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Adiponectin in Fresh Frozen Plasma Contributes to Restoration of Vascular Barrier Function after Hemorrhagic Shock

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Abstract

Hemorrhagic shock is the leading cause of preventable deaths in civilian and military trauma. Use of fresh frozen plasma (FFP) in patients requiring massive transfusion is associated with improved outcomes. FFP contains significant amounts of adiponectin, which is known to have vascular protective function. We hypothesize that FFP improves vascular barrier function largely via adiponectin. Plasma adiponectin levels were measured in 19 severely injured patients in hemorrhagic shock (HS). Compared to normal individuals, plasma adiponectin levels decreased to 49% in HS patients prior to resuscitation ($p < 0.05$) and increased to 64% post resuscitation (but not significant). In a HS mouse model, we demonstrated a similar decrease in plasma adiponectin to 54% but a significant increase to 79% by FFP resuscitation compared to baseline ($p < 0.05$). HS disrupted lung vascular barrier function, leading to an increase in permeability. FFP resuscitation reversed these HS-induced effects. Immunodepletion of adiponectin from FFP abolished FFP's effects on blocking endothelial hyperpermeability *in vitro*, and on improving lung vascular barrier function in HS mice. Replenishment with adiponectin rescued FFP's effects. These findings suggest that adiponectin is an important component in FFP resuscitation contributing to the beneficial effects on vascular barrier function after HS.

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Conflicts of Interest

The authors have no conflicts of interest to declare.

Keywords

Human subjects; mouse model; immunodepletion; endothelial cell hyperpermeability; pulmonary microvascular permeability

INTRODUCTION

Hemorrhagic shock (HS) is the leading cause of potentially preventable deaths in civilian and military trauma, accounting for up to 40% of the deaths associated with traumatic injuries (1). One of the primary clinical manifestations of HS is the disruption of the vascular barrier, which leads to microvascular hyperpermeability in vital organs such as the lungs (2) and contributes to the morbidity and mortality of shock (3). Efforts to control hemorrhage and to restore cardiovascular function form the core of the therapeutic approaches to traumatic injuries.

Clinical studies have shown that early resuscitation with fresh frozen plasma (FFP) is associated with improved outcomes including a survival benefit after trauma/HS (4-8). To investigate the mechanisms underlying FFP's protective effects, our group has recently examined the role of FFP resuscitation in vascular barrier function, independent of volume expansion and correction of coagulopathy. We have demonstrated that FFP suppresses inflammatory cytokine- and hypoxia-induced hyperpermeability in endothelial cells *in vitro* (9-11) and *in vivo* after HS (12). The protective effects of FFP resuscitation after HS on lung vascular barrier function are associated with restoration of syndecan-1 and the glycocalyx (12, 13).

Adiponectin, also referred as ACRP30, AdipoQ or gelatin-binding protein-28, is produced in adipocytes with a biological concentration in a range of 14-18 µg/ml (14). It has diverse functions including anti-diabetic, anti-atherogenic, and anti-inflammatory properties (15). Adiponectin also has vaso-protective effects in sepsis (16) and inhibits cytokine-induced endothelial cell hyperpermeability (17). However, the role of adiponectin in HS and resuscitation is unknown. We hypothesized that it is adiponectin in FFP that plays a protective role in restoring vascular barrier function after HS. We demonstrated that plasma adiponectin levels were significantly reduced in severely injured patients in HS and partially restored by FFP based resuscitation. As proof-of-concept, FFPs were depleted of adiponectin or replenished with adiponectin after the depletion, and their effects on vascular barrier function were investigated *in vitro* in human endothelial cells and *in vivo* in a mouse model of HS and resuscitation.

