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Hemorrhagic Shock and Surgical Stress Alter Distribution of Labile Zinc within High and Low Molecular Weight Plasma Fractions

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Abstract

Zinc ions (Zn^{2+}) are essential for tissue repair following injury or stress. We hypothesize that during such stresses Zn^{2+} is redistributed to labile pools in plasma components. Here we tested this hypothesis utilizing a novel assay to monitor labile Zn^{2+} in plasma in hemorrhagic shock. Adult rats in the Shock (S) group underwent hemorrhage and resuscitation. Blood samples were drawn at baseline, 1 hr, 4 hrs and 24 hrs. The Surgical Control (SC) group was anesthetized and instrumented, but not bled. Albumin, total Zn^{2+} , and labile Zn^{2+} levels were assayed in plasma. Binding capacity for Zn^{2+} was assessed in high (HMW) and low (LMW) molecular weight pools. Significant decreases in total Zn^{2+} were observed by 24 hrs, in both S and SC groups. Albumin levels were significantly reduced in the S group at 1 hr and 4 hr but restored at 24 hrs; significant changes were not observed in other groups. In whole plasma, labile Zn^{2+} levels were stable initially in the S and SC groups, but declined at 24 hrs. In the HMW pool, marked and significant impairment of binding was noted throughout all time periods following the shock period in the S group. Such changes were observed in the SC group of less intensity and duration. These experiments suggest that Shock alters affinity of plasma proteins for Zn^{2+} , promoting delivery to peripheral tissues during periods of increased Zn^{2+} utilization.

Keywords

Zinc; Surgery; Shock; Heavy metal ion; Zinc assay; Zinc binding; Stress

Introduction

Zinc is an essential trace element for all living cells and is a cofactor for many critical cellular functions, including protein synthesis, energy metabolism, nucleic acid synthesis, gene transcription, and programmed cell death [1–3]. A wealth of investigative work has documented acute decreases of zinc content in serum or plasma following various forms of surgical illness and systemic stress, including sepsis and endotoxemia, trauma, and burns [4–15]. In *in vivo* experiments and clinical studies, it has been shown that a pre-existing deficiency or dietary deprivation of zinc worsens organ injury and prevents or delays recovery [16] [17–20]. In some [21–24], but not all circumstances [11, 25], the magnitude of

the decrease is thought to be predictive of further complications and delays in recovery. In some circumstances [9, 26, 27] but not others [13, 27–30] supplementation with zinc may accelerate recovery and mitigate complications.

This body of literature indicates that ongoing availability of Zn^{2+} is vital in the response of the organism to injury, infection and systemic stress. In plasma, Zn^{2+} is tightly bound to plasma proteins such as albumin and alpha-2 macroglobulin [31], constituting more than 99% of the total content in the plasma. The component that is loosely bound, i.e., free to interact with cells and tissues, is designated as “labile” Zn^{2+} . Unanswered questions, however, include when and by what mechanisms does Zn^{2+} in the circulation becomes available to peripheral tissues during injury and surgical stress?

Investigation of the mechanisms of zinc delivery has been hindered by the absence of a rapid method for monitoring “labile” or “free” concentrations of Zn^{2+} in the circulation. We [32] recently reported that ZnAF-2, a fluorescent dye that binds zinc selectively, can be utilized to detect nanomolar concentrations of free zinc in the plasma, at levels thought to be physiologically relevant [33]. A modification of this assay was then used to detect changes in Zn^{2+} binding within high molecular weight ($>10\text{kD}$) and low molecular weight ($<10\text{kD}$) components in a rat model of anesthesia alone or with a modest acute surgical stress. The present study was undertaken to evaluate alterations in circulating Zn^{2+} levels in a rat model of acute hemorrhagic shock. As noted above, considerable attention has been focused on disturbances in the total content of Zn^{2+} in the circulation during endotoxemia, sepsis and different forms of traumatic injury. To our knowledge, however, there have been no studies evaluating the influence of acute but atraumatic blood loss and re-infusion, a condition that permits assessment of the systemic response to brief hypotension and systemic hypoperfusion with minimal alteration of circulating blood components or tissues. Thus, our goals were: first, to determine the magnitude and reversibility of disturbance in levels of total and labile Zn^{2+} in the circulation during mild surgical stress plus shock, as compared to milder surgical stress; second, to determine whether alterations in total and labile Zn^{2+} are due, at least in part, to changes in binding characteristics of high and/or low molecular weight components of the plasma.

