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Plasma-Mediated Gut Protection After Hemorrhagic Shock is Lessened in Syndecan-1^{-/-} Mice

Kechen Ban¹, Zhanglong Peng², Shibani Pati^{3,4}, Richard B Witkov⁵, Pyong Woo Park⁶, and Rosemary A Kozar²

¹Department of Pathology and Immunology, Baylor College of Medicine, Houston, TX

²Shock Trauma Center, University of Maryland, Baltimore, MD

³Blood Systems Research Institute, San Francisco, CA

⁴Department of Surgery, University of California, San Francisco, San Francisco, CA

⁵University of Texas Health Science Center at Houston, TX

⁶ Division of Respiratory Diseases, Children's Hospital, Harvard Medical School, Boston, Massachusetts

Abstract

We have shown in a rodent model of hemorrhagic shock (HS) that fresh frozen plasma (FFP) reduces lung inflammation and injury which are correlated with restitution of syndecan-1. Since the gut is believed to contribute to distant organ injury and inflammation after shock, the current study sought to determine if the protective effects of plasma would extend to the gut and to elucidate the contribution of syndecan-1 to this protective effect. We also examined the potential role of TNF α , and a disintegrin and metalloproteinase (ADAM)-17, both intestinal sheddases of syndecan-1. Wild-type (WT) and syndecan-1^{-/-} (KO) mice were subjected to HS followed by resuscitation with lactated ringers (LR) or FFP and compared to shock alone and shams. Small bowel and blood were obtained after 3 hours for analysis of mucosal injury and inflammation and TNF α and ADAM-17 protein expression and activity. After HS, gut injury and inflammation were significantly increased compared to shams. Resuscitation with LR decreased both injury and inflammation which were further lessened by FFP. KO mice displayed worsened gut injury and inflammation after HS compared to WT mice, and LR and FFP equivalently inhibited injury and inflammation. Both systemic and intestinal TNF α and ADAM-17 followed similar trends, with increases after HS, reduction by LR, and a further decrease by FFP in WT but not KO mice. In conclusion, FFP decreased gut injury and inflammation after hemorrhagic shock, an effect that was abrogated in syndecan-1^{-/-} mice. Plasma also decreased TNF α and ADAM-17, representing a potential mechanistic link to its protection via syndecan-1.

Corresponding Author: Rosemary Kozar, Shock Trauma Center, 22 South Green St, Room S4D07, Baltimore, MD 21201, 410-328-7611 phone 410-328-0687 fax, rkozar@umm.edu.

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INTRODUCTION

Hemorrhage remains a leading cause of early deaths in severely injured patients. (1) Recent efforts have focused on improved techniques for control of truncal and extremity bleeding while resuscitative strategies are attempting to optimize the timing and ratios of blood component administration.(2) The early and empiric use of fresh frozen plasma (FFP) in hemodynamically unstable patients with bleeding has led to a decrease in early hemorrhagic deaths.(3,4) This decrease in mortality from a plasma-based resuscitation strategy appears to extend beyond its ability to correct trauma-induced coagulopathy and provide hemorrhage control and is hypothesized to involve additional protective effects of plasma on a dysfunctional endothelium that follows trauma and hemorrhage.(5) Endothelial dysfunction leads to not only coagulation abnormalities but also to inflammation and breakdown of organ specific endothelial and epithelial barrier integrity. We have previously shown in a rodent model that hemorrhagic shock results in loss of pulmonary vascular integrity with subsequent hyperpermeability and edema that is mitigated by plasma based resuscitation.(6) Similarly, shock-induced lung inflammation and injury was significantly decreased by plasma. Concurrent with these improvements in lung injury were alterations in pulmonary syndecan-1 and the endothelial glycocalyx.(7)

