is indicated with the fluid net balance

Data are expressed as mean ± SEM. *p <0.05 vs. Control; §p <0.05 vs. Sham.

3.3.2. Undesired increase in plasma sodium was transitory with the intermediate dose and absent with the lower dose

The concentration of sodium in plasma (Napl) was decreased in the Control group (138.6 ± 0.82 mL at 24 hours) between 6 and 24 hours vs. Sham (144.7 ± 0.75 mL at 24 hours). In TLVP-M (142.9 ± 1.8 mL at 24 hours), Napl was elevated vs. Control between 6 and18 hours and vs. Sham between 9 and 18 hours. In TLVP-L (146.9 ± 1.5 mL at 24 hours), Napl was elevated vs. Control between 6 and 24 hours and was statistically similar to Sham. Compared to the results from section 3.1.19. using a higher dose of TLVP, here the intermediate dose had just a transitory increase in Napl and the lower dose had no increase. In Figure 3.3.2., we can also observe that the level of NApl

decreased in Control vs, Sham. However, both doses of TLVP were able to prevent the excessive loss of Napl.

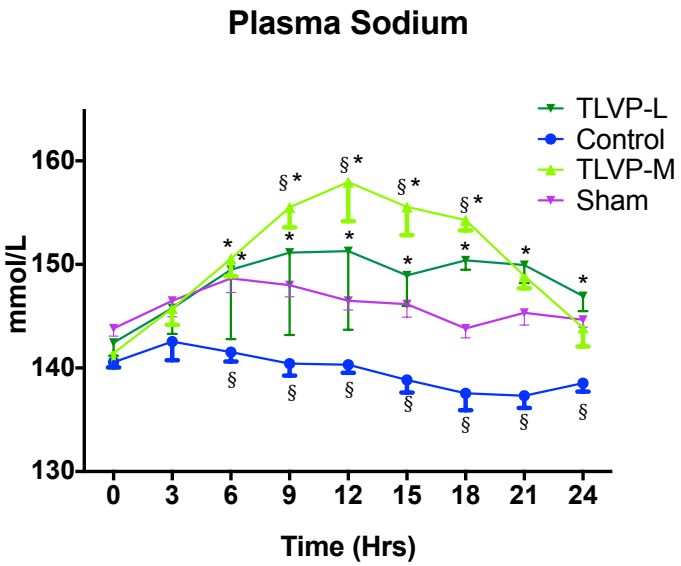


Figure 3.3.2. The plasma sodium level with lower doses of TLVP are shown

Data are expressed as mean ± SEM. *p <0.05 vs. Control; §p <0.05 vs. Sham.

3.3.3. Creatinine plasma levels were unchanged either at intermediate or the lower dose of TLVP

The concentration of creatinine in plasma (plCr) was augmented in Control group (1.0 ± 0.11 mg/dL at 24 hours) at 12 hours vs. Sham (0.6 ± 0.03 mL at 24 hours). At 24 hours, there was no difference among, Sham (0.6 ± 0.02 mg/dL at 24 hours), Control (0.8 ± 0.15 mg/dL at 24 hours), TLVP-M (0.8 ± 0.11 mg/dL at 24 hours) and TLVP-L (0.9 ± 0.01 mg/dL at 24 hours). Regarding the levels of BUN in plasma, a slight increase was observed at 12 hours in Control and TLVP-M group vs. Sham, although with no statistical relevance. The levels at 24 hours were 12.8 ± 1.3 mg/dL, 13.6 ± 1.3 mg/dL,

12.3 ± 1.5 mg/dL and 13.5 ± 1.5 mg/dL in Sham, Control, TLVP-M and TLVP-L respectively.