Materials and Methods

Animal Protocol

Male Sprague Dawley rats weighing 300–350g were used for all experiments. Rats were housed using standard animal care procedures (12:12 h light-dark cycle, food and water ad libitum). All animal care and experimental procedures used were consistent with National Institutes of Health Animal Care and Use Guidelines and were approved by the Institutional Animal Care and Use Committee at Harvard University. Rats were randomly assigned to Moderate Stress or Shock groups.

Rats in the Moderate Stress group were anesthetized, then underwent placement of catheters in the femoral artery and vein with 18 gauge tubing, as described previously [32]. After saline flush, the arterial catheter was used for invasive blood pressure measurement using a Spacelabs multichannel monitor (Spacelabs Healthcare, Issaquah, Washington) and the venous catheter was used for infusion and sampling. A 15 minute recovery and equilibration period was observed, following which samples were obtained at intervals corresponding to those chosen for the control group: baseline (*Time Point A*), 1 hr (*Time Point B*), and 5 hr (*Time Point C*). They were then sacrificed after Time Point C, or in some cases, awakened, re-anesthetized for sampling under anesthesia 24 hr after the initial induction (*Time Point D*), then euthanized using intravenous pentobarbital. Figure 1 summarizes the time course of the experiment.

Rats in the Shock Stress group were anesthetized and underwent cannulation of femoral vessels, as had been done for the Moderate Stress group. In this group, samples were obtained at baseline (**Time Point A**), just before induction of shock. Each animal in the group then underwent gradual withdrawal of blood until a mean arterial pressure (MAP) of 40 mmHg was reached. This blood pressure was maintained for 60 minutes by withdrawal or return of shed blood as needed. Shed blood was kept in heparinized syringes at room temperature with gentle agitation. After 60 minutes of shock, a blood sample was drawn (**Time Point B**), and then the rats were resuscitated using shed blood and normal saline as needed to maintain MAP >90 mmHg. Animals were observed for the next 4 hours, at which point samples were again obtained (**Time Point C**). They were then sacrificed after Time Point C, or in some cases, awakened, recovered and then, re-anesthetized for sampling under anesthesia 24 hr after the initial induction (**Time Point D**), then euthanized using intravenous pentobarbital. A schematic of the Shock Protocol time line is shown in Figure 1

Blood Processing

Blood specimens were collected in heparinized 1 mL aliquots and centrifuged at $10,000 \times G$ for 10 minutes. The plasma was removed and immediately assayed for total and labile zinc, total protein and albumin. Immediate processing was needed to eliminate confounding factors introduced by plasma storage. For plasma fractionation experiments, a 500-mL aliquot of plasma from each sample was filtered through a 10- kDa plasma filter (Amicon Ultra; Millipore, Billerica, Mass) at $14,000 \times G$ for 15 min at 4°C. The Filtrates (enriched for low molecular weight macromolecules) were retained and the Concentrates (enriched in high molecular weight macromolecules) were resuspended in zinc-free 18-MQ Milli-Q water. For experiments in zinc buffering, $8\mu M$ $ZnCl_2$ was added to the wells. All chemicals and reagents were obtained from Sigma Aldrich (St. Louis MO) unless otherwise noted.

Total Zinc assay

Plasma total zinc was determined using the Quantichrom Zinc Assay kit (BioAssay Systems, Hayward CA) according to the manufacturer's instructions. This zinc assay is a colorimetric assay based on zinc binding to a chromogen that reports at 425 nm. Solutions were plated in 96-well plates and absorbance was measured in a Synergy 2 microplate reader (Biotek Inc, Winooski, VT).

Monitoring Plasma levels of Labile Zinc

As described previously [32], labile Zn^{2+} in plasma was monitored in plasma samples ($50\mu L$) placed in 96 well plates, to which $1.5\mu L$ of a stock 1mM solution of ZnAF-2 (Axxora, San Diego, Calif) solution for a final concentration of 30nM. Samples were mixed by gentle shaking for 10 seconds and fluorescence readout was performed at 37°C (Excitation 485nm, Emission 528nm) on a Synergy 2 microplate reader (Biotek Inc, Winooski, VT).