Syndecans are cell surface or transmembrane co-receptors that modulate binding and signaling of cytokines, chemokines, and adhesion molecules, among other heparin-binding molecules. Syndecan-1 has been a focal area of our investigations into plasma's systemic protective effects on the endothelium. Syndecan-1 is found on both endothelial and epithelial cells although much of the literature refers to shed syndecan-1 as a potential surrogate marker for the endothelial glycocalyx.(8-11) Epithelial cells also possess a glycocalyx structure and perturbations of epithelial syndecan-1 can lead to intestinal inflammation, edema and injury after gut ischemia/reperfusion.(12,13) Therefore, in the current study we investigated the role of intestinal syndecan-1 on gut injury and inflammation after plasma-based resuscitation following trauma and hemorrhage. We hypothesized that plasma would lessen injury to the gut through restoration of syndecan-1 on the intestinal epithelium. We tested this hypothesis using a clinically relevant coagulopathic mouse model of trauma and hemorrhage in WT and Sdc1-KO mice. We also examined the potential role of TNF α and a disintegrin and metalloproteinase (ADAM)-17, as intestinal sheddases of syndecan-1 that may contribute to the loss of syndecan-1 after hemorrhage and restitution by plasma.

MATERIALS AND METHODS

Mouse model of hemorrhagic shock

All procedures performed were protocols approved by the University of Texas Houston Medical School and the University of Maryland School of Medicine Animal Welfare Committees. The experiments were conducted in compliance with the National Institutes of Health guidelines on the use of laboratory animals. All animals were housed at constant room temperature with a 12:12-h light-dark cycle with access to food and water ad libitum. Male syndecan-1^{-/-} mice (generously provided by Dr. Pyong Park) on the C57BL/6J background and wild type littermates, 8-10 weeks of age and weighing approximately 20 grams, were used for all experiments. An established coagulopathic mouse model of trauma-

hemorrhagic shock was utilized.(6) Under isoflurane anesthesia, a midline laparotomy incision was made, the organs inspected, then the incision closed. Bilateral femoral arteries were cannulated for continuous hemodynamic monitoring and blood withdrawal or resuscitation. After 10-minute period of equilibration, mice were bled to a mean arterial pressure (MAP) of 35 ± 5 mmHg and maintained for 90 minutes. Shams underwent anesthesia and placement of catheters but were not subjected to hemorrhagic shock. Mice were resuscitated with either lactated Ringer's (LR) at 3X shed blood volume or fresh frozen plasma at 1× shed blood and compared to animals that underwent shock alone as we have described.(6) Fresh frozen plasma was obtained from Gulf Coast Regional Blood Bank, Houston,TX.

Upon completion of resuscitation, vascular catheters were removed, incisions closed, and the animals were awoken from anesthesia. After three hours, animals were sacrificed by exsanguination under isoflurane anesthesia. Blood was obtained at the time of sacrifice and the distal small intestine was harvested for further analysis. The three hour time point was chosen based on our previous investigation showing partial restoration of the endothelial glycocalyx and pulmonary syndecan-1 by plasma at this time point.(7)

Small intestine mucosal damage

Intestinal tissue was embedded, placed on slides and stained with hematoxylin and eosin. Five random fields with 100-250 villi from each mouse were analyzed in a blinded fashion by means of light microscopy at 100X magnification. Villi were identified as having damage if any one of the following were present: development of a subepithelial space, extension of the subepithelial space with moderate lifting of the epithelial layer from the lamina propria, massive epithelial lifting down the sides of the villi, denuded villi with lamina propria and dilated capillaries and/or digestion and disintegration of the lamina propria with hemorrhage, and ulceration.(14)

Small intestine neutrophil infiltration

Intestinal tissue was placed on slides, and neutrophils were stained using naphthol ASD chloroacetate esterase. Tissue was stained via hematoxylin counterstain and neutrophils counted per area of tissue in a blinded fashion by means of light microscopy at 100X magnification.

