Sepsis: Pathogenesis, Biomarkers, and Treatment

Guest Editors: Baoli Cheng, Andreas H. Hoeft, Malte Book, Qiang Shu, and Stephen M. Pastores
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Sepsis is an infection-initiated systemic inflammatory syndrome with an estimated incidence of 18 million cases annually worldwide. Despite advances in intensive care and supportive technology, the mortality rate of sepsis still ranges from 15% to 80%, reminding scientists and clinicians that it remains to be a major clinical challenge. The key to winning the “campaign” to combat sepsis is improved understanding of the epidemiology, pathogenesis, and biomarkers of sepsis and discovery of novel therapies. The present special issue shows several encouraging results and provides comprehensive reviews of the latest advances in this field.

The effector cells from the innate and adaptive immune systems play a crucial role in sepsis. Dendritic cells, in particular, serve as professional antigen presenting cells and are involved in the aberrant immune response to sepsis. In this special issue, X. Fan et al. discuss the effects of sepsis on the amount, surface molecule expression, cytokine secretion, and T-cell activating function of dendritic cells and the underlying mechanisms in their review "Alterations of Dendritic Cells in Sepsis: Featured Role in Immunoparalysis."

Recent postmortem studies of patients who died of sepsis showed that depletion of CD4 and CD8 lymphocytes is an important characteristic. Thus, knowledge of these circulating lymphocyte abnormalities is relevant for the understanding of sepsis pathophysiology. R. de Pablo et al., who have previously reported on the alteration of B cells, natural killer cells, and T-cell function in septic patients, summarize their latest findings on the role of blood lymphocytes in sepsis and discuss the different kinetic patterns of lymphocyte subsets and their relationship to outcome in their review “Role of Circulating Lymphocytes in Patients with Sepsis.”

Both the clinical and basic researches have shown that sepsis-associated immunosuppression is associated with adverse outcomes. A novel heterogeneous population of immature myeloid cells that possess immunosuppressive activities, termed myeloid-derived suppressor cells (MDSCs), has gained much attention in recent sepsis studies. D. Lai et al. discuss the complex functions of MDSCs in the pathogenesis of sepsis. Their review “Myeloid-Derived Suppressor Cells in Sepsis” also proposes that the overall role of MDSCs involves much more than simply being an immunosuppressive cell population. These 3 review articles provide a comprehensive analysis of the major important immune cells in sepsis and highlight potential therapeutic targets.

As a group who have investigated the function of the family of defensins in sepsis for nearly 10 years, G.-H. Xie et al. summarize the in vitro, in vivo, and genetic studies on the effects of defensins as well as the corresponding mechanisms within sepsis. Their review, “Defensins and Sepsis,” also points out that the function of defensins reflects both their immunomodulatory and broad-spectrum antimicrobial effects.

Although the international Surviving Sepsis Campaign guidelines have been released for 10 years, sepsis remains a fatal syndrome due to the lack of efficient biomarkers and novel treatments. D. N. Nguyen et al. investigated plasma
cortisol levels in septic patients with delirium and coma and found that cortisol is a potential biomarker of brain dysfunction in their article “Cortisol Is an Associated-Risk Factor of Brain Dysfunction in Patients with Severe Sepsis and Septic Shock.” F. Song et al. and P. Madhusudan et al. discuss two important but controversial issues related to the Surviving Sepsis Campaign Guidelines. In a meta-analysis of 12 randomized trials involving 4100 septic patients, “Intensive Insulin Therapy for Septic Patients: A Meta-Analysis of Randomized Controlled Trials,” F. Song et al. reported no benefit and a higher incidence of hypoglycemia with intensive insulin therapy compared with conservative glucose management. P. Madhusudan et al. discuss the current debate on the choice, amount, and end points for fluid resuscitation in sepsis in their review “Fluid Resuscitation in Sepsis: Reexamining the Paradigm.” K. Xie et al. investigated the therapeutic function of hydrogen gas in a septic animal model for several years, and, in their present review, “Hydrogen Gas Presents a Promising Therapeutic Strategy for Sepsis,” they summarize the progress of hydrogen treatment in sepsis. J. Zhou et al. and X. Li et al. explore novel drugs for sepsis from the perspective of the neuroendocrine network in sepsis in their two studies, “Epinephrine Enhances the Response of Macrophages under LPS Stimulation” and “Agmatine Protects against Zymosan-Induced Acute Lung Injury in Mice by Inhibiting NF-κB-Mediated Inflammatory Response.”

In this present special issue about the pathogenesis, biomarkers, and treatment of sepsis, the authors provide comprehensive reviews and attractive research perspectives on the mechanisms of sepsis which we hope will inspire researchers investigating novel biomarkers and therapeutic sepsis targets.

Acknowledgment

This study was supported by National Natural Science Foundation of China (no. 81102226).

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Review Article

Alterations of Dendritic Cells in Sepsis: Featured Role in Immunoparalysis

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Received 15 February 2014; Revised 25 May 2014; Accepted 28 July 2014

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Sepsis, the leading cause of mortality in intensive care unit, is characterized by hyperinflammatory response in the early stage and followed by a period of immunosuppression. This immune disorder is believed to be the potent factor that is tightly associated with high mortality in sepsis. Dendritic cells (DCs) serve as professional antigen-presenting cells that play a vital role in immune response by activating T lymphocytes. During the progression of sepsis, DCs have been reported to take part in the aberrant immune response and be necessary for survival. Therefore, a better understanding of the DCs pathology will be undoubtedly beneficial for resolving the problems occurring in sepsis. This review discusses effects of sepsis on DCs number and function, including surface molecules expression, cytokines secretion, and T cell activation, and the underlying mechanism as well as some potential therapeutic strategies.

1. Introduction

Sepsis is high lethal public disease. In 2012, over 20 million people are affected by sepsis worldwide [1]. The mortality from septic shock and severe sepsis both in Europe and in USA is around 30% and this value is still elevated [1, 2]. Recently, sepsis is defined as the systemic inflammatory response syndrome (SIRS) due to infection [3], which indicated that SIRS and infection are two important factors in determination of sepsis.

When the host receives an infection, both pro- and anti-inflammatory responses are initiated. The inflammatory response is partly mediated by innate immune cells through recognition with invading pathogens or microorganisms [4]. These cells can decide the trend of inflammatory response toward pro- or anti-inflammatory state by producing proinflammatory cytokines (interleukin- (IL-) 1β, tumor necrosis factor- (TNF-) α, and interferon- (IFN-) γ) or anti-inflammatory cytokines (interleukin- (IL-) 10, transforming growth factor- (TGF-) β) [5, 6]. At the early stage of sepsis, there is a large amount of proinflammatory mediators termed as cytokines storm in the host. Therefore, various therapeutic methods have been used to treat sepsis by downregulation of proinflammatory cytokines expression. But in fact it does not bring good news in the clinical setting. There is one possibility that the animal model, such as cecal ligation and puncture (CLP), cannot entirely reflect the real state of septic patients, in which the gender, hormone, age, and other interference factors cannot be neglected [7, 8]. Another possibility is correlated with sepsis progression. Observation from clinical studies showed that about 80% septic patients had a persistence of infectious focus at the day they died [9]. Some other studies also found that the active cytomegalovirus normally existed in thesepticpatient without resolution [10, 11]. These results indicate that the host immunity exhibits a tolerance status, which makes the patients at an increased risk of subjection to secondary pathogen infection. The immunosuppression is found to be accompanied with immune cells deactivation and apoptosis, impaired antigen-presentation, suppression of proliferation of lymphocytes, and high levels of anti-inflammatory cytokines (IL-10). Moreover, polarization of T helper (Th) cells is toward to the Th2 type that results
in an increase in susceptibility to infection. The aberrant immune response will further lead to multiple organ failure and death.

Among the innate immune cells, dendritic cells (DCs), firstly discovered by Ralph in the early 1970s, are the most potent antigen-presenting cells and central component for linking the innate and adaptive immunity [12–14]. DCs originate from bone marrow CD34+ stem cells and home to all tissues via the blood stream where they developed into immature cells [15]. Immature DCs have high phagocytic properties and readily take up antigen and present the antigen to T cells. In response to endogenous danger signals or microbial antigens, DCs mature and migrate into the T cell area of lymphoid tissues, where CD4+ T cell will be activated. During the maturation, the phagocytic receptor will be lost, the surface molecules (e.g., MHCII, CD80, and CD86) involved DCs migration, and T cells activation will be upregulated [16, 17]. Although many different classification manners have been described, two major subsets of DCs are recognized: myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) [18, 19]. The former is derived from bone marrow precursor and the latter is believed to evolve from circulating lymphoid precursor [20, 21]. These two types of DCs have a similar molecular phenotype except for CD8α+, which is present in pDCs but absent in mDCs [22]. Based upon the importance of DCs in immune system and its central role in sepsis [23], this review will focus on the pathology changes of DCs during the evolution of sepsis.

2. The Effect of Sepsis on DCs Numbers

At first, large amounts of studies on animals or patients had featured obvious loss of CD4+ and CD8+ T cells in sepsis [24–27]. Due to the importance of DCs in the immune system, more and more investigators have focused on the change of DC numbers and its role in depletion of T cells. In general, CD11c+ DCs is believed to be the common marker of murine DC for its steady state. A profound loss in the number of CD11c+ DCs was observed in spleen after sepsis and the time ranging from 12 h to 3 d [28–32]. When the CD11c+ DCs are further divided into CD8−CD4+, CD8−CD4+, and CD8−CD4+, it is found that CD8−CD4+ and CD8−CD4+ subsets were lost 36 h after CLP, but the number of CD8−CD4+ DCs was increased [33]. Thus it could be demonstrated that the reduced number of splenic DCs was mediated by a selective loss of CD8−CD4+ and CD8−CD4+ subtypes.

In addition to spleen, sepsis was also found to reduce the percentage of CD11c+ DCs present in local mesenteric nodes beginning 12 h after CLP and reach a 50% decline by 24 h. This phenomenon was also observed in systemic inguinal nodes, but not in popliteal nodes [34]. Moreover, another study was performed on the mice with CLP, which were subsequently intravenously challenged with Schistosoma mansoni eggs to develop granulomas. Results showed that there was a significant loss of DC in lung during the granulomatous response [35]. However, it should be noted that gradual reconstitution of DC numbers was found on postsepsis day 28 [30].

In clinical settings, the number of DCs in blood was lower in severe septic or septic shock patients in comparison with healthy controls [36, 37]. For two distinct populations of DCs, mDCs and pDCs, their numbers was markedly reduced in patients with sepsis when compared with controls, and both cell counts recovered slightly until day 28 [38]. But data from another clinical study of twenty-six patients showed that decreased mDC and increased pDC were observed at day 1, and the number of mDCs was not different in survivors and nonsurvivors of septic patients, while pDCs were obviously higher in nonsurvivors [39]. This discrepancy between these two study groups may be due to the different severity of illness. Moreover, reduction of circulating DCs can become a predictive factor for the development of septic complication after pancreatectomy [40]. Besides the adult patients, flow cytometric assay showed that the levels of pDCs and mDCs were also significantly lower in pediatric patients with sepsis [41].

In conclusion, sepsis causes the loss of DCs occurring in various lymphoid and nonlymphoid tissues from septic patients and septic mice. This phenomenon does not result from the inhibition of de novo generation of DCs from progenitors [42, 43], although these monocyctic progenitors display characteristics of immunosuppressive properties [44] (Figure 1).

3. The Effect of Sepsis on DCs Function

3.1. Surface Molecular Expression. Upon the stimulation of microbial antigens or danger signals, DCs rapidly mature and migrate through the lymphatic system to lymphoid organs to stimulate T cells mediated immunity response. During this process, DCs will upregulate the presentation of cell surface proteins involved in T cell priming, including MHC, CD40, CD80, and CD86. In the CLP model, no obvious changes of CD40, CD80, and CD86 expression were discovered in CD11c+ splenocytes when compared with control group by 24 h after surgery. Similarly, peritoneal DCs showed CD40 and CD80 did not change in addition to an increase trend in CD86 expression [28]. However, splenic DCs from another study showed that levels of CD40 and CD86 were obviously enhanced by 15 h and 36 h after CLP while MHCII expression was much higher than control at 36 h following CLP. Only slight changes were observed in the expression of CD80 and MHCII [33]. For the DCs in lymph nodes, the percentage of CD40, CD80, CD86, and MHCII did not differ within 24 h between CLP-operated mice and sham-operated mice. Similarly, peritoneal DCs showed CD40 and CD80 did not change in addition to an increase trend in CD86 expression [28]. However, splenic DCs from another study showed that levels of CD40 and CD86 were obviously enhanced by 15 h and 36 h after CLP while MHCII expression was much higher than control at 36 h following CLP. Only slight changes were observed in the expression of CD80 and MHCII [33]. For the DCs in lymph nodes, the percentage of CD40, CD80, CD86, and MHCII did not differ within 24 h between CLP-operated mice and sham-operated mice, but there was a much higher expression of these molecules 36 h after sepsis [33, 34]. In addition, sepsis did not cause the change of CD40 and CD80 in the lung until 7 d after CLP [45]. B and T lymphocyte attenuator (BTLA), a coinhibitory receptor, has been demonstrated to inhibit T cell activation and thus contributed to many diseases [46]. BTLA and its primary ligand, herpes virus entry mediator (HVEM), expressions were found to increase in immature and mature DCs in peritoneum by 24 h after CLP, while HVEM+ DCs were significantly decreased in bone marrow [47].
3.2. Cytokine Secretion. A large number of studies have reported that septic DCs exhibit an aberrant cytokine secretion pattern, in which levels of proinflammatory cytokines (TNF-\(\alpha\), IL-1\(\beta\), and IL-12) are significantly depressed and anti-inflammatory cytokines (TGF-\(\beta\), IL-10) are enhanced [33, 38, 45] (Figure 1). DC-derived IL-12 is believed to be a key host defense cytokine and it is a heterodimeric cytokine composed of an IL-12p40 and IL-12p35 subunit [30, 52]. Flow cytometric analysis of splenic DCs from LPS-primed mice revealed that the percentage of DCs able to produce IL-12 p40 was dramatically decreased from 1.7% to 0.3% [53]. When DCs were stimulated with TLR2 agonist (Pam3Cys) or TLR4 agonist (LPS) or TLR9 agonist (CpG-DNA), mRNA levels of both \(\text{Il}12\ p40\) and \(\text{Il}12\ p35\) from sepsis splenic DCs were significantly lower than that from sham splenic DCs [30]. Sepsis also resulted in a lower intracellular expression of IL-12 p40 induced by CpG-DNA compared with sham group [33]. In addition, only a small amount of IL-12 p70 was secreted from DC being stimulated with CpG or LPS + CD40L [33]. A similar trend was also seen in lung DCs. The DCs from lungs of postseptic mice with developing granulomas had a lower IL-12 p40 mRNA and IL-12 p70 protein levels compared with controls [35]. Moreover, they also exhibited defective IL-12 synthesis after TLR agonist challenge [45].

IL-10 is a pleiotropic cytokine possessing both anti-inflammatory and immunosuppression properties [54]. In the acute phase of sepsis, endogenous IL-10 production and exogenous administration can reduce the magnitude of the inflammation. Therefore, injection of recombinant adenovirus expressing IL-10, which limits DC maturation and associated T cell activation, could attenuate acute sepsis [55, 56]. However, the upregulation of IL-10 will result in the immunity tolerance that fails to defend the secondary pathogen challenge. 36 h after CLP, DCs from septic mice produced increasing amounts of IL-10 [33]. Upon incubation with TLR agonist, the higher level of IL-10 at both of mRNA and protein level was observed in splenic and lung DCs from postseptic mice in contrast to control [30, 35, 45]. The increased concentration of IL-10 in blood from septic patients is associated with worsened clinical outcome [57]. Furthermore, endogenous IL-10 has been reported to regulate...
IL-12 synthesis of DCs in an autocrine manner [58, 59]. DCs from sham mice could increase LPS-induced IL-12 expression in the presence of anti-IL-10 antibody. However, blocking of IL-10 could not rescue the production of IL-12 of postseptic DCs, which suggests that the low production of IL-12 during sepsis is not dependent on IL-10 expression [30].

3.3. T Cell-Stimulatory Capacity. The impact of DCs on T cells proliferation during sepsis was determined in a mixed leucocyte reaction (MLR). IL-2 plays a crucial role in the proliferation of T cells. It was found that the percentage of IL-2-secreting T cells was significantly lower when cultured with DC from septic mice as compared with control mice [33]. This finding was also confirmed when OT-II CD4⁺ T cells were incubated with DCs in the presence of antigen [60]. However, peritoneal DCs and splenic DCs from CLP mice both showed higher capacity to trigger proliferative response of T cells than those from sham group [28]. In addition, an increased activation of CD3⁺CD4⁺ T cell was also seen in the inguinal nodes and popliteal lymph nodes [34]. For septic patients, immature DCs from patients and health donors had a similar ability to induce T cells proliferation, but mature DCs from patients did not enhance T cell response [43].

Studies on polarization of T cells had showed that OVA peptide-specific CD4⁺ T cells secreted markedly higher levels of Th2 cytokines such as IL-5, IL-13, and IL-4 but a lower amount of Th1 cytokine IFN-γ when cocultured with postseptic splenic DCs that pulsed with OVA, indicating that Ag-loaded DCs direct T cells toward a Th2-dependent response during severe sepsis [30]. This is consistent with another study in which adoptive transfer of bone-marrow derived DC from septic mice impaired Th1 priming [42]. In addition, the expression of Foxp3 in T cells cocultured with patient or control DCs suggested that CD1a⁺ DCs from septic patients made the T cells have a stronger regulatory function, because the percentage of naïve T cells expressing Foxp3 when cultured in patient DCs was much higher than that induced by control DCs (93% versus 40%) [61], which suggested that sepsis led to an increase in regulatory T cells (Tregs).

In short, though controversy still exist, DCs will engender apoptotic or anergic T cells after sepsis. These anergic T cells, in turn, may disrupt DCs function.

4. The Potential Mechanisms Involving Changes of DC during Sepsis

4.1. Apoptosis-Dependent Mechanism. Studies by numerous groups have suggested that apoptotic death of immune cells plays a vital role in contributing to the immune hyporesponsiveness and organ injury during sepsis [62–64]. 24 h after CLP, a significant increase of apoptotic and dead DCs was found in mesenteric and inguinal nodes through the staining of annexin V [34]. This result was also confirmed by immunohistochemical staining for active caspase 3, a crucial mediator of apoptosis [29]. However, a high false-positive result may occur, because DCs have phagocytic properties and the positive signal may form the apoptotic debris that is phagocyted by DCs [65, 66]. To further clarify the relationship between apoptosis and the loss of DC, study from the transgenic mice which could overexpress the Bcl-2 reported that overexpression of Bcl-2 could dispel sepsis-induced DCs depletion. Furthermore, Bim+/− mice exhibited remarkably less sepsis-induced loss in the DCs population [67]. Thus these proapoptotic and antiapoptotic proteins play a central role in DC loss during sepsis. In addition to DC loss, uptake of apoptotic DC would make viable DC display tolerogenic state that induces generation of Foxp3⁺ Treg [68].

The mechanisms by which sepsis caused DC apoptosis are at present not fully explored. A previous study has found that mechanism of apoptosis induced by LPS required activation of acid sphingomyelinase (A-SMase). Inhibition of this enzyme activity and ceramide generation could prevent apoptosis induction [69]. Furthermore, mammalian toll-like receptors (TLR)-dependent pathway is also found to involve in the process of sepsis-induced apoptosis, which was confirmed by several studies: (i) apoptosis of spleen DCs from CLP performed on TLR4⁻/⁻, TLR2⁻/−, and TLR2⁻/−TLR4⁻/− was inhibited [31]. (ii) TNF-α, a production of stimulation of TLRs, could impair mitochondrial integrity and induce apoptosis [70]. (iii) Interferon regulatory factor-1 (IRF-1) whose activation is dependent on intact TLR4 signaling was reported to trigger immune cells apoptosis [71]. However, a recent study showed that LPS-induced activation of nuclear factor of activated T cells (NFAT) via CD14 is necessary for DCs apoptosis, which was independent of TLR4 engagement [72].

4.2. Peroxisome Proliferator-Activated Receptors-Mediated Mechanism. Peroxisome proliferator-activated receptors (PPARs) are a superfamily of ligand-activated nuclear transcription factors and are involved in the regulation of lipid metabolism, glucose homeostasis, and cellular differentiation [73–75]. So far, three subtypes have been identified in human: PPAR-α, β(δ), and γ. Peripheral blood monocytes express high levels of PPAR-α and PPAR-β with low expression of PPAR-γ [76]. During the generation of DCs from monocytes and its maturation, PPAR-γ becomes the abundant subtype while the levels of other two subtypes are below the detection limit [76]. It was found that activation of PPAR-γ significantly increased the surface expression of CD36 and CD86 on LPS- and CD40 ligand-challenged DCs, whereas the synthesis of CD80, CXCL10, and CCL5 was reduced [77]. Moreover, it could depress the production of IL-12 with no effect on expression of IL-1β, TNF-α, IL-6, and IL-10 [77]. Studies also showed that PPAR-γ activation inhibited TNF-α induced DC migration from epithelia and subsequent accumulation in the draining lymph nodes [78]. Adoptive transfer of PPAR-γ-activated Ag-presenting DCs resulted in the impaired production of Th1 and Th2 cytokines, so as to induce CD4⁺ T cell anergy which fail to expand the secondary clone upon restimulation [79]. More interestingly, PPAR-γ was reported to be restricted to CD1a⁻ cells in the process of cytokine-induced DC differentiation. PPAR-γ transcriptional activity was higher in CD1a⁻ cells.
but not in CD1a+, indicating that the generation of CD1α− cells might be associated with PPAR-γ [80]. However, a large number of CD1α− cells were generated from peripheral blood monocytes of septic patients and the percentage of this type of cells reached 68% after 7 d [61]. So it is not difficult to hypothesize whether the changes of DC in progression of sepsis are correlated to PPAR-γ. But there is no paper to clarify the connection between PPAR-γ and DCs in sepsis. Hepatic PPAR-γ mRNA expression and protein levels were reported to decrease at 20 h after CLP [81], but the results from another study showed that PPAR-γ expression of peritoneal cells was elevated significantly at both gene and protein levels 6 h after CLP [82]. Additionally, PPAR-γ expression in peripheral blood mononuclear cells from children patient with septic shock was also decreased but its activity was increased when compared to controls [83]. PPAR-γ activation could also promote T cell apoptosis in sepsis [84, 85]. Besides PPAR-γ, PPAR-α expression was reduced in patients with septic shock which was correlated to severity of  illness [86]. Cell surface markers and cytokines production were decreased in PPAR-α knockout mice [86]. These data indicate the absence of PPAR-α is not beneficial for treating sepsis.