Fractionation and measurements of binding capacity

In a subset of experiments, a 500- μL aliquot of plasma from each sample was filtered through a 10-kDa plasma filter (Amicon Ultra; Millipore, Billerica, Mass) at $14,000 \times G$ for 15 min at 4°C. The filtrates were retained and concentrates were resuspended in zinc-free 18-MQ Milli-Q water to achieve the original volume of 500 μL . For measurements of labile $ZnCl_2$, whole or fractionated samples of plasma (50 μL /sample) were plated in duplicate or triplicate on black 96-well plates (NUNC; Thermo Scientific, Roskilde, Denmark). After an initial baseline fluorescence reading (ex. 485, em. 528), ZnAF-2 (1.5 μL of a 1-mM solution in dimethyl sulfide) was added to each well for a final concentration of 30 nM. Samples were mixed by gentle shaking for 10 seconds, and fluorescence was read at baseline and

following addition of a 2 μ L aliquot of ZnCl₂, sufficient to increase the concentration by 8 μ M. Results were read on the microplate reader.

Albumin assay

Plasma albumin was assayed using the BCP Albumin Colorimetric Assay Kit (BioAssay Systems, Hayward, Calif) without modification, and results were read at 610 nm on the plate reader.

Statistics

Data were analyzed using SigmaStat statistical software. Continuous variables are expressed as mean plus or minus standard error. ANOVA was used for statistical comparison of all groups, and Student's t-test was used for specific groups for before and after comparison where appropriate.

Results

Shock-associated alterations in total [Zn²⁺] in the plasma

Shown in Figure 2A is a comparison of time dependent changes in levels of total Zn²⁺ in plasma samples from animals before and after mild surgical stress or mild surgical stress plus shock from blood loss. As noted in our prior study [32], these levels decrease following mild surgical stress and are significant ($p < 0.01$) at Time Point C and Time Point D. In contrast, the decrease is delayed for several hours in animals subjected to shock, not becoming significant until Time Point D. Shown in Figure 2B are summaries of the corresponding changes observed in levels of labile Zn²⁺ in plasma, as monitored semi-quantitatively by fluorescence of ZNAF-2. Of interest, levels of labile Zn²⁺ did not decrease during mild surgical stress, until the end of the experiment. In contrast, these levels were not only sustained, but appeared to increase after resuscitation during the third period of study, before declining again at the end of the experiment.

Shock-associated alterations in albumin levels in the plasma

Shown in Figure 3 are measurements of plasma albumin levels in subsets of animals ($n = 6$ to 7) responding to surgical stress alone or surgical stress in combination with bleeding and hypotension. As is well recognized [7, 9, 10, 14, 23], plasma albumin levels declined acutely, becoming significant at Time Point C even with mild surgical stress. This decrease was accelerated and enhanced following induction of shock. Importantly, at Time Point D, albumin levels in the circulating plasma had been restored, suggesting that depletion of total Zn²⁺ levels in the plasma is not attributable to the absence of carrying proteins such as albumin.

Shock-induced alterations in Zn²⁺ binding capacity of high and low molecular weight protein fractions in the plasma

In the last part of the study, we evaluated the possibility that decreased content of Zn²⁺ in the plasma is due to disturbances in the capacity of its binding proteins. We assessed binding capacity in plasma subjected to fractionation into high (HMW) and low-molecular weight (LMW) components, by adding known aliquots of ZnCl₂ to each fraction reconstituted to a standard volume of 500 μ L at pH 7.4. After equilibration of the sample, exogenous Zn²⁺ not taken up by plasma components would be free to interact with the reporter, ZNAF-2. Thus, increased fluorescence signal corresponds to decreased binding capacity of the fraction.

Summarized in Figure 4 is a comparison, at each time point, of binding capacity in the LMW fraction (< 10 kD) from animals subjected to surgical stress (**Panel 3A**) alone and those

subjected additionally to bleeding and hypotension (**Panel 3B**). Samples from both groups were highly responsive to addition of $8\mu\text{M Zn}^{2+}$, indicating relatively low binding capacity in the LMW fraction. In addition, there was relatively little variation in such responsiveness, over the 24 hr course of the experiment in the Surgical Stress group, indicating that, in the LMW fraction, binding capacity is not materially altered by stress, shock, resuscitation, or recovery.