MATERIALS AND METHODS

Reagents and antibodies

Citrated FFPs of mouse or human origin (designated mFFP or hFFP) were obtained from Innovative Research (Novi, MI) and the Gulf Coast Regional Blood Center (Houston, TX), respectively. For both *in vivo* and *in vitro* experiments, FFPs were pooled from three individual donors to minimize donor-to-donor variations. The FFP was thawed and used on the same day (<6 hours), which is defined as day 0 (9). Evans blue dye was purchased from

Sigma-Aldrich (St. Louis, MO). Anti-adiponectin antibody (derived from rabbit, specific for both human and mouse species) and the isotype-matched rabbit IgG were obtained from Abcam (Cambridge, MA). Human or mouse recombinant globular adiponectin (rgAdpn) was obtained from MBL International (Woburn, MA).

Human subject study

The human subject study was approved by the University of Texas Health Science Center at Houston (UTHSC-H) Committee for the Protection of Human Subjects as waiver of informed consent. Severely injured patients in hemorrhagic shock with a systolic blood pressure <90 mmHg and/or a base deficit of >5 meq/ml who received at least one unit of blood after Emergency Department (ED) arrival but prior to Intensive Care Unit (ICU) admission were eligible for the study. Subject demographics, laboratory values and outcomes were obtained from patient records. Blood samples were obtained upon admission to the ED (referenced as pre-resuscitation) and subsequently upon arrival to the ICU (referenced as post-resuscitation) at Memorial Hermann Hospital, Houston, TX, a Level I trauma center. Blood samples were also obtained from healthy subjects following obtainment of consent. After collection, blood samples were centrifuged and plasma aliquots prepared and stored at -80°C for subsequent analysis. Levels of total human adiponectin in plasma were measured using Quantikine Human Immunoassay kit from R&D Systems (Minneapolis, MN).

Preparation of immunodepletion and replenishment of adiponectin from plasma

To deplete adiponectin from FFPs, 1 ml of human or mouse FFPs was incubated with 20 µg of an anti-adiponectin antibody for 20 min followed by incubation with 50 µl of Dynabeads Protein G for 40 min at 4°C. Control FFPs were prepared by incubation with an isotype IgG. The supernatant, designated FFP(-Adpn) or FFP with an isotype control FFP(Ctrl), was collected and used for measurement of the adiponectin level using the Human or Mouse Total Adiponectin/Acrp30 Immunoassay kit from R&D Systems (Minneapolis, MN). Additionally, 20 µg/ml of human or mouse recombinant global adiponectin (rgAdpn) was added to hFFP(-Adpn) or mFFP(-Adpn) respectively, and designated as hFFP(-Adpn +rgAdpn) or mFFP(-Adpn+rgAdpn). All three types of FFPs were used for both *in vitro* and *in vivo* experiments.

Cell culture and *in vitro* permeability assay

Primary human pulmonary endothelial cells (HPMECs) were purchased from PromoCell (Heidelberg, Germany) and cultured in Growth Media MV2 (containing 10% fetal bovine serum (FBS) and the growth supplements) in 37°C incubator with 5% CO₂. The cells were used for experiments within passage 10.

As reported in our published work, FFP inhibits endothelial permeability shown by decreased transwell solute flux and by increased transendothelial electrical resistance measurements over time (9, 12). Based on the transwell solute flux assay, 30 min fluorescence readings were chosen in our published work (9), and in the current study for comparing the effects of different FFP groups on endothelial permeability. *In Vitro* Vascular Permeability Assay kit (Millipore, ECM640, in 24-well format) was used as described (10).

Twenty four well transwell inserts were coated with 1 mg/ml of collagen/insert and incubated at room temperature for 1 hour. The cells were seeded at 40,000 cells per insert well and cultured until formation of integrated cell monolayer. An integrated cell monolayer was verified by microscopic monitoring. A hypoxiareoxygenation (HR) protocol was employed to mimic HS under *in vitro* conditions (9). The Cells were incubated in hypoxia chamber with 2% O₂ for 18 hours and then treated with hFFPs for 1 hour under normoxia with 21% O₂. Cells cultured under normoxia were used as controls. Permeability was tested by adding fluorescein isothiocyanate-conjugated Dextran to the upper chamber of each well. Readings were taken from the bottom chamber at 30 min, and the measurements were determined using a fluorimeter (excitation/emission wavelengths of 485 nm/530 nm).

