Coagulation changes following traumatic brain injury and shock

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1. STUDIES INCLUDED

This thesis is based on the following three studies, performed as a Harvard Medical School Research Fellow at the Massachusetts General Hospital in Boston, USA:

<u>Sillesen M</u>, Johansson PI, Rasmussen LS, Jin G, Jepsen CH, Imam A, Hwabejire J, Lu J, Duggan M, Velmahos G, DeMoya M, Alam HB: Platelet activation and dysfunction in a large animal model of traumatic brain injury and hemorrhage. J Trauma Acute Care Surg. 2013 May;74(5):1252-9

<u>Sillesen M,</u> Rasmussen LS, Jin G, Jepsen CH, Imam A, Hwabejire JO, Halaweish I, DeMoya M, Velmahos GC, Johansson PI, Alam HB: Assessment of coagulopathy, endothelial injury and inflammation after traumatic brain injury and hemorrhage in a porcine model. J Trauma Acute Care Surg. 2014 vol 76(1): 12-9.

<u>Sillesen M,</u> Rasmussen LS, Jin G, Jepsen CH, Imam A, Hwabejire JO, deMoya M, Velmahos GC, Johansson PI, Alam HB: Valproic acid attenuates platelet dysfunction following neurotrauma. Submitted to Injury May 2014.

2. ABBREVIATIONS

Abbreviation	Meaning
ТВІ	Traumatic Brain Injury
HS	Hemorrhagic shock
MAP	Mean arterial blood pressure
TEG	Thrombelastography
LY30%	TEG lysis index

VPA	Valproic acid	
PF 1+2	Prothrombin fragment 1+2	
C5a	Activated complement factor 5	
hcDNA	Histone complexed DNA	
TF	Tissue Factor	
sTM	Soluble Thrombomodulin	
sVCAM-1	Soluble vascular cell adhesion	
	molecule 1	
vWF	Von Willebrand Factor	
PAI-1	Plasminogen activator inhibitor 1	
TNF-α	Tumor necrosis factor alpha	
IL-6	Interleukin 6	
IL-8	Interleukin 8	
ATIII	Antihrombin III	
aPC	Activated protein C	
sP-Selectin	Soluble P-Selectin	
TGF-β	Transforming growth factor beta	
CD40L	CD40 Ligand	
TLR	Toll Like Receptor	
РІЗК/АКТ	Phospharidyinositol-4,5-	
	biophosphate 3-kinase/ Protein	
	Kinase B	
MAP kinase	Mitogen activated protein kinase	

3. INTRODUCTION

Despite recent treatment advances, trauma remains the leading cause of death in those younger than 40 years of age(1). The risk of adverse outcome depends on the modality of the injury, but traumatic brain injury (TBI) and hemorrhagic shock (HS) have consistently been associated with mortality and morbidity in trauma patients.

Indeed, TBI has been estimated to be a contributing factor to up to one third of injury related deaths(2) while almost 50% of patients with non-compressible hemorrhage succumb to their injuries in the civilian settings(3). Most of these trauma related deaths occur within the first day after injury(4), and targeting this early phase of injury thus holds the potential to significantly increase survival and reduce morbidity in these critically injured patients. Successful development of such targeted treatment strategies does however hinge upon knowledge of the complex immunological interplay between pathways occurring early after trauma, but our insight into this field remains incomplete at best. Recent evidence suggests that trauma elicits a massive immunological response from the host, with almost 80% of the leucocyte genome responding at the time of hospital arrival or shortly thereafter(5). These results thus suggest that the majority of the immunological response to injury is already mounted upon hospital arrival, and it is thus difficult to elucidate pathways amendable to early treatment from human studies alone. A better understanding of these early responding pathways could, however, help identify novel therapeutic targets since early responding pathways likely exerts a ripple effect and further drive the dysregulation of the immune response, ultimately leading to adverse outcome.

The coagulation system and downstream effects

One such promising pathway could be the coagulation system. Several studies have thus indicated that both isolated brain injury and shock cause early activation of this pathway(6-9). In the case of TBI, this is classically described to be the result of tissue factor release from the injured brain parenchyma or exposure of blood to subendothelial collagen, catalyzing a series of enzymatic reactions through either the intrinsic or extrinsic pathway activation. This differentiation is, however, likely more relevant when investigating coagulation in the in-vitro environment only. In contrast, rather than being simply a series of enzymatic reactions restricted to the coagulation cascade, it is increasingly recognized that coagulation is a cell-based reaction occurring on the negatively charged surfaces of activated cells such as platelets(10). This procoagulant surface promotes the enzymatic cascade reactions classically associated with the coagulation system, and provides the foundation for the thrombin burst(11) as well as the integration of coagulation factors V and VIII into the prothrombinase and tenase complexes, respectively(12). Furthermore, cell surface expression of coagulation activators such as tissue factor (TF) has been described in multiple different cell lines such as monocytes(13), endothelial cells(14), platelets(15) as well as cell surface derived microparticles(16). Coagulation is, however, not only activated by vessel injury. A multitude of different mediators thus have the capacity to activate the coagulation system, including but not limited to complement(17), bacterial byproducts(18), the sympatho-adrenal system(19) as well as cellular debris such as histones(20). Activation of receptors such as the Toll-Like family (TLR's) through these mediators as well as coagulation related products such as fibrinogen(21), furthermore provide strong links between the coagulation and the innate immune defenses.

Activation of the coagulation system thus further modulates important downstream pathways, in part directly related to the pluripotent effects of thrombin. Thrombin directly activates complement factors C3 and C5, which in turn acts in a reciprocal manner to further amplify the coagulation response(22). The conversion of prothrombin to thrombin also promotes a proinflammatory response through its actions on the family of protease-activated receptors (PARs), and studies have indeed demonstrated that activation of receptors such as PAR-1 are key drivers of inflammation, including promoting the interleukin 6 (IL-6) response(23, 24).

Thrombin furthermore promotes the activation of natural anticoagulation and fibrinolysis(25), as well as regulate endothelial(26) and platelet activation(27).

