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Increased TGF- β Contributes to Deterioration of Refrigerated Fresh Frozen Plasma's Effects *in vitro* on Endothelial Cells

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Abstract

Resuscitation with fresh frozen plasma (FFP) is associated with improved outcomes after hemorrhagic shock (HS). Many trauma centers are using thawed plasma which has been stored for up to 5 days at 4°C (refrigeration), yet the effect of refrigeration on FFP is relatively unknown. Previously our group showed that refrigeration of FFP changed its coagulation factors and diminished its beneficial effects on endothelial cell (EC) function and resuscitation in an animal model of HS. We hypothesize that growth factor composition of FFP is altered during refrigeration, leading to a diminished beneficial effect on EC. Transforming growth factor (TGF)- β is a potent inhibitor of EC migration and is released during refrigeration of platelets. We found increased TGF- β 1 protein levels and greater activation of downstream mediators Smad2/3 during refrigeration of FFP. Both Day 0 FFP (used on the same day after being thawed) and Day 5 FFP (used after being thawed and refrigerated for 5 days) stimulated EC migration *in vitro*; however, the EC migration in Day 5 FFP was significantly reduced. Inhibition of TGF- β type I receptor blocked FFP-induced Smad3 signaling in EC cells and restored the effectiveness of Day 5 FFP on EC migration to a comparable level seen in Day 0 FFP. These data suggest that the increased TGF- β levels during FFP refrigeration contribute to the deterioration of refrigerated FFP's effects on EC migration. This study identifies a novel molecular mechanism contributing to the reduced efficacy of refrigerated FFP.

Keywords

Hemorrhagic shock; resuscitation; Smad; TGF- β type I receptor

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Introduction

Hemorrhagic shock (HS) is a leading cause of death in trauma patients worldwide (1). Based on the data from both military and civilian centers, new resuscitation strategies have been developed, including earlier and increased use of fresh frozen plasma (FFP) in addition to packed red blood cells and conventional crystalloids, which is collectively referred to “damage control resuscitation” (2). Aggressive administration of FFP was associated with improved survival in trauma patients (2-4). Data from casualty care in the Iraq war revealed a reduction in mortality as the ratio of packed red blood cells to plasma approached 1:1 (5). Civilian studies have shown mixed results, but predominantly support the use of FFP in massively transfused patients (6-9).

Conceptually, the beneficial effects of FFP in HS have been attributed to the hemostatic potential that is capable of replacing lost coagulation factors. Recently, our group has shown that FFP has protective effects on vascular endothelial cells (10). The endothelium is an extensive organ, reaching to all recesses of the human body. It plays a critical role in homeostasis and participates in the regulation of vasomotor tone, leukocyte trafficking, coagulation, permeability, antigen presentation, innate immunity, and angiogenesis (11). After HS and severe trauma, there is endothelial dysfunction that can be broadly defined as a disruption of the normal function of the vasculature. This dysfunction includes shifts in the hemostatic balance, increased cell adhesion and leukocyte trafficking, altered vasomotor tone, loss of barrier integrity, and programmed cell death (12). The impaired endothelial function can lead to multiple organ dysfunction syndrome and eventually to multiple organ failure (13). HS following trauma has been shown to activate apoptotic signaling, leading to hyperpermeability due to disrupted vascular barrier integrity (14-15). EC migration is essential for vascular repair or healing after injury by endothelialization. This repair process can be delayed in areas of endothelial damage by anti-migratory action (16). In addition to restoring hemostasis, FFP is hypothesized to attenuate HS-induced endothelial dysfunction, leading to improved clinical outcome. This hypothesis is supported by our previous *in vivo* and *in vitro* studies that exhibit FFP's beneficial effects on endothelial permeability, vascular stability, and resuscitation in rats after HS (10).

To provide immediate access to FFP, it is increasingly common in busy trauma centers or blood banks to thaw FFP and to store it between 1-6°C (refrigeration) for up to five days (17). Approved by the American Association of Blood Banks, this approach has the obvious advantage of easy access for rapid administration during emergency situations. However, the clinical effectiveness of using stored FFP is unknown. As with other blood components, the question of storage stability has been raised for FFP. FFP is a complex biological material which contains a plethora of bioactive molecules (18). Although optimal conditions for storage are distinctive for each protein, it is well known that proteins generally alter structural integrity and activity as a result of refrigeration due to proteolysis, aggregation, and suboptimal buffer conditions (19). We have recently shown that refrigeration of FFP decreased its coagulation factors and diminished its beneficial effects on endothelial function and resuscitation in an animal model of HS (10). However, the underlying molecular mechanisms leading to decreased efficacy of refrigerated FFP are unknown.

