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Intravenous Injection of Mesenteric Lymph Produced During Hemorrhagic Shock Decreases RBC Deformability in the Rat

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Abstract

Objective—To test the hypothesis that gut-derived factors carried in trauma-hemorrhagic shock (T/HS) lymph are sufficient to induce red blood cells (RBC) injury, to investigate their potential mechanisms of action, and to define the time post-T/HS that these factors appear in the lymph.

Methods—Mesenteric lymph collected from T/HS or trauma-sham shock (T/SS) rats over different time periods was injected intravenously into male rats at a rate of 1 mL/h for 3 hours. RBC deformability was measured using laser-assisted ektacytometer to calculate the elongation index. From the shear-stress elongation curve, the stress required for the erythrocytes to reach 50% of their maximal elongation was also determined. RBC deformability was measured before lymph infusion and at 1 hour and 3 hours after the initiation of lymph infusion. The effect of the lymph samples (5% v/v) was also determined in vitro by incubating naïve whole blood with the lymph samples. The potential role of T/HS lymph-induced RBC oxidant injury mediated by inducible nitric oxide synthase (iNOS)-generated oxidants and/or white blood cells (WBC) was investigated using iNOS inhibitors and WBC depletion, respectively. In all the in vivo studies, five to seven rats were studied per group.

Results—The intravenous injection of T/HS lymph but not T/SS lymph caused in vivo RBC injury. The biological activity of T/HS lymph varied over time with the RBC-injurious factors being produced only during the first 3 hours postshock. The in vivo inhibition of iNOS did not prevent lymph-induced RBC injury. T/HS lymph incubated in vitro with naïve whole blood resulted in RBC injury, but this injury was not observed in blood depleted of WBC.

Conclusions—These results indicate that T/HS lymph produced during the initial 3-hour postshock period is sufficient to induce RBC injury in otherwise normal rats and that the lymph-induced RBC injury is not dependent on activation of the iNOS pathway but seems to require WBC.

Keywords

Red blood cell dysfunction; MODS; Gut-derived factors; iNOS; RBC injury; WBC

Increasing evidence from both preclinical and clinical studies-indicate that red blood cells (RBC) alterations or dysfunction plays a role in the pathogenesis of the multiple organ

dysfunction syndrome (MODS) in patients sustaining major trauma, especially when associated with significant hemorrhage.¹⁻⁷ This notion is supported by clinical studies showing that large-volume transfusions of stored blood (>6 U) is a major risk factor for the development of MODS¹ as well as by preclinical and clinical studies showing that stored blood impairs microvascular blood flow and thereby potentiates organ injury.^{2,3,7} This concept that RBC abnormalities may contribute to MODS is also supported by clinical and laboratory observations, showing that RBC deformability is impaired after trauma-hemorrhage^{4,5} and that these changes in RBC deformability are in turn associated with impaired microvascular and nutrient blood flow.^{6,7} Specifically, using the current rat model of trauma-hemorrhagic shock (T/HS), we documented that the exchange transfusion of T/HS RBC into naïve rats resulted in decreased organ blood flow.⁷ The mechanism by which decreased RBC deformability leads to impaired microcirculatory blood flow is related in part to the mechanical need of the 7- μ m RBC to deform and change its shape from a biconcave disc to an elongated structure to successfully pass through 4- μ m capillaries.⁸ Failure of RBC to deform as it passes through capillaries would lead to microvascular stasis and blockade with resultant decreases in cellular oxygen delivery.⁹ This phenomenon is recognized in primary RBC disease states, such as sickle cell anemia, where stress-induced changes in RBC deformability leads to decreased microcirculatory blood flow and clinically significant vasoactive crises.¹⁰

Because of the potential clinical importance of loss of RBC deformability (i.e., increased rigidification of the RBC membrane), we have carried out a series of studies investigating the pathogenesis of trauma-shock-induced loss of RBC deformability. These studies suggest that trauma-hemorrhagic shock (T/HS)-induced alterations in RBC deformability and shape are mediated to a significant degree by gut-derived humoral factors carried in the mesenteric lymph,¹¹ because ligation of the mesenteric lymph duct largely abrogates T/HS-induced RBC rigidification. In addition, we have recently shown that the loss of RBC deformability is associated with decreased alpha-spectrin ubiquitination of the RBC membrane.¹² Although preventing mesenteric lymph from reaching the systemic circulation by ligating the mesenteric lymph duct prevents T/HS-induced RBC deformability and shape changes, it remains unclear whether T/HS lymph itself is sufficient to induce RBC injury or requires the activation of other factors during the T/HS period to exert its effect. Therefore, we tested the hypothesis that whether gut-derived factors carried in T/HS lymph is sufficient to induce RBC injury by infusing T/HS lymph into normal rats and measuring RBC deformability.