It is thus clear that activation of the coagulation system, rather than solely being a hemostatic event, is an integral part of the early immune response to trauma. As such, treatment strategies modulating this response could from a theoretical standpoint also alter the subsequent downstream effects such as inflammation, classically associated with adverse outcomes in trauma patients.

Dysregulation of coagulation – the acute traumatic coagulopathy

Many of these adverse downstream effects are observed in the setting of a dysregulated coagulation response immediately following trauma, an entity that has been termed acute traumatic coagulopathy (ATC). This may be defined as abnormal levels of markers of coagulation cascade potential, including increased international normalized ratio (INR), prothrombin time (PT) or activated partial thromboplastin time (aPTT).

This devastating complication of injury affects up to 25% of severely injured patients(28) and is characterized by a reduced coagulation capacity coupled with increased activation of natural anticoagulation and fibrinolysis(29). The effects of ATC are, however, not only restricted to the coagulation cascade as evidenced by a fourfould increase in mortality in ATC patients(28) as well as an association between ATC and the development of late multi organ failure(30). In contrast, ATC has on the cellular level been associated with dysregulation of many of the above mentioned pathways, including endothelial activation(31), platelet dysfunction(32), sympatho-adrenal activation, cell death and inflammation(33, 34).

Collectively, this suggests that although the underlying pathophysiological mechanisms have yet to be determined, coagulopathy likely presents an entity in the extreme end of the immune response continuum following injury, with tight associations to dysregulation of the immunological response as a whole. Early modulation of the coagulation response may thus potentially affect the development of coagulopathy

The role of histone acetylation and histone deacetylase inhibitors

Acetylation and deacetylation of lysine structures on core histones and other proteins were first identified in 1968(35). Depending on the location, these events regulate numerous cellular processes such as gene transcription, proliferation, metabolism and differentiation.

When cellular signaling pathways results in altered gene transcription, messenger signals enter the nucleus in order to regulate transcription from the chromatin. In order to facilitate transcription, the chromatin needs to be maintained in an uncoiled and transcriptionally active formation. This process is subject to epigenetic regulation by acetylation of lysine residues on histones around which the DNA is coiled.

Acetylation of these lysine residues thus promote uncoiling of the DNA from the histones as well as to facilitate the binding of transcription factors to DNA strands.

The effect of acetylation is thus in essence to uncoil the DNA and render it accessible to transcription factors, thus promoting increased genomic transcription and protein production from cells.

The acetylation is catalyzed by the activity of a family of enzymes known as the histone acetylases (HATs), whereas deacetylation is catalyzed by the histone deacetylases (HDACs). HDACs are classically divided into four groups, based on their homology to yeast(36). Class I HDACs include HDAC 1-3 and 8, whereas class II HDACs include HDAC 4-7 and 9-10. Class III HDACs are referred to as Sirtuins and include SIRT1-7. Finally, class four HDACs include HDAC11.

In normal cells, the balance of HAT and HDAC activity is maintained in a harmonic equilibrium and the term "acetylation homeostasis" has previously been coined to describe this phenomenon(37). Studies have, however, confirmed that insults such as hemorrhagic shock and related events such as low oxygen states promote the upregulation of HDAC activity(38, 39). This disrupts the aforementioned equilibrium, essentially inhibiting genomic transcription and protein production(40).

These findings have sparked an interest in investigating pharmaceutical modulation of the HDAC activity in an effort to promote genetic transcription and pro-survival pathways in various shock states.

Recent studies have thus focused on a group of histone deacetylase inhibitors (HDACI), including valproic acid (VPA). VPA and other HDACIs such as suberoylanilide hydroxamic acid (SAHA) and trichostatin A, are pan-HDAC inhibitors and do as such exert inhibitory effects on Class I-IV HDAC enzymes.

Valproic acid was first approved by the US food and drug administration for the treatment of epilepsy, mood disorders and migraine in 1978 and remains in widespread clinical use. Studies have, however, indicated that the administration of VPA increase survival in both large and small animal models of hemorrhagic shock(41-44) as well as provide neuroprotection following TBI(45).

While the effects of HDACIs are likely multifactorial, these positive results have largely been attributed the histone acetylation regulating properties of VPA and other HDACI's and are classically associated with a downregulation of cell death as a result of necrosis and apoptosis through a plethora of different signaling pathways, including a modulation of TLR, PI3K/AKT and MAP kinase signaling pathways (43, 46, 47).

At present, little is known of the potential interactions between VPA and the coagulation system. From a theoretical standpoint, the reduced activation of key proinflammatory pathways and associated cell death could lead to a reduction in activators of coagulation through pathways discussed above and thereby mediate an attenuation of many of the adverse downstream effects previously described. Alternatively, the beneficial effects of VPA could be attributed to unknown interactions with the coagulation system.

A better understanding of the effects of VPA treatment on the early coagulation response may thus yield insight into the beneficial effects of VPA, thus facilitating the development of novel targeted HDACI's. Furthermore, knowledge of the interplay between HDACI's and the coagulation system could unveil new potential areas of use, such as coagulopathy.

Aim and hypothesis

The overall aim of these three studies was to describe the early immunological response to a combination of TBI and shock as well as to investigate the effects of VPA treatment on this response using a previously validated large animal model of these combined insults(48)

Study 1 and 2 – effect of injury

The aim of these two studies was to investigate the early temporal relationship between activators of coagulation and downstream effects, including platelet, endothelial, complement, sympatho-adrenal and inflammation activation following combined TBI and shock.

Study 3 – effect of intervention with valproic acid

The aim of this study was to investigate whether treatment with the histone deactelylase inhibitor valproic acid would attenuate the activation of the in study 1 and 2 investigated pathways following combined TBI and shock.

Hypotheses

We hypothesized that coagulation, platelet and endothelial activation would be detectable early after injury and would be associated with subsequent platelet and endothelial dysfunction as well as inflammation.

We furthermore hypothesized that treatment with VPA would attenuate these changes.

3. METHODS

Group allocation and overview of studies: A total of 42 Female Yorkshire swine (40–50 kg; Tufts Veterinary School, Grafton, MA, USA) were used for the purpose of all experiments. Depending on the study, animals were allocated to groups as described in table 1.