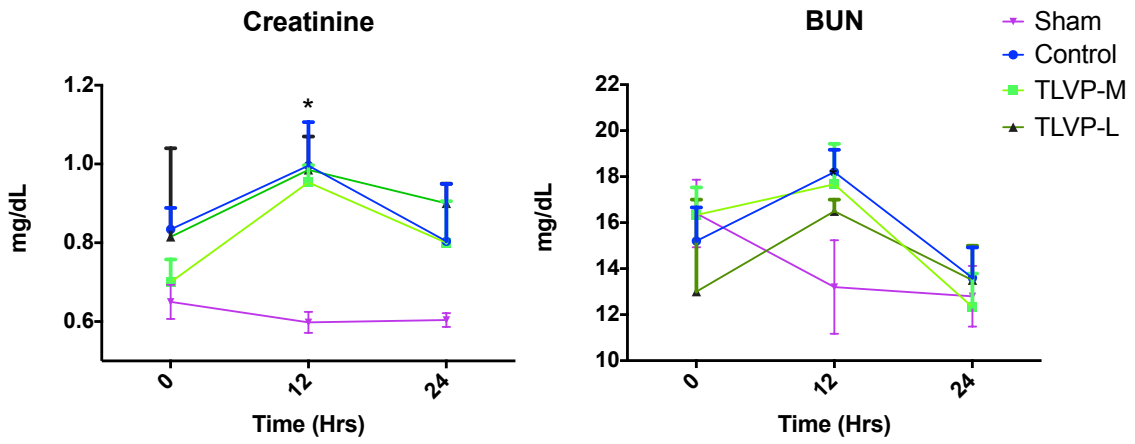


Figure 3.3.3. Kidney function determined by Creatinine and BUN in plasma

Data are expressed as mean ± SEM. *p <0.05 vs. Control; §p <0.05 vs. Sham.

3.3.4. The therapeutic effect of AVP vs. TLVP: the MAP was maintained with AVP, although it had no effect to prevent fluid retention

By comparing a sheep treated with AVP vs. the Sham, Control, TLVP-M and TLVP-L groups we found that blood pressure was slightly higher in AVP vs. the rest. However, the fluid balance after AVP treatment was clearly higher vs. the rest of the groups.

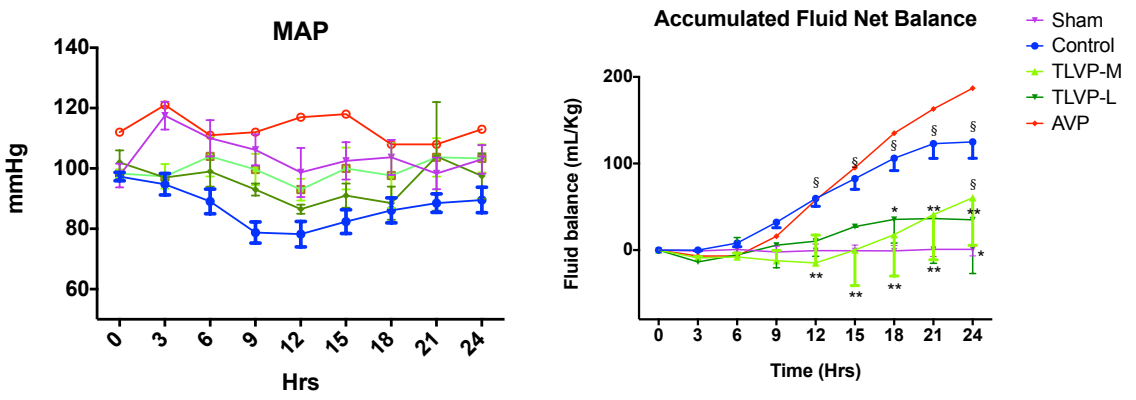


Figure 3.3.4. Mean arterial pressure and fluid balance in a sheep treated with AVP vs. Sham, Control and TLVP treated groups.

Data are expressed as mean ± SEM. *p <0.05 vs. Control; §p <0.05 vs. Sham.

4. DISCUSSION

4.1. Primary endpoints

The finding of the present project is that activation of V_2R plays a critical role in the pathophysiology of vascular hyperpermeability during severe sepsis and septic shock. This concept is supported by the results of both *in vivo* and *in vitro* cell culture studies. First, treatment of septic sheep with the V_2R antagonist tolvaptan significantly attenuated the sepsis-induced fluid retention and markedly reduced lung water content. These pathological changes were not affected or augmented by the treatment with V_2R agonist desmopressin (DDAVP). Secondly, the incubation of cultured HMVECs with DDAVP or with VEGF (the latter served as a positive control of our assay) significantly increased paracellular permeability. Moreover, endothelial cells subjected to MRSA also augmented endothelial permeability. Finally, both the DDAVP- and MRSA-induced elevated hyperpermeability were significantly attenuated by treatment with the V_2R antagonist, tolvaptan (TLVP).