4.3. Wnt Signal Pathway-Mediated Mechanism. Wnt family is a highly conserved secreted signaling pathway that regulates developmental and homeostatic processes [87, 88]. Wnt proteins activate canonical or noncanonical signal pathway in a context-dependent manner [89, 90]. The former primarily takes part in cell fate determination and the latter is responsible primarily for cell movement and tissue polarity [91]. Wnt and their receptors are found to be expressed in hematopoietic progenitor cells (HPCs) [92], indicating that Wnt may be involved in HPCs differentiation. There was a remarkable expansion of hematopoietic progenitor cells after activation if Wnt canonical pathway. Wnt signaling pathway plays a central role in DCs differentiation in means of promotion on conventional DCs differentiation and inhibition on pDCs differentiation [93]. During the differentiation process of DCs from HPCs in vitro, Wnt signaling was upregulated characterized by accumulation of β-catenin and upregulation of Wnt target gene expression [94]. Activation of Wnt canonical pathway by Wnt 3a could promote the degeneration of CD11c+ DCs and enhance their capacity to stimulate T cells proliferation [94]. However, the activation of noncanonical Wnt pathway by Wnt 5a was shown to inhibit DC differentiation [94]. Wnt 5a-treated DCs had worse ability of capturing antigen. Wnt 5a had no effect on LPS-induced DC maturation but impaired the production IL-12p70 and TNF-α while increasing levels of IL-10. Furthermore, Wnt 5a inhibited the T cell proliferation and fail to prime T cell response [95]. So the two types of signal pathway display an opposite effect and sustain the regulation of DCs differentiation by crosstalking to each other. During sepsis, Wnt 5a concentration in sera of patients was elevated and Wnt 5a was also found to induce macrophage differentiation to a tolerogenic phenotype, which was related to induction of IL-10 and suppression of NF-κB signaling [96, 97]. Therefore, Wnt signal pathway may be a factor that contributes to the dysfunction of DCs during sepsis.

4.4. Epigenetic Mechanisms. Epigenetic regulation refers to external modification on gene activity without any changes in DNA sequence. Epigenetic mechanisms have been involved in the maintenance of various genes expression during embryogenesis and caner [98, 99]. In eukaryotic cells, nucleosome is the basic unit of chromatin, consisting of a short length of DNA wrapped around eight histone protein cores (duplicated in H2A, H2B, H3, and H4) [100, 101]. More and more investigators have discovered that histone modifications, including acetylation, ubiquitylation, methylation, and phosphorylation, are important epigenetic mechanisms of gene expression [101]. It is reported that maintenance of Th1/Th2 memory and gene Il17 expression are associated with acetylation and methylation of histone [102]. Histone methylation, especially for the methylation of histone H3 at lysine-4 (H3K4) and at lysine-27(H3K27), is known as a critical mechanism correlated with transcriptional activation and repression [103, 104]. Methylation at H3K4 mediated by MLL family histone methyltransferase (HMT) complex, in conjunction with several structural proteins including WD40-repeat proteins WDR5, RbBP5, and Ash2L, contributed to transcription activation [102, 105]. Methylation at H3K27 is mediated by polycomb repressive complex 2 (PRC2) which contains several core components including EZH2, suppressor of Zeste 12 (SUZ12) and embryonic ectoderm development (EED) [104]. It is correlated with transcription silencing. The production of IL-12 as discussed above, an important cytokine directing Th1 immune response, was dramatically depressed in DCs from both septic patients and mice. To test if the aberrant change of IL-12 is correlated with epigenetic mechanism. Chromatin immunoprecipitation techniques were performed and data show that the reduction of IL-12 is mediated by decreasing the H3K4 trimethylation and increasing H3K27 dimethylation at Il12p35 and Il12p40 promoter, which result from the suppression in recruitment of MLL complex (WDR5 and RbBP5) and enhancement in recruitment of PRC2 complex (EED and SUZ12) on promoter, respectively [30]. These results indicate that epigenetic modification may be one potential mechanism of long-term immunoparalysis.

5. Potential Therapeutic Modulation of DC Aberrant Function

Given the central role of DCs in the immune response and survival in sepsis, it seems natural that DCs are the hopeful target for improving the aberrant immune response and prolonging the life during sepsis progression. To date many strategies for correcting the DC impaired function have been discovered, as shown in Table 1.

5.1. Increase the Number of DC. It has been mentioned that the loss of DCs is partly dependent on cell apoptosis, so the methods that can inhibit the apoptosis are thought to be beneficial for sepsis. IL-15 is a pluripotent cytokine that can
not only coordinate the innate and adaptive immune system but also inhibit apoptosis by inducing the antiapoptotic proteins Bcl-2 and Bcl-xL in immune cells [106–108]. After the CLP operation, mice were injected s.c. with IL-15 or vehicle. Results showed that IL-15 administration significantly inhibited the apoptosis of splenic CD4, CD8, NK, and DCs induced by sepsis. During this process, IL-15 treatment increased Bcl-2 protein expression in all cells. The level of circulating IFN-γ was increased after IL-15 treatment, whereas both TNF-α and IL-6 production was decreased. Within the observation of 7 days, CLP mice treated with IL-15 had more than three-time improvement in survival compared with CLP only mice [109]. These data demonstrate that IL-15 may be a novel therapy of sepsis. Based upon the antiapoptotic molecules, TAT-Bcl-xL fusion protein and TAT-BH4 peptide were obtained and they have the ability to prevent sepsis-induced lymphocyte apoptosis, and high level of Bcl-xl improved the survival in sepsis [110]. Besides apoptosis, Fms-like tyrosine kinase-3 ligand (Flt3L) treatment was found to increase the number of CD11c+ DC populations by accelerating its expansion, so as to be able to reverse the endotoxin-induced tolerance [111, 112].

5.2. Change the DC Distribution. C5a is a potent chemottractant among the complement products and possesses a number of functions including the modulation of cytokine and adhesion molecules expression, causing oxidant burst and granule enzymes [113–115]. C5a was reported to be excessively activated and its high expression was harmful for host during sepsis [116, 117]. After treatment with anti-C5a antibody, the IL-12+ DCs in peripheral blood and lymphoid nodes were decreased but were increased in peritoneal cavity in which IL-12+ DCs play a protection role in sepsis. Furthermore, anti-C5a antibody-treated mice had a higher survival rate than that in sham mice [118].

5.3. Promote DC Maturation and Increase Proinflammatory Cytokines Release. This function is the most potent in improving the immunoparalysis status in sepsis. It is known that TLR family play a critical role in the clearance of pathogen by promoting proinflammatory response. However, the activation of TLR during this process requires the interaction with coreceptor CD14 which can amplify the inflammatory signal primed by bacterial pathogen [119, 120]. So CD14 is thought to be a potential target for skewing Th1 response in sepsis. TLR2-derived peptide enhances the DC maturation by upregulation of MHCII, CD80, and CD86 expression. The peptide also increased the release of IL-12 and IFN-γ which are key factors for activating Th1 cell. At the same time, TGF-β release was inhibited. It was indicated that the TLR2-derived peptide promoted a Th1 adaptive immune response and improved the status of immunosuppression [121]. In addition, the introduction of phospholipase A2 (PLA2) enhanced expression of HLA-DR, CD86, CD80, CD83, and CD40 on DCs. PLA2 also improved the ability of DCs to secrete IFN-γ when cocultured with allogeneic T cells [122]. Moreover, microRNA is also a potential target of immune modulation. Silencing of miR-142-3p which targets the IL-6 3’untranslated region significantly promoted the IL-6 expression and reduced endotoxin-induced mortality [123].

6. Conclusion

DCs are crucial in pathogen recognition and induction of specific immune response to protect host from the invading infection. When sepsis develops, DCs from lymphoid and nonlymphoid tissues are lost, which mostly result from the apoptosis. Several surface molecules associated with DCs maturation are changed, in which the most obvious one is HLA-DR. Upon the stimulation of external antigen or danger signal, IL-12 expression is suppressed while IL-10 production is increased, which results in the polarization of Th cell toward Th2 or Treg. During sepsis Wnt or PPAR or epigenetic-mediated mechanism may be involved (Figure 1). Several therapies that focus on improving DCs function have been shown to be able to mitigate the disease symptom. It is known that septic patients need to undergo two stages: a hyperinflammatory state and the secondary occurrence of immunosuppression. However there is no clinical parameter able to point out what the undergoing mechanism is. Therefore, specific biomarkers responsible for reflecting the immune status need to be discovered in future. Furthermore,
it is imperative to find out the ideal therapeutic target that only directs to one phase without affecting the other one.

**Conflict of Interests**

The authors declare that there is no conflict of interests regarding the publication of this paper.

**Acknowledgment**

This work was supported by a Grant from National “973” Project (no. 2012CB518102).

**References**


Clinical Study

Incidence and Risk Factors of Postoperative Pulmonary Complications in Noncardiac Chinese Patients: A Multicenter Observational Study in University Hospitals

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Received 9 January 2014; Revised 1 July 2014; Accepted 6 August 2014

Academic Editor: Stephen M. Pastores

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Purpose. To assess the incidence of postoperative pulmonary complications (PPCs) in Chinese inpatients, and to develop a brief predictive risk index.

Methods. Between August 6, 2012, and August 12, 2012, patients undergoing noncardiac operations in four university hospitals were enrolled. The cohort was divided into two subsamples, cohort 1 to develop a predictive risk index of PPCs and cohort 2 to validate it. Results. 1673 patients were enrolled. PPCs were recorded for 163 patients (9.7%), of whom the hospital length of stay (LOS) was longer ($P < 0.001$). The mortality was 1.84% in patients with PPCs and 0.07% in those without. Logistic Regression modeling in cohort 1 identified nine independent risk factors, including smoking, respiratory infection in the last month, preoperative antibiotic use, preoperative saturation of peripheral oxygen, surgery site, blood lost, postoperative blood glucose, albumin, and ventilation. The model was validated within cohort 2 with an area under the receiver operating characteristic curve of 0.90 (95% CI 0.86 to 0.94).

Conclusions. PPCs are common in noncardiac surgical patients and are associated with prolonged LOS in China. The current study developed a risk index, which can be used to assess individual risk of PPCs and guide individualized perioperative respiratory care.

1. Introduction

More than 230 million major surgical procedures are undertaken each year worldwide [1] and postoperative complications imposed a significant clinical and economic burden to surgical patients as well as the public health systems [2, 3]. Postoperative pulmonary complications (PPCs) are common postoperative complications that occur in 2% to 40% of patients and are associated with increased morbidity, mortality, and length of stay (LOS) [3–9]. In noncardiac patients, PPCs occur more frequently than cardiac complications [10]. Though it came to wide attention in recent years, the literature investigating the incidence and outcome of PPCs in Chinese inpatients remains scarce.

It is known that PPCs have a multifactorial etiology and had been defined broadly, including respiratory tract infection, pneumonia, respiratory failure, atelectasis, pleural effusion, pneumothorax, bronchospasm, and aspiration pneumonitis [11]. Previous studies demonstrated that PPCs were associated with a series of perioperative risk factors, such as age, smoking, chronic obstructive pulmonary disease (COPD), type of surgery, and serum albumin [4, 6, 7, 11–14]. A majority of these risk factors can be intervened and improved [15–17]. Therefore, identifying perioperative risk factors of PPCs is an important step toward improving quality of care in surgical patients, which has been already explored in several studies [11, 12, 18].
Table 1: Definitions of postoperative pulmonary complications.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Respiratory infection</td>
<td>Treatment with antibiotics for a respiratory infection, plus at least one of the following criteria: new or changed sputum, new or changed lung opacities, fever, and leukocyte count &gt; 12,000/mm³</td>
</tr>
<tr>
<td>Respiratory failure</td>
<td>Postoperative PaO₂ &lt; 60 mmHg on room air, a ratio of PaO₂ to inspired oxygen fraction &lt; 300, or SaO₂ &lt; 90% and requiring oxygen therapy</td>
</tr>
<tr>
<td>Pleural effusion</td>
<td>Chest radiograph demonstrating blunting of the costophrenic angle, evidence of displacement of adjacent anatomical structures, or (in supine position) a hazy opacity in one hemithorax with preserved vascular shadows</td>
</tr>
<tr>
<td>Atelectasis</td>
<td>Collapse of the alveoli, lung opacification with shift of the mediastinum, hilum, or hemidiaphragm toward the affected area, and compensatory overinflation in the adjacent nonatelectatic lung</td>
</tr>
<tr>
<td>Pneumothorax</td>
<td>A collection of air in the pleural space (the area with no vascular bed surrounding the visceral pleura)</td>
</tr>
<tr>
<td>Bronchospasm</td>
<td>Newly detected expiratory wheezing treated with bronchodilators</td>
</tr>
<tr>
<td>Aspiration pneumonitis</td>
<td>Acute lung injury after the inhalation of regurgitated gastric contents</td>
</tr>
</tbody>
</table>

In the United States, Arozullah et al. developed a multifactorial risk index to predict the postoperative pneumonia after major noncardiac surgery [12]. In Canada, McAlister et al. paid attention to the nonthoracic surgery [7]. They found the incidence of PPCs is 8% and identified the preoperative risk factors. Dupont et al. also investigated five independent predictive factors of postoperative pneumonia in France [19]. A recently published risk-prediction equation for PPCs was a significant advance in this field, which identified seven independent risk factors for PPCs by Canet et al. [11]. The researchers further tested this predictive risk score in a large European cohort and found this risk score performed differently between geographic areas [20]. In China, the most populous developing country in the world, however, very limited information about PPCs has been reported so far. Since China is different from the USA and European countries in geographic areas, race, disease spectrum, and social psychological background, investigations assessing the incidence and characteristics of PPCs in Chinese surgical cohorts are indispensable. Hence we conducted the present study to assess the incidence and risk factors of PPCs after noncardiac surgery in China and to develop a risk index of PPCs which is applicable for Chinese inpatients. The results of the present study would help health care providers to understand the existing situation of PPCs in China, to identify high-risk patients from the generic surgical population, and to guide individualized perioperative respiratory care.

2. Materials and Methods

2.1. Study Settings and Patients. We conducted a prospective, multicenter, and observational study of inpatients undergoing noncardiac surgical procedures. This study was performed at 4 university hospitals located in Zhejiang province, China (the First Affiliated Hospital, Zhejiang University School of Medicine, the Sir Run Run Shaw Hospital, Zhejiang University School of Medicine, the First Affiliated Hospital, Wenzhou Medical College, and the Second Affiliated Hospital, Wenzhou Medical College). The inpatients undergoing surgical procedures between August 6, 2012, and August 12, 2012 were enrolled into the study. All patients would receive routine care, and no research-related intervention would be introduced.

2.2. Inclusion and Exclusion Criteria. All noncardiac operations performed under general, spinal, epidural, or regional anesthesia were eligible for inclusion. The exclusion criteria were as follows: (1) younger than 18 years; (2) pregnancy; (3) organ transplantation; (4) procedures performed under local nerve anesthesia; (5) procedures outside the operating room; (6) outpatient procedures (who had an LOS in hospital less than 24 hours); (7) reoperation related to a previous surgical complication; (8) patients with preoperatively intubated trachea.

2.3. Data Collection. Two trained anesthesiologists were assigned at each center to collect the following data: (1) generic information: date of surgery and hospital admission/discharge, age, gender, American society of anesthesiologists (ASA) physical status, height and weight, smoking status, alcohol use, and chronic comorbid disease; (2) preoperative variables: respiratory infection in the last month, antibiotic use, nasogastric tube, saturation of peripheral oxygen (SpO₂), and laboratory results (leucocytes count, neutrophil, hemoglobin, serum creatinine, serum albumin, and fasting blood glucose); (3) intraoperative variables: anaesthetic technique, surgery (type, site, and duration), nasogastric tube, bladder catheter, central venous catheter, blood loss, blood
Table 2: Demographic and clinical characteristics.

<table>
<thead>
<tr>
<th></th>
<th>Overall (n = 1673)</th>
<th>Cohort 1 (n = 902)</th>
<th>Cohort 2 (n = 771)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male, n (%)</td>
<td>770 (46.0)</td>
<td>436 (48.3)</td>
<td>334 (43.3)</td>
<td>0.04</td>
</tr>
<tr>
<td>Age, yr</td>
<td>49 (37–60)</td>
<td>48 (36–60)</td>
<td>49 (39–60)</td>
<td>0.69</td>
</tr>
<tr>
<td>Education, yr</td>
<td>9 (6–9)</td>
<td>9 (6–9)</td>
<td>9 (9–14.5)</td>
<td>0.31</td>
</tr>
<tr>
<td>Smoking status, n (%)</td>
<td></td>
<td></td>
<td></td>
<td>0.85</td>
</tr>
<tr>
<td>Never smoker</td>
<td>1397 (83.5)</td>
<td>753 (83.5)</td>
<td>644 (83.5)</td>
<td></td>
</tr>
<tr>
<td>Former smoker</td>
<td>222 (13.3)</td>
<td>118 (13.1)</td>
<td>104 (13.5)</td>
<td></td>
</tr>
<tr>
<td>Current smoker</td>
<td>54 (3.2)</td>
<td>31 (3.4)</td>
<td>23 (3.0)</td>
<td></td>
</tr>
<tr>
<td>Drinker, n (%)</td>
<td></td>
<td></td>
<td></td>
<td>0.47</td>
</tr>
<tr>
<td>Never drinker</td>
<td>1487 (88.9)</td>
<td>804 (89.1)</td>
<td>683 (88.6)</td>
<td></td>
</tr>
<tr>
<td>Former drinker</td>
<td>152 (9.1)</td>
<td>83 (9.2)</td>
<td>69 (8.9)</td>
<td></td>
</tr>
<tr>
<td>Current drinker</td>
<td>34 (2.0)</td>
<td>15 (1.7)</td>
<td>19 (2.5)</td>
<td></td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>22.3 (20.3–24.3)</td>
<td>22.1 (20.3–24.2)</td>
<td>22.4 (20.3–24.3)</td>
<td>0.85</td>
</tr>
<tr>
<td>Preoperative SpO₂, %</td>
<td>98 (98-99)</td>
<td>98 (98-99)</td>
<td>98 (98-98)</td>
<td>0.45</td>
</tr>
<tr>
<td>Respiratory infection in the last month, n (%)</td>
<td>28 (1.7)</td>
<td>16 (1.8)</td>
<td>12 (1.6)</td>
<td>0.85</td>
</tr>
<tr>
<td>ASA physical status, n (%)</td>
<td></td>
<td></td>
<td></td>
<td>0.43</td>
</tr>
<tr>
<td>1</td>
<td>830 (49.6)</td>
<td>464 (51.4)</td>
<td>366 (47.5)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>722 (43.2)</td>
<td>380 (42.1)</td>
<td>342 (44.4)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>114 (6.8)</td>
<td>54 (6.0)</td>
<td>60 (7.8)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>5 (0.3)</td>
<td>3 (0.3)</td>
<td>2 (0.3)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>2 (0.1)</td>
<td>1 (0.1)</td>
<td>1 (0.1)</td>
<td></td>
</tr>
<tr>
<td>Emergency surgery, n (%)</td>
<td>102 (6.1)</td>
<td>55 (6.1)</td>
<td>47 (6.1)</td>
<td>0.99</td>
</tr>
<tr>
<td>Anesthesia, n (%)</td>
<td></td>
<td></td>
<td></td>
<td>0.24</td>
</tr>
<tr>
<td>General and combined¹</td>
<td>1251 (74.8)</td>
<td>664 (73.6)</td>
<td>587 (76.1)</td>
<td></td>
</tr>
<tr>
<td>Neuraxial/regional</td>
<td>422 (25.2)</td>
<td>238 (26.4)</td>
<td>184 (23.9)</td>
<td></td>
</tr>
<tr>
<td>Surgical site, n (%)</td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Peripheral</td>
<td>1015 (60.7)</td>
<td>620 (68.7)</td>
<td>395 (51.2)</td>
<td></td>
</tr>
<tr>
<td>Abdominal</td>
<td>600 (35.9)</td>
<td>243 (26.9)</td>
<td>357 (46.3)</td>
<td></td>
</tr>
<tr>
<td>Intrathoracic</td>
<td>58 (3.5)</td>
<td>39 (4.3)</td>
<td>19 (2.5)</td>
<td></td>
</tr>
<tr>
<td>Duration of surgery, h</td>
<td></td>
<td></td>
<td></td>
<td>0.63</td>
</tr>
<tr>
<td>≤2 h</td>
<td>1238 (74.0)</td>
<td>659 (73.1)</td>
<td>579 (75.1)</td>
<td></td>
</tr>
<tr>
<td>2-3 h</td>
<td>235 (14.0)</td>
<td>132 (14.6)</td>
<td>103 (13.4)</td>
<td></td>
</tr>
<tr>
<td>&gt;3 h</td>
<td>200 (12.0)</td>
<td>111 (12.3)</td>
<td>89 (11.5)</td>
<td></td>
</tr>
<tr>
<td>LOS, d</td>
<td>8 (5–14)</td>
<td>9 (6–14)</td>
<td>8 (5–13)</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Data are median (quartile) unless otherwise specified.
SpO₂: saturation of peripheral oxygen; ASA: American society of anesthesiologists; LOS: length of stay.
* Cohort 1 versus cohort 2; ¹ this category included general anesthesia alone and general anesthesia combined with regional blockade.

Transfusion, pulmonary, and cardiovascular complications; (4) postoperative variables: clinic (SpO₂, mechanical ventilation, vasoactive drugs) and laboratory results within 2 hours after surgery. After operation, surgeon and nurses visited the patients every day and recorded incidence of PPCs. The PPCs were defined in Table 1 [11]. Postoperative mortality was defined as death within 60 days of surgery.

2.4. Outcomes. The primary outcome was the occurrence of PPCs, the postoperative LOS, and the postoperative mortality rate. The secondary outcome is the predictive risk index of PPCs.

2.5. Statistical Analysis. Quantitative data were presented as means and standard deviations (SD) or median and interquartile range (IQR; from 25th to 75th percentiles) as appropriate. Qualitative data were reported as N (%).

Student's t-test or Mann-Whitney U test were used for comparison of continuous variables and the chi-squared test or Fisher’s exact test for categorical variables to test the relationship between each potential risk factors and PPCs.

In order to develop a risk index of PPCs, enrolled patients were further divided into two cohorts. Cohort 1 composed of patients from two hospitals and was used to develop the predictive index, while cohort 2 was used to validate the index. In cohort 1, risk factors were firstly analyzed using univariate analysis (P < 0.05). Then a multivariate logistic regression was conducted in cohort 1 with the occurrence of PPCs as the dependent factor, incorporating all risk factors on the basis of correlation coefficients between variables lower than 0.4. The forward LR mode was adopted in the process of regression. The adjusted odds ratios (OR) and the confidence intervals (CI) were also calculated. A brief predictive index was then calculated by multiplying the regression (β) by 10 and rounding off to the nearest integer [12]. The brief
Table 3: Incidence of PPCs with LOS according to surgical specialties.

