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AUTHORSHIP

Y.L. and H.B.A. designed this study, for which H.B.A. secured funding. Z.L. performed the experiments as well as collected and analyzed the data. X.D. performed the pathologic examination of ALI. B.L., T.Z., and W.C. provided experimental support. D.K.D. and P.Z. performed the article's revision. G.C.V. advised on the study, while Y.L., B.L., X.D., and H.B.A. contributed methodological advice. Z.L. and Y.L. wrote the article, which was critically revised by Y.L. and H.B.A. All authors read and approved the final article.

DISCUSSION

Dr. Peter Rhee (Tucson, Arizona): This study examines the synergistic effects of hypertonic saline and valproic acid in the setting of bowel perforation after hemorrhagic shock and resuscitation.

Synergy usually means that two or more things work together to have a combined effect that's greater than the simple sum of the individual effects. The data showed that there were some positive effects due to hypertonic saline and valproic acid, but when given together there was synergy and the survival figure tells the whole story. All animals without this combination of therapy die, but given a cocktail you live. It is quite impressive, indeed.

I have two comments and questions. Dr. Alam, you and I learned about histones from our laboratory neuroscientist Dr. Koustova about 15 years ago. She kept asking that I let her study histones in the setting of hemorrhagic shock. It took her a long time to convince me and you mainly because I couldn't understand what histones did and sadly, I still don't. We have published on this many times and I still don't understand what histones do so I will ask you a difficult question.

By which mechanism does it work? I know how hypertonic saline works. It works by resuscitating without all that toxic water and by making the pickled neutrophils not work so well for a temporary period of time.

But could you explain to me how histones work as if I was a 12-year old or even, better yet, as if I was a trauma surgeon post-call. Is it an immune modulator? Healer? Anti-inflammatory agent? Pressor agent? Endothelial enhancer? Antimicrobial? Immune enhancer? I just don't know. How does it save lives? Explain it to me slowly and in English.

I know, Dr. Alam, that you have received FDA approval for use in clinical trials. Could you please provide for us a preview of what is to come? In what setting do you think the clinical trials will be done? Will it be hemorrhagic shock, septic shock, or both? Your guess, please. Thank you.

Dr. Hasan B. Alam (Boston, Massachusetts): Well, thank you very much. It is actually a pleasure to have Dr. Rhee to comment on the paper.

I started as post-doctoral fellow in his lab many years ago, and we published quite a few papers together about hypertonic saline that time. And now 12 years later I am presenting a paper on hypertonic saline, which he is critiquing, so it seems like everything has come around full circle.

Let me talk about the mechanism. The well-described, conventional mechanism for how histone deacetylase inhibitors work is by changing the function of the histone proteins which are nuclear proteins. It is a fairly sophisticated system and, depending on the activation/deactivation of the histone proteins and the exact activation patterns, you can precisely control the transcription of genes minute to minute. So the conventional wisdom is that these drugs can be used to change the acetylation of the protein at many sites. This creates almost an "acetylation code," almost like a combination lock. However, in the setting of rapidly fatal injuries, the survival benefit provided by these drugs is unlikely to be due to this well-defined pathway, as it takes time to transcribe new RNA and make new protein.

What we've discovered over the last 10 years or so is something happens much more quickly than that, within minutes. In addition to the changes in the nuclear histones, HDAC inhibitors also add acetyl groups to pre-formed cytoplasmic proteins and immediately alter their function. If these proteins are involved in cell survival pathways, like PI3/AKT pathway, then activation to the pathway provides an immediate benefit. In addition, these drugs blunt the inflammatory response and activate the endothelial cells. In short, we should think about acetylation the same way we understand phosphorylation. These are broad post-translational protein modifications that modulate numerous cellular functions. Importantly, acetylation is completely reversible and almost instantaneous in its onset. Over the last 10 years, our team has identified a number of these pathways, and many more remain undiscovered. We have written a number of review papers trying to summarize these findings. I should mention that the response to these drugs is organ-specific, cell type-specific, and insult-specific. So what happens in septic shock is not exactly the same as what happens in hemorrhagic shock but there are a lot of similarities. Similarly, cancer cells, injured cells, and normal cells respond differently.