Transforming growth factor (TGF)- β is a multifunctional growth factor that is critically important for normal vascular development and physiology (20). TGF- β signals via binding to type I (T β RI) and type II (T β RII) serine/threonine kinase membrane receptors. Activation of TGF- β receptors leads to phosphorylation and activation of Smad2 and Smad3, the downstream key mediators of TGF- β signaling. The phosphorylated Smad2 and Smad3 transduce TGF- β signaling from cell surface to the nucleus (21). TGF- β is a known inhibitor of EC migration (22-23), and EC migration plays an important role in restoring endothelial

barrier function after endothelial damage (24). Under most *in vitro* culture conditions, TGF- β inhibits EC migration (23). TGF- β is released to the plasma during platelet storage at 4°C (25); however, whether TGF- β level is altered during refrigeration of FFP is largely unknown and highly relevant to the storage of blood components. Since improved endothelial cell function is the mechanism of FFP mediated beneficial effects, we chose EC migration assay as an outcome measure in this study to test our hypothesis. We hypothesize that FFP promotes EC migration and that refrigeration of FFP increases TGF- β levels, leading to decreased FFP efficacy on EC migration.

Materials & Methods

Fresh frozen plasma

Citrated FFP prepared as platelet-poor-plasma was obtained from the Gulf Coast Regional Blood Center in Houston, Texas, and stored at -80°C. FFP was thawed at 37°C water-bath and then stored at 4°C up to 5 days. FFPs were pooled from three donors and the pooled FFPs were used for TGF- β measurement and for *in vitro* migration assay. FFP used on the same day of being thawed was designated Day 0 FFP and used after being thawed and refrigerated up to 5 days were designated Day 1, 2, 3, 4 and 5 FFP.

Measurement of TGF- β protein levels

TGF- β 1 and TGF- β 2 levels in FFP were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Quantikine kits, R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Three pools of three donors each were used for TGF- β measurement. Triplicate measurements were performed for all samples.

Primary endothelial cell culture

Two primary human endothelial cell lines, human pulmonary microvascular endothelial cells (HPMECs) and human dermal lymphatic endothelial cells (HDLECs), were used in this study. The lung is one of the end organs of injury after HS, is commonly associated with post injury multiple organ failure (26), and contains approximately half of the body's ECs (27). Therefore, we used HPMECs for examining the effects of FFPs on TGF- β signaling and EC migration. The HDLECs were used to confirm the key findings from HPMECs. These cells were purchased from PromoCell (Suttart, Germany), cultured in growth medium recommended by the manufacturer, and incubated at 37°C with 5% CO₂. All cells were used within ten passages for the experiments. The cells were seeded overnight and then treated with FFP at the concentration of 0, 2.5, 5, 10, and 30% for 30 min for western blotting analysis or for 16 h for a migration assay. Cultured medium alone was used as a baseline control. Lactated Ringer's (LR; L-isomer of lactic acid, purchased from Baxter Healthcare Corporation, Houston TX), a crystalloid fluid used clinically for resuscitation, was used at equal volume as a control.

Western blotting analysis

The cells were seeded at the density of 1.6×10^5 /35 mm dish and grown overnight to reach a confluence of 60-70% before treatment. Whole cell lysates were prepared from the treated cells, and western blotting was performed as described previously (28). Blots were probed with the primary antibodies against phospho-Smad2 (pSmad2), phospho-Smad3 (pSmad3) (1:1000, Cell Signaling Technology Inc., Danvers, MA), Smad2, Smad3 (1:500, Invitrogen, Carlsbad, CA), or β -actin (1:2000, Sigma-Aldrich Inc., St Louis, MO) followed by incubation with horseradish peroxidase (HRP)-coupled secondary antibodies. Signals from the target proteins were detected using luminol substrate PicoWest (Pierce Biotechnology

Inc, Rockford, IL), visualized after exposure to x-ray films (Eastman Kodak Company, Rochester, NY), and quantified by densitometric analysis (Kodak 1D).