Small intestine and plasma TNF α and A disintegrin and metalloproteinase (ADAM)-17

Levels of TNF α and ADAM-17, also known as TNF α converting enzyme (TACE), in plasma and small intestinal tissue were measured by mouse TNF α ELISA kit and mouse ADAM-17 ELISA kit (MyBioSource, San Diego, CA). The activity of ADAM-17 in the intestinal tissue was measured by SensoLyte 520 TACE (α -secretase) activity assay kit (AnaSpec, Fremont, CA) according to manufacturers' instructions. Results were normalized to protein concentration, and there were no significant differences between groups (data not shown).

Data Analysis

All data was analyzed by one way analysis of variance (ANOVA) with Bonferroni correction. P values < 0.05 were considered significant. Data are expressed as mean \pm SEM, n=8 per group.

RESULTS

Plasma mitigates gut injury and inflammation in wild type but not syndecan-1^{-/-} mice

In wild type mice, gut injury (Figure 1) and inflammation (Figure 2) were significantly increased after hemorrhagic shock (46.3 \pm 3.7% injury and 62.1 \pm 3.9 inflammation) compared to shams (2.6 \pm 1.2% injury and 5.0 \pm 1.2 and inflammation). Resuscitation with LR decreased both injury (25.6 \pm 2.9%) and inflammation (44.3 \pm 2.0) and these parameters were further decreased by FFP (15.4 \pm 1.8% injury and 29.5 \pm 2.5 inflammation). Additionally, KO mice had worsened gut injury (64.4 \pm 3.0%) and inflammation (118 \pm 5.9) after HS compared to WT mice. While LR decreased these parameters in KO mice (45.9 \pm 3.0% injury and 88.1 \pm 3.2 inflammation), there was no further protection by FFP (40.3 \pm 3.1% injury and 80.6 \pm 3.3 inflammation).

Plasma lessens systemic and gut TNF α and ADAM-17 in wild type but not syndecan-1^{-/-} mice

There was a low but measurable level of systemic TNF α in WT and KO sham animals (WT 20.9 \pm 1.9 pg/ml and null 23.9 \pm 1.5 pg/ml) that was significantly increased after hemorrhagic shock in WT (155.8 \pm 6.3 pg/ml) mice and further increased in KO mice (207.9 \pm 11.9 pg/ml) (Figure 3A). TNF α significantly decreased with LR resuscitation in WT (79.7 \pm 4.0 pg/ml) and KO (142.8 \pm 6.9 pg/ml), though remained significantly higher in KO mice. FFP further decreased TNF α in WT (49.3 \pm 1.8 pg/ml) but not KO mice (126.6 \pm 7.2 pg/ml FFP).

ADAM-17, a protein that cleaves TNF α on the cell surface, was found to be present at protein levels that systemically mirrored those of TNF α with an increase after shock (189.1 \pm 8.7 pg/ml WT) and a further increase in KO mice (243.4 \pm 13.5 pg/ml vs shams 11.0 \pm 1.2 pg/ml WT and 14.1 \pm 1.5 pg/ml null) (Figure 3B). There was a decrease with LR in both WT (90.5 \pm 7.4 pg/ml) and KO mice (166.6 \pm 7.3 pg/ml) though ADAM-17 remained significantly increased in KO mice compared to WT. Similarly, FFP decreased ADAM-17 in WT mice (61.8 \pm 5.2 pg/ml) but not in KO mice (147.0 \pm 7.4 pg/ml). Lastly, to ensure that ADAM-17 protein levels accurately reflected ADAM-17 activity, plasma ADAM-17 activity was measured (Figure 4A) and confirmed the protein measurements.

The potential contribution of intestinal TNF α and ADAM-17 to plasma's protection was next assessed. The overall protein levels of both were markedly increased compared to systemic levels but the pattern of change was similar between serum and small intestinal TNF α and ADAM-17 protein (Table 1). ADAM-17 intestinal activity is shown in Figure 4B. In WT mice, ADAM-17 activity was significantly increased after hemorrhagic shock (303 \pm 15%), lessened by LR (223 \pm 16%), but further decreased by plasma (173 \pm 11%). These findings are suggestive of a role of FFP in the regulation of ADAM-17 (or TACE) activity. In KO mice, there was a further increase in ADAM-17 activity after hemorrhagic shock

(388±14%) compared to WT, with a reduction by LR (292±15%) but no further decrease by plasma (256±21%).