Our group previously described that the retention of fluid was abolished in septic sheep treated with a V_1R selective agonist compared to other groups treated with AVP (V_1R and V_2R nonselective agonist) or vehicle (33). We have also described that co-treatment with DDAVP abolished the salutary effects of V_1R agonist, suggesting a possible role of V_2R activation in the severity of vascular leakage in ovine sepsis (34). Some of these results were reproduced recently by He *et al*. (35). In summary, our present

and previous studies demonstrate a critical role of V_2R activation in vascular leakage during sepsis.

In the present proposal, we have also investigated the downstream mechanisms of V_2R mediated vascular leakage.

First, we tested the hypothesis whether the V_2R-induced microvascular hyper-permeability is mediated by the potent permeability factor angiopoietin-2, which has been linked to the severity and prognosis of sepsis in numerous clinical reports (70, 114, 115). Interestingly, the MRSA-induced angiopoietin-2 increase was significantly reduced in heart tissue collected from the TLVP treated animal group. This was supported by the *in vitro* results showing that both angiopoietin-2 gene and protein secretion were elevated by the addition of the V_2R selective agonist, DDAVP. Furthermore, pretreatment with the V_2R antagonist TLVP proved to inhibit the upregulation of angiopoietin-2 in HMVECs exposed to DDAVP and MRSA. In our previous studies, we have also demonstrated that increases in heart and lung tissue of MRSA septic sheep were significantly inhibited by V_1R selective agonist (33).

Multiple *in vitro* and *in vivo* studies have confirmed the major role of angiopoietin-2 as an endothelial barrier-disrupting factor (24, 116). Angiopoietin-2 is an endogenous competitor of angiopoietin-1, an endothelial pro-survival and barrier-enhancing agent. In sepsis, the ratio of angiopoietin-2 / angiopoietin-1 increases, leading to a gradual loss of the protective role of angiopoietin-1. Angiopoietin-2 is localized in the

WPBs, and it has previously been demonstrated that the activation of endothelial V_2R releases vWF, a major resident of the WPBs (51, 78, 117); therefore, as stated in our sub-hypothesis, it is feasible to consider that activation of endothelial V_2R may release angiopoietin-2 as well. So, our research is the first to demonstrate the strong connection between V_2R activation-induced microvascular hyperpermeability and angiopoietin-2 release.

To further extend our findings, we aimed to elucidate the mechanisms through which V_2R activation causes release of angiopoietin-2. Utilizing a multigene assay to determine the expression of the most important GPCRs, we found that the PLCB4 gene was upregulated in cultured HMVECs after DDAVP or MRSA treatment. Phospholipase C beta (PLCβ) enzyme is a target molecule of the Gq subunits. PLCβ activation catalyzes the formation of diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3) from the cleavage of phosphatidylinositol 4,5-bisphosphate (PIP_2). IP3 is directly involved in the release of intracellular calcium (118). As we described in section 1.4.1., V_2R is coupled to Gs but not Gq. However, it has been demonstrated that V_2R has a particular ability to activate PLC in a Gq independent manner (119). Therefore, we speculated that in endothelial cells, V_2R might not just use cAMP as a secondary messenger, but also IP3 and calcium. Additionally, it is well known that both tissue plasminogen activator (TPA) and angiopoietin-2 are common residents of the WPBs (40) and Muldowney *et al*. demonstrated that the release of TPA in endothelial cells is mediated by PLCβ (120). Therefore, it is quite possible that the release of angiopoietin-2 occurs in a PLCβ dependent manner.

Other important intermediates such as p38MAPK, AP-1, JNK and ERK1 also have a role in the regulation of endothelial function (121, 122), and we first speculated that those intermediates might participate in the V_2R activation-induced angiopoietin-2 release. However, the multi gene expression analysis showed no gene upregulation of p38MAPK, AP-1, JNK or ERK1.