Migration assay

EC migration was performed by transwell assay. The transwell with filter membrane of 8- μ m pore size was inserted in 24-well cultured plates (Corning, Life Sciences, Lowell, MA), which created an upper and a low chamber. Filter membrane was coated with 0.1% gelatin overnight at 4°C. The cells were seeded (3×10^4 cells/well) in the transwell in the upper chambers. The lower chambers were filled with Day 0 FFP, Day 5 FFP, or LR diluted in culture medium. Culture medium alone was the baseline control. Sixteen hours later, the cells migrating from inside of the transwell through the pores of the filter were analyzed. The non-migrating cells in the transwell were removed using a cotton swab. Cells on the filter membranes were fixed with 70% ethanol for 10 min and stained with propidium iodide (30 μ g/ml) for 30 min at 37°C. Fluorescence images of the migrating cells stained with propidium iodide were taken with a Nikon ECLIPSE TS 100 microscope (Melville, NY). The migrating cells were counted using NI-Element AR Imaging software (2.3) (Melville, NY).

Blockade of TGF- β signaling

The TGF- β type I receptor activin receptor-like kinase (ALK) 5-specific inhibitor, SB431542 (SB, from Tocris Bioscience, Ellisville, Missouri) was used to block TGF- β signaling. SB was dissolved in dimethyl sulfoxide (DMSO) vehicle (Sigma-Aldrich, St. Louis, MO). SB was added to the cultured medium with the indicated concentrations for 45 min followed by treatment with FFP for 30 min for further western analysis and for 16 h for a transwell migration assay. DMSO was used as the vehicle control.

Statistical analysis

Data are presented as mean $\pm$ SE. Generalized estimating equation (GEE) was used to analyze correlated data of TGF- β s. EC migration is expressed as % of medium (or vehicle DMSO control in blockade experiments). Student *t*-test was used for the analysis of pSmad2 and pSmad3 signaling in western blots. Two way analysis of variance (ANOVA) was used for migration assays on dose-dependent experiments and the blockade experiments with outcomes of Day 0 and Day 5 FFPs. When significant, Holm-Sidak test was applied for comparisons of differences between groups. *p* values of less than 0.05 were considered significant.

Results

TGF- β 1 level in FFP increases during refrigeration

To determine whether TGF- β s in FFP were altered during FFP refrigeration, we quantified TGF- β levels in FFP stored at 4°C for up to 5 days. TGF- β 1 protein levels increased in a time-dependent fashion at a rate of 244.31 pg/ml/day ($p < 0.05$) with an intercept of 5366.81 pg/ml ($p < 0.01$) (Figure 1A). In contrast, TGF- β 2 levels in FFP were not altered during refrigeration (Figure 1B). These data indicate a selective increase of TGF- β 1 in FFP during refrigeration.

Refrigeration of FFP enhances its activation of TGF- β /Smad2/3 signaling

To examine the effects of Day 0 and Day 5 FFP on phosphorylation of Smad2 and Smad3 (pSmad2 and pSmad3), HPMECs and HDLECs were treated with different concentrations of FFP (0, 2.5, 5, 10, and 30%). pSmad2 and pSmad3 were measured by western blotting analysis. We found that FFPs induced pSmad2 and pSmad3 in both HPMECs and HDLECs

in a dose-dependent fashion compared to medium control (Figure 2). Compared to Day 0 FFP, Day 5 FFP induced greater pSmad2 and pSmad3 in all the doses tested. However, LR, a commonly used crystalloid resuscitation reagent, did not induce pSmad2 and pSmad3 in both HPMECs and HDLECs compared to the medium control. The increased Smad2/3 activation associated with refrigeration of FFP demonstrates greater activation of the TGF- β signaling pathway.

FFP induces EC migration which diminishes during refrigeration

Using the transwell assay, we examined the effect of FFP refrigeration on EC migration. Both Day 0 FFP and Day 5 FFP increased EC migration in HPMECs and HDLECs in a dose-dependent fashion compared to LR. However, Day 5 FFP had a reduced effect on EC migration in both cell types at all the doses tested compared to Day 0 FFP (Figure 3). There was no difference in EC migration between LR and the medium control. The results of the transwell assay were confirmed by the scratching assay in HPMECs (data not shown). These results demonstrate that FFP induced EC migration and that this effect significantly diminished with refrigeration of FFP for 5 days.