DISCUSSION

The current data demonstrates for the first time that resuscitation with plasma after hemorrhagic shock provides protection to intestinal epithelial cells, an effect that is mediated in part by syndecan-1. Gut injury and inflammation were increased after hemorrhagic shock and lessened by plasma in WT but not syndecan-1 KO mice, suggesting a critical role for syndecan-1 in FFP mediated gut protection. The primary focus of most syndecan-1 studies has been based on its endothelial, rather than epithelial, location.(8-11, 15) However, recent reports have shown that epithelial syndecan-1 is important to intestinal pathologies.(12, 16) We have shown in an in-vitro model of hypoxia/reoxygenation that intestinal epithelial cell syndecan-1 shedding was significantly increased and cell surface syndecan-1 immunostaining reduced.(13) Clinical studies in patients with inflammatory bowel disease have shown that Crohns patients had significantly reduced intestinal syndecan-1 that correlated with disease severity.(17) Yablecovitch et al demonstrated elevated levels of soluble syndecan-1 in serum of patients with both Crohn's and ulcerative colitis.(18) These studies highlight the important and newly appreciated role of syndecan-1 in intestinal pathology. Hemorrhagic shock, though not an intestine specific insult, is clearly a systemic insult that results in vascular and epithelial barrier compromise in multiple organs (19)

In our past work, we have shown in a mouse model of LPS that syndecan-1 shedding protects the host from dysregulated inflammation by facilitating resolution of neutrophilic inflammation.(20) In these studies ablation of syndecan-1 resulted in significantly increased neutrophil accumulation in multiple organs. Syndecan-1 did not affect systemic or local expression of inflammatory factors such as TNF α but specifically facilitated removal of CXC chemokines. This is in distinct contrast to the current findings in a mouse model of hemorrhagic shock where both local (gut) and systemic levels of TNF α were markedly elevated in syndecan-1^{-/-} mice compared to wild type mice. The pro-inflammatory phenotype of null mice after hemorrhagic shock and endotoxemic shock suggests that syndecan-1 shedding confers protection, though the precise mechanism remains unclear. We have previously reported in a pilot study of severely injured patients that three pro-inflammatory cytokines with endothelial activity; IFN gamma, fractalkine, and IL-1 β , all correlated with systemic levels of shed syndecan-1. (21) We postulated that hemorrhagic shock-induced syndecan-1 shedding which exposes the underlying injured endothelium to these pro-inflammatory cytokines. This in turn can lead to endothelial activation and attraction of pathologic neutrophils to the site of injury. IFN gamma then binds to heparan sulfate moieties on the shed syndecan-1 ectodomain, facilitating resolution of inflammation at the injured endothelium. These data suggest a dual role for syndecan-1 where shedding can be detrimental but also aid in resolution and restoration. Stratt et al recently reported that transfusion of FFP to nonbleeding critically ill patients was associated with a reduction in systemic TNF α and syndecan-1, similar to the results of the current laboratory study. Importantly, FFP did not aggravate the inflammatory response but rather suggested an endothelial stabilizing effect. (22)

Ectodomain shedding of syndecan-1 is regulated by multiple signaling pathways converging on a diverse group of metalloproteinases, referred to as sheddases. The ADAM family comprises a large group of these sheddases.(23) We sought to specifically investigate the role of ADAM17, also known as TNF α converting enzyme (TACE), as a modulator of syndecan-1 shedding. ADAM-17 is co-expressed with syndecan-1 on epithelial cells and has been shown to mediate syndecan-1 shedding in lung epithelial cells.(24, 25) In wild type mice intestinal ADAM 17 was increased after hemorrhagic shock, lessened by resuscitation with LR and further decreased by plasma. Additionally, ADAM-17 protein and activity were higher in syndecan-1 KO mice after shock and resuscitation and not lessened by plasma when compared to LR resuscitation. These data suggest that there may be complex pathways involved that interlink syndecan-1 to ADAM17 expression and activity.