<table>
<thead>
<tr>
<th>Surgical specialty</th>
<th>All patients</th>
<th>PPCs, n (%)</th>
<th>LOS (day)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>With PPCs (n = 163)</td>
<td>Without PPCs (n = 1510)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>1673</td>
<td>163 (9.7)</td>
<td>16 (10–23)</td>
<td>8 (5–13)</td>
</tr>
<tr>
<td>Orthopaedics</td>
<td>303</td>
<td>10 (3.3)</td>
<td>22 (14–31)</td>
<td>11 (6–17)</td>
</tr>
<tr>
<td>Breast</td>
<td>72</td>
<td>2 (2.8)</td>
<td>20 (19–21)</td>
<td>4 (3–8)</td>
</tr>
<tr>
<td>Gynaecology</td>
<td>208</td>
<td>16 (7.7)</td>
<td>10 (7–19)</td>
<td>6 (4–8)</td>
</tr>
<tr>
<td>Vascular</td>
<td>33</td>
<td>1 (3.0)</td>
<td>16 (16–16)</td>
<td>9 (7–13)</td>
</tr>
<tr>
<td>Upper gastrointestinal</td>
<td>47</td>
<td>19 (40.4)</td>
<td>18 (16–21)</td>
<td>17 (13–20)</td>
</tr>
<tr>
<td>Lower gastrointestinal</td>
<td>136</td>
<td>16 (11.8)</td>
<td>17 (15–28)</td>
<td>11 (6–15)</td>
</tr>
<tr>
<td>Hepatobiliary</td>
<td>229</td>
<td>31 (13.5)</td>
<td>24 (16–33)</td>
<td>8 (5–14)</td>
</tr>
<tr>
<td>Urology</td>
<td>168</td>
<td>7 (4.2)</td>
<td>13 (10–17)</td>
<td>10 (7–14)</td>
</tr>
<tr>
<td>Kidney</td>
<td>56</td>
<td>8 (14.3)</td>
<td>12 (10–15)</td>
<td>14 (9–16)</td>
</tr>
<tr>
<td>Head and neck</td>
<td>147</td>
<td>9 (6.1)</td>
<td>4 (3–7)</td>
<td>7 (5–9)</td>
</tr>
<tr>
<td>Thoracic</td>
<td>49</td>
<td>19 (38.8)</td>
<td>12 (10–15)</td>
<td>14 (9–16)</td>
</tr>
<tr>
<td>Neurosurgery</td>
<td>36</td>
<td>8 (22.2)</td>
<td>22 (12–27)</td>
<td>18 (12–25)</td>
</tr>
<tr>
<td>Endocrinology</td>
<td>147</td>
<td>14 (9.5)</td>
<td>8 (7–14)</td>
<td>7 (6–8)</td>
</tr>
<tr>
<td>Others</td>
<td>41</td>
<td>4 (2.4)</td>
<td>10 (7–26)</td>
<td>7 (5–9)</td>
</tr>
</tbody>
</table>

Data are median (quartile) unless otherwise specified.
PPCs: postoperative pulmonary complications; LOS: length of stay.

predictive index then validated in cohort 2 to evaluate the model's discriminatory capability, and the area under the receiver operating characteristic (ROC) curve was displayed (c-statistic).

Data were analyzed using SPSS 16.0 (SPSS inc., Chicago, USA). All these tests were two-tailed and statistical significance was considered when a P value was less than 0.05.

3. Results

3.1. Demographic and Clinical Characteristics. Between August 6, 2012, and August 12, 2012, 2001 patients were undergoing noncardiac surgery, of which 328 were excluded according to the inclusion criteria or lost. Consequently, the final sample included in the statistical analysis consisted of 1673 patients, 902 in cohort 1 and 771 in cohort 2 (Figure 1). The basic characteristics of the study subjects are detailed in Table 2.

3.2. PPCs, LOS, and Mortality. A total of 163 (9.7% of the 1673 patients) patients were recorded with 237 episodes of PPCs. Postoperative respiratory failure developed in 30 patients (1.8%), bronchospasm in 48 (2.9%), pleural effusion in 7 (0.4%), respiratory infection in 131 (78%), atelectasis in 19 (11%), cardiopulmonary edema 1 (0.06%), and pneumothorax in 1 (0.06%). Most PPCs occurred after upper gastrointestinal surgery (40.4%), followed by thoracic (38.8%), neurosurgery (22.2%), kidney (14.3%), hepatobiliary (13.5%), and lower gastrointestinal surgeries (11.8%). The median postoperative LOS was longer in patients with PPCs (16 days, IQR 10–23 days) than in those without PPCs (8 days, IQR 5–13 days) (P < 0.001), especially in orthopaedics (P = 0.03), breast (P < 0.001), gynaecology (P < 0.001), lower gastrointestinal (P < 0.001), and hepatobiliary (P < 0.001) surgery. The detailed information on the characteristics of PPCs and LOS was shown in Table 3.

Four (0.24%) patients died in the hospital, 3 (1.84%) of the 163 patients with PPCs and 1 (0.07%) of the 1510 patients without PPCs; the mortality was significantly higher in patients with PPCs than those without (P < 0.001).

3.3. Risk Factors and PPCs. The variables having a statistically significant impact on the incidence of PPCs detected from the cohort 1 are shown in Table 4. Then the independent variables were entered into the logistic regression model, except for these have high collinearity with others (intraoperative blood
Table 4: Distribution of results of independent variables in cohort 1.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Number of patients</th>
<th>Number (%) of patients with PPCs</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;59</td>
<td>670</td>
<td>65 (9.7)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>≥60</td>
<td>232</td>
<td>47 (20.3)</td>
<td></td>
</tr>
<tr>
<td>ASA physical status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>464</td>
<td>20 (4.3)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>2</td>
<td>380</td>
<td>77 (20.3)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>54</td>
<td>13 (24.1)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>1 (33.3)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>1 (100.0)</td>
<td></td>
</tr>
<tr>
<td>Smokers</td>
<td></td>
<td></td>
<td>0.001</td>
</tr>
<tr>
<td>No</td>
<td>753</td>
<td>79 (10.5)</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>149</td>
<td>33 (22.1)</td>
<td></td>
</tr>
<tr>
<td>Respiratory infection in the last month</td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>No</td>
<td>886</td>
<td>101 (11.4)</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>16</td>
<td>11 (68.8)</td>
<td></td>
</tr>
<tr>
<td>Diabetes</td>
<td></td>
<td></td>
<td>0.001</td>
</tr>
<tr>
<td>No</td>
<td>846</td>
<td>97 (11.5)</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>56</td>
<td>15 (26.8)</td>
<td></td>
</tr>
<tr>
<td>COPD</td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>No</td>
<td>890</td>
<td>106 (11.9)</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>12</td>
<td>6 (50.0)</td>
<td></td>
</tr>
<tr>
<td>Cirrhosis</td>
<td></td>
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<td>0.014</td>
</tr>
<tr>
<td>No</td>
<td>895</td>
<td>109 (12.2)</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>7</td>
<td>3 (42.9)</td>
<td></td>
</tr>
<tr>
<td>Stroke/transient ischaemic attack</td>
<td></td>
<td></td>
<td>0.001</td>
</tr>
<tr>
<td>No</td>
<td>887</td>
<td>106 (12.0)</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>15</td>
<td>6 (40.0)</td>
<td></td>
</tr>
<tr>
<td>Preoperative antibiotic use</td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>No</td>
<td>298</td>
<td>19 (6.4)</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>604</td>
<td>93 (15.4)</td>
<td></td>
</tr>
<tr>
<td>Preoperative SpO₂, %</td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>≥96</td>
<td>845</td>
<td>92 (10.9)</td>
<td></td>
</tr>
<tr>
<td>&lt;96</td>
<td>57</td>
<td>20 (35.1)</td>
<td></td>
</tr>
<tr>
<td>Preoperative anemia</td>
<td></td>
<td></td>
<td>0.007</td>
</tr>
<tr>
<td>No</td>
<td>837</td>
<td>97 (11.6)</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>65</td>
<td>15 (23.1)</td>
<td></td>
</tr>
<tr>
<td>Preoperative LOS, d</td>
<td></td>
<td></td>
<td>0.002</td>
</tr>
<tr>
<td>&lt;2</td>
<td>126</td>
<td>5 (4.0)</td>
<td></td>
</tr>
<tr>
<td>≥2</td>
<td>776</td>
<td>107 (13.8)</td>
<td></td>
</tr>
<tr>
<td>Surgery site</td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Peripheral</td>
<td>620</td>
<td>36 (5.8)</td>
<td></td>
</tr>
<tr>
<td>Abdominal</td>
<td>243</td>
<td>56 (23.0)</td>
<td></td>
</tr>
<tr>
<td>Intrathoracic</td>
<td>39</td>
<td>20 (51.3)</td>
<td></td>
</tr>
<tr>
<td>Anesthesia</td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Regional</td>
<td>238</td>
<td>10 (4.2)</td>
<td></td>
</tr>
<tr>
<td>General</td>
<td>664</td>
<td>102 (15.4)</td>
<td></td>
</tr>
<tr>
<td>Intraoperative nasogastric tube</td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>No</td>
<td>847</td>
<td>85 (10.0)</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>55</td>
<td>27 (49.1)</td>
<td></td>
</tr>
</tbody>
</table>
Table 4: Continued.

<table>
<thead>
<tr>
<th>Number of patients</th>
<th>Number (%) of patients with PPCs</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intraoperative bladder catheter</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>466</td>
<td>28 (6.0)</td>
</tr>
<tr>
<td>Yes</td>
<td>436</td>
<td>84 (19.3)</td>
</tr>
<tr>
<td><strong>Intraoperative central venous catheter</strong></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>No</td>
<td>821</td>
<td>85 (10.4)</td>
</tr>
<tr>
<td>Yes</td>
<td>81</td>
<td>27 (33.3)</td>
</tr>
<tr>
<td><strong>Intraoperative blood loss, mL</strong></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>&lt;100</td>
<td>636</td>
<td>32 (5.0)</td>
</tr>
<tr>
<td>≥100</td>
<td>266</td>
<td>80 (30.1)</td>
</tr>
<tr>
<td><strong>Intraoperative blood transfusion</strong></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>No</td>
<td>875</td>
<td>102 (11.7)</td>
</tr>
<tr>
<td>Yes</td>
<td>27</td>
<td>10 (37.0)</td>
</tr>
<tr>
<td><strong>Duration of surgery, h</strong></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>≤2 h</td>
<td>659</td>
<td>47 (7.1)</td>
</tr>
<tr>
<td>&gt;2 to 3 h</td>
<td>132</td>
<td>27 (20.5)</td>
</tr>
<tr>
<td>&gt;3 h</td>
<td>111</td>
<td>38 (34.2)</td>
</tr>
<tr>
<td><strong>Postoperative SpO₂, %</strong></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>≥96</td>
<td>891</td>
<td>101 (11.3)</td>
</tr>
<tr>
<td>&lt;96</td>
<td>11</td>
<td>11 (100.0)</td>
</tr>
<tr>
<td><strong>Postoperative leucocyte, 10⁹/L</strong></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>&lt;4</td>
<td>6</td>
<td>2 (33.3)</td>
</tr>
<tr>
<td>4–10</td>
<td>665</td>
<td>47 (71)</td>
</tr>
<tr>
<td>&gt;10</td>
<td>231</td>
<td>63 (27.3)</td>
</tr>
<tr>
<td><strong>Postoperative anemia</strong></td>
<td></td>
<td>0.009</td>
</tr>
<tr>
<td>No</td>
<td>793</td>
<td>90 (11.3)</td>
</tr>
<tr>
<td>Yes</td>
<td>109</td>
<td>22 (20.2)</td>
</tr>
<tr>
<td><strong>Postoperative fasting blood glucose, mmol/L</strong></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>≤6.1</td>
<td>778</td>
<td>50 (6.4)</td>
</tr>
<tr>
<td>&gt;6.1</td>
<td>124</td>
<td>62 (50.0)</td>
</tr>
<tr>
<td><strong>Postoperative creatinine, μmol/L</strong></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>≤115</td>
<td>872</td>
<td>99 (11.4)</td>
</tr>
<tr>
<td>&gt;115</td>
<td>30</td>
<td>13 (43.3)</td>
</tr>
<tr>
<td><strong>Postoperative albumin, g/L</strong></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>&lt;35</td>
<td>244</td>
<td>85 (34.8)</td>
</tr>
<tr>
<td>≥35</td>
<td>658</td>
<td>27 (4.1)</td>
</tr>
<tr>
<td><strong>Postoperative ventilation</strong></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>No</td>
<td>878</td>
<td>97 (11.0)</td>
</tr>
<tr>
<td>Yes</td>
<td>24</td>
<td>15 (62.5)</td>
</tr>
<tr>
<td><strong>Postoperative vasoactive drug</strong></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>No</td>
<td>893</td>
<td>105 (11.8)</td>
</tr>
<tr>
<td>Yes</td>
<td>9</td>
<td>7 (77.8)</td>
</tr>
</tbody>
</table>

Smoking: defined as patients who smoked up to 1 year before surgery; Anemia: defined as hemoglobin <100 g/L. PPCs: postoperative pulmonary complications; ASA: American society of anesthesiologists; COPD: chronic obstructive pulmonary disease; SpO₂: saturation of peripheral oxygen; LOS: length of stay.

loss and duration of surgery; intraoperative blood loss and central venous catheter; intraoperative blood loss and blood transfusion; postoperative ventilation and vasoactive drug; pre- and intraoperative nasogastric tube; pre- and postoperative hemoglobin. Multivariable logistic regression indicated that 9 of those potential predictors were present in the final model. The raw and adjusted odds ratios for the nine variables are shown in Table 5, which also shows the brief predictive index derived from the β coefficient for each variable. This nine-variable regression model had good discrimination and calibration values in cohort 1 (c-statistic 0.91, 95% CI, and from 0.89 to 0.94). The ROC curves and the c-statistics for the validation subsamples (cohort 2) are shown in Figure 2. The brief predictive index developed in the present study has
Table 5: Independent predictors of risk factors for PPCs.

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>OR (95% CI)</th>
<th>β coefficient</th>
<th>Risk score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Respiratory infection in the last month</td>
<td>7.03 (1.66–29.80)</td>
<td>1.950</td>
<td>20</td>
</tr>
<tr>
<td>Smokers</td>
<td>2.37 (1.27–4.42)</td>
<td>0.861</td>
<td>9</td>
</tr>
<tr>
<td>Preoperative antibiotic use</td>
<td>0.238 (0.11–0.54)</td>
<td>–1.436</td>
<td>–14</td>
</tr>
<tr>
<td>Preoperative SpO2 &lt;96%</td>
<td>5.56 (2.38–12.98)</td>
<td>1.715</td>
<td>17</td>
</tr>
<tr>
<td>Surgery site</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peripheral</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abdominal</td>
<td>2.88 (1.49–5.59)</td>
<td>1.058</td>
<td>11</td>
</tr>
<tr>
<td>Intrathoracic</td>
<td>12.20 (4.61–32.28)</td>
<td>2.501</td>
<td>25</td>
</tr>
<tr>
<td>Intraoperative blood loss ≥100 mL</td>
<td>3.00 (1.64–5.50)</td>
<td>1.100</td>
<td>11</td>
</tr>
<tr>
<td>Postoperative fasting blood glucose &gt;6.1 mmol/L</td>
<td>2.60 (1.91–3.54)</td>
<td>0.956</td>
<td>10</td>
</tr>
<tr>
<td>Postoperative albumin &lt;35 g/L</td>
<td>4.21 (2.24–7.92)</td>
<td>1.438</td>
<td>14</td>
</tr>
<tr>
<td>Postoperative ventilation</td>
<td>7.20 (1.96–26.45)</td>
<td>1.975</td>
<td>20</td>
</tr>
</tbody>
</table>

PPCs: postoperative pulmonary complications; SpO2: saturation of peripheral oxygen.

Potential advantage in predicting PPCs in Chinese inpatients (better c-statistic than the risk index in the study of Canet et al.) [11]. The most relevant cut point was the score of 13 (sensitivity 90.2%, specificity 79.4%). Table 6 shows the incidence of PPCs by risk index score, the number of patients in each risk class, and the actual incidence of PPCs in cohort 1 and cohort 2.

3.4. Strategies to Reduce PPCs. Opportunities to reduce risk for PPCs occur throughout the perioperative period. Table 7 summarizes perioperative interventions that have been recommended to decrease the risk of PPCs [15, 16, 21, 22].

4. Discussion

This prospective study assessed the 9.7% incidence of PPCs after noncardiac surgery in Chinese university hospitals and found increased hospital LOS by 8 days in patients with PPCs than those without. Furthermore, we identified the perioperative risk factors of PPCs and developed a brief risk index for predicting PPCs in Chinese inpatients.

In the current study, the incidence of PPCs after noncardiac surgery and the hospital LOS in patients with PPCs were comparable to the incidence observed in some previous studies [11, 23–25]. The high incidence of PPCs and the increased hospital LOS indicating PPCs is also an important public health issue demanding nationwide attention in China. Unexpectedly, we found the postoperative mortality in patients with PPCs was lower than several previous results [3, 11]. This may be in association with the higher percentage of ASA class 1 to 2 patients and younger median age. Firstly, the medical resources in China were mainly concentrated in larger hospitals, especially the university hospitals and tertiary hospitals. So the patients always chose to go to large hospitals directly, even if they only have a cold or want to remove a small lipoma on body surface. Secondly, the percentage of emergency surgery in the present study was low, since none of the four studied hospitals had a major trauma center.

Nine independent risk factors were finally selected to participate in the brief predictive index for PPCs, including smoking, respiratory infection in the last month, antibiotic
Table 6: Distribution of PPCs risk index scores in patients.

<table>
<thead>
<tr>
<th>Risk class</th>
<th>Low risk (&lt;13 points)</th>
<th>Intermediate risk (13–30 points)</th>
<th>High risk (31–42 points)</th>
<th>Extremely high risk (&gt;42 points)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cohort 1, n (%)</td>
<td>672 (74.5)</td>
<td>127 (14.1)</td>
<td>67 (7.4)</td>
<td>36 (4.0)</td>
</tr>
<tr>
<td>Cohort 2, n (%)</td>
<td>577 (74.8)</td>
<td>110 (14.3)</td>
<td>54 (7.0)</td>
<td>30 (3.9)</td>
</tr>
<tr>
<td>Rate of PPCs in cohort 1, n (%)</td>
<td>21 (3.1)</td>
<td>22 (17.3)</td>
<td>38 (56.7)</td>
<td>31 (86.1)</td>
</tr>
<tr>
<td>Rate of PPCs in cohort 2, n (%)</td>
<td>5 (0.9)</td>
<td>17 (15.5)</td>
<td>12 (22.2)</td>
<td>17 (56.7)</td>
</tr>
</tbody>
</table>

PPCs: postoperative pulmonary complications.

Table 7: Interventions to reduce the risk of PPCs.

Preoperative interventions
- A careful history taking and physical examination
- Encourage cessation of smoking for at least 2 months
- Appropriate use of antibiotics and delay surgery if respiratory infection is present
- Recommend a regular exercise program (e.g., walking, upper limb exercises, swimming, pool exercises, etc.)
- Treat patients with established asthma with inhaled corticosteroids
- Treat patients with established COPD with regular bronchodilators

Intraoperative interventions
- Substitute less ambitious procedure for upper abdominal or thoracic surgery when possible
- Minimize blood loss
- Limit duration of surgery to less than 3 hr
- Whenever possible, use spinal or epidural anesthesia

Postoperative interventions
- Recommend regular lung expansion modalities such as deep breathing exercises
- Perform selective decompression of abdominal contents using nasogastric tube if patient is experiencing symptomatic gastric distension
- As soon as possible after surgery, have the patient sit up in a chair

PPCs: postoperative pulmonary complications; COPD: chronic obstructive pulmonary disease.

use, $\text{SpO}_2$, surgery site and blood lost, blood glucose, albumin, and ventilation.

Among the preoperative risk factors, smoking, a history of respiratory infection in the last month, $\text{SpO}_2$, and antibiotic use are strong PPC risk factors. Smoking and respiratory infection may lead to local changes in airway reactivity, pulmonary function, and residual impairment of immunity, which could increase the risk of PPCs for several folds [6, 7]. $\text{SpO}_2$ is an easily recorded objective measure, which reflects the respiratory function as well as cardiovascular functional status [11]. Antibiotic use is a protective factor in the current cohort. It seems to be helpful to use prophylactic antibiotic, especially in these high-risk patients.

The intraoperative risk factors were identified in the present study including surgery site and blood loss, which were similar to many previous studies [4, 7, 14, 26]. Thoracic and abdominal surgeries are always performed via a large painful incision associated with obviously changes in lung compliance and functional residual capacity. As a result, the incidence of PPCs in these surgeries is much higher than others. Significant intraoperative blood loss gives rise to haemodynamic instability and relative ischaemia and so causes ischaemia-reperfusion injury, which can lead to organ dysfunction.

Factors in early stage of postoperative may have a better performance in predicting PPCs. We found postoperative mechanical ventilation, albumin, and blood glucose could increase the risk of PPCs. Among them, ventilation is the most important risk factor for PPCs. Researchers found an increased risk of respiratory events in patients with ventilation [26, 27]. It is important to follow the guidelines for the management of ventilation and the prevention of nosocomial infection.

A brief risk score based on these factors was calculated to predict the incidence of PPCs. In Spain, Canet and coworkers also developed a similar risk index of PPCs [11]. Three of these risk factors (preoperative $\text{SpO}_2$, respiratory infection in the last month, and surgery site) were also identified in the current study. What is more, the brief predictive index developed in the present study has potential advantage in predicting PPCs in Chinese inpatients (better $c$-statistic than the risk index of Canet). This disparity may be due to the different geographic areas, ethnic, cultural, socioeconomic, and political differences between China and Spain. In fact,
the risk index developed by Canet also performed differently between the Western Europe sample and the Eastern Europe sample [20]. In general, the current brief predictive index predicted the risk of PPCs well in both the development and validation cohorts. These findings suggest patients with high risk of PPCs should be closely monitored and early intervened if the risks factors could be modified.

Several limitations in our study should be acknowledged. First of all, only four university hospitals participated. This may result in some bias. Further studies targeting general Chinese hospitals with a larger population are still needed. Another limitation is that the 7-day study period was arbitrarily decided. All patients were enrolled in August. Englesbe et al. demonstrated a significant seasonal variation in surgical morbidity and mortality [28]. They found a dramatic worsening of surgical mortality in July, which was attributed to the influx of inexperienced trainees. However, Ehler and colleagues refuted the “July Phenomenon” in a larger population [29]. In most hospitals in China, July is marked by an influx of intern. Instead of managing patients directly, they only do the paperwork. Therefore, they may have little effect on the present results. Further study is needed to confirm the impact of seasonal variation in surgical morbidity and mortality.

5. Conclusion

The present prospective, multicenter study found there was high incidence of PPCs which increased hospital LOS in noncardiac surgical inpatients in China. We identified nine objective and easily assessed factors associated with the occurrence of PPCs. A simple risk index based on these factors predicted the development of PPCs. This brief predictive index may be useful for clinicians in estimating patients’ risk for PPCs and guide individualized perioperative respiratory care.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

This research was supported by National Natural Science Foundation of China (no. 81301652) and Zhejiang Provincial Program for the Cultivation of High-level Innovative Health Talents.