The clinical trial question—we have received approval by the FDA to proceed with a phase I trial in hemorrhagic shock patients at this time. We are not looking at septic shock at this time, and traumatic brain injury patients will be excluded to keep the study population focused. Our approval is for a Phase I dose escalation trial so we can start with a 15 milligram per kilogram dose, which is the lowest dose of valproic acid, and then go up to about 180 milligram per kilogram. And then the two highest doses that are well tolerated in healthy human volunteers will be tested in trauma patients.

We do have some very compelling translational data that HDAC inhibitors decrease the sign of brain lesion following TBI, so this might be another study in the future. Sepsis we have left alone just because it's too complicated to tackle at this stage.

Thank you very much for the kind comments, and once again I thank the AAST for the privilege of presenting the paper.

Synergistic effects of hypertonic saline and valproic acid in a lethal rat two-hit model

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Abstract

BACKGROUND—Hemorrhagic shock (HS) followed by an infection (“second hit”) can lead to severe systemic inflammatory response and multiple-organ failure. Studies have shown that resuscitation with hypertonic saline (HTS) can blunt the inflammatory response. We demonstrated that large doses of valproic acid (VPA, 300 mg/kg), a histone deacetylase inhibitor, improves survival in a rodent two-hit model (HS followed by cecal ligation and puncture [CLP]). In the present study, we examined whether combination of HTS with VPA would allow us to achieve survival advantage at a lower dose of VPA (200 mg/kg).

METHODS—Male Sprague-Dawley rats were subjected to HS (50% blood loss) and randomized into five groups (n = 7–8 per group) as follows: (1) isotonic sodium chloride solution (ISCS), (2) 7.5% saline, (3) VPA, (4) ISCS + VPA, and (5) HTS + VPA. After 24 hours, they underwent CLP, followed by the same doses of ISCS, HTS, and/or VPA and were monitored for 10 days. In a parallel experiment, blood, peritoneal irrigation fluid and lung homogenate were subjected to enzyme-linked immunosorbent assay 3 hours and 24 hours after CLP to measure myeloperoxidase activity and proinflammatory cytokines tumor necrosis factor α and interleukin 1β levels. Western blotting was performed to investigate the expression of pentraxin 3 protein in the lung homogenate at 24 hours after CLP. Hematoxylin and eosin staining of lungs at the 24 hours were performed to quantify the degree of acute lung injury.

RESULTS—HTS + VPA treatment significantly improved survival (87.5%), compared with the other groups (14.3%; $p < 0.05$), while attenuating peritoneal myeloperoxidase levels and proinflammatory cytokine tumor necrosis factor α and interleukin 1β levels in the serum, peritoneal cavity, and lung. The degree of acute lung injury and expression of pentraxin 3 in the lung were significantly reduced in the HTS + VPA group.

CONCLUSION—This is the first study to show that VPA and HTS can work synergistically to attenuate inflammation and improve survival in a lethal two-hit model.

Keywords

Hemorrhagic shock; sepsis; valproic acid; hypertonic saline; rats

Hemorrhagic shock (HS) is the foremost etiology of early deaths in trauma patients, whereas infections remain the leading cause of late morbidity and mortality. Patients who survive the initial hemorrhage often pass through periods of systemic inflammatory response syndrome (SIRS) and immune dysfunction where they die of infectious complications.¹ This combined (“two-hit”) insult creates an exaggerated injury pattern that can lead to multiple-organ failure.² Goal-directed resuscitation remains important in the management of these patients, but what exactly is the most appropriate early resuscitation strategy remains controversial.^{3,4} Although fluids are capable of restoring intravascular volume, they have been implicated in worsening of the postshock inflammatory response.^{4–6} Compared with conventional isotonic crystalloids (such as isotonic sodium chloride solution [ISCS]), hypertonic saline (HTS) has several theoretical advantages, including more effective expansion of intravascular volume and a blunting of the inflammatory response.^{3–5,7,8} However, the largest and the most recent randomized clinical trial has failed to show an overall survival advantage in the patients that were resuscitated with hypertonic fluid.⁹ In fact, this trial was stopped ahead of schedule owing to an increase in early deaths in the hypertonic group.¹⁰