Taken together, the current results indicate that V_2R activation during sepsis causes microvascular hyperpermeability through angiopoietin-2 and PLCβ-4 mediated signaling pathways. Furthermore, MRSA also proved to stimulate the V_2R- PLCβ-4-angiopoietin-2 axis.

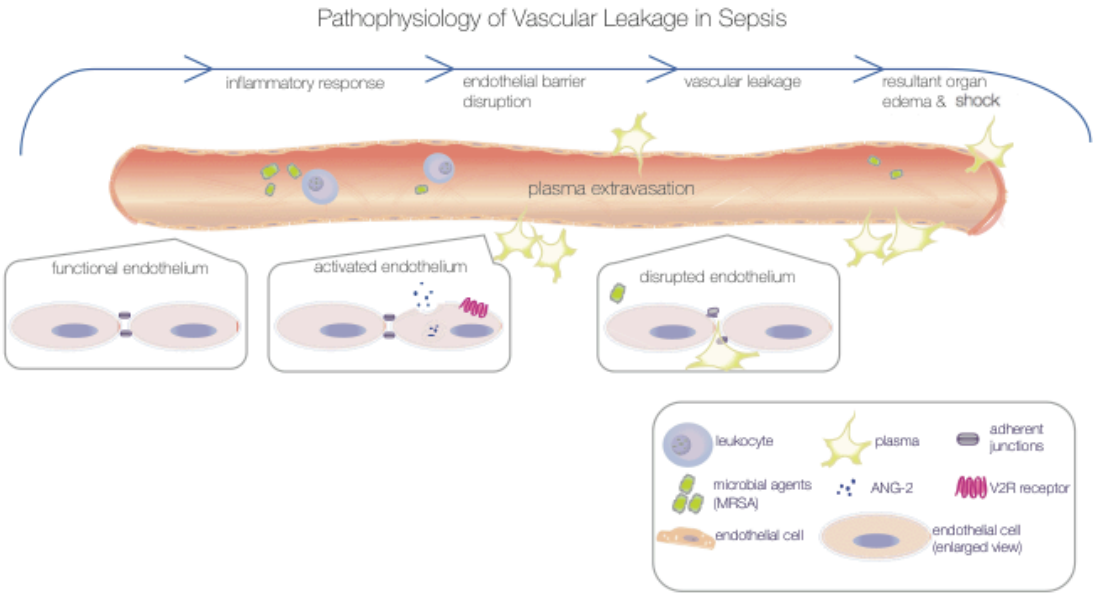


Figure 4.1. Schematic representation of the role of arginine vasopressin receptor 2 in the pathophysiology of vascular leakage during sepsis.

As mention in this section, during MRSA-sepsis, the activation of V_2R promotes the release of angiopoietin-2, which triggers the disruption of the endothelial barrier. The result is an increase in plasma extravasation leading to cardiovascular collapse.

4.2. Secondary endpoints

Heart and lung variables

Parallel to the results explained above, we found a noteworthy therapeutic effect of TLVP in lung. This was supported by the reduction of the lung water content (indicated by W/D) in combination with the reduced lung capillary hydrostatic pressure and pulmonary resistance (indicated by the P_c and PAP).

Our first interpretation of this favorable event was that the lung endothelial barrier integrity was preserved based on the antileak effect of TLVP. We also found a significant decrease in LAP in the TLVP treated sheep, suggesting that V_2R antagonist improves heart performance during sepsis. Interestingly, treatment with TLVP significantly inhibited increases in circulating brain natriuretic peptide (BNP). As we know, BNP is a marker of myocardial stress and clinical trials have shown its correlation with sepsis-induced myocardial dysfunction (123, 124). These results suggest that V_2R antagonist treatment may reduce, at least in part, pulmonary edema by improving heart muscle performance during sepsis. The fundamental mechanism of this salutary effect of TLVP on heart performance remains unknown and needs to be further clarified in future studies. However, these intriguing cardiopulmonary outcomes are consistent with other animal and clinical studies of heart failure that have showed similar reductions of PAP, BNP, and LAP following TLVP treatment (84, 93, 125, 126).