References


Review Article

Role of Circulating Lymphocytes in Patients with Sepsis

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Received 23 February 2014; Revised 15 July 2014; Accepted 29 July 2014; Published 28 August 2014

Academic Editor: Baoli Cheng

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Sepsis is a systemic inflammatory response syndrome due to infection. The incidence rate is estimated to be up to 19 million cases worldwide per year and the number of cases is rising. Infection triggers a complex and prolonged host response, in which both the innate and adaptive immune response are involved. The disturbance of immune system cells plays a key role in the induction of abnormal levels of immunoregulatory molecules. Furthermore, the involvement of effector immune system cells also impairs the host response to the infective agents and tissue damage. Recently, postmortem studies of patients who died of sepsis have provided important insights into why septic patients die and showed an extensive depletion of CD4 and CD8 lymphocytes and they found that circulating blood cells showed similar findings. Thus, the knowledge of the characterization of circulating lymphocyte abnormalities is relevant for the understanding of the sepsis pathophysiology. In addition, monitoring the immune response in sepsis, including circulating lymphocyte subsets count, appears to be potential biomarker for predicting the clinical outcome of the patient. This paper analyzes the lymphocyte involvement and dysfunction found in patients with sepsis and new opportunities to prevent sepsis and guide therapeutic intervention have been revealed.

1. Introduction

Sepsis is a systemic inflammatory response that occurs during infection [1]. Septic shock is the leading cause of multiple organ failure and death in intensive care units, and the incidence is increasing worldwide [2–4]. The pathogenesis of sepsis is a result of a complex network of events involving immune-inflammatory and anti-inflammatory processes triggered by the infection agent [5]. This host response is complex and variable, in which both proinflammatory and anti-inflammatory mechanisms can contribute to either clearance of infection and tissue recovery or organ injury. Early and appropriate intervention is critical for improving the patient’s outcome, reducing morbidity and mortality [6]. It is generally accepted that the clinical strategy for improving the outcome of sepsis patients includes the advancement in the knowledge of the pathogenesis of this syndrome as well as the identification of biomarkers to establish risk assessment, predicting the development of individual or multiple organ dysfunctions, guiding antimicrobial therapy, and establishing new and individualized treatments.

Sepsis is initiated when the host responds to pathogen insult. The first line of defenses is constituted by the innate immune system response. Several effector cells are involved in this antimicrobial response including different leukocyte populations. Monocyte-macrophage cells and dendritic cells play a key role in the innate immune response. These cells have the ability to phagocytose bacteria and interact with their products through an interaction with their pattern-recognition receptors. These activated phagocytic cells release proinflammatory mediators, such as cytokines, chemokines, lipid mediators, nitric oxide, and oxygen radicals [7–9]. Activated neutrophils also promote clearance of bacteria, and they subsequently contribute to tissue inflammation and injury through respiratory burst, cytotoxicity, degranulation, increased vascular permeability, and organ injury by releasing several proinflammatory mediators, myeloperoxidases, and proteases [10]. Thus, a “cytokine storm” is generated, which is
responsible for triggering the inflammation. But the immune system, including cells of the adaptive immune response, may also harbor humoral and cellular mechanisms that attenuate the potentially harmful effects of the proinflammatory response. However, the release of anti-inflammatory cytokines also appears to be exacerbated, as illustrated by the strong relationship between high levels of these mediators and poor outcome [11]. Indeed, our group and other researchers have described that an early response to continuously elevated anti-inflammatory cytokine serum levels was better predictor of mortality than the classic proinflammatory cytokines in patients with septic shock [12]. It is important to remark that this compensatory anti-inflammatory response syndrome named CARS is a two-wave process that follows SIRS (systemic inflammatory response syndrome) in experimental animals, but in most of patients both events are concomitant, [13] and it is often found when patients are admitted to the ICU [12]. Furthermore, the adaptive immune response has a relevant role to control of bacterial infection [14]. Adaptive immunity is driven by innate immune cells through sensing microorganisms and presenting antigens in the context of major histocompatibility complex class II (MHC class II) and costimulatory molecules. The recent discovery of subsets of lymphocytes that are defined by their limited antigen receptor variability and are restricted to specific tissue may prove a link between immune activation and antibacterial defense during sepsis [15]. “Innate lymphocytes” are defined by their limited antigen receptor variability, and, therefore, these T cells have a memory phenotype in the absence of deliberate immunization [16]. The innate-like lymphocytes include natural killer T cells, gamma delta T cells, and mucosal-associated invariant T (MAIT) cells. MAIT cells are already primed to gastrointestinal flora and work in cooperation with the innate response to stave off infections [15]. Furthermore, T lymphocytes play a critical role in the regulation of antimicrobial phagocytic and cytotoxic activity of the innate immune response cells [17]. Interferon (IFN)-γ and granulocyte macrophage colony stimulating factor (GM-CSF), mainly produced by T lymphocytes, increase this defensive activity but other cytokines such as interleukin (IL)-10 have inhibitory effects [17, 18]. B lymphocyte response also plays an important role in the defensive host response. B cells produce cytokines, present antigens to T lymphocyte, and differentiate into antibody producing cells [19]. Antibodies bound to bacteria may increase bacteria opsonization and favor phagocytosis [17]. However, abnormal bacterial induced activation of T and B cells may be followed by inflammation and endothelial and tissue damage [20, 21].

Blood lymphocyte dysfunction during sepsis has long been recognized with significant lymphopenia and decreased lymphocyte T CD4+, CD8+, and natural killer (NK) cells [5]. However, recently a renewed interest in lymphocyte dysfunction during sepsis emerged from studies demonstrating that immunosuppression was present not only in peripheral blood cells but also locally in organs in patients who died of sepsis [22].

In this review, we try to highlight the role of the main populations of blood lymphocytes in sepsis and we discuss how different kinetic patterns of lymphocyte subsets are involved and their relationship to the surviving outcome. This knowledge in the future may have important therapeutic implications for patients with septic shock. Furthermore, circulating lymphocyte abnormalities might have also potential prognostic biomarker signification.

2. Lymphopenia and Anergy

B- and T-lymphopenia is a hallmark of sepsis that can be mimicked in human volunteers receiving a bolus of lipopolysaccharide [11]. Extensive lymphocyte apoptosis is seen in animal models of sepsis and in patients with sepsis [23]. In a model of cecal ligation and puncture, prevention of lymphocyte apoptosis with caspase inhibitors results in a marked improvement in animal surviving [24]. Other studies on animals also suggest that immune depression resulting from the loss of lymphocytes may be the key factor in inability to survive sepsis [24–27].

Anergy is a tolerance mechanism in which the lymphocyte is intrinsically functionally inactivated following an antigen encounter, but it remains alive for an extended period of time in a hyporesponsive state [5]. T-cell anergy relates to a decreased proliferation to mitogen stimulation, a shift toward a TH2 profile of cytokine secretion, an increased apoptosis, and an increased percentage of CD4+CD25+ regulatory T lymphocytes (Tregs) [28]. The occurrence of a state of lymphocyte anergy has been described in patients with major trauma or burns, associated with mortality rate and with the development of secondary septic complications [5, 29]. Meakins et al. described that surgical patients who had failure of delayed hypersensitivity response had an increased risk of sepsis and related mortality [30]. Therefore, defective T-cell proliferation and secretion of IL-2 and TNF correlated with sepsis mortality [31].

3. T Lymphocytes

T-cell compartment plays a critical role in regulating the effector stage of the immune response. CD3+CD4+ T lymphocytes or T helper (TH1) cells are mainly involved in the regulation of the immune response [32].

It has been recognized that different CD4+ T cells subsets play a critical role in response to microbial challenges. The first subsets recognized were denoted by TH1 and TH2 cells based on the selective production of 2 cytokines, IFN-γ and IL-4, respectively [33]. TH1 cytokines exert a positive feedback on antigen-presenting cells, whereas TH2 cytokines promote downregulation of the immune response. This TH1 paradigm was reasonably useful for the initial categorization of mechanisms involving elimination of microbial pathogens. Previous works showed a shift from TH1 to TH2 cytokine profiles following severe aggression such as trauma, burns, cardiac arrest, and infection [14, 34].

It has been proposed that the lack of a shift from TH1 to TH2 response increases survival among patients with sepsis [35]. Reductions in circulating CD4+ T-lymphocytes and their shift to a TH2 phenotype characterize aspects of sepsis-induced immunosuppression [36]. The associations between
complicated clinical course and unfavorable prognosis of septic patients with the decline of peripheral blood CD4+ T-lymphocytes were established in a majority of trauma victims or surgical patients with secondary sepsis [37, 38]. We have described that T lymphopenia found in patients with septic shock persisted during the first week of follow-up in the intensive care unit (ICU) and was independent of the outcome [39]. These findings are consistent with reports demonstrating significant lymphopenia early in the course of disease [40, 41]. However, at the end of the second week of follow-up, we observed that the absolute number of circulating CD3+CD4+ T cells had clearly normalized in surviving patients with septic shock [36].

Nowadays, we know that the opportunities for TH9 lymphocytes diversity are far greater than just TH1 and TH2 profiles. The newly described TH9 cell subsets include TH17, TH9, and TH12 cells; follicular helper T (Tfh) cells; and different types of regulatory T (Treg) cells [42]. TH17 has relatively recently been characterized as an IL-17-producing subset of CD4+ T cells. Naive CD4+ T cells, in the presence of IL-6, IL-21, or TGF-beta, can proceed to a TH17 phenotype [43, 44]. Their proliferation and differentiation are supported by IL-23 and IL-1 secreted from antigen-presenting cells [43, 45]. Once differentiated, TH17 cells are capable of producing not only IL-17, but also IL-21, IL-22, TNF-α, and IFN-γ [46–48], suggesting plasticity of these cells, with an ability to produce different cytokines depending upon environmental stimulus [46, 48]. IL-17 plays a major role in linking adaptive and innate immune responses. IL-17 is a potent proinflammatory cytokine which induces the production of many other proinflammatory cytokines, chemokines, and other inflammation mediators such as prostaglandin E2 and nitric oxide [49]. Information about its role in human sepsis is scarce [14]. TH17 cells contribute to host defense against extracellular bacteria, such as *Staphylococcus aureus* and *Klebsiella pneumoniae* as well as fungi [50, 51]. In humans, TH17 lymphocyte count on day 1 and after 6 days in survivors with severe sepsis was higher than that in nonsurvivors [52]. Salomao et al. observed an increased proportion of CD4+ lymphocytes producing IL-17 in patients with sepsis [14]. Thus, TH17 differentiation appears to contribute significantly to the surviving in patients with severe sepsis, and it represents one exception in the overall downregulation of T-cell immune functions in these patients.

Treg is one of the T-cell subsets that have strong immunosuppressive activity, playing an essential role in controlling both adaptive and innate immune responses. These cells can downregulate effector activities mediated by CD4+ T cells, CD8+ T cells, NK cells, and also dendritic cells and B cells [37, 52–57]. Recently, Wu et al. found that the circulating Treg lymphocyte counts on day 1 were higher in surviving patients with severe sepsis than those in nonsurviving ones [35]. This finding confirms results found by other authors [58, 59]. The relative increase in circulating Treg might play a role in lymphocyte anergy described after septic shock [60]. This, altogether, strongly suggests that Treg cells not only represent a reliable marker of immunoparalysis in sepsis, but also may play an important role in its pathogenesis.

CD8+ T lymphocytes are effector cytotoxic cells. We have found a decrease of CD8+ T lymphocytes in patients with septic shock at ICU admission [36]. In survivors, CD8+ T lymphocytes showed a further drop on day 3 of followup, followed by a gradual recovery although numbers failed to reach the count recorded in healthy controls. Importantly, CD3+CD8+ T lymphocyte count in survivors was significantly diminished with respect to nonsurvivors on day 3. A drop in circulating CD3+CD8+ T cells has been described by other authors [52, 54, 61–63].

CD45 is essential in T-cell differentiation and antigen receptor signaling [64]. When inflammatory agents activate non-effector CD45RA+CD45RO− T lymphocytes, such as bacterial infection, the isoform CD45RO is upregulated and CD45RA is downregulated [65]. CD28 is a costimulatory molecule that plays a key role in regulating the activation and surviving of T lymphocytes [66–68]. It has been reported that patients with severe sepsis showed a significant reduction in T lymphocyte CD28 expression [69]. The migration of circulating T lymphocytes to peripheral lymph nodes depends on the expression of the CD62L homing receptor [70]. We observed downregulation of L-Selectin expression on CD3+CD8+ cells in patients with septic shock and it was associated with a better prognosis. When we analyzed the phenotype of the circulating CD3+CD8+ T cells according to the activation criteria in patients with septic shock at ICU admission, all patients showed low count of CD3+CD8+CD45RA+CD45RO− T lymphocytes (naive cells), and survivors also have low CD3+CD8+CD45RA−CD45RO+ lymphocytes (memory cells) at day 3 of the follow-up, associated with a lower count of CD3+CD8+CD28+ T lymphocytes. Furthermore, survivors also show lower count of CD3+CD8+CD62L+ T lymphocytes. These findings may suggest that the rapid migration of activated CD8+ T cells to peripheral lymph nodes may be a mechanism contributing to patient survival and, therefore, delayed tissue response could determine the failure of the immune system in patients with the worst outcome [36]. It is known that cellular immune responses play a critical role in the defense against infections and strong T-cell responses have been reported in patients who clear infection [71].

As we have described above, the innate-like lymphocytes include natural killer T (NKT) cells, gamma delta T (γδ-T) cells, and MAIT cells. The main characteristic of these cells is their limited antigen receptor variability, and, therefore, these T cells have a memory phenotype in the absence of deliberate immunization [16]. NKT cells are activationally restricted by the MHC class I-like molecule called CD1d [72]. NKT cells are potent producers of proinflammatory mediators such as IFN-γ; they are capable of activating macrophages, NK cells, dendritic cells, and effector T cells and possess cytotoxic effector activity [73]. Altogether, they have been thought to be significant promoters of the dysregulated septic response [74]. Recently, Heffernan et al. have demonstrated that invariant NKT (iNKT) cells, a type of NKT cells that express an invariant Vα24/δι8 chain and a restricted β chain [72], are increased in sepsis and this is most pronounced in geriatric nonsurviving patients [75]. However, Grimaldi et al.
did not observe any quantitative changes in circulating NKT cells in critically ill patients with severe infections [76].

γδ-T cells are preferentially localized in mucosal organs containing epithelia and are known to regulate macrophages [77]. Circulating γδ-T cells count is reduced in patients with sepsis [78, 79], and this reduction seems to become more intense as the septic process becomes more severe [80]. Thus, these studies suggest the key role of γδ-T cells in the defense against infection and open up the possibility of initial explorations of new therapeutic strategies [80].

MAIT cells have the ability to be activated in the presence of antigen-presenting cells infected with Gram-positive (except streptococcal and enterococcal bacteria), Gram-negative bacteria and yeasts [81]. They display fast activation upon microbial infection and rapidly express effector mechanisms including high amounts of proinflammatory cytokines production such as INF-γ and IL-17. A recent study by Grimaldi et al. showed an early and marked decrease in MAIT cell counts in patients with severe sepsis and a relationship between this reduction throughout the first 4 days of ICU admission and the development of ICU-acquired infections [76]. These findings suggest that MAIT cells are involved in sepsis-induced immunosuppression. Therefore, the understanding of innate-like lymphocytes may be crucial for the development of potential therapies to restore immune system function in patients with sepsis.

4. Natural Killer (NK) Cells

Recently, several works have highlighted a key role of natural killer cells during sepsis [82, 83]. NK cells have effector cytotoxic activities and immunoregulatory functions such as the production of cytokines such as IFN-γ, TNF-α, and granulocyte-macrophage colony-stimulating factor (GM-CSF) [84, 85]. NK cells are also engaged in crosstalks with other immune cells, such as dendritic cells [86], monocytes, macrophages [87], and neutrophils [88]. Furthermore, NK cells are probably directly involved in the antibacterial response of the innate immune system due to their capacity to recognize pathogen-associated molecular patterns [89]. It is possible that all NK cell subsets are not equivalent in their antibacterial activity [90].

In sepsis, severe lymphopenia also affects circulating NK cells [38, 82, 83]. Andaluz-Ojeda et al. have reported that patients with the highest NK cell number had the lowest probability to survive [83]. However, we do not find higher percentages of NK cells in nonsurviving patients with septic shock in agreement with other authors [82, 91, 92]. Sepsis is also associated with an activation of NK cells. CD69 is rapidly induced in NK cells and its role in NK cytotoxic has been demonstrated in humans [93]. An increase in the counts and percentage of the CD3−CD56+CD69+ cells in nonsurvivors at ICU admission and 48 hours later has been shown [82]. We also found a higher percentage of the expression of CD57, a marker of long-lived and highly differentiated effector NK cells, in patients with septic shock who died. Our data demonstrate that surviving patients with septic shock exhibited more NK cells depletion than nonsurviving ones and that these NK cells are early activated and rapidly differentiated in patients with septic shock.

Functions of circulating NK cells in critically ill patients have been poorly studied. Unexpectedly, and in apparent contradiction with murine data [94–100], Forel et al. found that patients with sepsis exhibited decreased production of IFN-γ, especially those who presented with septic shock [92].

5. B Cells

Classically, B lymphocytes are characterized by their ability to differentiate into immunoglobulin secreting plasma cells. However, B cells also play critical immunoregulatory roles as antigen presenting cells and also as cytokine producing cells. Currently, it is accepted that B cells have an important role in both adaptive and innate immune responses [101]. During the immune response against infectious agents, the production of antibodies by antigen activated B lymphocyte clones is critical for the efficient eradication of many agents. B cells may also act as effective antigen presenting cells of the microorganism antigens to T lymphocytes [102]. Furthermore, the interaction of several bacterial products with B cells may also cause their activation and cytokine secretory function [101]. Interestingly, the activation of B cells by microorganisms takes place not only by antigen recognition, but also through the activation of Toll-like receptors (TLR). Dual antigen-specific B-cell receptors (BCR) and TLR engagement can fine-tune functional B-cell responses, directly linking cell-intrinsic innate and adaptive immune responses [103]. Moreover, it has been demonstrated that B cells also release a broad variety of cytokines. Pivotal to B cells is IL-10 production, which inhibits proinflammatory cytokines and restrains the excessive inflammatory responses that occur during autoimmune diseases or that can be caused by unresolved infections [104, 105].

The role of B lymphocytes in the pathogenesis of sepsis has not been established. Recently, it has been proposed that B cells are involved in the early innate immune response during experimental bacterial sepsis [101] and Darton et al. reported that adults who have recovered from an episode of invasive pneumococcal disease demonstrate defective B-cell activation [106]. More recently, Rauch et al. have demonstrated that innate response activator B cells (IRA-B cells) play a critical role in the response to sepsis, as mice lacking B-cell-derived GM-CSF are unable to clear bacteria, elicit exaggerated inflammatory responses, and, more likely, succumb to infection [107]. Moreover, they have already developed an in vitro system to expand IRA-B cells from their precursors and then inject them into the patient to boost their immune response [108]. Thus, B cells appear to play a role in the sepsis immunodisturbance [109] and one may expect that, by restoring their function, the overall immune response could be improved.

We have reported that patients with septic shock suffer from a severe retraction of peripheral blood B lymphocytes [110]. Furthermore, circulating B cells show increased expression of CD95 antigen. As described in T cells [111], the increased expression of CD95 on B cells from patients with septic shock might contribute to the observed reduction
of circulating B cells in these patients. Several studies have shown an inverse correlation between lymphocyte count and survival [23, 111]. We also found higher percentage of CD95 expression on B cells from nonsurvivors than that from survivors [110].

The retraction of circulating B cells affects heterogeneously the different B-cell subsets in patients with septic shock [100]. In patients with septic shock, the numbers of circulating CD19+CD69+ remain normal, but CD19+CD23+ B lymphocytes are clearly decreased. Furthermore, higher percentages of circulating CD19+CD23+ are associated with better clinical outcome of the patients [110]. CD23 is involved in different regulatory functions such as enhancing antigen presentation, improving B-cell differentiation, and growth [112]. Some authors have reported that CD23 is expressed on activated B cells whereas others have suggested that peripheral blood CD23 B cells resemble classic memory cells [112].

CD80 and CD86 are critical molecules in the B-cell antigen presentation function. In murine studies of sepsis, an important role for CD80 and CD86 antigens in the response to sepsis has been established [113, 114]. Our results showed higher percentage of CD86 expression on B cells from patients with septic shock. Furthermore, at ICU admission, nonsurvivors had more elevated percentages of CD19+CD80+ B cells than those found in survivors [110].

6. Peripheral Blood Lymphocytes as Biomarkers in Sepsis

Leukocyte phenotyping might also have a predictive value for the development of immune-supportive or immunostimulatory therapies in the management of septic shock patients [115, 116]. We have also studied the predicting value for the outcome of combining different T-cell, B-cell, and NK cell markers in patients with septic shock. According to cytomics methodology [117], we have found a set of five immunophenotypic variables (CD3+CD8+CD28+, CD3+CD8+CD45RA+CD45RO−, CD19+CD80+, CD56+CD69+, and CD3+CD11A br+CD11B+ lymphocyte subsets) which are able to improve the prediction for outcome in septic shock patients to a sensitivity of 94% and a specificity of 100% [118].

7. Therapeutic Approaches for Restoring Lymphocyte Functions

Despite the extraordinary developments in understanding the immunopathology of sepsis, advances in immunotherapy have been very disappointing. Recognizing the pivotal role of lymphocytes in orchestrating the body’s immune response against infection, based on their ability to interact with cells of innate and adaptive immune system, it might be possible to decrease mortality in sepsis by therapies to augment host immune response through restoring lymphocytes function.

Therapeutic strategies for treating lymphocyte alterations in patients with sepsis should include the restoration of lymphocyte count and function or the blockade of inhibitory signals (Figure 1). Currently, the most promising immunotherapeutic agent is purified interleukin (IL)-7. IL-7 is an essential cytokine that affects both T and B cells and induce T lymphocyte development, survival, expansion, and maturation in humans [119]. In experimental models of sepsis, IL-7 treatment increased the production of CD4 and CD8 cells, restored delayed type hypersensitivity responses, blocked lymphocyte apoptosis, reversed the impaired IFN-γ production leading to macrophage activation, increased expression of cell-adhesion molecules leading to improved T cells recruitment to sites of infection, and increased T-cell receptor diversity leading to more potent immunity against pathogens [120]. Furthermore, IL-7 can mediate the crosstalk between T_H11 and T_H17 lymphocytes during sepsis such that neutrophil recruitment and bacterial clearance are improved [121].

IL-15 is also a pleiotropic cytokine having promising results in experimental models of sepsis. The administration of IL-15 improved survival in two different models of sepsis and was associated with an increase in lymphocyte survival, decreased apoptosis of NK cells, dendritic cells, and T cells, and increased IFN-γ secretion [122].

Fms-like tyrosine kinase 3 ligand is a cytokine capable of enhancing the sensitivity of antigen-specific B and T-cell responses upon bacterial challenge, and it would be another potential treatment in infectious disease [123].