The pathophysiology of sepsis is classically attributed to hyperinflammatory responses that mediate the excessive production of cytokines, which can lead to cellular injury and organ dysfunction.¹¹ We now know that trauma, even in the absence of sepsis, can cause inflammation through multiple mechanisms.^{12,13} This sterile inflammation/SIRS is often clinically indistinguishable from its infectious counterpart (sepsis and septic shock).¹⁴ Traditional treatment strategies have largely been supportive, but recent research has focused on developing more specific treatments that normalize the underlying affected pathways.¹⁵ We have shown that shock alters the acetylation status of the cell, which in turn impairs gene transcription and the function of multiple cell survival pathways.¹⁶ Acetylation is a rapid and reversible posttranslational protein modification that is controlled by two opposing enzyme families, histone deacetylases (HDACs) and histone acetyl transferases. The balance of acetylation in a cell can be therapeutically altered by a class of drugs called HDAC inhibitors (HDACIs). HDACs remove acetyl groups from lysine residues of the target proteins, and treatment with their inhibitors (HDACIs) can tilt the balance in favor of histone acetyl transferases. This results in increased acetylation, which has been shown to regulate numerous biologic functions, including inflammation and immune responses.^{15,16} At the molecular level, it has been reported that both hemorrhage and sepsis lead to an imbalance in protein acetylation and that treatment with HDACIs can restore this balance and improve outcomes.^{17,18}

Recently, our team has demonstrated that treatment with a large dose (300 mg/kg) of valproic acid (VPA), which works as an HDACI, can significantly decrease inflammation and improve survival in a lethal two-hit rodent model of hemorrhagic and septic shock (data presented at the 92nd annual meeting of the New England Surgical Society, September 2011). Although effective, this high dose of VPA is associated with increased chances of

toxicity, which has prompted us to explore various strategies that could lower the effective dose of the drug. One such approach is to combine VPA with another anti-inflammatory agent, such as HTS, to see if the prosurvival benefits could be achieved at a lower dose. In the present study, we tested the hypothesis that VPA and HTS would work synergistically to attenuate inflammation and improve survival in a rodent two-hit model: HS followed by septic shock from cecal ligation and puncture (CLP).

MATERIALS AND METHODS

All the research was conducted in compliance with the Animal Welfare Act and other Federal statutes and regulations related to animal experimentation. The study adhered to the principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, and was approved by the Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (250–300 g) were housed in their cages, allowed food and water, and kept at room temperature before the experiment.

Animal Model and Experimental Design

Hemorrhage was induced as previously described by Fukudome et al.¹⁹ Briefly, rats were anesthetized with 0.7% to 1.2% isoflurane (Abbott Laboratories, North Chicago, IL) mixed with air, which was administered via a nose cone scavenging system and allowed to breathe spontaneously, using a veterinary multichannel anesthesia delivery system and vaporizer (Kent Scientific Corporation, Torrington, CT). At the end of experiment or the time for blood collection and tissue harvest, the animals were killed with pentobarbital (200 mg/kg, intraperitoneally administered).

The femoral artery and vein were cannulated with polyethylene 50 catheters (Clay Adams, Sparks, MD). The venous cannula was used for hemorrhage and fluid resuscitation, while the arterial catheter was connected to the Ponemah Physiology Platform (Gould Instrument Systems, Valley View, OH) for continuous hemodynamic monitoring. To induce HS, baseline arterial blood samples were obtained, and then additional blood was withdrawn to a target of 50% of the estimated total blood volume (total blood volume [mL] = weight [g] × 0.06 [mL/g] + 0.77) over 20 minutes (40% over 10 minutes, then 10% over 10 minutes). After 30 minutes of unresuscitated shock, animals were randomly assigned to two different experiments.