It is worth noting that TLVP also showed a therapeutic effect in lung function, indicated by a decrease in the mean airway pressure and oxygenation index. The exact

mechanism of how TLVP improves pulmonary function during sepsis is not completely understood. It can be partially related to the improved cardiac function. However, it is unlikely that this salutary effect is solely attributable to the improved cardiac function, as our *in vitro* studies in cultured HMVECs demonstrated that the MRSA induced permeability is significantly inhibited by TLVP and augmented by DDAVP. The fact that TLVP significantly reduced the systemic fluid retention strongly suggests that V_2R activation is a major causative factor for pulmonary edema rather than sole role of improved cardiac performance.

Low dose of TLVP

In this project, we obtained favorable effects attributed to TLVP treatment in sepsis. However, we also found an undesired increase in plasma sodium associated with the administration of TLVP. The increase of sodium is a well-documented effect of TLVP in animal experiments and clinical trials (93, 112, 113). However, the levels of sodium raised approximately four standard deviations above the normal range. In addition, the levels of creatinine and urea in both urine and plasma were marginally altered compared to the control group. In patients, both alterations in electrolytes and renal functions are common clinical manifestations of TLVP, which are reversed after drug cessation (112, 113). The animals were euthanized before cessation of the drug, and this above-mentioned concept was not proved in our ovine model. However, we tested lower doses of TLVP to elucidate its therapeutic safety. We tested the effect of TLVP at one third (~120 mg/day) and one fifth (60 mg/day) of the first tested dose (~320 mg/day). Both low doses demonstrated to be equally efficacious attenuating vascular leakage. In contrast, there was

no increase in plasma sodium with 60 mg/day TLVP, and only a transitory increase associated with 120 mg/day TLVP. The levels of creatinine and urea were also unaffected with the lower doses of TLVP.

5. CONCLUSIONS

In conclusion, the results of the present work show that endothelial V_2R activation increases vascular permeability in an angiopoietin-2 and PLCβ dependent manner. Proven by *in vivo* and *in vitro* experiments, the V_2R antagonist can effectively mitigate the MRSA-induced microvascular hyperpermeability.

Based on the present results and the contributions of previous investigations, we conclude that in septic patients, V_2R requires further attention and perhaps a closer modulation could improve the outcome of septic patients, particularly in those with severe fluid retention and tissue edema. In the current study, we showed that the V_2R antagonist, tolvaptan can achieve this goal in a clinically correlated animal model, although further evidence would be required before this concept could move into a clinical trial.

Future directions for the management of sepsis

The current findings will help researchers to find new directions for the development of an effective therapeutic option to modulate V_2R activation in the management of severe sepsis and septic shock. We determined that, TLVP treatment prevented the capillary leak and improved heart and lung hemodynamics.

To effectively ensure a normotensive state, the use of TLVP as an adjunct therapy to other first line vasopressor agents could be a better choice. This may help to achieve the goal of maintaining blood pressure at a desired level, reducing the requirement for both large volume of fluid resuscitation and high doses of vasopressors, and reducing associated side effects, such as augmentation of fluid and ischemia/reperfusion injury.

Based on the current contribution, a V_2R antagonist, such as TLVP, at an optimized dose could improve the outcome of septic patients alone or as adjuvant therapy to vasopressors.

In addition, the results of our study also point out the necessity for pilot clinical studies to test the efficacy of TLVP treatment in septic patients.

Vita

Ernesto Lopez was born in La Piedad, Mexico in March 26th 1985. In 2003, he entered to medical school at the University of Monterrey in Monterrey, Mexico. In 2010, he graduated from medical school and in the same year he joined the neuroscience graduate program at the University of Texas Medical Branch. Dr. Lopez conducted his dissertation project at the translational intensive care unit under the mentorship of Dr. Perenlei Enkhbaatar.

Permanent address:
482 Leona Vicario,
La Piedad, Michoacan, Mexico, 59300.
This dissertation was typed by Ernesto Lopez

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