The development of lymphocyte apoptosis is markedly more increased in septic patients than in critically ill nonseptic controls [23]. Strategies to block programmed cell death in lymphocytes are suggested to be beneficial in sepsis [119]. Also, amplified antiapoptotic signals might be of therapeutic value. A genetically manipulated T-cell resistant to apoptosis and polyclonal for a variety of pathogens could be transfused during immune dysfunction to restore patient immunity. For instance, mice transfected with the human gene Bcl-2 were protected from death after cecal ligation and puncture [24]. Moreover, the transfer of T cells from Bcl-2 overexpressing mice into wild type septic mice also improved survival [124]. More recently, cell penetrating peptides (CPPs) have been used to deliver the antiapoptotic Bcl-xl-derived BH4 peptide to prevent injury-induced apoptosis both in vitro and in vivo [125]. Furthermore, administration of ritonavir, a HIV protease inhibitor which is known to prevent apoptosis in vitro, improved survival in mice with sepsis [126].

The development of blocking antibodies to multiple inhibitory receptors involved in sepsis represents another innovative therapeutic strategy. PD-1 (programmed death 1) is a negative costimulatory molecule expressed on immune effector cells. It is upregulated in sepsis and impairs immunity by inducing apoptosis, increasing production of IL-10, preventing T-cell proliferation, and causing T-cell exhaustion. Guignant et al. showed that PD-1 overexpression on circulating T cells from patients with sepsis, correlated with decreased T-cell proliferation, increased secondary nosocomial infections and mortality [127]. In animal models of bacterial and fungal sepsis, blockade of the PD-1 pathway improves survival [128–130]. Additional receptors associated with cell exhaustion such as BTLA, TIM-3, LAG-3, and CTLA-4 may be good potential therapeutic targets [131].
Figure 1: Therapeutics approaches to counteract apoptosis and recovery lymphocyte functions. Based on lymphocyte alterations described in this review, two main therapeutic strategies must be taken into account in patients with sepsis: to block lymphocyte apoptosis for recovery of lymphocyte count or to restore effector lymphocyte functions. Abbreviations: IL: interleukin; IFN: interferon; Bcl-2: B-cell lymphoma 2 gen; PD-1: programmed cell death protein 1; BTLA: B- and T-lymphocyte attenuator; TIM-3: T-cell immunoglobulin and mucin protein 3; CTLA-4: cytotoxic T-lymphocyte-associated protein 4; LAG-3: lymphocyte activation gene 3.

Table 1: Main lymphocyte types' alterations in sepsis.

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<th>Lymphocyte type</th>
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Abbreviations: T\(^{1H}\): T helper; IL: interleukin; TNF: tumor necrosis factor; IFN: interferon.
IFN-γ plays a pivotal role in regulating the adaptive immune response mediated by T-lymphocytes and dendritic cells and in controlling the NK and phagocytic cells of the innate host immune antimicrobial defense system. Our group showed that surviving patients exhibited significantly higher levels of IFN-γ than the healthy controls during the first 14 days of monitoring [12]. Thus, it could be that the increased circulating IFN-γ levels noted here in survivors might be linked to a better immune response against the microorganisms causing septic shock. Döcke et al. treated patients with sepsis showing low monocyte HLA-DR expression with IFN-γ and observed the recovery of the deficient HLA-DR expression. Clearance of sepsis was achieved in eight out of nine patients. IFN-γ is a critical immunoregulatory cytokine [132].

IL-12 is a cytokine that induces expression of the TH1 lymphocyte phenotype [133]. IL-12 increased survival in an animal model of burn injury after a septic challenge. It acts, at least in part, through IFN-gamma [134]. On the other hand, thymosin alpha-1 (Tα1) is a molecule with known immunostimulating properties [135], and it can induce T-cell and dendritic cell maturation as well as increasing IL-12 expression. Wu et al. showed a reduction in 28-day mortalities in patients with severe sepsis with an associated increase in mHLA-DR [136].

In this review, we have described the main changes in circulating lymphocytes from patients with sepsis (Table 1). Do not forget that these cells perform their function in peripheral tissues. Boomer studied postmortem spleen and lung tissue from patients with sepsis who died in ICU and they found that circulating blood cells showed similar findings to those in previous studies [22]. However, the analysis of circulating lymphocytes to be clinically relevant has to be confirmed in a large-size population, with standardized methods, [133] and finally—and most importantly—any targeted therapeutic intervention to restore immune system function must be demonstrated in large randomized studies.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

This work was partially funded by Grants from Comunidad de Madrid, Spain, MITIC-CM (S-2010/BMD-2502) and Instituto de Salud Carlos III, Spain, MEC (PI051871, CIBERehd).

References


Research Article

Agmatine Protects against Zymosan-Induced Acute Lung Injury in Mice by Inhibiting NF-κB-Mediated Inflammatory Response

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Received 16 February 2014; Revised 8 July 2014; Accepted 29 July 2014; Published 27 August 2014

Academic Editor: Baoli Cheng

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Acute lung injury (ALI) is characterized by overwhelming lung inflammation and anti-inflammation treatment is proposed to be a therapeutic strategy for ALI. Agmatine, a cationic polyamine formed by decarboxylation of L-arginine, is an endogenous neuromodulator that plays protective roles in diverse central nervous system (CNS) disorders. Consistent with its neuromodulatory and neuroprotective properties, agmatine has been reported to have beneficial effects on depression, anxiety, hypoxic ischemia, Parkinson’s disease, and gastric disorder. In this study, we tested the effect of agmatine on the lung inflammation induced by Zymosan (ZYM) challenge in mice. We found that agmatine treatment relieved ZYM-induced acute lung injury, as evidenced by the reduced histological scores, wet/dry weight ratio, and myeloperoxidase activity in the lung tissue. This was accompanied by reduced levels of TNF-α, IL-1β, and IL-6 in lung and bronchoalveolar lavage fluid and decreased iNOS expression in lung. Furthermore, agmatine inhibited the phosphorylation and degradation of IκB and subsequently blocked the activation of nuclear factor (NF)-κB induced by Zymosan. Taken together, our results showed that agmatine treatment inhibited NF-κB signaling in lungs and protected mice against ALI induced by Zymosan, suggesting agmatine may be a potential safe and effective approach for the treatment of ALI.

1. Introduction

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS), which is the severest form of injury, are the leading causes of morbidity and mortality in critically ill patients [1]. ALI is characterized by the development of hypoxemia, damage to the alveolar capillary membrane barrier, pulmonary edema, and the resultant respiratory failure [2]. Current therapeutic strategy includes protective ventilation and supportive fluid conservative [3]. Although extensive studies about the pathogenesis of ALI have been conducted, the mortality of ALI remains very high [4]. Thus, it is critical to explore the innovative therapies and effective medications for ALI.

Among the animal models established for the investigation of mechanism involved in ALI, intraperitoneal injection of Zymosan (ZYM) is one of the most commonly used models of ALI [5]. Zymosan is a substance derived from the cell wall of the yeast Saccharomyces cerevisiae. When injected into animals, ZYM induces inflammation by a series of mechanisms. Reports show that the onset of ZYM-induced inflammatory response in mouse lung is associated with the gas exchange barrier and that it culminates with maximal neutrophil accumulation, exudate formation, and proinflammatory cytokines production [6, 7]. ZYM is recognized by toll-like receptor 2 (TLR-2) on immune cells (e.g., neutrophils), which subsequently trigger signal cascade for nuclear factor-κB (NF-κB) activation [8]. NF-κB activation is required for maximal expression of many proinflammatory cytokines and chemokines and iNOS involved in the pathogenesis of acute lung injury [9].

Agmatine, a biogenic amine formed by arginine decarboxylation, is widely but unevenly distributed in mammalian tissues. Agmatine has been reported to have various biological actions. It attenuates morphine withdrawal syndromes, inhibits inducible nitric oxide synthase (NOS), and
contributes to polyamine homeostasis [10]. Additionally it is known to exert antidepressant, anxiolytic, antitumor cell proliferative and anticonvulsive effects [11]. However, the precise working mechanisms of agmatine are not yet fully understood. The aim of our study was to investigate the protective effects of agmatine on Zymosan-induced acute lung injury and to assess its relative mechanisms.

2. Materials and Methods

2.1. Reagents. Zymosan and agmatine were obtained from Sigma-Aldrich. Antibodies against iNOS, NF-κB p65, IκB-α, and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and those against phosphor (p)-IκB and NF-κB p65 were from Cell Signaling Tech (Danvers, MA). Mouse TNF-α, IL-1β, and IL-6 enzyme-lined immunosorbent assay (ELISA) kits were purchased from Boster Biotechnology (Wuhan, China). All suspensions were freshly made before use.

2.2. Animals and Treatments. Male C57Bl/6 mice (weighing 18–22 g) were used in this study. Animal procedures were approved by the Ethics Committee for Animal Experimentation of Third Military Medical University. An inflammation-associated lung injury model was established by aseptic intraperitoneally (IP) injection of ZYM (30 mg/mL suspended in normal Saline (NS)) into mice, at a dose of 600 mg/kg of body weight, as previously described [12]. The same volume of NS was injected through the same route as the sham control.

2.3. Histologic Examination. Lungs were harvested for observing morphologic alterations at 24 hrs after ZYM or NS administration. The subjects were fixed with 10% formalin for 8 hrs at room temperature, embedded in paraffin, and sectioned at 5 μm thickness. After deparaffinization and rehydration, the sections were sequentially stained with hematoxylin and eosin. Histologic changes were evaluated by two independent pathologists, who had no knowledge of the treatment regimen received by each respective animal. The degree of lung injury was scored on a subjective scale ranging from 0 to 3; 0 = absence, 1 = mild, 2 = moderate, and 3 = severe. The ranging scale was used for each of histologic features: congestion, edema, inflammation, and hemorrhage. The final score will be the adding of the single evaluation.

2.4. Wet/Dry Weight Ratio. To quantify the magnitude of pulmonary edema, we evaluated lung wet/dry (W/D) weight ratio at 24 hrs after NS or ZYM administration. The harvested wet lung was weighed and then placed in an oven for 24 hrs at 80°C and weighed when it was dried. The ratio of wet lung to dry lung was calculated [13].

2.5. BALF Collection. At 24 hrs after administration of ZYM or NS, BALF collection was performed by the methods described previously [14]. The mice were anesthetized with pentobarbital, tracheas were cannulated after exsanguination, and lungs were gently washed with 2 mL of PBS. The amount of exudate was calculated by subtracting the volume injected (2 mL) from the total volume recovered. BALF samples were centrifuged at 500 g at 4°C for 12 mins, and the supernatant was stored at −70°C for subsequent analysis of protein and cytokine levels.

2.6. Measurement of Lung MPO Activity. Myeloperoxidase (MPO) activity was measured as an indicator of neutrophil infiltration into the lung tissue as previously described [15]. At 24 hrs after ZYM or NS injection, all animals (n = 8 for each group) were sacrificed with pentobarbital. Lungs were obtained and perfused with cold PBS to remove all blood, and homogenated lung supernatants were prepared to detect the activity of MPO. MPO activity was defined by the change in absorbance measured by spectrophotometer at 590 nm and expressed in unit per gram weight of wet tissue. The activity of MPO was measured by using commercial kits purchased from Boster Biotechnology (Wuhan, China).

2.7. Measurement of Cytokine Production. At 6 hrs after ZYM or NS injection, the cytokines levels in BALF and lung tissue were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits (mouse TNF-α, IL-1β, and IL-6 ELISA kits are from Boster Biotechnology, Wuhan, China). The optical density (OD) was measured on an ELISA plate scanner. All experiments were performed according to the manufacturers’ instructions.

2.8. Immunohistochemistry. Immunohistochemistry was performed as previously described [16]. At 24 hrs after ZY or NS injection, the lung tissues were fixed in 10% PBS-buffered formalin, and 5 μm sections were prepared from paraffin-embedded tissues. After deparaffinization, endogenous peroxidase was blocked with 0.3% (volume/volume [v/v]) hydrogen peroxide in 60% (v/v) methanol for 30 mins. The sections were permeabilized with 0.1% (v/v) PBS-buffered Triton X-100 for 20 mins. Incubate the section in 3% (v/v) normal goat serum in PBS for 20 mins to minimize the nonspecific adsorption. Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 mins with avidin and biotin (BD Biosciences, CA, USA). The sections were then incubated overnight with rabbit anti-iNOS mAb (Santa Cruz, CA, USA, 1:500 in PBS, v/v) or with control solutions. A biotin-conjugated specific secondary anti-immunoglobulin G and avidin-biotin peroxidase complex were used to detect the specific labeling. To verify the binding specificity for iNOS, some sections were also incubated with primary antibody only (no secondary antibody) or with secondary antibody only (no primary antibody). In these situations, no positive staining was found in the sections indicating that the immunoreactions were positive in all the experiments carried out.

2.9. Western Blot Analysis. The lung tissues were harvested, after mice were killed, and homogenized immediately. Cytoplasmic proteins were extracted from the lungs using Cytoplasmic Protein Extraction Kit (Beyotime Biotechnology, Jiangsu, China) according to the manufacturer’s protocol.
For extraction of nucleoprotein, lung tissue were homogenized and lysed in the lysis buffer (10 mM Hapes pH 7.9, 1.5 mM MgCl$_2$, 10 mM KCl, 0.5 mM DTT, 2% NP-40, and 1 mM PMSF) for 30 min. After that, the lysis buffer was centrifuged at 1,2000 × g for 15 minutes. Then the supernatant was collected as cytoplasmic protein. Precipitation was washed twice and lysed in the lysis buffer containing Triton X-100 as nucleoprotein. Protein concentrations were determined by BCA protein assay kit. The protein samples were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were transferred to a polyvinylidene difluoride membrane. The membrane was then incubated overnight with antibodies against iNOS (1:400), p-κB-α (1:1000), β-actin (1:1000), NF-κB p65 (1:800), and LaminB1 (1:1000). The membrane was then incubated with the secondary antibodies (anti-rabbit or antimouse IgG peroxidase conjugated, 1:10000). The blots were visualized with ECL-Plus reagent (Sigma).

2.10. NF-κB DNA Binding Activity Assay. At 6 hrs after ZY or NS injection, nuclear extracts of homogenated lung tissue were prepared. The DNA binding activity of NF-κB in lung tissues was quantified using the TransAM NF-κB p65 transcription factor assay kit (Active Motif, Carlsbad, CA). According to the manufacturer's instructions, all standards and samples were run in duplicate.

2.11. Statistical Analysis. Data were expressed as mean ± SEM. Differences between groups were examined for statistical significance using one-way analysis of variance with Student's t-test. A P value less than 0.05 was considered statistically significant.

3. Results

3.1. Agmatine Relieves Zymosan-Induced Lung Injury in Mice. Lung injury was characterized by alveolar thickening, infiltration of neutrophils into the lung interstitium, and alveolar space as well as alveolar hemorrhage. As shown in Figure 1(a), the mice in the control group or AGM-treated alone group showed no significant morphologic damages, indicating that intraperitoneal administration with Saline did not induce additional inflammation response in this protocol. However, ZYM-challenged mice appeared to have significant neutrophil infiltration into lung interstitium, alveolar wall thickening, and alveolar hemorrhage. Interestingly, agmatine treatment reduced infiltrated inflammatory cells and improved lung architecture in ZY-challenged mice. A scoring system was used to grade the degree of lung injury by evaluating congestion, edema, inflammation, and hemorrhage. Lung histologic scores significantly increased in ZY-challenged mice (P < 0.05) but were reduced by agmatine treatment (P < 0.05) (Figure 1(b)).

3.2. Agmatine Downregulates Zymosan-Induced TNF-α, IL-1β, and IL-6 in Lung and BALF. To test whether agmatine modulates the inflammatory process through the regulation of secretion of proinflammatory cytokines, we detected the levels of TNF-α, IL-1β, and IL-6 in lung and BALF. Six hours after ZYM or Saline injection, the lung and BALF were obtained and measured by ELISA analysis. In the Zymosan group, the concentration of TNF-α, IL-1β, and IL-6 in lung (1521.5 ± 128.4, 718.5 ± 67.2, and 917.4 ± 89.5, resp.) and BALF (157.5 ± 13.4, 124.7 ± 23.9, and 197.1 ± 24.3, resp.) increased significantly compared with that of the sham group in lung (421.1 ± 38.4, 114.8 ± 16.4, and 135.2 ± 17.4, resp.) and in BALF (8.1 ± 1.2, 6.5 ± 2.1, and 13.5 ± 4.2, resp.) (all P < 0.05). However, in the ZYM + AGM group, the levels of TNF-α, IL-1β, and IL-6 in lung (926.2 ± 89.4, 495.5 ± 54.2, and 424.3 ± 74.7, resp.) and BALF (56.7 ± 17.5, 35.02 ± 4.7, and 70.5 ± 33.6, resp.) were significantly lower compared with that of the Zymosan group (all P < 0.05) (Figure 2).

3.3. Effects of Agmatine on Zymosan-Induced Wet/Dry Weight Ratio, Protein in BALF, and MPO Activity. The pathogenesis of ALI involves increased permeability of the alveolar-capillary membrane, accumulation of protein-rich fluid in the airspaces, pulmonary edema, and pulmonary infiltration of neutrophils. In our study, twenty-four hour after Zymosan challenge, the lung tissues were obtained to employ lung weight and dry ratio (W/D), protein concentration in BALF, and MPO activity. In the Zymosan group, the W/D ratio (6.2 ± 0.6) was significantly increased compared with that of the Saline group (2.9 ± 0.4; P < 0.05). However, the ratio was significantly decreased in the ZYM + AGM group (4.1 ± 0.5; P < 0.05) compared with that of the Zymosan group (Figure 3(a)). Besides, the protein concentration in bronchoalveolar lavage fluid (BALF) was also increased in Zymosan group (0.71 ± 0.22) compared with that of the Saline group (0.06 ± 0.01; P < 0.05), whereas its level in ZYM + AGM group (0.29 ± 0.11) was significantly lower than that of the Zymosan group (P < 0.05) (Figure 3(b)). MPO activity, a biochemical marker of neutrophil infiltration, rose to 25.2 ± 1.8 in the lung of the Zymosan group compared with that of the Saline group (5.2 ± 1.4; P < 0.05). Treatment with agmatine resulted in a significant reduction in the lung MPO activity of the ZYM + AGM group (11.0 ± 3.4; P < 0.05) compared with that of the Zymosan group (Figure 3(c)).

3.4. Agmatine Reduces iNOS Expression in Lung. To understand the iNOS expression in lung, the lung tissues obtained at 24h after Zymosan administration were detected by immunohistochemistry (Figure 4(a)) and Western blot analysis (Figure 4(b)). A significant increase of iNOS expression in ZYM group was detected compared to that in Saline group by evaluating gray level ratio of iNOS/β-actin (0.78 ± 0.12 versus 0.05 ± 0.03, P < 0.05). However, agmatine treatment significantly attenuated iNOS expression in the lung compared to that in Saline group (0.23 ± 0.12 versus 0.78 ± 0.12, P < 0.05).

3.5. Agmatine Inhibits Zymosan-Induced NF-κB Activation and DNA Binding Activity in Lung. Nuclear factor (NF)-κB signaling plays a central role in the initiation and regulation of cellular inflammatory response to bacterial stimuli. Thus, to investigate the mechanisms in which agmatine enhanced...
l lung inflammation resolution, we assessed the effects of agmatine on the degradation of IκB-α and activation of NF-κB by Western blot analysis, and further we detected its effect on DNA binding activity. The mice treated with ZYM exhibited significant degradation of IκB-α in lungs, whereas agmatine treatment at the dose of 200 mg/kg prevented the IκB-α degradation. In contrast, ZYM challenge induced the nuclear translocation of NF-κB p65 subunit, compared with basal group. Agmatine treatment inhibited the nuclear translocation of NF-κB p65 (Figure 5(a)). Furthermore, the Zymosan administration significantly increased NF-κB p65 DNA binding activity in ZYM group compared with that in Saline group (2.70 ± 0.25 versus 1.10 ± 0.12, P < 0.05), whereas its activity in ZYM + AGM group was inhibited compared with that in ZYM group (1.82 ± 0.21 versus 2.70 ± 0.25, P < 0.05) (Figure 5(b)).

4. Conclusion

Zymosan has been well recognized in the pathogenesis of ALI. Experimental administration of Zymosan, both systematically and intratracheally, has been used to induce neutrophil...
Figure 2: Agmatine downregulates Zymosan-induced TNF-α, IL-1β, and IL-6 in lung and BALF. At 6 hr after ZYM or Saline injection, the cytokines levels in lung and BALF tissue were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits. *P < 0.05 compared with NS group. #P < 0.05 compared with Zymosan group.
infiltration and develop pulmonary inflammation in animal models [17, 18]. In the present study, we demonstrated that agmatine protected against ZYM-induced lung inflammation and lung injury in mice. Agmatine treatment inhibited the neutrophil infiltration and lung endothelial permeability in lung of ZYM-challenged mice. Histopathological examination showed that ZYM-induced congestion, edema, inflammation, and hemorrhage in lung were relieved by agmatine. In addition, ZYM induced upregulations of proinflammatory mediators and cytokines in BALF and lung were partially inhibited by agmatine. Furthermore, agmatine treatment inhibited the degradation of IκB-α and subsequent activation and DNA binding activity of NF-κB in lung. These results demonstrate that agmatine exerts a protective effect on ALI via inhibiting NF-κB-mediated inflammatory response.

ALI is characterized by excessive neutrophil infiltration, release of proinflammatory mediators, and loss of vascular barrier integrity [19]. During airway inflammation, neutrophils are the first cells to be recruited and are the predominant cause of tissue damage [20]. Activated neutrophils induce extensive lung inflammation and the destruction of basement membrane and increase the permeability of alveolar capillary membrane [21]. Besides, neutrophils can release damaging mediators, such as cytokines and oxidants, leading to the injury of epithelial-vascular barrier [22]. In the present study, Zymosan-induced mice group caused excessive production of MPO, an enzyme located mainly in the primary granules of neutrophils. Agmatine treatment significantly reduced neutrophil infiltration by determining MPO activity in lung. Furthermore, we detected less protein concentration in BAL and lung W/D weight in mice treated with agmatine, showing the protective effect of agmatine against lung endothelial permeability injury.