Mouse model of hemorrhagic shock and resuscitation

All animal procedures were approved by the Animal Welfare Committee at the UTHSC-H. All animal experiments were performed according to the guidelines of the Animal Welfare Act and the Guide for Care and Use of Laboratory Animals from the NIH. C57BL/6 mice (male, 10-12 weeks old, 25-28 g) were purchased from Harlan Laboratories (Indianapolis, IN). Total 42 mice were used and randomly divided into the following groups: Sham n=8, HS n=8, HS+mFFP(Ctrl) n=10, HS+mFFP(-Adpn) n=10, HS+mFFP(-Adpn+mrgAdpn) n=6. A previously described two-event model of mouse laparotomy/HS (18) was used in this study with modifications. Briefly, soft-tissue trauma was induced by performing a 2-cm long ventral midline laparotomy before the onset of hemorrhage. After trauma, animals were stabilized for 5-10 min and then bled rapidly to a mean arterial pressure of 35 mmHg. Blood pressure was maintained at 35 mmHg for 90 min (hemorrhage period). At the end of the hemorrhage period, the mice were resuscitated over a period of 20 min with one to one volume of the blood lost for HS+FFPs group, or left unresuscitated for HS group. Sham-operated animals underwent laparotomy but were neither hemorrhaged nor resuscitated. At two hours after the initiation of resuscitation, the mice were injected with Evans Blue dye. Three hours after resuscitation, the mice were sacrificed; blood and the lung tissue samples were collected. The blood samples were centrifuged and plasma aliquots were prepared and stored at -80°C. We reported that FFP decreases vascular endothelial growth factor A (VEGF)- or tumor necrosis factor (TNF)- α -induced leukocyte adhesion and endothelial hyperpermeability (11, 12, 19). These results demonstrate that FFP has inhibitory effects on inflammation that directly affect vascular barrier function. In the current study we chose to measure lung vascular barrier function to assess FFP's beneficial effects since the lungs are the most sensitive organs to HS-induced disruption of vascular barrier function. Pulmonary microvascular permeability was measured using the dye extravasation technique as a measurement of vascular barrier function (20). Wet-to-dry ratios of the lung samples from the lower right lobe were measured as an indication of tissue edema (21).

Statistics

Data are presented as mean $\pm$ SEM. Statistical significance between multiple groups is determined by one-way ANOVA followed by Holm-Sidak test using SigmaPlot 11.0 (Systat Software, Chicago, IL). *p* values less than 0.05 are considered significant.

RESULTS

Adiponectin levels decrease in patients with hemorrhagic shock

We evaluated the plasma levels of adiponectin in 19 patients that presented with a systolic blood pressure <90 mmHg and/or a base deficit of >5 meq/ml, and received at least one unit of blood in Emergency Department. Their demographics, laboratory values and outcomes are presented in Table 1. Based upon the admission base deficit, these subjects were in severe shock and 26% (5/19) subsequently died. They all received blood products prior to ICU admission. We found significantly lower plasma adiponectin levels in HS patients at the time of admission to the Emergency Department (7.3 ± 1.0 $\mu\text{g/ml}$) compared with healthy donors ($n=13$, 15.0 ± 2.4 $\mu\text{g/ml}$, $p<0.05$). In patients with HS, there was a slight increase of plasma adiponectin levels upon admission to the ICU (9.5 ± 1.0 $\mu\text{g/ml}$, post-resuscitation) although not significant compared to pre-resuscitation.