Inhibition of TGF- β /ALK5 signaling pathway enhances FFP-induced migration

To examine whether the increased TGF- β levels in Day 5 FFP contributed to the decreased effectiveness of Day 5 FFP on EC migration, we used SB, a selective pharmacological inhibitor of TGF- β receptor type I ALK5 (29-30). At concentrations from 1 to 10 μ M, SB significantly inhibits TGF- β signaling with pretreatment for 15 min to 4 h (29-30). Based on the literature and our own experiences in using chemical inhibitors, we chose the concentration of SB 1-3 μ M and pretreatment for 45 min for inhibitor studies. In addition, the FFP dose curve studies on EC migration in Figure 2 clearly demonstrate that all concentrations of FFP tested (2.5, 5 and 10%) revealed a significant reduction of EC migration in Day 5 FFP groups compared to Day 0 FFP groups. We chose the lowest effective concentration of FFP (2.5%) for inhibitor studies in order to achieve complete signaling inhibition by SB.

The optimal dosage of SB in inhibition of ALK5 signaling was determined by pretreatment with SB for 45 min followed by treatment with Day 0 FFP for 30 min. Pretreatment with SB dose-dependently inhibited FFP-induced pSmad3 in both HPMECs and HDLECs. SB at 3 μ M abolished FFP-induced pSmad3. SB alone did not induce pSmad3 compared to the vehicle control (Figure 4). Therefore, the concentration of 3 μ M of SB was chosen for EC migration assays.

HPMECs were pretreated with SB (at 3 μ M) for 45 min followed by Day 0 FFP or Day 5 FFP treatment at the concentration of 2.5% for 16 h. EC migration was analyzed by transwell assay. As shown in Figure 5A, both Day 0 FFP and Day 5 FFP induced EC migration compared to the vehicle control, and Day 5 FFP had reduced EC migration by 50% compared to Day 0 FFP. These confirm our earlier findings (Figure 3). SB alone did not alter basal EC migration, compared to the vehicle control. However, SB pretreatment enhanced Day 0 FFP-induced-EC migration by 30% and restored Day 5 FFP-induced EC migration to a similar level as that of Day 0 FFP. These findings were confirmed in HDLECs (Figure 5B).

Taken together, our findings suggest that: (1) TGF- β detected in FFP is functionally active and inhibits EC migration; (2) the inhibitory effect on EC migration by TGF- β may function through the ALK5 signaling pathway; and (3) the increased levels of TGF- β 1 during refrigeration of FFP are responsible for the decreased effectiveness of Day 5 FFP on EC migration.

Discussion

In this study, we found that FFP contained a significant amount of TGF- β , which increased during refrigeration. Correspondingly, Day 5 FFP elicited stronger activation of Smad2/3 signaling than Day 0 FFP. Although both Day 0 and Day 5 FFPs induced EC migration, there were significant differences in EC migration. Day 5 FFP showed a diminished effect on EC migration in both HPMECs (around 20% reduction) and HDLECs (around 50% reduction). Both Day 0 and Day 5 FFPs were superior to LR, which had no effect on EC migration. Blocking TGF- β /ALK5 signaling restored EC migration induced by Day 5 FFP to a comparable level induced by Day 0 FFP. These support our hypothesis that refrigeration of FFP increases TGF- β levels, leading to diminished FFP efficacy on EC migration.

Clinical resuscitation volumes vary greatly based on the volume of blood loss and the type of resuscitation fluids used. In this study, we examined the direct effects of FFP on ECs using FFP concentrations up to 30% since these concentrations should be able to be achieved clinically with FFP resuscitation. Our dose response studies (Figure 2) demonstrate that FFP from all concentrations tested (2.5, 5, 10, and 30%) generated significant effects on TGF- β signaling, with 10% and 30% of FFPs being similar in inducing signaling activation. Therefore, we chose the lower concentrations (10% and less) for most of the studies. These *in vitro* studies are not intended to replicate clinical studies, especially as clinical dilution volumes of FFP and circulating whole blood would be highly variable. However, we do feel that the 10% figure is well within what would be expected (2 units of FFP in an adult blood volume).