Except for neutrophils and the other inflammatory cells, the release of proinflammatory mediators has been reported to be involved in inflammatory cascade [23]. Among them, TNF-α, IL-1β, and IL-6 were considered the most important inflammatory mediators in innate immune response. It has been reported that resident alveolar macrophages release TNF-α and IL-1β in early phase of ALI in response to ZYM stimulation, resulting in the subsequent inflammatory cascade and tissue injury [24]. It is reported that TNF-α elevates intracellular reactive oxygen species which causes mitochondrial damage or ion exchange dysfunction across

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**Figure 3:** Effects of agmatine on Zymosan-induced wet/dry weight ratio, protein in BALF, and MPO activity. 24 hour after Zymosan challenge, the lung tissues were obtained to detect lung weight and dry ratio (W/D) (a), protein concentration in BALF (b), and MPO activity (c). *P < 0.05 compared with NS group. **P < 0.05 compared with Zymosan group.
Figure 4: Agmatine decreases iNOS expression in lung. (a) Lung samples were obtained from NS group (A), agmatine group (B), Zymosan group (C), and Zymosan + agmatine group (D) for immunohistochemical staining. (b) Lungs were harvested for observing iNOS expression at 24 hrs after ZYM or NS administration by Western blot. *P < 0.05 compared with NS group. #P < 0.05 compared with Zymosan group.

the cell membrane [25, 26]. Besides, inhibition of TNF-α was proved to be protective in an animal model of ALI [27]. IL-1β also enhances the recruitment of inflammatory cells into airspaces and alters vascular permeability leading to fluid transport and subsequent lung edema formation [28]. During inflammation, elevated TNF-α and IL-1β are associated with a poor ALI prognosis [29]. In the present study, the expressions of TNF-α, IL-1β, and IL-6 in lung and BALF were markedly induced by ZYM challenge, which was blocked by the treatment of agmatine. Our results confirmed the link
between the inflammatory cytokine levels and the extent of lung inflammation, demonstrating the anti-inflammatory effects of agmatine.

Inflammatory activity is mediated by the endogenous free radical NO, which is produced at high levels upon the induction of NO synthase by inflammatory stimulus [30]. In an experimental murine model, ZY administration increases iNOS expression and activity that exacerbates nonseptic shock and leads to cellular and tissue damage including lung injury if unchecked. However, iNOS inhibitors suppress airway inflammation in mice by downregulating proinflammation and chemokine expression that are detrimental to the lung [31]. iNOS-deficient mice also undergo less lung injury after ZY challenge [32]. In the present study, we demonstrated that agmatine treatment significantly reduced iNOS expression and its activity in the lung tissue, and this phenomenon significantly inhibited the inflammatory response. Moreover, NF-κB is a potent regulator of iNOS expression [33], and our results show that agmatine treatment inhibited NF-κB activity as well as iNOS expression in lung. Based on these findings, we speculated that agmatine probably inhibited iNOS expression and activity by blocking NF-κB activation.

It has been reported that NF-κB, a transcription factor, plays a pivotal role in the pathogenesis of immune and inflammatory responses [34]. In experimental animal models of ALI, NF-κB activation is increased [35]. Pharmacological inhibition of NF-κB pathway decreases the production of proinflammatory mediators and protects against endotoxin-induced ALI in animals [36]. Therefore, NF-κB pathway is involved in the pathogenesis of ALI and is known as an important target for anti-inflammatory molecules [37]. Our present study displayed that ZYM obviously enhanced the phosphorylation and degradation of IκB-α. However, agmatine treatment dramatically regulated this trend, which suggested that suppression of IκB-α activity may be the main reason of agmatine lessening ZYM-initiated pulmonary inflammation in mice. These results suggested that inhibition of NF-κB signaling plays a role in the protective effects of agmatine on ALI.

Agmatine is a cationic polyamine under physiological conditions and thus is thought not to be able to permeate biological membranes, although agmatine can cross the blood-brain barrier when administered peripherally in high doses. In mammalian tissues, agmatine binds to several receptors, including imidazoline, α2-adrenergic, and NMDA glutamate receptor. Sameer et al. reported that agmatine attenuated the acquisition of ethanol conditioned place preference by imidazoline (I1 or I2) receptors [38]. Bhalla et al. found that agmatine potentiated oxycodone antinociception in

Figure 5: Agmatine inhibits Zymosan-induced NF-κB activation and DNA binding activity in lung. 6 h after ZYM injection with or without agmatine treatments, mice were exsanguinated and their lungs were removed. (a) Western blot was performed to detect p-IκB-α and IκB-α in cytoplasm and NF-κB p65 in nucleus. Expressions of β-actin and LaminB1 were shown as loading controls. (b) DNA binding activity of NF-κB p65 was examined by a TransAM p65 transcription factor ELISA kit. Each bar represents the mean ± SD of 8 mice. *P < 0.05 compared with NS group. #P < 0.05 compared with Zymosan group.
mice via an imidazoline I$_2$ receptor-mediated mechanism [39]. Besides, agmatine was found to be protective against glutamate-induced necrotic neuronal cell death through NMDA receptor blockade by interacting with a site located within the NMDA channel pore [40]. Therefore, we speculated that agmatine may protect against zymosan-induced acute lung injury by interacting with its endogenous receptors, which will be tested and verified in the next round of experiments.

In summary, we have demonstrated the protective effects of agmatine on ZYM-induced ALI. It is evidenced by alleviating lung inflammation, reducing neutrophil infiltration, decreasing vascular leakage, and proinflammatory cytokine release, inhibiting NF-$\kappa$B activation and DNA binding activity in ZYM-challenged mice. These results suggest agmatine may be considered as an effective and safe drug for the potential treatment of ALI.

Conflict of Interests

The authors declare that no conflict of interests exists.

Acknowledgments

This work was supported by a Grant from National “973” Project (no. 2012CB518102).

References


Research Article

Early Response Roles for Prolactin Cortisol and Circulating and Cellular Levels of Heat Shock Proteins 72 and 90α in Severe Sepsis and SIRS

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Received 7 March 2014; Accepted 24 July 2014; Published 27 August 2014

Academic Editor: Baoli Cheng

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Objective. To evaluate the early heat shock protein (HSP) and hormonal stress response of intensive care unit (ICU) patients with severe sepsis/septic shock (SS) or systemic inflammatory response syndrome (SIRS) compared to healthy subjects (H).

Methods. Patients with early (first 48 hrs) SS (𝑛 = 29) or SIRS (𝑛 = 29) admitted to a university ICU and 16 H were enrolled in the study. Serum prolactin, cortisol, and plasma ACTH were determined using immunoassay analyzers. ELISA was used to evaluate extracellular HSPs (eHSP90α, eHSP72) and interleukins. Mean fluorescence intensity (MFI) values for intracellular HSPs (iHSP72, iHSP90α) were measured using 4-colour flow-cytometry.

Results. Prolactin, cortisol, and eHSP90α levels were significantly increased in SS patients compared to SIRS and H (𝑃 < 0.003). ACTH and eHSP72 were significantly higher in SS and SIRS compared to H (𝑃 < 0.005). SS monocytes expressed lower iHSP72 MFI levels compared to H (𝑃 = 0.03). Prolactin was related with SAPS III and APACHE II scores and cortisol with eHSP90α, IL-6, and lactate (𝑃 < 0.05). In SS and SIRS eHSP90α was related with eHSP72, IL-6, and IL-10. Conclusion. Prolactin, apart from cortisol, may have a role in the acute stress response in severe sepsis. In this early-onset inflammatory process, cortisol relates to eHSP90α, monocytes suppress iHSP72, and plasma eHSP72 increases.

1. Introduction

Severe sepsis and septic shock are leading causes of death in intensive care units (ICU) worldwide and despite efforts in understanding their pathophysiology and implementing effective treatment, their annual incidence has been projected to increase by 1.5% per year [1]. Sepsis is accompanied by major changes in the hypothalamus-pituitary-adrenal axis through multiple interactions between the autonomous nervous system and immune systems [2]. During early sepsis, initial activation of the pituitary adrenal axis depends on activation of hypothalamus and pituitary by cytokines, while in late sepsis a shift from neuroendocrine to local adrenal regulation of glucocorticoid production has been proposed [3]. The initial release of cytokines from immune cells participates in homeostasis of the body by acting as paracrine, autocrine, and hormonal agents and elevating corticotropin releasing hormone levels (CRH) [3]. Except for CRH, septic patients have increased ACTH, growth hormone, and prolactin levels in the early stage of sepsis [2].

The association of prolactin with modulation of the immune system during sepsis has been studied in septic mice, where administration of prolactin was associated with decreased survival and alterations in immune response [4].
It has been found that prolactin serum levels in critical ill children are usually low early after pediatric intensive care admission [5]. The role of prolactin as an early acute stress in adult ICU sepsis and trauma patients has not been elucidated yet [6]. In an animal model, a possible relationship between heat shock proteins (HSPs) and prolactin receptors has been previously reported [7].

Intracellular HSPs (iHSPs) are high evolutionary conservative proteins that play an important role in regulating host response against infections, thermal injury, oxidative damage, and hypoxia [8]. Particularly, the major heat shock proteins iHSP70 and iHSP90α confer tolerance to sepsis by maintaining the conformational homeostasis, exerting anti-apoptotic effects, and mediating LPS-signaling as a part of the LPS receptor cluster [9]. However, although animal studies have demonstrated a protective effect of iHSP72 in sepsis, human studies are inconclusive showing either protection or relation to mortality and infections [10]. To add more questions about the extracellular HSPs (eHSPs) function and their role in sepsis [11, 12], eHSPP90α levels were recently shown to decline in controls and remain increased in septic patients, contrasting eHSP72, which increased over time in both groups [13]. Thus, the involvement of extracellular bound HSPs as signals for activation of the immune system and especially macrophages [14] raises interest about the role of these proteins in sepsis. Apart from septic patients, serum levels of eHSP72 measured early after injury in trauma patients correlated with survival, with significantly higher levels in trauma patients who survived compared to nonsurvivors [15].

In this study, we evaluated the early (first 48 hours) serum levels of prolactin, cortisol, and interleukins and plasma levels of ACTH, eHSP90α, and eHSP72 and measured mean fluorescence intensity (MFI) of iHSP72 and iHSP90α in ICU patients with severe sepsis and septic shock (SS) or systemic inflammatory response syndrome (SIRS) compared to healthy control subjects (H). We also correlated their expression with interleukins (ILs), severity scoring systems, clinical and laboratory data, and outcome.

2. Materials and Methods

2.1. Patients. The study was approved by the institutional review board of Evangelismos Hospital and was performed during a 14-month period between October 2012 and December 2013. Informed consent form was obtained from the relatives of patients admitted to the ICU. Consecutively admitted patients >18 years and <75 years with early (<48 h) severe sepsis, septic shock, or SIRS admitted to the ICU were eligible for enrolment and were divided in two groups. The SIRS group included trauma patients (n = 29) who met at least two of the four conventional criteria for SIRS. The severe sepsis or septic shock group (SS) included patients (n = 29) with an identified source of infection. Sepsis, severe sepsis, septic shock, and SIRS were defined according to the Surviving Sepsis Campaign Guidelines [16]. The third group included healthy volunteers (H) (n = 16) matched for age and sex to the ICU patients. Exclusion criteria were (a) malignancy, (b) autoimmune diseases, (c) prior use of corticoids, (d) immunosuppressive illness, and (e) late sepsis or SIRS 48 h after admission. Acute physiology and chronic evaluation (APACHE II) [17], sequential organ failure assessment (SOFA) [18], and simplified acute physiology score III (SAPS III) [19] scores were recorded on admission. Demographics, date of hospital, and ICU admission, ICU and in-hospital mortality, length of stay, and laboratory tests were also recorded for all patients.

2.2. Laboratory Assays

2.2.1. Prolactin, Cortisol, and ACTH. Blood was drawn between 8 and 9 a.m. in the first 48 h after ICU admission. For ACTH measurement, blood was collected into ethylenediaminetetraacetic acid (EDTA) containing tube, immediately centrifuged at 4°C, plasma-pooled, and finally stored at −80°C until measurement. For cortisol and prolactin measurement blood was collected in tubes containing clot and gel for serum separation and centrifuged at 4°C and serum was also stored at −80°C until measurement. Serum cortisol and prolactin levels were determined using the ADVIA Centaur Immunoassay Analyzer (Siemens Healthcare Diagnostics, Tarrytown, NY, USA) while plasma ACTH was measured using the Immulite 2000 Immunoassay Analyzer (Siemens Healthcare Diagnostics, Tarrytown, NY, USA).

2.2.2. Cytokines and Extracellular Heat Shock Proteins. Cytokine levels of serum IL-6, IL-10, IL-17, and IFN-γ were measured by ELISA as mentioned by the kit instructions and extracellular plasma levels of HSPs (eHSP72 and eHSP90α) were analyzed by ELISA assay according to the manufacturers’ instructions (Invitrogen Carlsbad, CA, USA, and Enzo Life Sciences, Ann Arbor, MI, USA, resp.). The inter- and intra-assay CV for each analyte were as follows: 6.2 and 7.8 for IL-6, 3.25 and 2.75 for IL-10, 3.7 and in process for IL-17, 3.5 and 7.3 for IFN-γ, 7.1 and 15.2 for hsp72, and <10 for hs90α. The sensitivities of the assays were <2 pg/mL for IL-6, <1 pg/mL for IL-10, 2 pg/mL for IL-17, 0.03 IU/mL for IFN-γ, 90 pg/mL for hsp72, and 50 pg/mL for hs90α.

2.2.3. Intracellular HSPs. EDTA-anticoagulated blood (100 μL) was used for flow cytometric analysis of fresh peripheral blood mononuclear cells (PBMCs). Monocytes iHSP72 and iHSP90α expressed as mean fluorescence intensity (MFI) were determined after staining with 5 μL surface antigens CD33-PE/Cy5 (BioLegend, San Diego, CA, USA) and 5 μL CD45 PE/Cy7 (BioLegend, San Diego, USA) followed by either 5 μL HSP72-FITC (Enzo Life Sciences, Ann Arbor, MI, USA) or 5 μL HSP90α-PE (Enzo Life Sciences, Ann Arbor, MI, USA) intracellular staining. Assays were performed according to the manufacturer’s instructions using 4-colour flow cytometry FC-500 (Beckman Coulter, Miami, FL, USA).

2.2.4. Statistical Analysis. All results are presented as means ± standard deviation. The results were analyzed using SPSS software (version 21.0, SPSS, Chicago, Ill). Group comparisons were performed using the Kruskal-Wallis test. The variables
that showed differences among groups were compared group by group by the Mann-Whitney test. Paired differences for continuous variables in the same subjects were analyzed using the Wilcoxon signed-rank test. The correlation between variables was analyzed by the Spearman correlation test. The level of significance between groups was set on $P < 0.05$.

3. Results

Anthropometric characteristics and severity scores of the (H), (SIRS), and (SS) groups are summarized in Table 1. Hormonal profile, extracellular heat shock protein and cytokine measurements, and mean fluorescence intensity of heat shock proteins in monocytes are summarized in Table 2.

Prolactin, cortisol, and ACTH levels differed significantly between groups (Figure 1). Prolactin was correlated with SAPS III ($r = 0.42, P = 0.004$) and APACHE II ($r = 0.3, P = 0.04$) scores; cortisol was correlated with eHSP90α ($r = 0.47, P = 0.013$), IL-6 ($r = 0.25, P = 0.05$), and maximum admission day lactate ($r = 0.30, P = 0.03$) and negatively with HCO$_3$ ($r = -0.50, P = 0.001$).

The eHSP90α levels in the SS group were increased in comparison to the H and SIRS groups. The eHSP72 levels in the SS group were increased compared to H (Figure 2). Both eHSP90α and eHSP72 were significantly increased in SIRS compared to H ($P < 0.02$).

Extracellular HSP72 in the SS and SIRS groups correlated with the severity of illness scores (Figure 3): APACHE II ($r = 0.45, P = 0.034$), SOFA ($r = 0.6, P = 0.002$), and SAPS III ($r = 0.5, P = 0.004$). There was a positive correlation of eHSP72 and eHSP90α levels in all groups (Table 3). The eHSP90α levels showed a positive correlation with IL-6 and IL-10 but not with IL-17 or IFN-γ. Also, eHSP72 levels were only correlated with IL-10 (Table 3).

Septic monocytes expressed significantly lower iHSP72 MFI levels compared to the H group (Table 2). Although a positive correlation of eHSP90α with iHSP72 monocyte levels was found in the SS and SIRS groups, only iHSP72 correlated negatively with severity of illness scores: APACHE II ($r = -0.32, P = 0.004$), SOFA ($r = -0.32, P = 0.017$), and SAPS III ($r = -0.34, P = 0.012$).

4. Discussion

In this study we evaluated the early inflammatory and hormonal stress response in SS and SIRS patients in an
ICU setting. We showed that in these critically ill patients prolactin levels, along with cortisol, were significantly higher in SS compared to H and SIRS groups. Hormonal increase was related to the severity of illness but only cortisol, not prolactin, was correlated with eHSP90α. Thus, our study demonstrated that stress response of prolactin and its relation to the severity of sepsis might not have been induced through the iHSP72 or iHSP90α “danger signal” pathways.

Patients suffering early sepsis induce elevation of baseline cortisol levels and decrease in blood cortisol to ACTH ratio compared to nonseptic patients admitted to the ICU [20]. Plasma ACTH and prolactin are increased within the few minutes following the insult of a pathogen [2]. Our findings of significantly increased ACTH, cortisol, and prolactin levels in severe sepsis and septic shock also support the hypothesis that prolactin, apart from cortisol, may have a role in the acute stress hormonal response in the early-onset inflammatory process. It has been previously suggested that, with nitric oxide as a key mediator, sepsis elicits a very reproducible pattern of pituitary hormone secretion, with plasma ACTH and prolactin increasing within a few minutes following the insult and with a rapid inhibition of secretion of luteinizing and thyroid-stimulatory hormone [2]. This is further supported by a strongly positive relationship of prolactin with the evaluated in this study’s severity scoring systems.

Cortisol and ACTH levels have extensively been studied in a critical ill setting. On the contrary, prolactin’s role as an immunomodulator in septic patients needs to be further elucidated. A recent study has suggested an association of increased prolactin mRNA expression in monocytes with better outcome in hematopoietic septic patients [21]. It has been also found that anterior pituitary cells recognize and respond to fungal cell wall glucans by appropriately stimulating the secretion of prolactin, a hormone that plays an important role in the response to fungal infection [22]. In addition, the increased prolactin levels in our trauma patients might
P = 0.001

Severe sepsis
SIRS
Healthy subjects

200
150
100
50
0

(a)

P = 0.003

P = 0.02

Severe sepsis
SIRS
Healthy subjects

P = 0.001

P = 0.018

Severe sepsis
SIRS
Healthy subjects

Figure 2: Extracellular heat shock protein (eHSP) levels of (a) eHSP90α and (b) eHSP72 in healthy subjects, SIRS, and severe sepsis patients. The P value was calculated using the Mann-Whitney U test and a P value < 0.05 (two sided) was considered statistically significant. The box-whisker plots show the median (horizontal line within the box) and the 10th and 90th percentiles (whiskers). The box length is the interquartile range. Solid circles represent outliers and stars extremes.

Table 3: Pearson’s coefficient correlations of hormones, extracellular heat shock proteins, and interleukins.

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eHSP: extracellular heat shock protein; iHSP: intracellular heat shock protein; IL: interleukins; IFN-γ: interferon gamma.

further suggest that the prolactin’s response role is equally important to that of cortisol in homeostasis not only in infectious but also in noninfectious SIRS.

Although none of the eHSPs correlated with prolactin levels, cortisol was correlated with eHSP90α. It has been previously shown that eHSP90α plays a crucial role in antigen presenting in dendritic cells through the major histocompatibility complex (MHC) type 1 [23] and stimulates monocytes [24]. To the best of our knowledge, this is the first time that a significant early increase of plasma eHSP90α is shown in septic patients compared to healthy subjects and severely injured patients. In addition, the strong correlation of eHSP90α with eHSP72 found in our study might further suggest a common pathophysiologic mechanism of their expression. Thus, extracellular levels of both HSPs were significantly elevated in severely septic patients as compared to healthy volunteers; moreover, eHSP90α levels of SS were higher when compared to trauma (SIRS) patients. Importantly, in both SS and SIRS groups, eHSP72 levels correlated with the severity of illness, further supporting the hypothesis that eHSPs may act as danger signals to modulate the immune system [8].

Increased eHSP72 levels have been found in inflammatory myopathy [25], lung injury [26], acute coronary syndrome and stable angina [27], trauma [15], inflammation,
and sepsis [12]. Only two in vivo studies of eHSP72 and none of eHSP90α were identified in a PubMed database research in human severe sepsis (1992–2012) [12, 28]. Wheeler et al. [28] found that serum levels of eHSP72 in children with septic shock were significantly elevated as compared to noncritically ill children undergoing elective surgical procedures and that the increase was related to mortality. Analysis of oxidative parameters in septic patients by Gelain et al. revealed that serum levels of eHSP72 were modulated according to oxidative stress [12]. In our study we further showed a significant early increase of eHSP72 in both septic and traumatic adult patients compared to controls.

It has been suggested that stimulation of toll-like receptors (TLR)-dependent signaling pathways by lipopolysaccharides (LPS) increases iHSP72 expression and its release in the extracellular environment both in in vitro cell cultures and in vivo [29]. The release of eHSPs appears to be a very complex phenomenon encompassing different alternative pathways containing nonconsensus secretory signals [30]. Proposed mechanisms for their release are translocation across the plasma membrane, release associated with lipid vesicles, passive release after cell death by necrosis via extracellular vesicles [II], and through nonclassical exosomal nonsecretory pathways [31]. Although in septic patients stimulation of LPS pathways has been proposed to explain the extracellular release of eHSPs, it might not clarify the increased eHSPs levels in our SIRS patients. Instead, it has been hypothesized that eHSPs may be released from damaged tissue and necrotic cells after trauma contributing to trauma induced immunosuppression [32].

In severe sepsis, in contrast to the extracellular monocyte-HSPs, intracellular monocyte-HSPs had a tendency to decrease in comparison to control subjects. These findings are in accordance with those of another prospective observational study in patients with severe sepsis demonstrating a reduced iHSP72 expression in PBMCs and a LPS dose-dependent inhibition of its expression [33]. In patients with inflammation, however, iHSPs were found increased compared to healthy control subjects [34]. Although SIRS was shown to increase iHSP72 and iHSP90α expression in monocytes indicating a protective function of iHSPs during the acute phase of stress, the downregulation of iHSP-positive cells in SS seems to be a result of (mal-)adaptation mechanism to severity of illness [35]. Preliminary data showed that iHSP72 and iHSP90α monocyte expressions follow different longitudinal courses in SS, not related to the metabolic response to stress [36].

The main limitations of our study are the small number of patients and an age difference among groups. Enrollment of septic patients has been proven to be difficult since most of them met exclusion criteria (immunosuppression, cancer). In addition, since that was a prospective study with consecutively admitted patients in ICU, the mean age of trauma patients was, as expected, lower than the mean age of septic patients. The mean age of healthy subjects, however, was matched with the mean age of ICU patients included in our study. Finally, we did not find any correlation between hormones and age, so that the importance of the age difference might not have significantly influenced results.

5. Conclusions
Prolactin, apart from cortisol, may have an early role in the acute stress hormonal response in severe sepsis and septic shock. Extracellular levels of HSP72 and HSP90α are significantly elevated in septic patients as compared to healthy volunteers supporting the hypothesis that eHSPs may act as danger signals to activate the immune system. Simultaneously, however, monocytes suppress iHSP72 expression early in septic patients. To the best of our knowledge, this is the first time that a significant early increase of plasma eHSP90α is shown in septic patients compared to healthy subjects and severely injured patients and that this increase is related to increased cortisol levels.