Immunodepletion of adiponectin from FFP abrogates its ability to inhibit hypoxia-induced hyperpermeability in endothelial cells

The role of adiponectin in mediating FFP's vascular barrier protective effects *in vitro* in human endothelial cells was investigated by immunodepletion of adiponectin. The adiponectin level was decreased by 92% in human FFPs immunoprecipitated of adiponectin (hFFP(-Adpn)) compared to FFPs immunoprecipitated with an isotype control hFFP(Ctrl) (1.30 ± 0.14 vs 18.03 ± 0.63 $\mu\text{g/ml}$). The concentration of adiponectin in hFFP(Ctrl) was similar to that observed in normal subjects. These FFPs were then used to examine the effects on hypoxia-induced endothelial hyperpermeability *in vitro*. In addition, human rgAdpn was added back to hFFP(-Adpn) termed as hFFP(-Adpn+rgAdpn) for rescue of depleted adiponectin in FFP. hFFP(Ctrl) blocked HR-induced endothelial hyperpermeability by 73%. Adiponectin depletion in hFFP(-Adpn) resulted in a 67% reversal of FFP(Ctrl)-inhibited hyperpermeability, and the effects were restored by rgAdpn replenishment (Fig. 1).

Adiponectin levels decrease in HS mice but are elevated after FFP resuscitation

We used the mouse model of laparotomy/HS and FFP resuscitation as depicted in Figure 2, and analyzed changes of plasma adiponectin levels. A reduction of plasma adiponectin levels was observed in the same group of mice before (as baseline) and after HS (22.5 ± 0.8 vs 12.2 ± 0.5 $\mu\text{g/ml}$, $p<0.05$). Adiponectin levels were elevated after HS and FFP resuscitation compared to HS only (17.6 ± 0.5 $\mu\text{g/ml}$ vs 12.2 ± 0.5 $\mu\text{g/ml}$, $p<0.05$). Thus both human and mouse data demonstrate an inverse association of adiponectin levels with HS.

Adiponectin is an important component in FFP to improve lung vascular barrier function in mice after shock and resuscitation

To investigate *in vivo* effects of adiponectin depletion, mouse FFPs were immunodepleted of adiponectin. The adiponectin level was decreased by 87% in mouse FFPs immunoprecipitated of adiponectin (mFFP(-Adpn)) compared to FFPs immunoprecipitated with an isotype control mFFP(Ctrl) (2.49 ± 0.16 vs 19.58 ± 0.88 $\mu\text{g/ml}$). Two different FFPs, mFFP(Ctrl) and mFFP(-Adpn), were used for resuscitation in the mouse laparotomy/HS

model. In addition, mouse rgAdpn was added back to mFFP(–Adpn) termed as mFFP(–Adpn+rgAdpn) for rescue of depleted adiponectin in FFP resuscitation after HS.

Resuscitation with mFFP(Ctrl) increased plasma adiponectin levels by 44% compared to HS alone, while resuscitation with mFFP(–Adpn) resulted in 60% reduction of plasma adiponectin levels compared to mFFP(Ctrl). Replenishment with rgAdpn restored plasma adiponectin to a level similar to that of the mice resuscitated with FFP(Ctrl) (Fig. 3A). HS alone induced significant pulmonary Evans blue dye extravasation compared to sham group. Resuscitation with mFFP(Ctrl) resulted in a significant decrease in pulmonary Evans blue dye extravasation compared to HS alone. Compared with mFFP(Ctrl), mFFP(–Adpn) resuscitation resulted in a significant increase in pulmonary Evans blue dye extravasation, the effects of which were negated by replenishment with rgAdpn (Fig. 3B). Wet-to-dry ratio did not show significant differences among all groups (Fig. 3C). However, a positive correlation coefficient is observed between the results of the Evans blue extravasation and the wet-to-dry ratio ($r=0.664$, $p<0.05$), indicating they tend to increase together. In contrast, a negative correlation coefficient is observed between the adiponectin levels and the Evans blue extravasation values ($r=-0.7140$, $p<0.05$), indicating the adiponectin levels tend to decrease while the Evans blue extravasation values increase. The reason for insignificant changes of the wet/dry ratio in the current study is likely due to large variations of the assay.