The first experiment was aimed at assessing the survival effect. All animals were subjected to HS (50% blood loss) and randomized into five groups as follows: (1) ISCS (32 mL/kg, intravenously administered, n = 7), (2) 7.5% HTS (4 mL/kg, intravenously administered, n = 7), (3) VPA (200 mg/kg, intraperitoneally administered, n = 7), (4) ISCS + VPA (ISCS 32 mL/kg, intravenously administered, VPA 200 mg/kg, intraperitoneally administered, n = 8), and (5) HTS + VPA (HTS 4 mL/kg, intravenously administered, VPA 200 mg/kg, intraperitoneally administered, n = 8). The volume of VPA in this study was 200 µL. After 1 hour of observation, postshock arterial blood samples were drawn, catheters were removed, vessels were ligated, skin was closed, and animals were recovered from anesthesia and returned to their cages. Twenty-four hours later, these rats were reanesthetized with isoflurane, and polymicrobial sepsis was induced by CLP as described by Rittirsch et al.²⁰ A

second (same) dose of ISCS, HTS, and/or VPA was given via subcutaneous injection or intraperitoneal administration, and animals were woken from anesthesia and transferred to their cages for observation. The animals were monitored for 10 days to document survival.

In the second experiment, we mainly examined some proinflammatory cytokines and proteins. A different set of rats were subjected to the two-hit as the same as the first experiment, and killed 3 hours ($n = 3$ per group) and 24 hours ($n = 3$ per group) after CLP. At the time of sacrifice, abdominal cavity was opened and irrigated with 2-mL ISCS, and this fluid was collected for analysis. Cardiac puncture was performed to collect blood for the measurement of circulating cytokines. Organs were harvested to analyze protein levels/activity. A rapid tracheal infusion method for routine lung fixation was used to preserve the left lung for histologic evaluation as previously described.²¹ As a control, tissue samples were also obtained from sham animals that were anesthetized and instrumented, without hemorrhage or CLP.

Myeloperoxidase Assay

Myeloperoxidase (MPO) levels in peritoneal irrigation fluid were determined using the Myeloperoxidase Assay Kit (Cell Sciences Inc., Canton, MA) according to the manufacturer's instructions. The peritoneal irrigation fluid samples were centrifuged at 3,000 G at 4°C for 10 minutes, and supernatants were analyzed for MPO levels.

Western Blot Analysis

The expression of pentraxin 3 (PTX3) protein (biomarker of lung inflammation) in the lung homogenate at 24 hours after CLP was evaluated by Western blot as previously described.²²

Cytokine Measurements

Tumor necrosis factor α (TNF- α) and interleukin 1 β (IL-1 β) levels in serum, peritoneal irrigation fluid and lung homogenate (lung whole cell lysate) were determined with commercially available enzyme-linked immunosorbent assay kits according to the manufacturer's instructions (R&D Systems Inc., Minneapolis, MN). The concentrations of cytokines were measured by optical densitometry at 450 nm in a SpectramaxPlus 384 microplate reader (Molecular Devices, Sunnyvale, CA). All of the analyses were performed in triplicate.

Acute Lung Injury Scoring

The acute lung injury (ALI) scoring was performed by a board-certified pathologist blinded to the treatment assignment of the samples. The method for objective quantification of the injury has been described previously.²³ In each animal, three separate lung sections were graded to generate the mean score.

Statistical Analysis

Survival rates were compared by Kaplan-Meier log-rank test in the program Prism (Graphpad Software, San Diego, CA). All continuous data were summarized as means and SD. Differences between three or more groups were assessed using one-way analysis of variance followed by Bonferroni post hoc testing for multiple comparisons. Student's t test

was used to compare the differences between two groups. A p value of less than 0.05 was considered to be statistically significant.