Conflict of Interests
The authors declare that they have no conflict of interests.

Acknowledgments
This research has been cofinanced by the European Union (European Social Fund (ESF)) and Greek national funds through the Operational Program “Education and Lifelong Learning” of the National Strategic Reference Framework (NSRF) Research Funding Program: THALES.

References


Research Article

Epinephrine Enhances the Response of Macrophages under LPS Stimulation

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Received 16 February 2014; Revised 8 July 2014; Accepted 29 July 2014; Published 26 August 2014

Academic Editor: Baoli Cheng

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Trauma associated with infection may directly trigger a neuroendocrine reaction in vivo while the hormone epinephrine is known to mediate immune responses to inflammation after injury. However, the role of epinephrine during the earliest stage of trauma still remains unclear. We therefore explored the role of epinephrine on activated macrophages under LPS stimulation in vitro as well as the mechanisms underlying its effect. Dose- and time-dependent effects of epinephrine on macrophage immune function were assessed after LPS activation. We also employed CD14 siRNA interference to investigate whether CD14 played a role in the mechanism underlying the effect of epinephrine on LPS-induced macrophage responses. Our results showed that epinephrine pretreatment (10 ng/mL) significantly promoted immune responses from LPS stimulated macrophages, including phagocytic rate, phagocytic index, TNFα/IL-1β/IL-10 secretion, and CD14 expression (P < 0.05). Moreover, TNFα/IL-1β/IL-10 levels attained their peak value 1 hour after incubation with 10 ng/mL epinephrine (P < 0.05), and CD14 siRNA transfection dramatically decreased phagocytosis and cytokine secretion by LPS-activated macrophages (P < 0.05). We therefore conclude that 10 ng/mL epinephrine enhances immune responses from macrophages under LPS stimulation and that the underlying mechanism may relate to CD14 upregulation on the surface of macrophages.

1. Introduction

A frequent complication arising from severe trauma is infection. Trauma-associated infection may directly trigger a neuroendocrine reaction in vivo. This complicated cross-interaction between the immune and neuroendocrine systems mediated by endogenous hormones can influence the homeostasis of host health [1].

Macrophages are the most important immune effector cells during the earliest stage of trauma and are activated by recognizing pathogen-associated molecular patterns (PAMPs) during the immune-defense reaction against infection. Gram-negative bacteria express a variety of PAMPs, including lipopolysaccharide (LPS), the main component of endotoxin that is present in their cell wall. Pattern recognition receptors (PRRs) expressed on macrophages, such as CD14, therefore play a decisive role in recognizing LPS and triggering the subsequent release of inflammatory factors that mediate cell activation.

Epinephrine, a major effector hormone of the sympathetic-adrenal medulla (SAM) axis, has gradually gained attention for its role in the innate immune response during the earliest stage of infection [2–4]. However, its influence on macrophage activation after trauma-associated infection remains unclear, especially its relationship with PRRs expressed on the surface of macrophages.

In this study, the role and mechanism underlying the effect of epinephrine on macrophages in the earliest stage of trauma-associated infection were examined in vitro using primary peritoneal macrophages from Sprague-Dawley (SD) rats. Their phagocytic and cytokine-secretion responses were evaluated after pretreatment with epinephrine at various doses and time points which was followed by stimulation with LPS (10 ng/mL) for 1 hour. To test whether CD14 played a
role in this response, the same methodology was also adopted under CD14 siRNA interference conditions.

2. Materials and Methods

2.1. Experimental Animals. Adult Sprague-Dawley (SD) rats were purchased from the experimental animal center at the Surgery Research, Daping Hospital, Third Military Medical University. All animals were bred in the animal facility under specific pathogen-free (SPF) conditions, and experimental procedures were performed in strict accordance with the guidelines set forth by the Research Council and Animal Use and Care Committee of the Third Military Medical University.

2.2. Dose-Dependent Effect of Epinephrine on Macrophage Responses under LPS Stimulation. Peritoneal macrophages were collected from rats and purified [5, 6]. Macrophages (2 × 10^6 cells/well) were cultured in RPMI 1640 containing 10% fetal bovine serum (FBS) (Hyclone, USA) in a 24-well plate overnight at 37°C and 5% CO_2. After cells were washed with PBS, epinephrine (Sigma, USA) was added at various concentrations (2, 10, 50, and 100 ng/mL) into respective wells and incubated for an additional hour at 37°C and 5% CO_2. Then, macrophages were stimulated with 10 ng/mL LPS (Escherichia coli O26:B6) for another hour under the same conditions. Supernatant was collected, and the expression of TNFα, IL-1β, IL-6, and IL-10 were detected by an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, USA; Boster Biotechnology, China) [5, 6]. Meanwhile, the adherent cell monolayer was washed with PBS, and 2 × 10^6 colony-forming units/mL of red-fluorescently labeled E. coli B21 was added. After a 30 min incubation, the plate was washed 3x with PBS and fixed with 4% paraformaldehyde. E. coli B21 phagocytosis was observed by confocal microscopy (TCSSP2; Leica Microsystems, Wetzlar, Germany) to acquire the phagocytic rate (% of macrophages containing at least one ingested bacterium) and the phagocytic index (the mean number of phagocytosed bacteria observed in the macrophage cytoplasm). In addition, total macrophage RNA was extracted by TRizol reagent (Invitrogen, Karlsruhe, Germany). One microgram of total RNA was transcribed into cDNA with Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, California, USA) by oligo dT priming. CD14 expression was measured by reverse transcription polymerase chain reaction (RT-PCR) (Table 1). PCR products were analyzed on a 2% (w/v) agarose-ethidium bromide gel with a computer-linked phosphomaging system (Gel Doc 2000; Bio-Rad, Hercules, California, USA). Semiquantitative analysis of CD14 expression was conducted with the software of GelDoc 2000 Imaging System.

2.3. Time-Dependent Effect of Epinephrine on Macrophage Responses under LPS Stimulation. The collection, purification, and incubation of primary rat peritoneal macrophages were performed as described above. In a second set of plates, 10 ng/mL epinephrine was added to each well, and the cells were incubated for varying periods of time (0, 0.5, 1, 2, 3, and 5 hours). Thereafter, 10 ng/mL LPS (E. coli O26:B6) was added into each well for another hour. Supernatant was collected, and the expression of TNFα, IL-1β, IL-6, and IL-10 were assayed by ELISA.

2.4. CD14 siRNA Interference Effect on Macrophage Responses. Peritoneal macrophages were acquired as described above. Macrophages (2 × 10^6 cells/well) were incubated in 24-well plate at 37°C and 5% CO_2 for 24 h before CD14 siRNA transfection. According to the recommended Lipofectamine reagent kit protocol (Santa Cruz, USA), we first determined the optimal time interval by observing cell morphology and CD14 expression level. The macrophages were then divided into the following 3 groups: negative control, siRNA control, and CD14 siRNA. At the optimal time interval by observing cell morphology and CD14 expression level, CD14 expression was evaluated by RT-PCR (Table 1), and semiquantitative analysis of CD14 was performed.

2.5. Statistical Analysis. The data were presented as mean ± standard deviation (SD) (x ± s). Origin 7.5 software was used to analyze the role of epinephrine in macrophage activation under LPS stimulation, while one-way analysis of variance (one-way ANOVA) by SPSS11.5 software was used to analyze normality, homogeneity of variance, and interblock contrast. The significance level was set at 95%.

3. Results

3.1. Epinephrine Effects on Phagocytosis Function by LPS-Stimulated Macrophages. Epinephrine at 10 ng/mL significantly increased the phagocytic rate of LPS-treated macrophages (P < 0.01). However, whether higher epinephrine concentrations (50–100 ng/mL) influenced the phagocytic rate was not clear. Meanwhile, lower epinephrine concentrations (2–50 ng/mL) enhanced the macrophage phagocytic index to some extent, especially at the 10 ng/mL concentration (P < 0.01), although this result was not found at the 100 ng/mL concentration (Figure 1).

3.2. Epinephrine Effects on TNFα/IL-1β/IL-6/IL-10 Secretion by LPS-Stimulated Macrophages. A series of inflammatory cytokines produced by macrophages, namely, TNFα, IL-1β, IL-6, and IL-10, were used to assess the ability of macrophages to produce cytokines. In the dose-dependent effect study, low epinephrine concentrations (2–10 ng/mL) significantly enhanced TNFα/IL-1β/IL-10 production, especially the 10 ng/mL dose (P < 0.05). With further increases in epinephrine concentration, however, TNFα secretion level did not continue to rise (Figure 2).

In the time-dependent effect study, the secretion of TNFα, IL-1β, IL-6, and IL-10 significantly increased during the first hour after exposure to 10 ng/mL epinephrine.
Figure 1: The role of epinephrine in macrophage phagocytosis under LPS stimulation. (a)–(d) Confocal images of the following groups are shown (magnification, (a) 200x; (b)–(d) 400x). (a) Control macrophages without LPS stimulation; macrophages pretreated with (b) 0 ng/mL, (c) 2 ng/mL, (d) 10 ng/mL, or (e) 100 ng/mL epinephrine under LPS stimulation. (f) Changes in phagocytic index and phagocytic rate at different epinephrine doses compared with the control group. *P < 0.01, †P < 0.05.

Table 1: RT-PCR primers.

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All primers were synthesized by Sangon (Shanghai, China) with the catalog numbers NM017008 and NM021744, respectively. PCR parameters for rat GAPDH and CD14 proceeded as follows: 94°C for the initial 3 min; 27 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 40 sec; and 72°C for the final 2 min.
Figure 2: Dose-dependent effect of epinephrine on macrophage secretion under LPS stimulation. The macrophages were pretreated with epinephrine at various concentrations (2 ng/mL, 10 ng/mL, 50 ng/mL, and 100 ng/mL) for 1 hour and then stimulated by 10 ng/mL LPS for another 1 hour. The levels of cytokines including (a) TNF-α, (b) IL-1β, (c) IL-6, and (d) IL-10 levels in each group were measured by ELISA. *P < 0.05, compared with the 0 ng/mL control group stimulated by LPS only.

(P < 0.05). However, this same epinephrine dose repressed these cytokines’ production when macrophages were exposed for longer time periods (2–5 hours for TNF-α, IL-1β, and IL-6; 3–5 hours for IL-10) (P < 0.05) (Figure 3).

3.3. Epinephrine Effects on CD14 Expression Levels in LPS-Stimulated Macrophages. CD14 expression level in LPS-stimulated macrophages significantly increased during the first hour of exposure to 10 ng/mL epinephrine (P < 0.05). However, higher epinephrine doses (50–100 ng/mL) did not induce the same increase in CD14 expression (Figure 4).

3.4. Effect of CD14 siRNA Interference on Epinephrine-Treated Activated Macrophages. RT-PCR analysis showed that CD14 expression by macrophages significantly decreased 48 hours after transfection with CD14 siRNA, which was maintained for 72 hours. This data demonstrated that the optimal time range for CD14 siRNA-mediated silencing in macrophages was between 48 and 72 hours, during which time the inhibition ratio exceeded 60%. Thus, 52 hours after CD14 siRNA transfection was chosen as the optimal time point to observe macrophage function in the following experiments.

At 52 hours after CD14 siRNA transfection, a 1 h exposure to 10 ng/mL epinephrine inhibited the phagocytic function of macrophages, where the phagocytosis of E. coli BI21 significantly decreased (P < 0.05) (Figures 5(a)–5(d)). Additionally, compared with the control group (P < 0.05) (Figure 5(e)), pretreatment with 10 ng/mL epinephrine led to significantly decreased TNFα secretion by peritoneal macrophages after CD14 siRNA transfection.

4. Discussion

Disease, trauma, inflammation, and infection may elicit a neuroendocrine reaction triggered by exciting the hypothalamic-pituitary-adrenal (HPA) and SAM axes [7, 8]. As major effector hormones secreted by the SAM axis, epinephrine, norepinephrine, and glucocorticoids participate in complicated cross-interaction with innate immunity and influence the homeostasis of host health [1]. Additionally, the movement of Gram-negative bacteria and toxins after severe trauma within hollow organs, such as intestines, is the main source of infection leading to traumatic sepsis. Specifically, bacterial endotoxin plays an important
role in pathogenesis of traumatic sepsis, as PRRs expressed on macrophages recognize LPS, a main component of endotoxin, during the innate immune defense response that leads to cellular activation [9, 10]. Thereafter, macrophages show immunological function, including phagocytosis and cytokine production [11]. However, the role and mechanism by which epinephrine affects macrophage function under LPS stimulation remain unknown.

The in vitro study presented here thus explored the relationship among epinephrine, LPS, and macrophages. The results showed that 10 ng/mL epinephrine promoted phagocytosis and TNFα/IL-1β/IL-10 secretion by macrophages, while higher doses of 50 and 100 ng/mL epinephrine inhibited them. Other studies also identified that lower epinephrine doses increased IL-1β secretion, while higher doses did not have the same effect [12–14]. Thus, these results imply that stress hormones at appropriate doses may enhance macrophage activation under LPS stimulation.

The mechanism may have to do with the types and pathways of adrenergic receptors participating in the regulatory course. It has been reported in several studies that the role of lower dose epinephrine is likely to be mediated by α2-adrenergic receptor (AR), which represses the function of adenylate cyclase and causes the amount of cyclic adenosine monophosphate (cAMP) to decrease. As a result, the phosphorylation level of the downstream proteins changes and inflammatory factors are released. On the other hand, it appears that β2-AR mediates the function of higher dose epinephrine and increases the concentration of cAMP. The phagocytosis is inhibited and anti-inflammatory effect is in place in those cases. In addition, the role of epinephrine is related not only to concentrations and pretreatment time but also to the density of AR in different cells. These factors may lead to a completely different immune regulation of epinephrine with various concentrations and pretreatment time. Moreover, the difference among animal strains, organs,
Figure 4: Dose-dependent effect of epinephrine on CD14 expression from macrophages under LPS stimulation. The macrophages were pretreated with epinephrine at various concentrations (2 ng/mL, 10 ng/mL, 50 ng/mL, and 100 ng/mL) for 1 hour and then stimulated by 10 ng/mL LPS for another 1 hour. The expression level of CD14 in each group was measured by RT-PCR. The data were showed in (a) electrophoresis result and (b) semiquantitative analysis. * \( P < 0.05 \), compared with the 0 ng/mL control group stimulated by LPS only.

This study further investigated the role of CD14 in macrophage activation under the above conditions by adopting CD14 siRNA technology. The results showed that CD14 siRNA interfered with CD14 expression in macrophages. Moreover, the phagocytosis and cytokine-production functions by macrophages were lower in the transfection group than in the control siRNA group, demonstrating that CD14 siRNA significantly inhibited the sensitivity of LPS-activated macrophages to lower doses of epinephrine. In addition, other studies illustrated that macrophage activation was significantly inhibited after treatment with an antagonistic anti-CD14 antibody [25, 26]. These data further confirm that CD14 plays an important role during epinephrine regulation of macrophage activation under LPS stimulation.

As to the relationship between CD14 and cytokines such as TNF\( \alpha \), it appears that the expression of cytokines (TNF\( \alpha \), IL-1\( \beta \), IL-6, and IL-10) could be effected by CD14 rather than the reverse. Our results showed that the pattern of CD14 expression was more or less similar to that of cytokines including TNF\( \alpha \), IL-1\( \beta \), IL-6, and IL-10. With the increasing of the concentration of pretreated epinephrine, the amount of their respective expression changed from low to high and then to low again. Furthermore, their expression levels all attained the peak value when they were pretreated by epinephrine at 10 ng/mL. Meanwhile, in our CD14 siRNA experiment, the expression level of TNF\( \alpha \) decreased when the function of CD14 was inhibited. The mechanism may be as follows. As an important pattern recognition receptor, CD14 mediates the recognition and signal transduction of...
macrophage on LPS. The latter in turn promotes the activation of macrophages and makes them secrete these cytokines. Although the expressions of these cytokines play a feedback role on CD14 to some extent, it appears that the role of CD14 is the most important.

Thus, epinephrine at appropriate concentrations, such as 10 ng/mL, may enhance responses from macrophages stimulated by 10 ng/mL LPS. The underlying mechanism likely relates to upregulated CD14 expression on the cell surface of macrophages. That being said, the precise regulatory mechanism remains unknown in light of the influence that many other stress hormone factors have on innate immunity after LPS infection. We will investigate this matter further in the future research. It must be noted that this investigation discusses a research in vitro and the goal is to explore whether the epinephrine pretreatment may influence the immunity function of peritoneal macrophage under LPS stimulation at the early stage (within 6 hours in general). The whole experiment does not involve the role of pretreated epinephrine in mortality and organ injury after LPS stimulated in vivo. Nonetheless, our results in this investigation will provide evidential support for the study on the role of epinephrine in vivo, especially under the severe trauma combined LPS stimulation.

**Conflict of Interests**

The authors declare that no conflict of interests exists.

**Acknowledgments**

The authors thank Dr. Xue Yan, Dr. Guoping Li, Ms. Qing Liu, Ms. Suna Huang, and Ms. Ying Chen for their kind assistance. This work was supported by the National Natural Science Foundation of China (81070624, 81273227), Natural Science Foundation Project of CQ CSTC (2011BB5034), and Open Foundation of State Key Laboratory of Trauma, Burns and Combined Injury (SKLKF201107).

**References**


Sepsis is a leading cause of mortality and morbidity in the critical illness. Multiple immune inflammatory processes take part in the pathogenesis of sepsis. Defensins are endogenous antimicrobial peptides with three disulphide bonds created by six cysteine residues. Besides the intrinsic microbicidal properties, defensins are active players which modulate both innate and adaptive immunity against various infections. Defensins can recruit neutrophils, enhance phagocytosis, chemotact T cells and dendritic cells, promote complement activation, and induce IL-1\(\beta\) production and pyroptosis. Previous publications have documented that defensins play important roles in a series of immune inflammatory diseases including sepsis. This review aims to briefly summarize in vitro, in vivo, and genetic studies on defensins’ effects as well as corresponding mechanisms within sepsis and highlights their promising findings which may be potential targets in future therapies of sepsis.

1. Introduction

Sepsis, severe sepsis, and septic shock represent a continuum of clinical syndromes which are common complications observed in patients with infection, trauma, and major surgeries [1–3]. These syndromes start with infection induced systemic inflammatory response syndrome (SIRS) and evolve to sepsis induced acute organ dysfunction and cardiovascular collapse. Epidemiology studies demonstrated that severe sepsis has a population prevalence of 300/100 000 in the United States and counts for 10–30% of the intensive care unit (ICU) patients [4–6]. And severe sepsis has already been acknowledged as the first cause of death in noncoronary ICUs with a high mortality rate of approximately 25–50% [7]. In the past one or two decades, steady progresses in treatment of sepsis have been made due to the advanced supportive care in ICU and the implementation of bundle therapies [7]. However, searching for specific remedies and reliable predictors within the pathophysiological mechanisms of sepsis is still the emphasis of today’s studies [8, 9].

Defensins are classified as a subfamily of cationic antimicrobial peptides, which are major components of the human innate immunity. They are small endogenous peptides with three disulphide bonds created by six cysteine residues. Defensins are categorized into three subtypes, \(\alpha\)-, \(\beta\)-, and \(\theta\)-defensin, based on the spatial structure and the locations of three disulphide bonds within the peptide. In the past decade, cumulative evidences have suggested that defensins play an important role and may be a potential intervention target in sepsis. This review hereby will summarize in vitro, in vivo, and genetic studies on defensins’ effects as well as corresponding mechanisms within sepsis and its sequential syndromes.

2. Antimicrobial Activities against Invading Pathogens in Sepsis

Defensins have broad spectrum antimicrobial activities against most pathogens in sepsis. The \(\alpha\)-defensins are constitutively expressed in human neutrophils (human neutrophil peptides [HNP] 1–4) or intestinal Paneth cells (human defensin [HD] 5-6) [10–12]. They can inhibit a large variety of Gram-positive bacteria, Gram-negative bacteria, and some species of fungi and viruses [11].

The \(\beta\)-defensins are mainly distributed in the epithelial cells of the respiratory system, digestive system, and genitourinary system [10–12]. They can effectively kill a number of Gram-negative bacteria, such as E. coli and P. aeruginosa, Gram-positive bacteria, such as S. aureus and...
Streptococcus pyogenes, and Candida albicans. β-Defensin-3 even has bactericidal effect towards multiresistant S. aureus and vancomycin-resistant Enterococcus [11, 13].

The θ-defensins, which have a unique macrocyclic structure, are isolated from leukocytes from some species of monkey and have not been detected in humans [14]. They are also reported to have antimicrobial activity against a spectrum of pathogens including E. coli, S. aureus, and C. albicans [15]. Also, they are found to have protective effect in a mouse model from a lethal pulmonary infection by a mouse adapted strain of SARS-coronavirus [16].

The classic mechanism of defensins' bactericidal effect is the "pore formation" theory. These positively charged antimicrobial peptides target negatively charged bacterial membrane components, such as lipopolysaccharides, teichoic acids, or phospholipids. Then they form transmembrane pores, disrupt cell integrity, and lead to bacteria lysis [10, 11]. Recently, another mechanism has been reported that defensins kill bacteria by inhibiting the synthesis of bacterial cell wall through interaction with certain precursors such as lipid II [17].

Defensins' bactericidal effect can be limited by high salt concentration of local environment where they encounter with the pathogens [18, 19]. Also, the antimicrobial action appears to be regulated by the redox response, as β-defensin-1 become more potent after reduction of disulfide bridges by thioredoxin or a reducing environment [20, 21].

3. Modulators and Alarmins in Immune Inflammatory Response of Sepsis

Defensins are also reported to have modulating effects on both innate and adaptive immune response. It is well known that HNPI-3 participate in the host immune defense via multiple mechanisms, including enhancing macrophage phagocytosis, facilitating neutrophil recruitment, modulating complement activation, and chemoattracting immature T cells and dendritic cells [12, 22].

In vitro studies showed that β-defensins have potent chemotactic effects, leading to the recruitment and maturation of naive dendritic cells and memory T cells in the inflammatory sites and the triggering of specific immune response in the host [23]. As the endogenous ligand of TLR-4, β-defensins interact with TLR-4 of the immune cells and regulate the expression of inflammatory mediators via the NF-κB pathway [18]. In vivo researches have revealed that the abnormal expression of β-defensins is associated with sepsis and various infectious diseases, as levels of β-defensins in both plasma and bronchoalveolar lavage fluid in patients with pulmonary infections are elevated [24–26], transcription of β-defensin-2 in leukocytes of severe septic patients is suppressed [27], expression of β-defensins in burn wound is reduced [28], and impaired expression of β-defensins is associated with inflammatory bowel diseases [29, 30]. In a mouse model of acute lung injury, Shu et al. expressed recombinant β-defensin-2 in lung tissue via recombinant adenovirus to study its protective effect against P. aeruginosa infection. Compared with control mice, they found considerably less P. aeruginosa in the transinfected lung tissue, as well as alleviated alveolar impairment, interstitial edema, and neutrophil infiltration [31, 32]. In subsequent studies, mice transinfected by adenovirus with or without β-defensin-2 genes received cecal ligation and puncture (CLP) twice to generate sepsis models. The impact of β-defensin-2 on the inflammatory response (e.g., the level of ICAM-1 expression), the severity of lung injury, and the sepsis outcome (7-day survival rate) were observed and evaluated. It was found that recombinant β-defensin-2 could down-regulate the expression of ICAM-1 in lung tissue 24 h, 36 h, and 72 h after CLP and significantly raised the 7-day survival rate in sepsis mice [31, 33]. In the clinical setting, Öhrich et al. found preterm neonates had lower levels of β-defensin-2 in cord blood when compared to term neonates [34]. And among these preterm neonates, lower β-defensin-2 level was associated with late-onset sepsis. These studies indicate that β-defensin-2 may play an important role in the immune inflammatory response in sepsis and might influence the outcome of sepsis.