Furthermore, hemorrhage resulted in a significant reduction in MAP. Resuscitation with FFPs increased MAP with no difference for the subsequent 120 minutes between different FFP groups (Fig. 4). These provide evidence that the results achieved aren't merely a result of resuscitative differences between the FFP groups.

Taken together with the *in vitro* results shown in Figure 1, our data suggest that adiponectin is an important component contributing to FFP's inhibitory effects on HS-induced pulmonary vascular hyperpermeability.

DISCUSSION

The results of this study demonstrate the beneficial role of FFP resuscitation in restoration of disrupted lung vascular barrier function after trauma/HS. More intriguingly, we have identified adiponectin as an important component in FFP contributing to the vascular protective effects by adiponectin depletion and replenishment from FFP.

Adiponectin belongs to the highest expressed proteins in adipocytes and represents ~0.01% of total plasma protein (22). In the past 20 years after its discovery, adiponectin has gained wide interests due to its insulin sensitizing, anti-inflammatory and anti-apoptotic actions. In general, adiponectin is considered as a protective molecule. Circulating adiponectin levels inversely correlate with multiple disorders including obesity, cardiovascular disease risk, and malignancies (15). On the other hand, increased plasma adiponectin levels are associated with elevated risks of cardiovascular mortality (23) and new onset heart failure (24). Mechanisms underlying these seemingly contradictory associations between adiponectin concentrations and individual disease risks or outcomes are not clear. Nevertheless, our data provide further evidence on an inverse association of plasma

adiponectin levels with trauma/HS in humans and in animal models. Furthermore, since circulating adiponectin is easily detectable and stable in blood, it has potential to be developed as a biomarker of endothelial dysfunction after trauma/HS.

Plasma is a complex biologic material that contains thousands of proteins covering a myriad of physiological and pathological functions (25). To identify whether adiponectin is a key component in FFPs for the protective effects, we performed a proof-of-concept study by depletion of adiponectin from FFPs and addition of recombinant adiponectin after depletion. We found that adiponectin depletion from FFPs abolished FFPs' protective effects *in vitro* and *in vivo*; adiponectin replenishment rescued these FFPs' protective effects. Thus, adiponectin is identified as an important component in FFP responsible for its vascular barrier protective effects in trauma/HS *in vitro* and *in vivo*. However, there were differences between adiponectin depletion efficiency (87-92%) and reversal of FFP-mediated inhibition of vascular hyperpermeability (52-65%) (Fig. 1 and 3), suggesting that other factors in FFP may still play a role in its vascular protective effects. These factors have not yet been identified. Overall, our findings support the protective roles of adiponectin, which are consistent with reports from other disease states. For instance, adiponectin is protective in hyperoxia-induced lung damage through attenuation of oxidative stress; overexpression of adiponectin in mice attenuated hyperoxia-induced lung injury, vascular leak, and lipid peroxidation *in vivo* (26). Administration of adiponectin protects from myocardial injury following ischaemiareperfusion in rats (27). Based on the literature, administration of adiponectin alone is likely to recapitulate FFP's beneficial effects at least in part, and is our future plan as therapeutic development for HS resuscitation.

In summary, our results have provided further evidence that FFPs exert vascular protective effects after trauma/HS and identified adiponectin as an important component in FFPs for the vascular beneficial effects. Understanding how FFPs protect against trauma/HS-induced vascular hyperpermeability at the molecular level provides the opportunity for the development of biomarker(s), as well as mechanism-based nonbiologic therapeutic interventions as surrogate(s) to FFPs for treatment of trauma/HS patients.