In contrast, responsiveness to exogenous Zn^{2+} in the HMW component of the plasma was quite low under baseline conditions (Figure 5), consistent with significant baseline buffering of Zn^{2+} by plasma proteins such as albumin or macroglobulins [9, 31, 32]. In response to surgical stress (**Panel 5A**) or shock (**Panel 5B**), there were significant decreases in binding capacity, consistent with the hypothesis that binding by the HMW component is impaired even by a moderate surgical stress. Disturbances were of greater magnitude in animals subjected to shock. In addition, these disturbances had started to reverse, as early as 5 hrs in animals subjected to surgical stress alone, but even at 24 hrs were not yet reversing in animals subjected to bleeding and hypotension. In both groups, despite the restoration of pre-stress levels of albumin to the plasma at 24 hrs (Figure 3), binding capacity in the HMW fraction was persistently altered as late as 24 hrs. Again, at Time Point D, albumin levels in the circulating plasma had been restored, suggesting that depletion of total Zn^{2+} levels in the plasma is not attributable to the absence of carrying proteins such as albumin.

In our previous study, Anesthesia controls demonstrated no significant change from baseline at any time point. These findings indicate that even moderate surgical stress induces a response in circulating plasma proteins that permit them to more easily give up their pools of bound Zn^{2+} .

Discussion

In a rat model, we have previously observed that anesthesia without acute surgical stress does not alter total levels of Zn^{2+} , nor does it lead to subacute or persistent alterations in circulating levels of albumin or labile Zn^{2+} [32]. Decreases in total content of Zn^{2+} in the plasma are a well recognized consequence of systemic and severe surgical stress [4–15]. Such decreases are observed within an hour, suggesting that zinc has exited the circulation to peripheral sites. Such states of intense systemic injury are associated with redistribution of Zn^{2+} from the circulation to peripheral, parenchymal tissues including spleen, lung, kidney, areas of injury and especially the liver [10, 34]. Perhaps paradoxically, such states may induce a general depletion of zinc within the parenchyma of such organs [35], suggesting that redistribution and demand for Zn^{2+} is markedly amplified throughout the body.

To our knowledge, kinetics and redistribution of Zn^{2+} in the plasma have not been explored in the setting of hemorrhagic shock. The current set of studies utilized a novel method for reporting labile Zn^{2+} and the binding capacity for Zn^{2+} in different size fractions in the plasma. The specific goal of these studies was to establish whether these decreases would be attributable to changes in plasma content of carrying proteins, the most prominent of which is albumin. The alternative hypothesis was that hemorrhagic shock leads to such decreases by altering the binding capacity of such carrying proteins.

Our findings indicate that labile Zn^{2+} is released from the HMW fraction of plasma, a novel observation in this model of systemic stress and hemorrhagic shock. In addition we find that the capacity of serum components to bind Zn^{2+} remains diminished well beyond the interval of stress with or without associated bleeding, hypotension and reperfusion. The early stress-induced decrease in total Zn^{2+} content and increases in labile Zn^{2+} concentration within the

plasma are thus attributable to decreases in circulating levels of binding proteins such as albumin and to decreases in overall binding capacity within the HMW fraction of the plasma. The persistent depletion of both total and labile Zn^{2+} levels at Time Point D (24 hours) indicates that repletion of Zn^{2+} pools in the plasma does not occur rapidly, even with restoration of circulating albumin levels in the plasma. Moreover, shock and resuscitation lead to sustained impairment of binding capacity and this late time point. It is important to note that the animals used in this experiment are not zinc deficient or malnourished, and were allowed access to food and water throughout the 24 hour time course. Food intake was not recorded during the experiment, however without specific measures to deplete zinc, results in this short course should not be significantly influenced by dietary intake.

There may be some effect on zinc binding from blood storage and blood preservation. In our experimental model, shed blood was stored during the shock phase and reinfused during resuscitation, and no chemical preservatives were used, in order to eliminate the effect of blood preservation solutions. The current experiment was designed to minimize these effects by keeping the blood extracorporeal for the shortest time possible. Alterations in zinc levels, especially in the short time course of our experiments, can be ascribed to rapid physiological responses, rather than deprivation or storage.