Study number	Objective	Groups
1	To asses platelet	TBI/Shock (n=27)
	activation and dys-	Controls (n=6)
	function following a	
	combination of TBI	
	and shock	
П	To asses activation of	TBI/Shock (n=27)
	coagulation,	Controls (n=6)
	endothelial and	
	inflammation activa-	
	tion following TBI and	
	shock	
III	To asses the effect of	TBI/Shock (n=27)
	VPA infusion on	TBI/Shock + VPA
	markers measured in	infusion (n=9)
	study I and II	

Table 1: Study description and group allocations

Animals in the TBI/Shock and TBI/Shock+VPA groups were subjected to a computer controlled TBI with subsequent 40% hemorrhage as described below. Animals in the TBI/Shock+VPA group furthermore received an infusion of VPA 100mg/kg/hour as described below.

Animals in the control group received similar anesthesia, instrumentation and observation, but no injury.

Animal preparation (All groups) A model overview is provided in figure 1.

Animal preparation is described in detail in the individual manuscripts (please see appendix). Briefly, animals were preanesthetized with in intramuscular injection of Tiletamine/Zolazepam and atropine prior to induction of anesthesia with inhalation of isoflurane. Animals were intubated and placed on ventilator support.

Instrumentation and monitoring (All groups): After induction of anesthesia, a cutdown technique was used to access the right and left femoral arteries for invasive blood pressure monitoring and blood draw respectively. The left femoral vein was cannulated for fluid administration, whereas the right external jugular vein was

used for the placement of a pulmonary artery (PA) catheter. A lower midline laparotomy was performed for the insertion of a cystostomy tube. Hemodynamic parameters were recorded in five-minute intervals.



Figure 1: Overview of the model. Valproic acid infusion was started one hour into shock in the TBI/Shock+VPA group (n=9) and continued for the duration of the experiment.

Traumatic brain injury and cerebral monitoring (TBI/Shock and

TBI/Shock+VPA groups): Controlled cortical impact was created as previously described in detail(48). Briefly, a 20 mm burr hole was made on the right side of the skull, next to the coronal and sagittal sutures over the frontal lobe to expose the dura. A computer-controlled cortical impact device was used for these experiments(49). A 15 mm cylindrical impactor tip was mounted on an electronic motor, and the dynamics were controlled to deliver 4 m/s velocity, 100 ms dwell time and 12 mm depth cortical penetration. After impact, the burr hole was sealed with bone wax to prevent leakage of cerebrospinal fluid. The control group underwent similar instrumentation, but did not receive the cortical impact.

Hemorrhage and resuscitation protocol (TBI/Shock and

TBI/Shock+VPA groups): Total blood volume was estimated, and 40% of it was withdrawn through the femoral arterial catheter. Bleeding was started concurrent with TBI at a rate of 3.15% total blood volume/min.

Following hemorrhage, animals were left hypotensive for 120 minutes and MAP was maintained between 30-35 mmHg by titrating the dose of inhaled isoflurane. After the 2 hours of hypotension, the current study was concluded.

The control group was not subjected to the hemorrhage protocol but was observed for a similar duration of time.

Blood sampling (All groups):

Blood samples were drawn from the femoral arterial line using a vacutainer[®] device (BD, Franklin Lakes, NJ) into collection tubes containing 0.109M buffered Sodium Citrate, Ethylenediaminte-traacetic acid (EDTA), Sodium Heparin (BD, Franklin lakes, NJ) or serum collection tubes at 4 discrete time points: Baseline, 3 min post injury, 15 min post injury and following 2 hours of hypotension. In the control group, injury time was arbitrarily set following instrumentation and postinjury times were calculated from this timepoint.

EDTA anticoagulated blood was centrifuged within 10 minutes of collection at 3000 rpms for 10 minutes at 4° and was subsequently

stored in 1ml aliquots (Eppendorf, Hauppauge, NY) at -80° pending analysis. Serum collection tubes were allowed to rest for 30 minutes prior to centrifugation and processing as described above.

Blood counts (EDTA anticoagulated blood, Hemavet 950, Drew Scientific, Dallas, TX) as well as arterial blood gasses (Heparinized blood, Profile Critical Care Xpress, Nova Biomedical, Waltham, MA) were analyzed at the same time points.

Platelet function:

In studies I and III, platelet function was assessed by whole blood impedance aggregometry using the Multiplate® platelet function analyzer (Verum Diagnostica Gmbh, Munich, Germany). Briefly, the Multiplate is an FDA approved platelet function analyzer that measures electrical impedance generated by activated platelets attached to two sets of electrodes (for internal control) in a heparinized whole blood sample(50). The result consists of two curves with arbitrary aggregation units, measured over a 6-minute activation period. Platelet function is measured as the area under the curve (AUC) and expressed in Units (U).

Heparinized blood samples were allowed 30 minutes to rest at room temperature after collection. Platelets were activated with agonists adenosine diphosphate (ADP, 0.2mmol/l, 20µl, final concentration 6.5µg/ml via P2Y₁ receptors), Collagen (COL, 100µg/ml, 20µl, final concentration 3.2µg/ml, via GpIa/IIb and GpVI receptors) and Arachidonic acid (AA, 15mM/ml, 20µl, final concentration 0.5mmol/l via the cyclooxygenase pathway).

Thrombelastography

Thrombelastography (TEG) was performed using the TEG-5000 system (Haemonetics, Braintree, MA) according to manufacturer instructions. Briefly, citrated whole blood was collected as mentioned above and was allowed to rest for 15 minutes prior to assaying. Each sample was activated with kaolin. Recorded values were reaction time (r time, time to initial fibrin formation), alpha angle (angle, speed of clot build-up indicating interaction between fibrin and activated platelets and thus an indirect marker of platelet function) and maximum clot strength (MA, final product of fibrin and platelet interaction and thus also a marker of clot strength and thereby indirectly platelet function).