To begin to investigate the underlying molecular mechanisms that contribute to the reduced effect on EC migration in response to the stored FFP, we focused on TGF- β , a key regulator of EC migration. We found that TGF- β protein level increased during refrigeration, which was associated with the increased activation of its intracellular signaling Smad2 and Smad3. In contrast, there was a significant decline in multiple coagulation factors (31), which may be due to protein degradation as a result of refrigeration. It has been reported that during storage of platelets, over 10-15% of platelets become activated, resulting in the release of α -granules that contain growth factors such as TGF- β (25). FFPs used in this study were found containing significant amount of residual platelets (3,500 - 21,500/ μ L) by our group although prepared as platelet-poor-plasma in the blood bank following the approved standard procedure. These platelets were activated during refrigeration (31-32). These activated platelets may be the source of the increased TGF- β 1 during FFP refrigeration. However, further experimentation is required to prove this concept and to exclude other possible mechanisms, such as the involvement of other residual blood cells. TGF- β is normally secreted as an inactive or latent form. The biological activity requires conversion of TGF- β from the latent form to the free form (33). The conversion can be done *in vitro* by acidification as indicated in the TGF- β 1 ELISA kit. Therefore, the TGF- β 1 ELISA kit used in this study measures total TGF- β 1 protein. In the physiological conversion of TGF- β 1, proteolysis is a probable step. Thus, increased latent form of TGF- β 1 in FFP during refrigeration may lead to increased conversion to free form, subsequently affecting biological processes.

In ECs, TGF- β can activate the ALK5/Smad2/3 pathway, resulting in the inhibition of EC migration (23). In our study, we found that Day 0 FFP induced activation of Smad2/3, which was blocked by SB, an inhibitor of T β RI ALK5. Day 5 FFP further enhanced activation of Smad2/3. Functionally, Day 0 FFP induced EC migration, and Day 5 FFP had reduced effect on induction of EC migration. Blockade of the ALK5 signaling pathway enhanced Day 0 FFP's effect on induction of EC migration and restored EC migration in response to Day 5 FFP to a comparable level of Day 0 FFP. Taken together, our data suggest that the amount

of TGF- β normally existing in FFP has an inhibitory effect on EC migration, and the additional TGF- β release during refrigeration augments this inhibitory effect. The ALK5 signaling pathway most likely mediates the inhibitory effect of TGF- β on EC migration.

Although the differences observed in cell migration between Day 0 and 5 FFP are modest, the significantly lower EC migration of Day 5 FFP compared to that of Day 0 FFP suggests that the prolonged refrigeration of FFP may decrease the efficacy of resuscitation during HS and trauma. While the logical prediction in clinical resuscitation in trauma patients is that Day 0 FFP would be more effective than Day 5 FFP, we feel that this is a very large leap from EC migration data to the much more complex clinical environment. Furthermore, these functional differences have not previously been described for the refrigeration of plasma. The magnitude of the changes in EC migration between Day 0 and Day 5 FFP (10-23% within the FFP dose of 2.5 to 10% in Figure 3) was in proportion to the changes in levels of TGF- β 1 (23% in Day 5 FFP vs Day 0 FFP in Figure 1A). However, the changes in phospho-Smad2 and -Smad3 (87-142% within the FFP dose of 2.5 to 10% in Figure 2) were relatively dramatic. This could be due to the amplified signaling cascade and the sensitive assay used for the detection. Although the changes in EC migration between Day 0 and Day 5 FFP were modest, our study identified a novel molecular mechanism contributing to the reduced EC migration efficacy of aged FFP. Our findings shed light on the differential effects of thawing and refrigeration on biologically active factors in plasma. Our study also provides rationale to identify other factors potentially altered during refrigeration and the biological consequences provoked by these changes.

There are no published studies correlating the age of plasma and clinical outcomes (10). Plasma proteins comprise a highly heterogeneous class of biological macromolecules, and many are unstable. As reported by our group, there was a significant decline in multiple coagulation factors in FFP during refrigeration (10). We also found that the refrigerated FFP reduced capacity to inhibit EC permeability, and reduced stimulation of EC migration in the current study. Furthermore, in a rat HS model, the refrigerated FFP demonstrated a diminished ability to effectively maintain mean arterial pressure after HS. In line with our findings, the age of transfused blood in trauma patients was reported as an independent risk factor for multiple organ failure after injury (34). Taken together, these findings suggest that the age of the stored blood products may result in reduced and/or undesirable effects in critically injured patients. Although the therapeutic significance of changes observed in aged versus fresh FFP are unclear to date, the present study raises potential clinical ramifications involving the use of aged FFP for resuscitation in trauma patients. Our study also suggests that clinical studies should be conducted to examine the impact of using aged FFP.