ADAM17 has diverse substrates which can include adhesion proteins and receptors, growth factors, and pro-inflammatory cytokines such as TNF α .(26) TNF α is elevated early after hemorrhagic shock in patients and in rodents, as shown in the current study.(27,28) There was a 65 fold increase in systemic TNF α in WT mice after hemorrhagic shock compared to sham mice. Whether the gut merely instigates a systemic response or directly contributes to the systemic response via gut-derived shed TNF α is unknown. There is data in the cancer literature that epithelial tumors shed syndecan systemically, suggesting that in pathologic states shed epithelial ectodomains can enter the vasculature.(29,30) It is therefore conceivable that some portion of cleaved intestinal TNF α contributed to the increase in systemic TNF α . Results of the current study support, but do not prove, syndecan-1 shedding after hemorrhagic shock by TNF α . In addition to functioning as a syndecan-1 sheddase, TNF α can also downregulate syndecan-1 expression. (31,32) Together, these observations suggest that after hemorrhagic shock TNF α induces syndecan-1 shedding in an ADAM17-dependent manner and that FFP inhibits this shedding mechanism.

Wild type mice expressed less gut and systemic TNF α and ADAM 17 than KO mice, consistent with FFP inhibiting sheddase activity or increasing expression of sheddase inhibitors. This does not, however, explain why KO mice expressed more TNF α and ADAM 17 than wild type mice and why plasma did not mitigate expression of TNF α or ADAM 17 in syndecan-1 KO mice. Results at least suggest that plasma is exerting its protection by directly interacting with syndecan-1. It could either restore cell surface syndecan-1 expression by mobilizing preformed syndecan -1 or contain a ligand which binds to syndecan-1 and inhibits subsequent shedding. Plasma contains over 1000 proteins and which, if any, function as effective syndecan-1 ligands has not been investigated. FFP could be exerting its protective effect by inhibiting ADAM-17. It is conceivable that plasma may contain protease inhibitors that could reduce the levels of ADAM-17. We have shown in preliminary studies that a gut protease inhibitor administered after hemorrhagic shock can decrease gut and systemic ADAM-17 (unpublished data).

The current studies represent preliminary studies of the complex interaction of intestinal and systemic syndecan-1, TNF α and ADAM-17, after hemorrhagic shock and their potential role in plasma's protection. Our proposed pathway for hemorrhagic shock-induced syndecan-1 shedding and protection by plasma is shown in Fig. 5. Our results suggest that regulation of syndecan-1 post-shock emerges as a potential therapeutic target for novel drug discovery in

the treatment of trauma patients. Further research should focus on the molecular pathways by which FFP elicits its protective effects. Additionally, further biochemical isolation of FFP's constituent proteins and elucidation of their effects upon regulation of syndecan-1 is warranted as is a better understanding of the role of sheddase inhibition by plasma.