Enzyme linked immunosorbent assay (ELISA)

All ELISA assays were done using citrated or EDTA anticoagulated plasma as described above or serum samples. Table 2 lists the assays performed for the individual studies

Stu	Assay	Abbreviati-	Marker of	Medi-
dy		on		um
П	Histone com-	hcDNA	Cell death	Serum
and	plexed DNA			
III				
П	Tissue factor	TF	Coagulation	EDTA
and			activation	plasma
III				
П	Prothrombin	PF1+2	Coagulation	Citra-
and	fragment 1+2		activation	ted
III				plasma
II	Activated com-	C5a	Complement	EDTA
and	plement factor		activation	plasma
III	5			
П	Soluble throm-	sTM	Endothelial	EDTA
and	bomodulin		activation	plasma

111				
II	Syndecan-1		Endothelial	EDTA
and			activation	plasma
Ш				
II	Soluble vascular	sVCAM-1	Endothelial	EDTA
and	cell adhesion		activation	plasma
Ш	molecule 1			
II	Von Willebrand	vWF	Endothelial	EDTA
and	factor		and platelet	plasma
Ш			activation	
П	D-Dimer		Fibrinolysis	EDTA
and				plasma
III				
II	Plasminogen	PAI-1	Fibrinolysis	EDTA
and	activator inhibi-			plasma
III	tor 1			
П	Tumor necrosis	TNF-α	Inflammation	EDTA
and	factor alpha			plasma
III				
П	Interleukin-6	IL-6	Inflammation	EDTA
and				plasma
III				
II	Interleukin-8	IL-8	Inflammation	Serum
and				
III				
Ш	Antithrombin III	ATIII	Natural	EDTA
and			anticoagula-	plasma
III			tion	
Ш	Activated prote-	aPC	Natural	EDTA
and	in C		anticoagula-	plasma
III			tion	
I	Soluble P-	sP-Selectin	Platelet	EDTA
and	Selectin		activation	plasma
III				
1	Transforming	TGF-β	Platelet	EDTA
and	growth factor		activation	plasma
III	beta			
I .	CD40 Ligand	CD40L	Platelet	EDTA
and			activation	plasma
Ш				
II	Epinephrine		Sympatho-	EDTA
and			adrenal	plasma
III			activation	
Table	2. Plasma or serum	n markers analy	zed hy FLISA in th	ne studi-

Table 2: Plasma or serum markers analyzed by ELISA in the studies

Statistical analysis and sample size considerations: Data are presented as median values with interquartile range. Groups were compared using Mann-Whitney's U test at relevant time points. No within-group comparisons were made. Correlations are reported as Spearmans rho. All statistical analyses were performed using SPSS 20.0 (IBM Corp. Armonk NY). We considered p< 0.05 as statistically significant. No correction for multiple comparisons was done.

The sample size calculation was based on human data as we had no data on the measured markers in pigs. We considered a difference in maximum TEG measured clot strength (TEG-MA) of 10mm to be clinically relevant. Assuming a standard deviation of 10mm, detection of this difference would require 32 animals.

5. RESULTS

STUDY 1 – PLATELET ACTIVATION AND DYSFUNCTION IN A LARGE ANIMAL MODEL OF TRAUMATIC BRAIN INJURY AND HEMORRHA-GE

Main results from study 1 are summarized in figures 2 and 3.

Clotting kinetics

Main results are summarized in figure 2. Coagulation activation as measured by the TEG showed an immediate activation of the coagulation cascade (TEG-r time) at 3 minutes post injury (3.8min vs. 5.1min, p<0.01). This activation was sustained at the 2-hour post shock time point (4.3min vs. 5.8min, p=0.05). Clot buildup speed (TEG angle) was not significantly different between groups at any time point; whereas clot strength (TEG-MA) was significantly lower in the TBI/Shock group at 15 minutes post injury (74.1mm vs. 79.8mm, p=0.01) as well as following the 2-hour shock phase (74.1mm vs. 79.4mm, p=0.05).

Platelet count and function

Results are summarized in figure 3 and table 3. Platelet count did not differ significantly between groups at any time point. Platelets activated with ADP showed a lower aggregation at 15 minutes post injury when compared to sham controls (65.1U vs. 80.4U, p=0.02).

This dysfunction was maintained at the 2-hour post shock observation time point (62.7U vs. 73.5U, p<0.01). In contrast no dysfunction of platelets activated with COL or AA could be identified. Dysfunction of ADP receptor induced aggregation was associated with increased levels of platelet activation marker TGF- β (1764.5pg/ml vs. 1252.0pg/ml, p<0.01), but no differences in levels of sP-Selectin or CD40L could be observed.



Figure 2: Kaolin activated thromboelastography measurements throughout the course of the experiment. Medians and interquartile range. Groups were compared at selected time points using the Mann-Whitney U test



Figure 3: Platelet count (bottom right) and aggregation when activated with collagen (top left), Arachidonic acid (top right) and adenosine diphosphate (bottom left). Medians with interquartile range

STUDY 2 - ASSESSMENT OF COAGULOPATHY, ENDOTHELIAL INJU-RY AND INFLAMMATION AFTER TRAUMATIC BRAIN INJURY AND HEMORRHAGE IN A PORCINE MODEL.

Main results from study 2 are summarized in figures 4 and 5

Coagulation, natural anticoagulation sympatho-adrenal system and complement

Results are summarized in figure 5. Activation of the coagulation system as evidenced by elevated levels of PF1+2 was identified 3 minutes after injury (289ng/ml vs. 232ng/ml, p=0.03). This was simultaneously mirrored by activation of complement (C5a, 2.83ng/ml vs. 2.05ng/ml, p=0.05) as well as activation of the sympatho-adrenal system (Epinephrine, 576ng/ml vs. 463ng/ml, p=0.01).

Activation of the protein C system only became evident following the 2-hour shock phase (57.7ng/ml vs. 26.1ng/ml, p=0.01), whereas levels at ATIII did not differ significantly between groups at any time point.

No differences in levels of circulating Tissue Factor or markers of fibrinolysis were detected.

Endothelial activation, inflammation and cell death.

Results are summarized in figures 4 and 6. Shedding of the endothelial glycocalyx layer was evident 15 minutes after injury (Syndecan-1, 851.0ng/ml vs. 715.5ng/ml, p=0.03) and was maintained at the 2-hour post shock time point (845.0ng/ml vs. 717.2ng/ml, p<0.01). In contrast, levels of vWF and sVCAM-1 only differed between groups at 15 minutes post injury.