In conclusion, we demonstrate that FFP promotes EC migration, which potentially plays an important role in early repair of injured vessels during trauma and HS. This potentially beneficial effect is decreased by using refrigerated FFP. Furthermore, the increase in TGF- β during FFP refrigeration has been identified in this study as one of the contributing factors to the diminished effect of FFP on EC migration, thus revealing a novel molecular mechanism underlying the reduced efficacy of refrigerated FFP. Together with our previous findings of FFP's effects on vascular endothelial function and resuscitation in an animal model of HS, our data further support the hypothesis that FFP has the capacity to "normalize" injured endothelium to restore the normal structure and function of the vessel, and that the beneficial effects of FFP diminish with increased refrigeration time of FFP.

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Figure 1. TGF-β1 protein level in FFP increases during refrigeration

TGF-β1 (A) and TGF-β2 (B) protein levels in FFP stored at 4°C up to 5 days were measured by Quantikine ELISA kits. The data are presented as mean $\pm$ SE (n=3 pools). Generalized estimating equation (GEE) was used to analyze the data using autoregressive of order 1 as the working correlation. TGF-β1 increases at a rate of 244.31 pg/ml/day ($p=0.002$, 95% CI 87.10 to 401.52 pg/ml). There is no statistical evidence that TGF-β2 changes with day ($p=0.7$).

Figure 2. Activation of TGF- β /Smad2/3 signaling by FFPs is dose-dependent and is enhanced during FFP refrigeration

The ECs were serum-starved with medium containing 0.1% serum for 4 h and then treated with FFP at the concentration of 2.5, 5, 10, and 30%, or 30% LR for 30 min. Medium (Med) alone was a baseline control. Whole cell lysates were prepared and subjected to western blotting. **A & B.** HPMEC; **C & D.** HDLEC. **A & C.** Representative western blots. **B & D.** Quantification of **A & C.** * $p < 0.05$ compared to Day 0 FFP.

Figure 3. FFP promotes EC migration and the refrigerated FFP has diminished effect on EC migration

HPMECs and HDLECs were seeded in transwell inserts and cultured with FFP or LR at the concentration of 0 (medium alone), 2.5, 5, and 10% for 16 h. EC migration was measured by transwell assay. **A & C.** Representative pictures showing migrating cells from transwell. “→” points to the migrating “white color” cells. “◀” points to the dark pores of the filter. **B & D.** Number of migrating cells from triplicate wells is expressed as mean $\pm$ SE (n=3/group). * $p < 0.05$ compared to LR control. # $p < 0.05$ compared the differences between Day 0 FFP and Day 5 FFP for all the doses.

Figure 4. Blocking ALK5 attenuates the activation of Smad3 by FFP

The cells were serum-starved with medium containing 0.1% serum for 4 h and then treated with ALK5 inhibitor SB431542 (SB, 1 μM or 3 μM) for 45 min followed by treatment with 2.5% Day 0 FFP for 30 min. Equal volume of DMSO was the vehicle control of SB. Medium alone was the baseline control (lane 1). The whole cell lysates were prepared and subjected to western blotting. **A.** HPMEC. **B.** HDLEC.

Figure 5. Blocking ALK5 restores EC migration capacity of Day 5 FFP comparable to that of Day 0 FFP

Transwell assay was used to measure EC migration. The cells were seeded in transwell inserts and treated with SB431542 (SB, 3 μ M) for 45 min followed by treatment with 2.5% of FFPs for 16 h. Equal volume of DMSO was the vehicle control of SB. **A.** HPMEC. **B.** HDLEC. Number of migrating cells from triplicate wells is expressed as mean $\pm$ SE (n=3/group). * $p < 0.05$ compared to vehicle (DMSO) control. # $p < 0.05$ compared between Day 0 FFP and Day 5 FFP. \$ $p < 0.05$ compared between -SB and +SB with vehicle, Day 0 FFP or Day 5 FFP.