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Figure 1. Depletion of adiponectin abrogates FFP's ability to inhibit hypoxia-induced endothelial hyperpermeability

HPMECs were incubated in hypoxia chamber with 2% O₂ for 18 hours and then treated with 30% of hFFP with an isotype control HR+hFFP(Ctrl), HR+hFFP(-Adpn), or HR+hFFP(-Adpn+rgAdpn) for 1 hour under normoxia with 21% O₂, and subjected to *in vitro* permeability assay. Data are presented as mean±SEM from triplicate samples. **p*<0.05 compared between HR, HR+hFFP(Ctrl), HR+hFFP(-Adpn), and HR+hFFP(-Adpn+rgAdpn). Adpn: adiponectin. rgAdpn: recombinant global adiponectin. EC: endothelial cells. RFUs: relative fluorescence units.

Figure 2. A diagram showing the mouse model of laparotomy/HS and resuscitation
Arrows underneath indicate the timing (in hour) after resuscitation. EBD: Evan's blue dye.

Figure 3. Depletion of adiponectin abrogates FFP's ability to inhibit hemorrhagic shock-induced pulmonary microvascular permeability in mice

A. The mice were subjected to laparotomy/HS as described in Figure 2, alone, or resuscitated with mFFP with an isotype control HS+mFFP(Ctrl), HS+mFFP(-Adpn), or HS+mFFP(-Adpn+rgAdpn). Total adiponectin levels were measured in mouse plasma samples harvested before HS as baseline, after HS alone, and after HS and resuscitation with different mFFPs. Baseline n=8, HS n=8, HS+mFFP(Ctrl) n=3, HS+mFFP(-Adpn) n=3, HS+mFFP(-Adpn+rgAdpn) n=6. **B.** Evans blue dye extravasation was measured from the mouse lung samples. Sham n=6, HS n=6, HS+mFFP(Ctrl) n=6, HS+mFFP(-Adpn) n=5, HS+mFFP(-Adpn+rgAdpn) n=4. **C.** Wet-to-dry ratio was measured from the mouse lung samples. Sham n=5, HS n=5, HS+mFFP(Ctrl) n=10, HS+mFFP(-Adpn) n=7, HS+mFFP(-Adpn+rgAdpn) n=5. Data are presented as mean±SEM. * $p<0.05$ compared between HS, HS+mFFP(Ctrl), HS+mFFP(-Adpn), and HS+mFFP(-Adpn+rgAdpn).

Figure 4. Mean arterial pressure (MAP) during hemorrhage and resuscitation

The mice were subjected to laparotomy/HS and resuscitated with mFFP with an isotype control HS+mFFP(Ctrl), HS+mFFP(-Adpn), or HS+mFFP(-Adpn+rgAdpn) as described in Figure 2. MAP was measured at the indicated time points. HS+mFFP(Ctrl) $n=10$, HS+mFFP(-Adpn) $n=10$, HS+mFFP(-Adpn+rgAdpn) $n=5$. Data are presented as mean $\pm$ SEM. There is no significant difference among the three groups. endHS: end of HS. endResus: end of resuscitation.

Table 1

Shock patient demographics and ED and ICU median (intra quantile range) admission values

Parameter	Value
Sample size	N=19
Median Age (yr)	35 (23, 50)
Males (%)	68%
Blunt (%)	89%
Median Injury Severity Score	34 (33, 38)
ED Systolic Blood Pressure (mmHg)	91 (75, 109)
ICU Systolic Blood Pressure (mmHg)	118 (102,140) *
ED Heart Rate (b/min)	107 (102, 124)
ICU Heart Rate (b/min)	98 (87, 115)
ED Base Deficit	7 (5, 11)
ICU Base Deficit	4 (2, 7) *
Pre-ICU Crystalloid (L)	4.7 (2.6, 5.0)
Pre-ICU RBCs (units)	5 (3.0, 9.5)
Pre-ICU Plasma (units)	5 (2.7, 8.3)

* $p < 0.05$, significantly different from ED