No difference in levels of sTM could be detected. Inflammation was evident following the 2-hour post shock phase as evidenced by elevated levels of IL-6 (15.8ng/ml vs. 0.1ng/ml, p<0.01) and TNF-α (81.1ng/ml vs. 50.8ng/ml, p<0.01). Cell death as measured by circulating hcDNA was also evident at this time point (0.22uM vs. 0.12uM, p=0.04).



Figure 4: Endothelial glycocalyx shedding (left) and natural anticoagulation (right) throughout the course of the experiment



Figure 5: Complement (top), coagulation cascade (middle) and sympatho-adrenal activation (bottom) throughout the course of the experiment

Activated complement factor 5 (c5a)



Figure 6: Inflammation (top left and right, bottom left) and cell death (bottom right) throughout the course of the experiment

Study 3 – VALPROIC ACID ATTENUATES PLATELET DYSFUNCTION FOLLOWING NEUROTRAUMA

Hemodynamics and arterial blood gases

The infusion of VPA had no significant effect on the hemodynamic status of the animals as measured by mean arterial pressures or cardiac output. No differences in lactate levels were observed. VPA infusion resulted in higher pCO_2 levels following the 2-hour shockphase (40.3mmHg vs. 34.8mmHg, p<0.01).

Coagulation, complement, natural anticoagulation and fibrinolysis

Activation of the coagulation cascade as measured by prothrombin split products prothrombin fragment 1+2 (PF 1+2) was increased less from baseline in the VPA group (-17 ng/ml vs. 18 ng/ml, p=0.03) Activation of the protein C system tended to change less from baseline in VPA treated animals (p=0.06). No difference in changes of complement activation (C5a), tissue factor, fibrinogen, D-dimer, plasminogen activator inhibitor (PAI-1) or antithrombin III (ATIII) was found.

Platelet function and activation

Less impairment of adenosine-diphosphate (ADP) induced platelet aggregometry was found in VPA treated animals (-3.10 U vs. -10.84U, p=0.03). No differences in circulating markers of platelet activation (sP-Selectin, CD40L and TGF- β) was found.

Endothelial and sympatho-adrenal activation, inflammation and cell death

Changes in shedding of the endothelial glycocalyx (Syndecan-1) was attenuated after shock in the VPA group (-68pg/ml vs. 62pg/ml, p=0.02). No differences in changes from baseline of hcDNA, soluble vascular adhesion molecule 1 (sVCAM-1), soluble thrombomodulin (sTM), epinephrine or inflammation - as measured by TNF- α , IL-6 and IL-8 - were found.

6. DISCUSSION

Main findings

These studies collectively indicate that the combination of TBI and shock induces immediate and sustained activation of the coagulation system as measured by both circulating markers (PF1+2) and by TEG. This activation was associated with simultaneous activation of the complement and sympatho-adrenal systems, but not with differences in circulating levels of tissue factor.

Within minutes of the activation of these pathways, shedding of the endothelial glycocalyx layer became evident, as did platelet dysfunction as assessed by ADP induced aggregation response. In contrast, activation of natural anticoagulation, inflammation and cell death appeared to be late modulating effects, as these were only observed following the two-hour shock phase. These results indicate that rather than being solely the effect of tissue factor released from the injured brain parenchyma, activation of coagulation following TBI and shock is likely the results of the reciprocal activation of multiple tightly interlinked pathways collectively activating and modulating the coagulation response. These may include an early response by pathways such as complement and the sympatho-adrenal system as observed here.

Interestingly, our results from study 3 indicate that especially the activation of coagulation and the endothelium is attenuated by VPA. Indeed, we observed a reduced level of coagulation cascade activation (PF1+2) and endothelial glycocalyx shedding (Synde-can-1). Improved platelet function in VPA treated animals mirrored this finding-

Furthermore, downstream effects of coagulation activation such as activation of natural anticoagulation (Protein C) showed a trend towards attenuation in VPA treated animals.

As such, our studies confirmed the initial hypotheses that the combination of TBI and HS would result in early activation of coagulation, complement and endothelial systems as well as produce subsequent platelet dysfunction and inflammation. We were furthermore able to demonstrate an attenuation of several of these pathways by treatment with VPA.

RELATION TO CURRENT EVIDENCE

Studies I and II – effect of injury

Several both clinical and animal studies have reported rapid activation of the coagulation system following TBI(6, 7). Classically, this is attributed to a release of TF from the injured brain parenchyma, a hypothesis first put forth by Goodnight et al. 40 years ago(51). According to this hypothesis, induction of coagulation following TBI is mechanistically different from non-TBI injury due to an abundance of TF in the brain.

Clinical studies have, however, failed to detect coagulation related differences in TBI vs. non-TBI injuries(33, 52) while other highly vascularized organs such as the lung also contains high concentration of TF(53).

While TF has been shown to induce coagulation and hyperfibrinolysis independently of hypoperfusion(54), recent evidence indicates that the source of TF may not solely be from injured tissue. As such, TF expressed on the surface of multiple cell lines and circulating cell-derived microparticles, could promote activation of coagulation(13-16).

Furthermore, significant cross talk exists between coagulation and other injury driven pathways such as inflammation, complement and the sympatho-adrenal system. Activation of complement, specifically complement factor C5a, directly promotes the expression of TF on neutrophils(55), endothelial cells(56) and monocytes(57) which again reciprocally upregulates complement activation in a self-perpetuating cycle(22). Inflammatory cytokines may also promote the upregulation of TF on endothelial cells(58). Other pathways not classically associated with coagulation, such as the sympatho-adrenal system, also holds the potential to further activate and propagate the coagulation/inflammation cycle. Indeed, platelets possess adrenergic receptors(59) and aggregate as a response to injury while the inflammatory properties of other important immune mediating cells such as neutrophils and macrophages also respond to adrenergic stimuli(60, 61). It is thus no surprise that elevated circulating levels of epinephrine following trauma has been associated with increased mortality in patients(62)

Finally, numerous damage-associated molecules such as histones have the capacity to activate coagulation, in part through the innate immunity defense associated family of toll like receptors (TLR)(63). Extracellular histones have the capacity to induce frank thrombocytopenia(20) and have been associated with mortality(64), but are interestingly not only the result of cell damage. In contrast, extracellularisation of histones and DNA has been identified as a key part of the innate immune defenses in the form of the so-called extracellular traps(65). Interestingly, these histone and DNA complexes not only serve to activate coagulation, but may be a necessary requirement for stable thrombus formation by providing a scaffold for fibrin deposition and crosslinking with platelets(66).