MATERIALS AND METHODS

Animals

Adult male specific pathogen-free Sprague-Dawley rats (Taconic Farms, Germantown, NY) weighing 325 g to 450 g were used in the experiments. Animals underwent a 7-day acclimatization period with 12-hour light/dark cycles under barrier-sustained conditions, during which time they had free access to water and Harlan Teklad laboratory chow (Teklad 22/5 Rodent diet [W] 8640, Harlan Teklad, Madison, WI). The animals were maintained in accordance with the guidelines of the National Institutes of Health guide for the Care and Use of Laboratory Animals, and the experiments were approved by the New Jersey Medical School Animal Care and Use Committee.

Experimental Design

The first goal of this study was to test whether T/HS mesenteric lymph was sufficient to recreate the changes in RBC deformability and morphology observed after actual T/HS. To do this, mesenteric lymph collected from male rats subjected to T/HS (laparotomy and hemorrhagic shock) or trauma-sham shock (T/SS; laparotomy plus vessel cannulation) was

injected into nonshocked rats that had undergone a laparotomy. In the initial experiment, lymph collected during the 90-minute shock or sham shock period and for 6 hours after the end of shock or sham shock period was injected into the nonshocked rats at a rate of 1 mL/h for 3 hours. This volume of lymph infusion was based on the fact that a total of about 3 mL of lymph are produced by rats during the shock (sham shock) and 6-hour postshock (sham shock) period. Blood samples for RBC deformability measurements were collected before lymph infusion and 1 hour and 3 hours after beginning the lymph infusions. In all studies, lymph from one individual rat subjected to T/HS or T/SS was injected into a second naïve rat. Only if the amount of lymph collected from a single rat was not sufficient, the lymph from two rats was pooled. In all the experiments, five to seven rats per group were studied.

Because our previous studies documented that RBC deformability was impaired within 3 hours after the end of the shock period,^{11,13} in a second set of experiments, we compared the biological activity of the lymph samples collected during the first 3 hours post-T/HS or T/SS with samples collected 4 hours to 6 hours after T/HS or T/SS. The goal of this study was to determine whether the biological activity of T/HS lymph changed over time.

As an additional proof-of-principle study that the acquired RBC deformability observed after actual T/HS is largely mediated by T/HS lymph, we performed an experiment where rats subjected to actual T/HS with mesenteric lymph duct ligation (LDL) were challenged with either T/HS or T/SS lymph. The idea behind this experiment is our previous studies showing that LDL largely limits actual T/HS-induced RBC rigidification by preventing gut-derived factors in mesenteric lymph from reaching the systemic circulation.¹¹ Thus, if T/HS lymph reaching the systemic circulation is the key factor in the pathogenesis of T/HS-induced RBC rigidification, the T/HS + LDL rats receiving T/HS but not T/SS lymph should manifest abnormal RBC deformability.

Next, we carried out a series of in vitro studies where T/HS or T/SS lymph were added to whole blood from naïve male rats. The goal of these studies was to test whether the in vitro exposure of whole blood to T/HS lymph was sufficient to cause the RBC deformability changes observed after the in vivo injection of T/HS lymph as well as after actual T/HS.

To begin to clarify the potential role of oxidants in T/HS lymph-induced RBC dysfunction, two groups of studies were performed. In the first group, we tested whether in vivo inducible nitric oxide synthase (iNOS) inhibition would prevent T/HS lymph-induced RBC damage. The rationale for this experiment is based on the following three lines of evidence: (1) that free radicals and nitric oxide decrease RBC deformability^{5,14,15}; (2) our previous work showing that T/HS results in increased nitric oxide levels and that increased nitric oxide levels correlated with organ injury¹⁶; and (3) that iNOS inhibition reduces T/HS lymph-induced lung injury.¹⁷ In this experiment, the iNOS inhibitor, aminoguanidine hydrochloride powder (Sigma, St. Louis, MO) dissolved in saline or a control injection of saline, was injected intravenously over 1 minute (100 mg/kg) just before the start of the lymph infusion. This dose of aminoguanidine was based on our earlier studies.¹⁷ In the second group of studies, we tested the hypothesis that circulating white blood cells (WBC) contribute to T/HS lymph-induced RBC dysfunction by determining whether WBC-depleted whole blood is more resistant to in vitro T/HS lymph-induced RBC damage.