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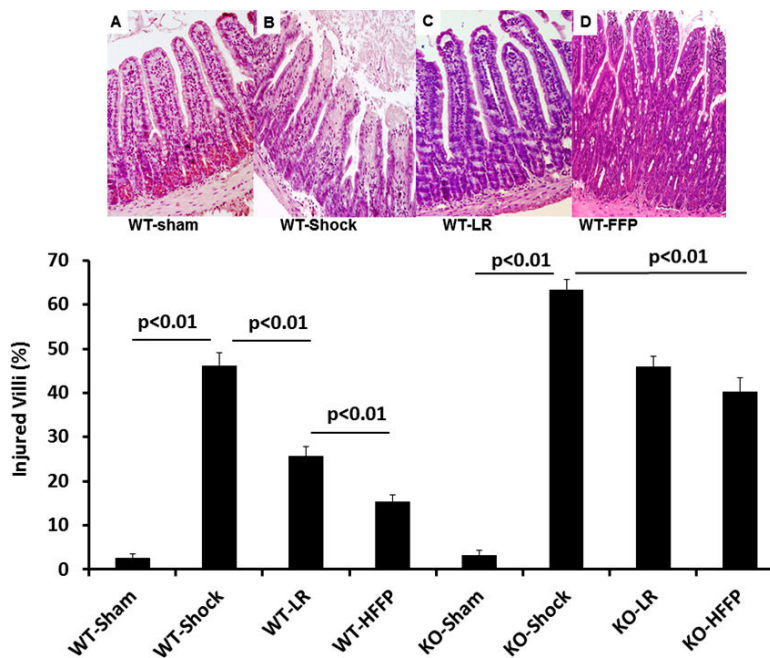


Figure 1. Plasma mitigates gut injury in wild type but not syndecan-1 null mice

Wild-type and syndecan-1 null mice underwent laparotomy and hemorrhagic shock followed by resuscitation with either lactated Ringers or fresh frozen plasma. After three hours small bowel was harvested and histopathologic injury assessed. Representative images from WT mice are shown for each group: (A) WT shams, (B) WT shock (C) WT LR and (D) WT FFP. The percentage of injured villi are shown in the graph. Data are expressed as mean \pm SEM, n=8/group with significance indicated by lines over the respective groups. WT= wild type; LR= lactated ringers, FFP= fresh frozen plasma

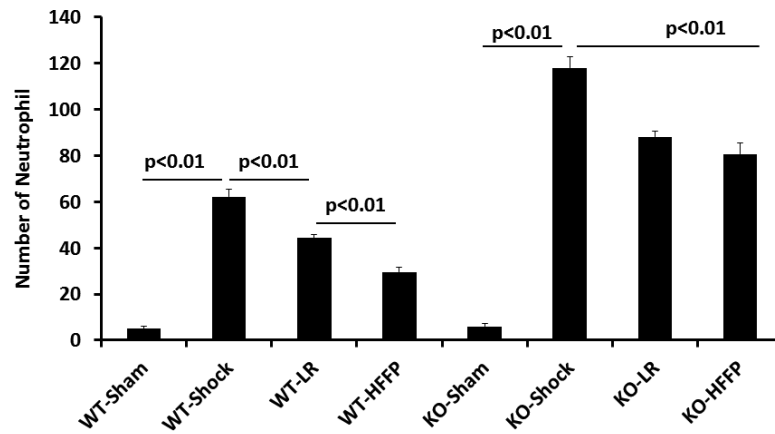


Figure 2. Plasma mitigates inflammation in wild type but not syndecan-1 null mice
Wild-type and syndecan-1 null mice underwent laparotomy and hemorrhagic shock followed by resuscitation with either lactated Ringers or fresh frozen plasma. After three hours small bowel was harvested for assessment of inflammation by quantifying neutrophilic infiltration. Data are expressed as mean \pm SEM, n=8/group with significance indicated by lines over the respective groups.