It is thus clear that the reductionist approach embraced when attributing activation of coagulation to the release of tissue factor from the injured brain parenchyma is too simplistic. Rather, multiple tightly interlinked pathways serve to activate, amplify and further modulate the coagulation response following injury. In light of this, our findings suggest that coagulation; complement and the sympatho-adrenal axis are amongst the first responders to injury, responding within minutes of the insult. This response is later modulated by the emergence of histones and DNA as well as inflammatory markers.

Finally, the activation of natural anticoagulation through the protein C pathway appears to be a late modulating response. Clinically, overt activation of the protein C system has been associated with coagulopathy in trauma patients(29) and likely presents a compensatory response to earlier activated pathways. As such, aPC not only serves to inhibit coagulation factors V and VIII as well as to induce fibrinolysis, aPC also counteracts the procoagulant and inflammatory properties of histones(64). Our finding of late activation of the protein C system is thus in line with previous findings, and suggests that mounting of the initial coagulation response promotes activation of the protein C system following TBI requires hypoperfusion(67), and it could thus be speculated that sufficient time under a low-flow state would be required for a response to be mounted.

Our results also indicate the early appearance of two major coagulation associated events, namely shedding of the endothelial glycocalyx and dysfunction of platelets.

The endothelial glycocalyx defines a layer comprised of membrane bound proteoglycans and glycoproteins as well as plasma proteins suspended in a non-circulating intravascular fluid phase of approximately 700-1500ml(68). Shedding of this glycocalyx layer has been described following both TBI and non-TBI trauma(31, 33) as well as been associated with mortality following injury(31). This event promotes coagulation by granting the cellular elements of the blood access to the surface of the endothelial cells(69).

The glycocalyx layer shed into the fluid phase of the blood in turn possesses endogenous heparin like properties, yielding a net effect of coagulation on the surface of activated endothelial cells with concurrent anticoagulation of the fluid phase. This has been proposed as a mechanism of maintaining microcirculatory flow during coagulation activation(70). Our results suggest that this response is manifested shortly after the activation of coagulation, and thus support the hypothesis that glycocalyx shedding is an early modulating response to intravascular coagulation.

Shedding of the endothelial glycocalyx layer may be a necessary prerequisite for platelet adhesion to the endothelial surface and

thus be tightly associated with platelet activation and dysfunction. It is thus interesting to note that our observation of platelet dysfunction coincided with endothelial glycocalyx shedding. Platelet dysfunction has been identified as a predictor of adverse outcome following trauma(32), although the underlying pathophysiology remain elusive. Human studies have identified dysor hypofunction of platelet aggregation as a response to ADP, AA, COL and TRAP(32, 71) while our studies only identified dysfunction of the ADP receptor pathway. This finding is mirrored in a recently published rodent model of isolated TBI(72). Interestingly, this latter study also found that pre-treatment with a thrombin inhibitor prior to injury attenuates platelet dysfunction. Furthermore, reports have indicated that fibrin fragments may directly inhibit ADP induced platelet aggregation(73-75) while both sub and supra normal fibrinogen levels may impact adversely on platelet function(76).

Collectively, these results suggest that the observed platelet dysfunction may be a compensatory attenuating response to the activation of coagulation. As such, our finding of early ADP induced platelet aggregation subsequent to activation of the coagulation system supports this line of thought.

In conclusion, our results from studies I and II are in line with current evidence indicating that multiple coagulation and noncoagulatory pathways respond early after injury. Furthermore, our findings coupled with the evidence described above suggests that adopting a reductionist approach by focusing on single biomarkers or individual pathways often utilized in laboratory research rather than embracing a systems-wide holistic view, may yield results that cannot be translated to clinical use due to the increased complexity and interaction of multiple pathways observed in trauma patients.

Study III – effect of Valproic acid

Several both large and animal studies have indicated that pharmacological treatment with VPA improves survival following trauma and hemorrhagic shock(41-43) as well as provide neuroprotection following TBI(45). The mechanism of action underlying this survival benefit is likely multifactorial, but modulation of several key survival cellular pathways has been identified to date. Studies have thus shown that activation of the phosphoinostide 3-kinase (PI3K)-Akt/PKB pathway play a role in enhancing cell survival and reducing apoptosis in multiple different cell lines(77). Furthermore, this process is regulated in part through acetylation, and studies have indeed confirmed that VPA exerts an antiapoptotic effect in cells by modulating this pathway following hemorrhagic shock(42), an effect that may be related to a direct inhibitory effect of VPA on glycogen synthase kinase 3β (GSK-3β), a key element of the PI3K/Akt pathway.

Other potential mechanism of action may include induction of Heat shock protein 70 (Hsp70). Hsp70 is implicated in multiple areas of cell-protection as well as exert an anti-inflammatory effect, and studies have indeed confirmed that HDACI treatment increase the expression of Hsp70(78). Furthermore, blocking of Hsp70 in VPA treated cells abolished the cell protective response, thus further strengthening the key role of Hsp70 signaling in VPA associated cell protection(79).

Evidence from models of septic shock furthermore suggests that VPA exerts an anti-inflammatory effect, in part by modulating the response of nuclear factor kappa B (NF-κB)(80). NF-κB is a gene transcriptional effector of several inflammatory pathways, including the aforementioned TLR family of receptors reacting to ligands such as lipopolysaccharide (LPS) as well as damage and inflammation associated molecules such as histones and fibrinogen.

As such, treatment with HDACIs has consistently been associated with reduced levels of circulating inflammation markers such as TNF- α , IL-1 β and IL-6 following injury or sepsis(45, 46). These effects could, however, not be confirmed by our studies which is likely related to sampling shortly after VPA treatment where a potential effect on inflammatory pathways is not fully manifested.