T/HS and Lymph Cannulation Models

To obtain mesenteric lymph for injection into the naïve rats, separate groups of rats underwent mesenteric lymph duct cannulation followed by T/HS or T/SS, as previously described.^{11,13} Briefly, the rats were anesthetized with intraperitoneal pentobarbital (50 mg/kg) following which the femoral artery and jugular veins were cannulated. After the lines were placed, laparotomy was performed, and the efferent mesenteric lymph duct draining

the majority of the small intestine, cecum, and the proximal one-half of the colon was identified and cannulated. The abdomen was closed in two layers. In the animals that were subjected to hemorrhagic shock, blood was withdrawn into a heparinized syringe via the jugular vein catheter until the mean arterial pressure was reduced to 30 mm Hg to 40 mm Hg and was maintained at this pressure for 90 minutes by reinfusing or withdrawing blood as needed. The shed blood was kept at 37°C. At the end of the shock period, the rats were resuscitated with their own shed blood. T/SS rats underwent the same procedure but no blood was withdrawn or infused. The animal's temperature was kept above 36.3°C with the use of heating pads or lamps as necessary. Mesenteric lymph was collected continuously into heparin-wetted sterile tuberculin syringes placed on ice for up to 6 hours in hourly aliquots. The lymph samples were centrifuged for 15 minutes at 400g to remove all cellular components and stored at -80°C until tested. A total of 50 rats were used for lymph collection.

Lymph Infusion Protocol

Male Sprague-Dawley rats underwent a laparotomy as well as femoral artery and internal jugular vein cannulation. Pooled T/HS or T/SS mesenteric lymph specimens collected during various T/HS or T/SS periods were then injected intravenously via the jugular vein catheter at rate of 1 mL/h for 3 hours. Continuous blood pressure monitoring was performed via femoral artery catheter throughout the experiment.

RBC Deformability

RBC deformability was determined by laser diffraction analysis using an ektacytometer (LORCA; RR Mechatronics, Hoorn, The Netherlands) as previously described.^{11,13} Briefly, shear stress was applied to RBC samples, and the degree of RBC deformability was measured. In this system, a laser beam is projected through the sample and the RBC diffraction pattern produced is analyzed by a microcomputer. RBC deformability was assessed by calculating the elongation index (EI) at shear stresses ranging from 0.3 Pa to 30 Pa. From the shear-stress elongation curve created above, the data were further analyzed using the Lineweaver-Burk analysis to determine the overall degree of deformability changes as we described earlier.¹⁸ The calculated maximal elongation (K_{EI}) is the level of shear stress (in Pascal) that is required for the erythrocytes to reach 50% of their maximal elongation. Thus, RBC deformability alterations were assessed either by the EI measured at low shear stress similar to that occurring during low-flow conditions in the microcirculation (0.3 Pa) or by calculating K_{EI} , which is an indicator of the overall status of RBC deformability. Because EI is a direct measure of RBC deformability, the smaller the number, the less deformable are the cells. In contrast, because the K_{EI} is the amount of stress needed to deform the RBC to half-maximal deformation, an increase in K_{EI} indicates a decrease in RBC deformability. All the EI and K_{EI} data are expressed as Pascal units (Pa).

Scanning Electron Microscopy

Scanning electron microscopic studies of RBC were performed by means of a JEM-100C electron microscope. The blood samples were fixed in phosphate-buffered (pH, 7.2–7.4) 2.5% glutaraldehyde for 1 hour, washed two times in 0.1 mol/L phosphate buffer (pH, 7.2–7.4), and mounted on adhesive glass slides. The glass slides were kept in a moist atmosphere for 1 hour, washed in phosphate buffer, postfixed in 1% osmium tetroxide for 1 hour, rinsed in distilled water, and dehydrated in graded ethanol (50%–70%–90%–100%). After critical point drying with liquid carbon dioxide in a vacuum apparatus and covering with a gold-palladium layer, the samples underwent scanning electron microscopic analysis.