RESULTS

HTS + VPA Improves Survival in a Rat Two-Hit Model

The sublethal HS (50% estimated blood volume) was selected to ensure that most rats would survive after the first hit. We found that animals had an average baseline mean arterial pressure around 105 mm Hg, which dropped to approximately 36 mm Hg to 40 mm Hg after 50% blood loss and came back to approximately 50 mm Hg 30 minutes after HS. Treatment of rats with ISCS or HTS rapidly increased mean arterial pressure to near normal levels, but 1 hour later, it drifted down to approximately 90 mm Hg.

To assess whether HTS + VPA could improve survival in this two-hit model, we compared the survival rates among different groups. As shown in Figure 1, more than 85.7% of rats from ISCS (53, 66, 70, 87, 80, 91, and 120 hours), HTS (87, 112, 118, 122, 128, 137, and 160 hours), VPA (18, 22, 24, 26, 47, 63, and 240 hours), ISCS + VPA (21, 24, 24, 36, 39, 41, 154, and 240 hours) groups died within 7 days, with most of the deaths occurring between 24 hours and 48 hours. The rats treated with HTS had a longer survival time compared with the ISCS animals, but they had identical long-term survival rates. However, HTS + VPA-treated animals (23, 240, 240, 240, 240, 240, 240, and 240 hours) displayed a significantly higher ($p < 0.05$) survival rate (87.5% 10-day survival; Fig. 1).

HTS + VPA Decreases CLP-Induced MPO Levels

MPO level is a marker of neutrophil-mediated oxidative damage. To examine whether HS + VPA treatment has any effect on local inflammation/oxidation, we measured MPO levels in peritoneal irrigate collected 3 hours after CLP. The activity of MPO was not significantly different among ISCS, HTS, VPA, ISCS + VPA groups. However, HTS + VPA treatment significantly ($p < 0.05$) attenuated the CLP-induced oxidative damage (Fig. 2).

HTS + VPA Suppresses Production of Proinflammatory Cytokines

TNF- α and IL-1 β are cytokines that are secreted by the immune cells during inflammation. To determine whether HTS + VPA affects the production of these cytokines, we collected peritoneal fluid, blood and lung homogenate at 3 and 24 hours after CLP and measured the concentration of these cytokines. When compared with the respective cytokine levels after ISCS treatment, HTS + VPA treatment significantly decreased the production of TNF- α and IL-1 β at both time points ($p < 0.05$). The cytokine levels were statistically similar between the ISCS and ISCS + VPA treatments, and the cytokine levels following HTS treatment were lower than those after ISCS treatment. The TNF- α levels after the HTS + VPA treatment was significantly lower than those after all other treatments; similarly, pulmonary IL-1 β levels after HTS + VPA treatment were significantly lower than after other treatments ($p < 0.05$) (Tables 1 and 2).

HTS + VPA Attenuates ALI

To determine whether HTS + VPA affects ALI in this model, lung samples were harvested 24 hours after CLP and evaluated under light microscopy. The lungs of the ISCS group and the ISCS + VPA group were significantly damaged, with interstitial edema, hemorrhage, thickening of the alveolar wall, and inflammatory cell infiltration. These histologic changes were found to be attenuated in the HTS + VPA group (Fig. 3). Quantitative scoring, performed by a pathologist blinded to the treatment, confirmed that HTS + VPA treatment significantly reduced the degree of ALI ($p < 0.05$).

HTS + VPA Decreases PTX3 Protein in the Lung

PTX3 is an acute phase protein with important regulatory functions in inflammation, which is elevated in patients with adult respiratory distress syndrome^{24,25} and is considered to be a biomarker of ALI.^{25,26} At 24 hours, the levels of pulmonary PTX3 protein were significantly reduced in HTS + VPA group ($p < 0.05$; Fig. 4).

DISCUSSION

In the present study, we have demonstrated for the first time that coadministration of HTS and VPA significantly improves survival in a rodent two-hit model (hemorrhage followed by CLP-induced septic shock). Further analysis showed that this synergistic treatment inhibits peritoneal oxidative activity, attenuates local and systemic levels of inflammatory cytokines (TNF- α and IL-1 β), reduces acute phase reaction in distant organs (pulmonary PTX3), and prevents the early development of ALI.