Multiple lines of evidence thus suggest that VPA and other HDA-CIs in part exert their pro-survival effects through modulation of cell death, apoptosis and inflammation. Our results collectively indicated that VPA treatment reduced activation of the coagulation cascade and the endothelium, conserve platelet function as well as potentially reduce activation of the Protein C system. Whether this was a direct effect on coagulation or a derived effect of reduced cell death, apoptosis and inflammation can, however, not be concluded due to our experimental setup. As discussed under studies one and two, an overall reduction in cell death and inflammation would likely result in an attenuation of the coagulation response in part due to a reduced circulating level of cellular debris such as histones and DNA, as was indeed observed in our study. This would again promote less activation of TLR receptors and platelets, thereby promoting less surface expression of TF on cells and ultimately resulting in a reduced activation of coagulation. This in turn would attenuate compensatory mechanisms such as activation of the protein C system and endothelial glycocalyx shedding, thus collectively explaining our observed findings.

While this effect may in part account for our findings, it is interesting to note that we observed an early attenuation of coagulation and endothelial related pathways, before any effect on inflammation and cell death could be detected. It is thus tempting to speculate that the observed effect of VPA may in part be due to a more direct effect on these systems.

Interestingly, several studies have however indicated that HDACI's may exert effects that more directly affect activation of the coagulation system. Primarily acetylation and de-acetylation of key coagulation proteins regulate the coagulation cascade function(81, 82), suggesting a potential direct effect of VPA treatment on the coagulation cascade. While this area is largely unstudied, it could thus be speculated that the acetylation regulation properties of VPA and other HDACI's were not limited to activity on histones, but rather extended to circulating proteins such as coagulation factors. If such a connection could be demonstrated, this would provide an interesting link between the observed protective effects of VPA in animal models and the coagulation system. Furthermore, it would provide a framework for studying a potential effect of acetylation control on the development of coagulation derangements following trauma such as the acute coagulopathy of trauma.

On the cellular level, several coagulation related effects of HDACI treatment have been demonstrated. HDACIs have been shown to directly downregulate tissue factor expression on monocytes and endothelial cells(83), supporting our observation of reduced thrombin split products (PF1+2) in VPA treated animals. HDACIs may also modulate fibrinolysis through an effect on tissue plasminogen activator (tPA)(84, 85).

GSK-3 β activation, inhibited by VPA treatment(42), also promotes tissue factor expression on endothelial cells(86). Collectively, these findings may thus account for part of the attenuation of the coagulation response observed in our study.

Attenuation of coagulation cascade activation and tissue factor expression would again directly reduce platelet activation, as was observed in our study. This reduced activation may thus be related to a downstream effect of reduced coagulation activation rather than a direct effect on platelets. Reduced activation of platelets could then again result in a conservation of platelet function through reduced platelet exhaustion. Alternatively, a reduced activation of the coagulation cascade would promote less pathological fibrin split products and thereby less inhibition of platelets as previously described. The net result of these interlinked events would be increased platelet function, as was observed in our study.

Alternatively, VPA may exert a direct effect on platelets. Although anucleate cells, platelet activation and CD40L expression is dependent on NF-KB(87, 88) signaling, while inhibition of GSK-3β activation also inhibits platelet activity(89). These results are supported by clinical studies indicating a reduction in platelet function in chronic VPA treated patients(90). While the beneficial effects of reduced platelet function following trauma may seem counterintuitive, it is interesting to note that while trauma induced platelet function has been established as a predictor of adverse outcome(32), pharmacological inhibition of platelets may actually be associated with a favorable outcome following trauma(91). As such, an immediate downregulation of platelet activation *in-vivo* may translate into a late conservation the platelet function as measured by our assays *in-vitro*, potentially through a reduced level of platelet exhaustion.

Furthermore, VPA has been reported to promote megakaryopoiesis and may thus increase influx of new platelets into the circulation from the bone marrow during a state of consumption such as is observed during trauma and shock(92).

Finally, previous reports have indicated that acetylation/deacetylation of platelet tubulin structures play a key role in platelet mobility. As such, the HDAC6 isoform has been shown to catalyze the deacetylation in platelets, an event associated with decreased platelet migration. Although VPA may not directly inhibit HDAC6, the finding establishes a novel role of acetylation and deactylation in platelets, suggesting that regulation of this event may impact on platelet function.

No studies have to our knowledge identified a direct effect of VPA treatment on the endothelial glycocalyx, and it is thus difficult to conclude on whether VPA could have a direct effect on this event. Several studies have, however, found that VPA treatment attenuate endothelial activation, which would likely result in reduced shedding of the glycocalyx. Studies thus reported a reduced pathological endothelial gene expression profile in swine subjected to hemorrhagic shock when treated with VPA(93) as well as reduced endothelial cell apoptosis(94).

Furthermore, acetylation and de-acetylation of tubulin structures in endothelial cells appear to regulate endothelial barrier function decline in response to agonists such as thrombin and studies have indeed indicated that treatment with an HDAC6 inhibitor attenuates this event(95) Other studies have indicated that VPA may reduce adhesion of circulating tumor cells to the endothelium, suggesting reduced endothelial activation in this setting(96). While the observed effects of VPA on the shedding of the endothelial glycocalyx may be a derived effect of an attenuation of other pathways such as coagulation, it is thus likely that VPA also has a direct effect on the endothelial cells.

Collectively, our results indicate that VPA treatment is associated with an attenuation of multiple damage-associated pathways characterized in studies 1 and 2. These include coagulation, platelet and endothelial activation.

Our experimental setup does not allow us to conclude on whether these observed effects are due to a direct interaction or regulation of coagulation related acetylation/de-acetylation events or rather a derived effect. Rather, these findings provide the framework for subsequent studies addressing these issues. Regardless of mechanism, our findings support the current body of knowledge indicating a protective effect VPA treatment in animal models of trauma and sepsis (40, 42).