In Vitro Lymph-RBC Assay

The in vitro effect of T/HS and T/SS lymph on RBC deformability was tested by incubating whole blood from naïve male rats with the various lymph samples at 37°C for 1 hour at a 5% v/v concentration after which RBC samples were removed and their K_{EI} was measured as described above. WBC depletion was accomplished by centrifuging whole blood samples and subsequently removing the plasma and the WBC-containing buffy coat layer. The RBC were then resuspended in phosphate-buffered saline.

Statistics

Analysis of variance with the post hoc Tukey-Kramer multiple comparison test or the unpaired Student's t test was used for comparisons between groups based on whether multiple versus two groups were compared. Results are expressed as mean $\pm$ SD. p values <0.05 were considered statistically significant.

RESULTS

All the rats receiving intravenous T/HS or T/SS lymph survived, and the injection of these lymph samples was not associated with hypotension (data not shown). In the rats receiving T/SS lymph collected over the 6-hour post-sham shock period, RBC deformability did not change from baseline values whether measured as EI at low shear stress similar to low-flow microcirculatory conditions or as K_{EI} , which is a broader indicator of RBC deformability status (Fig. 1). In contrast, RBC deformability was significantly impaired in the rats receiving T/HS lymph collected during the shock and 6-hour postshock period (Fig. 1). Changes in RBC deformability were present as early as 1 hour after beginning the T/HS lymph infusion (Fig. 1). Electron micrographs of the RBCs showed that T/HS lymph caused significant changes in RBC shape and morphology, which was not observed in the RBC collected from the rats injected with T/SS lymph (Fig. 2). This association between T/HS lymph-induced changes in RBC deformability and shape were similar to what was previously observed after actual T/HS.¹³

Because our previous studies documented that the in vitro biological activity of trauma hemorrhagic lymph varies over time and that organ injury¹⁹ as well as RBC rigidification¹³ was present by 3 hours after the end of the shock period, in a second set of experiments, we compared the biological activity of lymph samples collected during the first 3 hours post-T/HS or T/SS with samples collected from 4 hours to 6 hours after T/HS or T/SS. The object of this experiment was to determine whether the biological activity of T/HS lymph changed over time. Infusion of T/HS lymph collected 4 hours to 6 hours after T/HS did not alter either the EI or the K_{EI} (Fig. 3) and was not significantly different from the rats receiving T/SS lymph. In contrast, both the EI and the K_{EI} was significantly increased in rats receiving T/HS lymph collected during 1 hour to 3 hours after T/HS. These results suggest that the biological activity of T/HS lymph changes over time and that the factors leading to changes in RBC deformability are primarily leaving the gut within the first 3 hours after the end of the shock period.

As a further proof-of-principle study that abnormal RBC deformability after actual T/HS is largely mediated by factors contained in T/HS lymph, we injected rats subjected to actual T/HS + LDL with either T/HS or T/SS lymph. The T/HS + LDL rats injected with T/SS lymph had minimal and nonstatistically significant changes in RBC deformability, whether measured as EI or K_{EI} (Fig. 4). Thus, LDL not only prevented actual T/HS-induced RBC rigidification, but the administration of T/SS lymph was not sufficient to reverse the protective effect of LDL. In contrast, the injection of T/HS lymph in the LDL rats was sufficient to induce impaired RBC deformability whether measured as EI or K_{EI} (Fig. 4).

Because oxidants have been implicated in the pathogenesis of RBC rigidification^{5,15} and we have documented a role for increased iNOS-induced nitric oxide in organ injury after actual T/HS or T/HS lymph injection,¹⁶ we tested whether the selective iNOS inhibitor, aminoguanidine, administered immediately before lymph infusion would limit T/HS lymph-induced RBC rigidification. Although T/HS lymph collected during the first 3 hours post-T/HS decreased RBC deformability (increased K_{EI} and decreased EI), its effects on RBC deformability were not prevented by aminoguanidine (Fig. 5), suggesting that increased nitric oxide is not involved in the pathogenesis of T/HS lymph-induced RBC injury.