Trauma and hemorrhage can cause SIRS, which can be especially lethal if it is further complicated by a second injury such as sepsis. The concept of two-hit insult has gained popularity in recent years to explain the development of multiple organ dysfunction syndrome in trauma patients, where the initial injury acts as the first hit, which primes the immune system while rendering the host susceptible to subsequent infections. In our study, we used a severe, but nonlethal, blood loss with no resuscitation as the first hit, followed by CLP to induce polymicrobial sepsis. CLP is the most widely used model of experimental sepsis in rodents and is often considered as the criterion standard.^{20,27,28} Fluid resuscitation plays an important role in the management of SIRS as well as sepsis, with the end point being the restoration of intravascular volume and normalization of tissue perfusion.³ However, the rationale behind early fluid resuscitation in trauma patients has been increasingly challenged in recent years.^{3-5,29,30} In addition to causing hemodilution, anemia, coagulopathy, acidosis, hypothermia, interstitial edema, and increased bleeding, isotonic fluids have also been shown to have proinflammatory properties.^{3-5,31-33} In contrast to isotonic fluids, HTS attenuates the inflammatory response^{32,34,35} and has been shown to ameliorate hepatic injury, neutrophil activation, endothelial dysfunction, and lung injury after HS and sepsis.³⁻⁵ Clinical studies, however, have shown that while the inflammatory response in trauma patients is blunted after HTS infusion,⁸ this does not translate into an improvement in survival.⁹ In the present study, we also discovered that HTS treatment decreased inflammatory markers without actually improving the survival rate. Interestingly,

combining HTS with a low-dose HDACI (VPA) changed the picture completely, with a significant and dramatic improvement in long-term survival.

In HS models, we have recently demonstrated that treatment with higher doses (300 mg/kg) of VPA normalizes cellular acetylation, modulates various critical pathways (e.g., Akt survival pathway members, heat shock proteins, early/immediate kinases), and improves survival.^{36–38} These results suggest that the anti-inflammatory effects of this HDACI are mediated through acetylation of various proteins, which alters their functions. However, lower doses of VPA may work through different mechanisms. We have previously shown that VPA in lower doses (200–250 mg/kg) when given with ISCS resuscitation do not increase protein acetylation.¹⁷ Similarly, in the current experiment, a dose of 200 mg/kg failed to improve survival and/or attenuate inflammation when used alone (without HTS). This proof-of-concept study was not designed to elucidate the specific mechanisms that are altered by the combined treatment (VPA + HTS). However, in follow-up studies, we are exploring a number of possible mechanisms. For example, we know that in addition to increasing acetylation, VPA also exerts its anti-inflammatory by decreasing oxidative damage. We have recently reported that VPA decreases 8-isoprostane expression in pulmonary tissues and attenuates inflammation in a rodent model of intestinal ischemia reperfusion.²¹ The 8-isoprostane is a prostaglandin-like compound formed in vivo from the free radical-catalyzed peroxidation of arachidonate independent of the cyclooxygenase. In addition to being an important marker of oxidative stress, 8-isoprostane also possesses potent biologic activities and likely mediates many aspects of the oxidative injury. Thus, inhibition of pulmonary 8-isoprostane may be another key mechanism by which VPA treatment prevents ALI.