The fraction of zinc unbound or loosely bound to plasma components, termed labile zinc, has been predicted to be in the nanomolar concentration range [33]. Heretofore there has been no direct assay to measure labile zinc, and thus there has been no method to determine zinc flux or zinc binding in the plasma. Recently we reported a labile zinc assay based on zinc binding to the fluorescent reporter dye, ZNAF-2[32]. The assay determines labile levels as fold changes from baseline plasma levels, is semiquantitative, and reports fluorescence intensities consistent with concentrations in the low nanomolar range. This assay is sensitive to small changes from baseline labile zinc measurements, and can be used to determine changes in labile zinc level resulting from changes in zinc binding to plasma proteins. A novel component of the current report is the finding that such an assay can be used to rapidly to discriminate between trends in labile concentrations and binding characteristics of Zn^{2+} in plasma under conditions of modest surgical stress or more intense systemic stress of hemorrhagic shock and resuscitation.

Binding characteristics of Zn^{2+} in plasma depend on physical characteristics, pH, competing divalent cations and the presence of specific proteins, including albumin and alpha-2 macroglobulin. Of these, the albumin pool has most capacity for binding of Zn^{2+} , accounting for 60% to 80% of the carrying capacity of the plasma [31, 36–39]. It seems likely that impairment of binding capacity in the HMW component of the plasma would be due, at least in part, to oxidant modification of albumin and other proteins. Binding of the divalent cation Co^{2+} in serum is impaired in ischemic and systemic inflammatory states, and is proposed for semiquantitative monitoring of modification of plasma albumin [40–43]. Since the Zn^{2+} binding sites in albumin include other divalent cations such as Cd^{2+} and the multivalent cation cisplatin [44, 45], it likely that the same site is modifiable by ischemic and oxidant stress in hemorrhagic shock. In conjunction with the findings of the current study, these considerations suggest that persistence of impaired Zn^{2+} binding state of the HMW plasma fraction. As zinc participates in a myriad of cellular functions, further study in the role of zinc in shock and resuscitation is likely to be fruitful. As the present study also indicates that alterations in zinc binding may reflect severity of shock or reperfusion injury; further study of zinc binding in conjunction with established markers of shock and in long term survival is warranted.

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Figure 1. Protocol Diagram

The phases of the protocol are shown as Time Points A, B, C, and D. Time Point A represents the Baseline, or initiation of the protocol for both Surgical Stress (■) and Surgical Stress with Shock (■). Time Point B represents the end of the Shock Phase, or one hour after the Baseline. Time Point C represents the end of the Resuscitation Phase, or five hours after Baseline. Time Point D represents the end of the protocol at 24 hours after Baseline.

Figure 2. Changes in Zn²⁺ dynamics in plasma before and after induction of Shock
 Panel 2A: levels of total Zn, measured by colorimetric assay (n=12). Panel 2B: levels of labile Zn²⁺, monitored by ZNAF-2 fluorescence (n=13). Results expressed as mean ± SEM; *p<0.05 compared to baseline (Time Point A), #p<0.05 compared to surgical stress group within the same period.

Figure 3. Changes in albumin levels in plasma before and after induction of Shock
Results expressed as mean $\pm$ SEM; * $p < 0.05$ compared to baseline (Time Point A), $n = 6$ animals in each group.

Figure 4. Changes in labile zinc signal with and without added ZnCl₂ in Low Molecular Weight (LMW) plasma fraction

Filled squares (■) summarize measurements of fluorescence without additional zinc and open squares (□) those obtained after addition of standard quantum of ZnCl₂ (8μM), the difference providing an assessment of buffering capacity. Results are expressed on a per volume basis, as mean ± SEM normalized to signal at baseline (Time Point A), n=6 animals in each group. *p<0.05 compared to corresponding untreated sample.

Figure 5. Changes in labile zinc signal with and without added ZnCl₂ in High Molecular Weight (HMW) plasma fraction

Filled squares (■) summarize measurements of fluorescence obtained prior to additional zinc, and open squares (□) those obtained after addition of standard quantum of ZnCl₂ (8μM), the difference providing an assessment of buffering capacity. Results are expressed on a per volume basis, as mean ± SEM normalized to signal at baseline (Time Point A), n=6 animals in each group. *p<0.05 compared to corresponding untreated sample