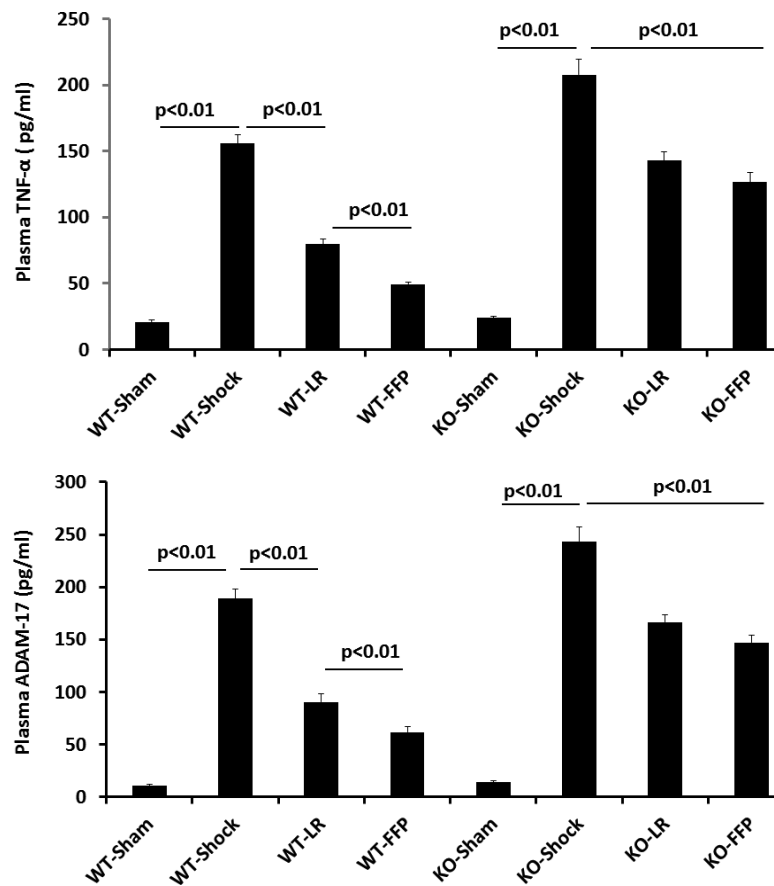


Figure 3. Plasma lessens systemic TNF α and ADAM-17 protein in wild type but not syndecan-1 KO mice

Wild-type and syndecan-1 KO mice underwent laparotomy and hemorrhagic shock followed by resuscitation with either lactated Ringers or fresh frozen plasma. After three hours animals were euthanized and blood obtained for measurement of TNF α and ADAM-17 protein by ELISA. Data are expressed as mean \pm SEM, n=8/group with significance indicated by lines over the respective groups.