The magnitude of proinflammatory and anti-inflammatory cytokine surge influences the degree and duration of the inflammatory response. Not surprisingly, therapeutic modulation of the inflammatory response to prevent organ dysfunction and improve outcome is a promising area of investigation.³⁹ However, owing to the redundancy in the inflammatory response with numerous overlapping pathways, any drug that blocks/modulates one specific cytokine is unlikely to change the outcome. A more broad-based approach that modulates multiple cellular pathways simultaneously (preferably upstream) is more likely to create a “prosurvival phenotype.” Recently, HDACIs have emerged as one such pharmacologic approach. These agents have potent prosurvival and anti-inflammatory properties, their effects are quick in onset, the cellular changes are reversible, and most importantly, many of these drugs are already approved for clinical use (nontrauma indication).¹⁶ Preclinical data so far are extremely promising. We have shown that treatment with HDACI improves survival in small and large animal models of HS, suppresses inflammation, activates prosurvival pathways, attenuates tissue damage, prevents distant organ injury, and prevents death in models of septic shock.^{16,18,19} Food and Drug Administration has recently approved our Investigational New Drug Application (IND #113010) application for a Phase I double blinded, placebo-controlled study to evaluate the safety and tolerability of VPA in healthy human volunteers and in trauma patients. However, as the prosurvival dose of VPA in preclinical studies is approximately six to eight times the clinically approved dose, we are also pursuing parallel studies to discover ways to reduce the effective dose. One such approach is to explore drug combinations that may have synergistic effects.

We have previously reported that the protective effects of HDACI occur through modulation of immune response. In that study, we established a two-hit model (initial hemorrhage in vivo followed by exposure to lipopolysaccharide in vitro) in rodents and humans and measured both TNF- α and IL-1 β levels.⁴⁰ To be consistent (and be able to compare) with the previous data, we measure the levels of TNF- α and IL-1 β in blood, peritoneal fluid, and lung tissue in the current experiment.

Like any other study, this study also has some limitations that must be acknowledged. While the sample size was statistically adequate, it may not have been large enough to show smaller intergroup differences. To minimize the number of blood draws during the observation period, we used survival as a primary end point without monitoring markers of organ function. Similarly, to reduce the total number of animals, we obtained tissue and blood samples only at two time points. Because this was a proof-of-concept study, we only tested limited combinations (VPA doses and concentrations/doses of HTS). It is entirely possible that additional adjustments in the total dose of HTS would further reduce the effective dose of VPA. Finally, for logistical reasons, we could not measure many other cytokines and regulatory pathways that may be involved. We are currently exploring a number of these pathways using chromatin immunoprecipitation, genomic DNA microarray, and high-throughput proteomic techniques.

In summary, our study presents the first direct evidence that combination of HTS with VPA has a synergistic effect in a lethal two-hit model. We have shown that addition of HTS allows us to use a much smaller (one-third lower) dose of VPA, to effectively reduce MPO levels, inhibit proinflammatory cytokines TNF- α and IL-1 β , attenuate ALI, and improve survival in a rodent model of HS followed by CLP.

Acknowledgments

DISCLOSURE

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Figure 1.

Effect of HTS + VPA on survival in a rat two-hit model. Male Sprague-Dawley rats (250–300 g) were subjected to sublethal HS and then randomized into five groups ($n = 7-8$ per group) as follows: (1) ISCS (32 mL/kg, intravenously administered), (2) HTS (7.5% HTS 4 mL/kg, intravenously administered), (3) VPA (200 mg/kg, intraperitoneally administered), (4) ISCS + VPA (ISCS 32 mL/kg, intravenously administered; VPA 200 mg/kg, intraperitoneally administered intraperitoneally administered), and (5) HTS + VPA (7.5% HTS 4 mL/kg, intravenously administered; VPA 200 mg/kg, intraperitoneally administered). The animals from different groups were treated with different agents as described in the Materials and Methods section. After 24 hours, all rats received CLP, followed immediately by injection of the same dose of the agents as the first time. Survival was monitored for 10 days. The Kaplan-Meier curve illustrates survival over the 10-day observation period. Treatment with HS + VPA significantly improved survival compared with other groups (*87.5% vs. 14.3% survival; $p < 0.05$).

Figure 2.