STRENGTHS AND LIMITATIONS

The presented studies provide novel insight into early responding immunological pathways associated with TBI and HS, as well as demonstrate a novel effect of VPA treatment on many of these pathways. By utilizing a previously validated large animal model of combined insults, we were able to create a clinically realistic scenario that allowed us to investigate these extreme early changes. This would not have been feasible in patients, as blood sampling at this early stage would be logistically impossible. Furthermore, the tight control of laboratory parameters provides a setting the allows for minimization of inter-subject variation and the ability disregard common confounders often found in trauma patients such as age, pre-injury medication and disease, hypothermia and different modalities of injury.

As is the case for any animal study, it is important to acknowledge that these strengths come at the price of other limitations. Primarily, the laboratory environment does not completely mirror the clinical reality and results may thus not be directly transferable. Secondly, it should be recognized that species differences might impact on results. Pigs are often utilized as large animal models in trauma studies, but it is well known that their coagulation system responds differently to insults than their human counterparts. As such, pigs tend to be hypercoagulable and rarely become coagulopathic even when subjected to extreme insults. The clinical relevance of the magnitudes of coagulation changes in pigs are only scantly reported in the literature, and it is thus impossible to ascertain whether our observed differences can be translated to clinical meaningful differences in patients. It is, however, important to underline that the observed changes were associated with life-threatening injuries in the animals and at the end of the studies, animals were in extremis. As such, one should be careful in overlaying human normal coagulation values on these results for the purpose of concluding on clinical relevance. Rather, even small coagulation changes in pigs might be associated with adverse outcomes.

In line with this, it can be argued that the pig does not provide an optimal model of trauma induced coagulation changes. While this is certainly a valid argument, it is however also important to consider the fact that while the porcine coagulation system might not mirror its human counterpart, it likely exhibits many common responses and thus provides a platform for studies that cannot otherwise carried out in humans.

While we describe the activation of multiple tightly interlinked pathways, it is important to acknowledge that our studies only observe associations and cannot conclude on causality. As such, we have to rely on previous studies to make the connection between coagulation, complement, endothelial and platelet systems. This limitation is inherent in all in vivo studies and is especially true for large animal studies where pre-study genetic modification and treatment with specific pathway blocking antibodies often is not economically or practically feasible. Furthermore, as is demonstrated by our studies, the combination of insults activates many interlinked pathways, which precludes us from identifying the primary effectors or most clinically relevant pathways. In light of these limitations, our studies should be viewed as exploratory investigations that provide the framework for further investigations in an either more controlled small animal model or alternatively in-vitro cell studies.

Also, it should be acknowledged that the use of VPA is still experimental. In this study, we utilized a dose of 100mg/kg. This, however, is more than the current maximal allowable dose of 60mg/kg in humans.

Differences in metabolic rate between species complicate a direct translation of dosage. As such, when converting from human to smaller animal species, the incrementing metabolic rates encountered in these species mandates higher drug concentrations than would be expected if a simple linear relationship could be determined between drug dose and weight. In short, due to a higher metabolic rate in pigs, a 40kg pig will require a higher dose of VPA than a 40 kg human.

Also, high dose VPA treatment might be associated with unwarranted side effects. Of these, somnolence and hypotension have previously been described. Especially the latter may preclude its use in trauma patients. Studies from our lab have, however, indicated that these side effects may be abrogated be the utilization of a slow infusion rather than a bolus injection. Finally, in order to address this important question, a phase I clinical dose escalation trial is currently underway to establish the maximum safe dose in humans.

Finally, it should be acknowledged that our sample size calculations as well as choice of statistical methods impact on the interpretation of our results. Upon entering into these studies, we neither had data on normal values of many of the measured parameters in pigs, nor information on how these parameters would react to trauma. As such, we were forced to base our sample size calculation on data extrapolated from human studies. Performing a series of pilot studies ascertaining the response of the observed parameters to trauma prior to engaging in the actual studies would have been preferential.

Also, it could be argued that our choice of the Mann-Whitney U test for comparisons between groups may be a drawback. Since we are assessing values over multiple time points, we are dealing with correlated measures. As such, an approach using for example a mixed model test for comparisons between groups might have provided a better statistical framework. We did, however, hypothesize that the combination of injuries would result in *early* changes in the observed parameters.

Utilizing a mixed model or a two-way ANOVA approach would only allow us to conclude on overall differences between groups and not on *when* these changes manifested themselves. As such, we opted to use a model that would enable us to detect such differences.

This choice of statistical model did, however, necessitate multiple statistical testing. This again obviously increased the risk of chance findings and thus the risk of having a type I error i.e. identifying differences not actually present. Classically, this is addressed by performing family-wise error rate post-hoc tests such as the bonferroni correction. It is, however, important to realize that utilizing such post-hoc tests again enforces a trade-off, since we would be reducing the risk of a type I error while dramatically increasing the risk of a type II error i.e overlooking differences actually present. As such, it is important to recognize that no perfect statistical solution exists for this problem.

Also, due to the nature of these studies as secondary use of animal models, a formal randomization was not possible, which again imposed drawbacks in terms of inequalities at baseline between animals in terms of certain of the measured markers. This problem was especially observed in study 3, which made it necessary to base our calculations on changes from baseline rather than absolute values.

Due to the above-described limitations, we were conscious of the fact that our studies could at best be hypothesis generating and only provide the framework for future more focused studies. As such, we opted to use the statistical method that provided the best framework for exploratory studies and thus in essence traded the risk of a Type I error over a Type II error.

It could, however, be argued that utilizing other less stringent post-hoc correction strategies such as the false detection rate correction pioneered in high-throughput gene microarray studies might have provided a more acceptable tradeoff.

7. SUMMARY AND OVERALL CONCLUSIONS

In these studies, we have shown that coagulation and innate immunity pathways respond to trauma within minutes. Furthermore, the appearance of dysfunction of platelets as well as activation of the endothelium is rapidly manifested. Interestingly, many of these changes were attenuated by treatment with VPA. These most notably included coagulation and endothelial activation as well as platelet dysfunction. These results add to the growing body of evidence indicating a protective effect of VPA following trauma, and suggests that this may in part be mediated through an attenuation of the abovementioned pathways. Furthermore, these results indicate that VPA treatment may be effective in other pathological settings such as coagulopathy following trauma or sepsis.

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