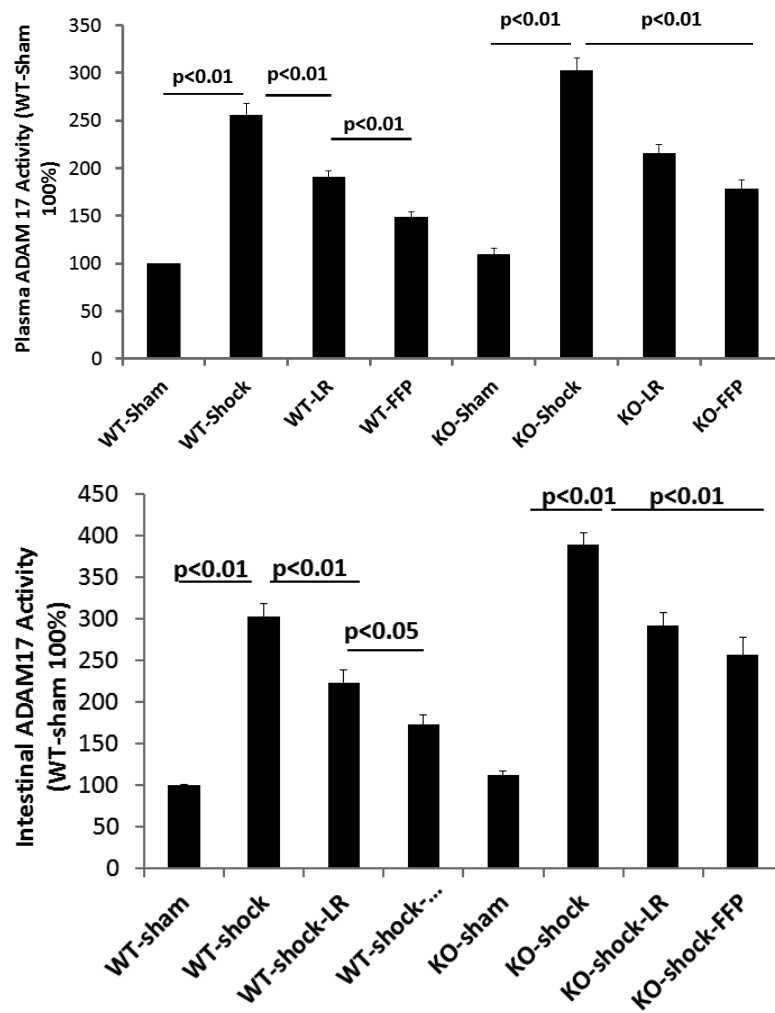


Figure 4. ADAM-17 activity in wild type and syndecan-1 null mice

Wild-type and syndecan-1 null mice underwent laparotomy and hemorrhagic shock followed by resuscitation with either lactated Ringers or fresh frozen plasma. After three hours animals were euthanized and small bowel and blood obtained for measurement of ADAM-17 activity. Data are expressed as mean \pm SEM, n=8/group with significance indicated by lines over the respective groups.

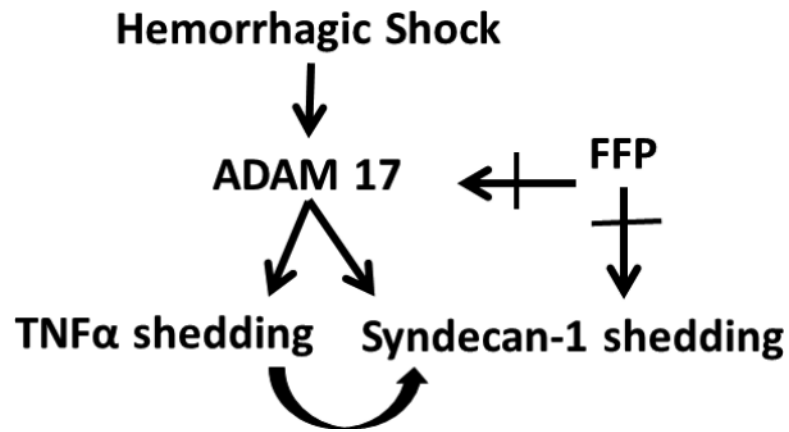


Figure 5. Proposed pathway of fresh frozen plasma's protection after hemorrhagic shock
After hemorrhagic shock, ADAM-17 is increased. ADAM-17 is a known sheddase of both TNF α and syndecan-1. Additionally, TNF α is a potent sheddase of syndecan-1. Fresh frozen plasma (FFP) may directly interact with ADAM-17 to reduce its activity and/or interact with syndecan-1 to reconstitute gut epithelial cell syndecan-1 and reduce systemic syndecan-1 shedding.

Table 1Intestinal TNF α and ADAM-17 protein following hemorrhagic shock and resuscitation.

	TNF α	TNF α	ADAM-17	ADAM-17
Group	WT	KO	WT	KO
Sham	20.9 \pm 1.0	22.0 \pm 1.3	1.31 \pm 0.06	1.28 \pm 0.07
HS	38.8 \pm 1.5 *	45.8 \pm 0.8 *	2.67 \pm 0.10 *	3.43 \pm 0.08 *
HS-LR	29.9 \pm 0.8 *#	39.3 \pm 1.2 *#	1.94 \pm 0.08 *#	2.37 \pm 0.10 *#
HS-FFP	24.5 \pm 1.0 *#^	36.9 \pm 0.8 *#	1.52 \pm 0.05 *#^	2.12 \pm 0.11 *#

WT= wild type; KO=knock out; HS= hemorrhagic shock; LR=lactated Ringers; FFP=fresh frozen plasma. Results are presented as ng/ml; mean \pm SEM, n=8/group.

* p<0.01 vs sham of respective WT or KO mouse

p<0.01 vs HS of respective WT or KO mouse

^ p<0.01 vs LR of respective WT or KO mouse