Because T/HS lymph activates neutrophils²⁰ and neutrophil-derived oxidants could be involved in the pathogenesis of RBC injury, we tested the role of WBC by comparing the in vitro effects of adding T/HS or T/SS lymph to naïve whole blood samples that had and had not been depleted of WBC. In this experiment, fresh whole blood collected from naïve rats was incubated with either T/HS or T/SS lymph (5% v/v) collected at 3 hours post-T/HS or T/SS. The K_{EI} value was significantly increased in the whole blood samples incubated with T/HS lymph (4.4 ± 0.7) as compared with T/SS lymph (2.9 ± 0.2 , $p < 0.01$). A similar observation was made for the EI values where the deformability of the whole blood samples incubated with T/HS lymph (0.048 ± 0.002) was significant less than that observed when whole blood was incubated with T/SS lymph (0.61 ± 0.002) ($p < 0.01$) (Fig. 6). WBC depletion totally abrogated the ability of T/HS lymph to alter RBC deformability, indicating that T/HS lymph-induced neutrophil activation is necessary for RBC injury (Fig. 6).

DISCUSSION

The role of the gut and gut-derived factors in the pathogenesis of systemic inflammation, acute respiratory distress syndrome, and MODS has evolved over time with new insights leading to the gut-lymph hypothesis of early post-trauma-shock acute organ failure.²¹ This hypothesis states that toxic products produced by the gut during shock states reach the systemic circulation via the mesenteric lymphatics rather than the portal blood and, once they reach the systemic circulation, they induce a number of proinflammatory and tissue injurious responses.²¹ The gut-lymph hypothesis is based on two principle observations. First, ligation of the mesenteric lymph duct prevents T/HS-induced acute lung injury,²² neutrophil activation,²⁰ bone marrow suppression,²³ endothelial cell activation and injury,^{24,25} and RBC injury.¹¹ Second, in vitro studies showed that T/HS lymph collected from rodents,²⁵ nonhuman primates,^{26,27} and pigs²⁸ were able to replicate the in vivo cellular changes caused by actual T/HS. Specifically, T/HS, but not T/SS, lymph causes endothelial cell injury, neutrophil activation, and bone marrow suppression. By showing that the injection of mesenteric lymph collected from rats subjected to trauma-hemorrhage into nonshocked rats is able to recreate the RBC changes in deformability and morphology observed after actual T/HS, this study provides data showing that T/HS lymph is sufficient to induce the RBC changes observed 3 hours after actual T/HS as well as provides additional support for the gut-lymph hypothesis of MODS. This notion that T/HS lymph is both necessary and sufficient to cause acute RBC dysfunction after actual trauma-hemorrhage is further supported by studies showing that T/HS lymph decreases RBC deformability in vitro. Of potential clinical significance is the fact that the RBC shape changes observed after T/HS lymph injection are similar to those observed in patients with severe trauma⁴ as well as after actual T/HS.¹³

A second potentially clinically important finding from this study is that lymph exiting the gut during the first 3 hours after the end of the shock period had the most biological activity. This observation, that the RBC-injurious biological activity in the T/HS mesenteric lymph is rapidly lost in the postshock period, is consistent with our studies on the effects of T/HS lymph injection on lung injury.¹⁷ These results would imply that therapeutic agents to limit

gut injury and the generation of toxic T/HS lymph or to neutralize the effects of T/HS lymph should be administered as early as possible during the immediate postshock period to be optimally effective. A corollary of this notion is that therapies begun during the later postshock period, hours after the initial insult, are less likely to be effective in limiting the proinflammatory and tissue-injurious effects of T/HS lymph.

Using the injection of T/HS lymph as a model to investigate the pathogenesis of T/HS-induced cellular and organ changes has some unique problems as well as important advantages. For example, one potential limitation of the T/HS lymph injection model, in contrast to other agents generally used to model and study the mechanisms of shock or sepsis, such as endotoxin or cytokines, is that lymph is a complex biological fluid containing many known and unknown factors. Being a complex biological fluid containing many factors could potentially complicate data interpretation because many diverse pathways and cell systems could be activated. However, because T/HS lymph is the fluid that is generated *in vivo*, we believe that the results obtained and the conclusions that are ultimately reached are more likely to have biological and clinical relevance.