Effect of HTS + VPA treatment on MPO levels in peritoneal irrigation fluid. MPO levels in peritoneal irrigation fluid were analyzed by enzyme-linked immunosorbent assay (described in the Materials and Methods section) 3 hours after CLP. All analyses were performed in triplicate. Values represent the means (SD) ($n = 3$ per group). HTS treatment can decrease MPO levels compared with ISCS, and HTS + VPA treatment can dramatically attenuate MPO levels. *Asterisk* indicates that a value significantly differs from the ISCS group ($p < 0.05$).

Figure 3.

HTS + VPA treatment attenuates ALI. Representative slides of lung sections from animal groups of the ISCS, HTS, VPA, ISCS + VPA, HTS + VPA treatment ($n = 3$ per group). The pathologic changes were examined using hematoxylin and eosin staining and light microscopy (original magnification $\times 40$) in the lung tissues 24 hours after CLP. HTS + VPA group had less alveolar congestion, neutrophil infiltration, and alveolar wall thickness. ALI was scored as described in the Materials and Methods section and expressed as mean (SD). Asterisk indicates that a value significantly differs from ISCS group ($p < 0.05$).

Figure 4.

HTS + VPA treatment decreases expression of PTX3 protein in the lung tissue. Whole-tissue lysate of rat lungs from sham, ISCS, HTS, VPA, ISCS + VPA, and HTS + VPA groups at 24 hours after CLP insult was subjected to Western blotting with anti-PTX3 and anti-actin antibodies. Specific bands were quantified by densitometry and expressed as mean (SD) ($n = 3$ per group). *Asterisk* indicates that a value significantly differs from the other four groups ($p < 0.05$).

TABLE 1HTS + VPA Treatment Decreases Levels of TNF- α in Plasma, Peritoneal Irrigation Fluid, and Lung

Group	Plasma		Peritoneal Fluid		Lung	
	3 h	24 h	3 h	24 h	3 h	24 h
ISCS	46.9 (2.4)	8.3 (1.2)	177.5 (15.9)	61.8 (4.0)	13.2 (1.3)	6.6 (0.4)
HTS	38.5 (2.1)	6.1 (0.4)	99.1 (1.5) [*]	50.0 (0.2)	7.8 (0.7) [*]	6.4 (0.1)
VPA	18.7 (11.1) [*]	12.3 (1.6)	137.9 (6.0)	42.3 (3.0)	4.2 (0.3) [*]	7.2 (0.3)
ISCS + VPA	37.2 (6.0)	11.2 (0.2)	114.9 (53.2)	57.9 (3.2)	8.5 (0.7)	8.0 (0.6)
HTS + VPA	22.0 (3.8) [*]	3.7 (0.7) [†]	64.3 (9.1) [*]	11.9 (7.9) [†]	4.9 (0.4) [*]	3.9 (0.6) [†]

^{*} $p < 0.05$ compared with ISCS group.

[†] $p < 0.05$ compared with other four groups.

TABLE 2HTS + VPA Treatment Decreases Levels of IL-1 β in Plasma, Peritoneal Irrigation Fluid, and Lung

Group	Plasma		Peritoneal Fluid		Lung	
	3h	24 h	3 h	24 h	3 h	24 h
ISCS	73.5 (3.5)	58.3 (5.824)	3,739.0 (70.7)	766.4 (20.9)	2,139.9 (11.5)	754.1 (19.0)
HTS	62.3 (4.9)	46.8 (7.1)	3,724.3 (102.9)	717.9 (12.2)	1,527.3 (74.4)	718.0 (2.0)
VPA	72.7 (0.7)	48.0 (5.4)	2,412.5 (26.5) *	781.6 (32.9)	1,233.1 (20.7) *	605.3 (31.6)
ISCS + VPA	68.1 (4.7)	54.5 (2.1)	3,185.2 (246.5)	830.1 (55.2)	1,933.9 (54.3)	1,093.7 (25.6)
HTS + VPA	41.9 (0.4) †	13.6 (3.2) †	2,720.7 (21.0) *	657.8 (12.7) †	1,275.8 (18.9) *	414.5 (11.5) †

* $p < 0.05$ compared with ISCS group.

† $p < 0.05$ compared with other four groups.