Because oxygen and nitric oxide-based free radicals have been shown to be capable of decreasing RBC deformability,^{5,14,15} we investigated the role of iNOS-generated oxidants as well as WBC in the pathogenesis of T/HS lymph-induced RBC dysfunction. Although our previous studies showed that actual T/HS results in increased nitric oxide levels and the increased nitric oxide levels correlate with organ injury,^{16,17} in this model of T/HS lymph injection, iNOS inhibition did not offer protection against RBC damage, suggesting that other mechanisms and pathways might be involved. In contrast to iNOS inhibition, depletion of WBC was sufficient to abrogate the ability of T/HS lymph to cause abnormal RBC deformability, indicating that RBC injury by T/HS lymph requires WBC. This observation that WBC activation by T/HS lymph seems to be involved in RBC dysfunction is consistent with earlier studies showing that T/HS lymph from rats, nonhuman primates, and pigs is able to directly activate neutrophils *in vitro*.^{20,27,28} Furthermore, the observations that T/HS lymph acts directly on endothelial cells and neutrophils whereas the effects of T/HS lymph on RBC seem to be mediated through WBC suggest that the injury of different cell populations exposed to T/HS lymph may occur via different mechanisms. Because T/HS lymph has been shown to increase PMN respiratory burst activity,^{20,27} one possible mechanism linking WBC to T/HS lymph-induced RBC injury would be through the generation of PMN products, including oxidants and proteases. These or other T/HS-induced WBC products could then lead to RBC dysfunction. Studies to investigate this possibility are currently being initiated.

In summary, we have documented that T/HS lymph injected into naïve rats leads to decreased RBC deformability and shape changes similar to those of actual T/HS shock and that the biological activity of lymph collected over the first 3 hours after the end of the shock period is greater than lymph samples collected at later time points. These observations are of potential clinical significance, because decreased red blood cell deformability renders the RBC to less able to traverse the microcirculation, thereby resulting in impaired microcirculatory flow and potentiating organ dysfunction. Consequently, the development of gut-protective and/or lymph-neutralizing therapeutic strategies aimed at preventing T/HS-induced RBC damage and subsequent organ dysfunction administered during the early resuscitation period may be of clinical benefit.

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Figure 1.

T/HS lymph (1–6 hours) decreased RBC deformability as demonstrated by a decrease in RBC deformability (EI) as well as an increase in the shear stress required to maximally deform (K_{EI}) the RBC population. Data expressed as mean $\pm$ SD. T/HS lymph, n = 7; T/SS Lymph, n = 5. * p < 0.01 versus T/SS lymph.

Figure 2. Scanning electron micrograph of red blood cells at the end of 3 hours of lymph infusion. (A) T/SS lymph infusion showing normal erythrocyte morphology. (B) T/HS lymph infusion showing irregularly shaped RBCs (spherochinocytes, spherocytes; magnification $\times 2,000$).

Figure 3.

Both the EI and the K_{EI} of the rats injected with T/HS lymph collected over the first 3 hours after the end of the shock period were abnormal. However, RBC deformability (EI and K_{EI}) was not significantly changed in the rats injected with T/SS lymph or T/HS lymph collected by 4 hours to 6 hours post shock. Data expressed as mean $\pm$ SD. $n = 6$ per group. * $p < 0.05$ versus all other groups.

Figure 4.

Injection of T/HS but not T/SS lymph into rats subjected to actual T/HS + LDL results in decreased RBC EI and increased K_{EI} values. Data expressed as mean $\pm$ SD. $n = 6$ per group.

* $p < 0.01$ versus T/SS lymph-injected group.

Figure 5.

Pretreatment with the iNOS inhibitor aminoguanidine (AG) did not protect against T/HS lymph-induced RBC injury, whether measured as EI or K_{EI} . Data expressed as mean $\pm$ SD. $n = 6$ per group. * $p < 0.05$ versus T/SS lymph.

Figure 6.

Incubation of whole blood with 5% v/v T/HS but not T/SS lymph for 1 hour resulted in a decrease in RBC deformability manifested as decreased EI and increased K_{EI} . Depletion of WBC abrogated the ability of T/HS lymph to impair the ability of RBC to deform. Data expressed as mean $\pm$ SD. $n = 4$ per group. * $p < 0.01$ versus all other groups.