Among the θ-defensins, rhesus macaque θ-defensin (RTD), which has six subtypes, has been extensively studied. Though not expressed in humans, RTDs were reported to significantly reduce levels of TNF-α, IL-1β, IL-6, IL-8, MIP1, and so on, in human peripheral blood leukocytes that are preincubated with various toll-like receptor agonists [35]. Furthermore, in vivo study showed that subcutaneously administration of 5 mg/kg RTD-1 could improve the survival rate and suppress the levels of a number of inflammatory cytokines and chemokines in two sepsis mouse models (received either intraperitoneal injection of E. coli or CLP). Although detailed mechanisms of the protective effect of RTD-1 have not been illuminated, the authors suggested that the interaction between RTD-1 and leukocyte is the critical determinant of TNF-α blockade [35]. The latter is a major proinflammatory cytokine and influences the consequent inflammatory cascades. These results indicate that θ-defensins may be a potential immune adjuvant in the treatment of sepsis, though they are not expressed in human.

In sepsis and other inflammatory disorders, defensins are among a group of rapidly-released host endogenous molecules, which are capable of both recruiting and activating APCs and are also termed the alarmins. Recently, in vitro studies have shown that alarmin HNPI-3 have the ability to boost host inflammatory response by promoting macrophage IL-1β production and pyroptosis via purinergic P2X7 receptor [36]. However, this effect is a double-edged sword in sepsis since it can promote pathogen elimination as well as mediate organ dysfunction such as acute lung injury [22, 37].

4. Genetic Polymorphisms and Sepsis Susceptibility

In molecular genetics and molecular biology, knock-out animal model is one of the most convincing means to determine the role of a specific molecule in the physiopathology of a certain disease. However, as members of the defensin
family have overlapped biological functions, the function of the knock-out gene in animal models may probably be compensated by other defensins. Since the gene cluster coding for the entire defensin family cannot be fully knocked out using the present techniques of molecular biology and genetics as well as human defensins lack of absolute animal analogues, genetic association analysis is a good alternative that can effectively explore the relationship between genetic polymorphism and sepsis.

In normal peripheral blood cells, mRNA levels of both \( \beta \)-defensin-1 and \( \beta \)-defensin-2 raise remarkably when stimulated by LPS or \( \text{P. aeruginosa} \) [23]. However, the upregulation of \( \beta \)-defensin-1 and \( \beta \)-defensin-2 varies among individuals, resulting in interindividual differences in host defense capacity and hence influencing the clinical progression of sepsis. Previous studies showed that single nucleotide polymorphism (SNP) of \( \beta \)-defensin-1 gene (DEFB1) correlates with chronic obstructive pulmonary disease, asthma, genetic allergy, HIV infection, and pseudomonas species infection in oral mucosa [38–42]. Since sepsis is a multifactorial disease caused by both environmental factors (pathogenic microbes) and host factors (comorbidities and genetic background), its occurrence and outcome are influenced with individual genetic background [43]. Chen et al. selected 5 SNPs in the promote region of DEFB-1 (–1816A/G, –390A/T, –52A/G, –44C/G, and –20A/G) and one in its exon (1654G/A) as candidate loci and studied 211 patients with severe sepsis and 157 healthy controls [44]. Distribution of alleles, gene types, and haplotypes associating with these loci were studied and compared between septic patients and controls, as well as between survivors and victims of severe sepsis. Association analysis, logistic regression, and linkage disequilibrium study showed that –44G allele was closely related with susceptibility to severe sepsis and poor outcome. And severe septic patients with haplotype –20G/-44G/-52G had even poorer outcome, while individuals with haplotype –20A/-44C/-52G were less susceptible to severe sepsis. The reason why –44C/G is correlated with the occurrence and outcome of severe sepsis may attribute to the following points. It located in the 5' untranslated region of DEFB1 and its polymorphism may result in changes in the space conformation of mRNA, which would alter the stability of mRNA and the efficiency of translation. And its impact on the protein function is more significant than nonsynonymous SNP in coding region [45], as the quantity of protein would change dramatically. However, as any other genetic association analysis, DEFB1 –44C/G may be only a surface marker of some unknown real genetic marker of sepsis in linkage disequilibrium. Although these hypothesis need to be proved by further researches, the above-mentioned study indicated that \( \beta \)-defensin-1 might be an influential factor in the process of immune defense and inflammation regulation in sepsis, and the locus of –44C/G may be an important genetic warning indicator of susceptibility to severe sepsis and its outcomes.

Copy number variation (CNV) is a kind of genetic polymorphism that accounts for approximately 12% of human genomic DNA. It refers to a large-scale duplication or deletion of certain DNA sections, which causes a variation in the number of copies of one or more genes. Previous publications reported that CNV is present in \( \beta \)-defensin-2 gene (DEFB4), \( \beta \)-defensin-3 gene (DEFB103), \( \beta \)-defensin-4 gene (DEFB104), \( \alpha \)-defensin-1 gene (DEFA1), and \( \alpha \)-defensin-3 gene (DEFA3) [18, 46–48]. And copy number of DEFB4 has a positive correlation with its mRNA level [35, 45]. Recently, Chen et al. screened 179 severe sepsis and 233 healthy controls for DEFA1 and DEFA3 [49]. An average DEFA1/DEFA3 copy number of 7 per genome was observed in the studied population, with a range of 2 to 15. The authors found that patients with high copy number of DEFA1/DEFA3 were predisposed to severe sepsis and tended to have lower level of plasma HNP1-3 as well as cytokines such as TNF-\( \alpha \), IL-6, and IL-10. They further validated their findings in an independent cohort. These results indicated that CNVs in the defensin gene may be potential genetic markers for identifying high risk patients or providing individual treatment in sepsis.

5. Perspective

Defensins are emerging therapeutic molecules against pathogens in sepsis because of their broad spectrum antimicrobial properties. In the past decade or two, a number of potent and salt insensitive defensins and their analogs have been screened, structurally modified, and synthesized. However, most of these studies are performed in vitro and not much is known about the in vivo roles of these molecules. In fact, chemoattracting and immunomodulating effects make defensins a double-edged sword in the pathogenesis of sepsis, which leads to facilitation of pathogen clearance as well as exacerbation of inflammation and injury of self-tissues. Recently, several investigations showed that the chemoattractant and antimicrobial activities of defensins could be separated, which shed light on the design of defensin-derived pharmaceuticals [50]. In addition, genetic studies help identify high risk patients with susceptibility to sepsis or its adverse outcome, which provides foundation for future individualized sepsis treatments that are targeting defensins.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (no. 81201495) and the research project of the Department of Education of Zhejiang Province (no. Y200909678).

References


Fluid Resuscitation in Sepsis: Reexamining the Paradigm

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Received 25 February 2014; Revised 8 July 2014; Accepted 20 July 2014; Published 11 August 2014

Academic Editor: Baoli Cheng

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Sepsis results in widespread inflammatory responses altering homeostasis. Associated circulatory abnormalities (peripheral vasodilation, intravascular volume depletion, increased cellular metabolism, and myocardial depression) lead to an imbalance between oxygen delivery and demand, triggering end organ injury and failure. Fluid resuscitation is a key part of treatment, but there is little agreement on choice, amount, and endpoints for fluid resuscitation. Over the past few years, the safety of some fluid preparations has been questioned. Our paper highlights current concerns, reviews the science behind current practices, and aims to clarify some of the controversies surrounding fluid resuscitation in sepsis.

1. Introduction

The incidence of severe sepsis varies between 20 and 30% in most intensive care units and is a leading cause of mortality [1]. Fluid resuscitation is one of the cornerstones of management. Though there is a consensus on the need for adequate fluid therapy, the timing, type, and quantity of fluid resuscitation remain controversial. Furthermore, the optimal monitoring technique to guide fluid therapy continues to be debated; with mounting and sometimes contradicting evidence, the ideal fluid strategy is increasingly elusive.

Contemporary understanding of the pathophysiology of sepsis supports intensive fluid resuscitation in the initial phase. SIRS and sepsis incite widespread inflammatory responses at tissue and cellular levels altering homeostasis. Resultant circulatory abnormalities (peripheral vasodilation, intravascular volume depletion, increased cellular metabolism, and myocardial depression) lead to an imbalance between oxygen delivery and demand, worsening end organ injury and failure.

In a landmark paper, Rivers and colleagues demonstrated early goal directed therapy, targeting a specific central venous pressure (CVP) and mixed central venous saturation (ScVO₂), improved mortality by 16% [2]. In response, the surviving sepsis guidelines recommend early aggressive fluid resuscitation during the “golden” hours [3]. Although adequate fluid resuscitation makes eminent physiological sense, the optimal amount, and type of fluid remain unclear. Our paper aims to clarify these issues by reviewing the latest evidence guiding these practices.

2. Monitoring Fluid Resuscitation

2.1. Static Monitors. In sepsis, it is important to identify which patients will respond to volume resuscitation. In the critically ill, this means identifying the patient whose cardiac output will improve with fluid administration, called preload responsiveness. Traditionally, static indicators such as CVP have guided therapy. However, historic and recent evidence suggest CVP is a poor predictor of fluid responsiveness. In a systematic review on the usefulness of the CVP, Marik et al. concluded that it is neither a good indicator of volume status nor a predictor of responsiveness to fluid therapy [4]. It has been suggested that CVP no longer be used to guide fluid therapy [5]; although it remains in the surviving sepsis guidelines, some authors suggest that these recommendations should be revisited [5]. In fact, recent
evidence suggests CVP guided fluid resuscitation leads to venous congestion increasing the incidence of pulmonary complications in septic shock [6]. However, removal of CVP parameters from the guidelines may result in inadequate volume resuscitation and many centres continue to use static CVP measurement, despite evidence that it is an unhelpful guide for fluid administration. Furthermore, respiratory variation in the CVP is useful for predicting fluid responsiveness in spontaneously breathing patients [7].

Similarly, the pulmonary artery catheter (PAC) is unable to predict fluid responsiveness. Perhaps this is partly why the PAC is not associated with improved outcomes and its use has declined over the past two decades [8]. Although hemodynamic variables available from the PAC, such as the pulmonary capillary wedge pressure (PCWP), cardiac output (CO), and derived variables, are helpful for determining the type of circulatory shock and assessing response to therapy, none of these parameters predict preload responsiveness [9]. Furthermore, recent evidence casts doubt on the accuracy of hemodynamic data obtained from PACs [10].

2.2. Dynamic Monitoring. The most useful indicators of preload responsiveness are phasic changes in stroke volume and systolic blood pressure during positive pressure mechanical ventilation [11]. Stroke volume variation (SVV) is the ratio of maximal stroke volume difference during several respiratory cycles and the mean stroke volume over the same period [12]. Since the arterial pulse pressure depends on the amount of blood ejected during each systole (stroke volume), the pulse pressure variation covaries with SVV [13]. During positive pressure ventilation, inspiration increases the intrathoracic pressure reducing the right ventricular (RV) filling and right ventricular output if the RV is volume responsive. This causes the left ventricular filling and left ventricular (LV) output to decrease over successive beats if the LV is also volume responsive [12]. A SVV of >15% in patients receiving a tidal volume of >8 mL/kg or an SVV of >10% in patients receiving a tidal volume of 6 mL/kg accurately predicts preload responsiveness in patients with a closed chest [14–16].

Commercially available monitors such as the PiCCO, LiDCOplus, Volume View/EV1000, and the FloTrac use pulse contour analysis to indirectly determine the cardiac output and stroke volume variation. Pulse contour analysis is based on the relationship of the stroke volume, aortic compliance, and systemic vascular resistance [17]. Complex algorithms that account for reflection waves and aortic impedance are used to analyse the arterial wave and derive the stroke volume. The LiDCO uses pulse power analysis to convert the arterial waveform into a volume-time waveform which makes it less dependent on the shape of the pulse wave [18]. Although these devices are dependent on accurate calibration to measure CO, SVV and PPV are not dependent on calibration and, therefore, less affected by reliability concerns associated with these devices.

2.3. Indicators of Tissue Perfusion. The ultimate goal of fluid resuscitation is adequate tissue perfusion. However, dynamic monitoring does not measure tissue perfusion. Indicators of adequate perfusion include SVO2, ScVO2, and lactate. The surviving sepsis group recommends targeting ScVO2 of 70% within the first 6 hours of recognition of sepsis [19]. However, ScVO2 may be normal or even elevated in sepsis, for example, in patients with chronic liver disease. In contrast, hyperlactatemia is a more consistent finding in severe sepsis [20]. Normalization of lactate can be a useful target, alongside other hemodynamic parameters. Jansen et al. demonstrated reduced hospital mortality when targeting normalisation of lactate in a multicentre RCT [21].

A potentially useful measure of tissue perfusion is gastric mucosal pH. Since splanchnic circulation is compromised early during hypoperfusion, gastric blood flow is reduced. Changes in gastric mucosal pH (pHi), measured using a tonometer, reflect the adequacy of splanchnic perfusion [22]. The pHi is determined using a fluid or air filled balloon tipped nasogastric tube. The balloon contents equilibrate with the gases in the gastric lumen; therefore, changes in carbon dioxide (CO2) in the balloon reflect gastric luminal CO2. The pHi is calculated from the gastric lumen CO2 and blood bicarbonate; lower values indicate greater hypoperfusion. Although it is useful in prognosticating multiorgan failure and death in several conditions such as acute pancreatitis [23], trauma [24], and other critically ill patients [25], technical difficulties and potential sources of error in manual tonometer monitoring have prevented its widespread use [22]. Other tissue perfusion monitors such as Sidestream Dark Field imaging technique (SDF) [26], sublingual capnometry [27–29], and near infrared spectroscopy (NIRS) [30–33] have also been studied in critically ill patients. Although some studies have shown benefit, these monitors are not widely available and their clinical utility for delivery of bedside critical care remains to be established [33, 34].

3. Which Fluid?

Intravenous fluid therapy originated during the great cholera outbreak of the nineteenth century [35–38]. Fluids of various compositions were used, and studies tracing their composition indicate they resembled balanced crystalloids [39]. Balanced solutions are those with an electrolyte composition similar to that of plasma. However, the most commonly used crystalloid is 0.9% saline, which is not balanced. About 10 million litres of saline are used each year in the UK and 200 million litres are sold every year in the United States [40].

3.1. Crystalloids (Saline and Balanced Solutions). 0.9% saline is frequently referred to as “normal” saline. However, Awad and colleagues elegantly showed that this term entered medical practice based on colloquialism rather than sound physiological or scientific data [39]. There is certainly nothing normal about “normal” saline. The first documented use of “normal saline” was in the Lancet in 1888 [41]; however the solution described bore no resemblance to 0.9% saline. The widespread adoption of 0.9% saline was likely based on its
isotonicity, as described in a single in vitro experiment on red
cell lysis as well as the convenience and low cost of production
[42].

Although there is no consensus on the superiority of balanced solutions over 0.9% saline, contemporary under-
standing of acid-base balance and recent observational evi-
dence favours balanced solutions. The Stewart physicochem-
ical approach [43] to acid-base balance that infusion of
large quantities of 0.9% saline will result in hyperchloremic
acidosis. The strong ion difference (SID-sum of all the strong
ions minus sum of all the strong anions) of plasma is
maintained by the greater concentration of sodium relative
to chloride in the plasma. Electroneutrality is maintained
by anions such as bicarbonate ($\text{HCO}_3^-$), weak acids (HA),
and hydroxyl ion (OH$^-$). Decreases in the SID decrease the
available "space" for these anions, ultimately reducing
[OH$^-$]. However, the dissociation of water (kw) must remain
constant. Since kw is directly proportional to the product of
[OH$^-$] and hydrogen ion concentration [H$^+$], decreases in
[OH$^-$] lead to increases in [H$^+$] causing acidosis. Infusing
0.9% saline provides relatively more chloride, compared to
sodium, resulting in a reduction in the strong ion difference
which in turn lowers the pH, causing hyperchloremic acidosis
[44].

Few studies have compared balanced crystalloids and
0.9% saline in patients with sepsis. However, there is sub-
stantial animal evidence that hyperchloremia causes harmful
effects. In dogs, hyperchloremia causes progressive renal
vasoconstriction and a fall in GFR in denervated kidneys
[45]. In animal sepsis models, infusions of 0.9% saline
increase inflammatory cytokines, worsen hypotension and
vasoconstriction and a fall in GFR in denervated kidneys
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[45].

3.2. Colloids. There are fundamental differences between
crystalloids and colloids. Crystalloids are predominantly
based on sterile water to which electrolytes have been added.
Colloids have an additional “colloidal” component that
does not freely diffuse across semipermeable membranes,
in theory making them more effective volume expanders.
Colloids are the preferred resuscitation fluids in Europe and
Australasia [54]. Albumin, hydroxyethyl starch (HES),
and gelatins are the three classes of colloid commonly
used.

However, the safety profile of certain colloids in patients
with sepsis has recently been challenged. In fact, safety
concerns have existed since their introduction. Scheinhart
and colleagues, in a meta-analysis of 37 RCTs in critically
ill patients, found that resuscitation with colloids (albumins,
gelatins, dextrans, and starches) increased risk of mortality
by 4% (95% CI 0–8%) [55]. A separate French multicentre
study found gelatins [odds ratio (OR) 4.81 (95% CI 2.01–
11.51 $P = 0.0005$]) and dextrans [OR 3.83 (95% CI 1.17–12.60
$P = 0.02$)] were independent risk factors for anaphylactoid
reactions [56]. Furthermore, Dextran 70 has been shown to
decrease Factor VIII procoagulant activity, factor VIII
related antigen, and ristocetin cofactor activity [57] resulting
in coagulopathies.

Based on adverse outcome reports, such as renal dysfunc-
tion and coagulopathy, high molecular weight starches have
already been phased out in favour of HES (130/0.42). The
adverse effects of HES were considered benign, transitory,
dose dependent, and related to only high molecular weight
starches. However, HES (130/0.42) is not readily excreted
and there is evidence it accumulates in the skin, liver, kidney,
and reticuloendothelial system [58]. It is suggested that the
lower degree of substitution and lower molecular weight
of HES (130/0.42) facilitate greater uptake in the tubular
epithelium leading to osmotic nephrosis and requirement
of renal replacement therapy and, therefore, could be more
harmful than its predecessors [58–60].

In the recent Crystalloid versus Hydroxyethyl starch Trial
(CHEST), the effect of fluid resuscitation with HES (130/0.4)
was compared with 0.9% saline among 7000 patients admit-
ted to an intensive care unit [61]. The study found no
difference in 90 day mortality between the groups; however,
patients receiving HES required renal replacement therapy
more frequently (RR 1.21, 95% CI 1.00–1.45, \( P = 0.04 \)). The study also demonstrated more adverse events with the use of HES.

A second trial recently randomized 804 patients with severe sepsis to receive either HES (130/0.42) or Ringer’s acetate, the 6S trial [62]. The primary outcome of death or severe sepsis to receive either HES (130/0.42) or Ringer’s acetate. The study also concluded that HES was associated with less volume expansion benefits and its use in sepsis cannot currently be recommended. Given the concerns with synthetic colloids, albumin has reemerged as a good alternative. The recent surviving sepsis campaign guidelines advocate the use of albumin for volume expansion after the use of crystalloids [19].

Apart from the hemodynamic efficacy that albumin confers, it is reported to have antioxidant and anti-inflammatory activity [65]. The postulated mechanisms include an increase in plasma thiol levels, modulation of cytokine activity, binding of endotoxin, and protection of glycocalyx. It also alters drug binding and reduces nitric oxide, attenuating vasodilatation [66].

The SAFE study compared albumin and saline resuscitation in 6997 patients [67]. Twenty-eight-day mortality was no different between both groups (726 albumin group versus 729 0.9% saline group, \( P = 0.87 \)). The study concluded that albumin and 0.9% saline are clinically equivalent for fluid resuscitation in the ICU. However, post hoc analysis of the sepsis subgroup indicates that resuscitation with albumin may reduce the mortality in patients with severe sepsis, confirming possible additional protective mechanisms conferred by albumin. Furthermore, a large meta-analysis showing resuscitation with albumin solutions in sepsis was associated with lower mortality [68]. Although many of the studies included had not used proper methodology, the results suggest that albumin does not have specific adverse effects in sepsis. Table 2 summarizes studies examining colloids.

A separate multicentre study in Italy, the ALBIOS trial, recruited 1800 patients with sepsis or septic shock and compared resuscitation with 20% albumin or a crystalloid the results of which are not yet published. Similarly, another large trial involving 800 patients with sepsis in France resuscitated either with 20% albumin or normal saline, conducted by the EARSS study group, is yet to publish its results. Together, the results of these two large trials will help confirm whether albumin has additional protective effects.

The recent CRISTAL randomized trial examined the effects of fluid resuscitation with colloids versus crystalloids on mortality in critically ill patients with hypovolemic shock [69]. It was a multicentre, open label randomized clinical trial stratified by case mix (sepsis, trauma, or hypovolemic

### Table 1: Summary of studies evaluating crystalloids.

<table>
<thead>
<tr>
<th>Author and Year</th>
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<th>Study fluid</th>
<th>Primary endpoint</th>
<th>Comments</th>
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</thead>
<tbody>
<tr>
<td>Wilcox [45] 1983</td>
<td>Animal experiment</td>
<td>48</td>
<td>Chloride rich solutions</td>
<td>Regulation of renal blood flow</td>
<td>Increased renal vasoconstriction and ↓GFR with chloride rich solutions</td>
</tr>
<tr>
<td>Waters et al. [49] 2001</td>
<td>Prospective randomized study</td>
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<td>0.9% Saline versus lactated Ringer</td>
<td>Multiple outcomes studied</td>
<td>Increased use of blood products and acidosis with 0.9% Saline</td>
</tr>
<tr>
<td>O’Malley et al. [50] 2005</td>
<td>Randomised clinical trial</td>
<td>51</td>
<td>0.9% Saline versus lactated Ringer</td>
<td>Creatinine concentration on POD3b</td>
<td>No difference; but Ringer’s was associated with less hyperkalemia and acidosis</td>
</tr>
<tr>
<td>Shaw et al. [51] 2012</td>
<td>Observational</td>
<td>31,920</td>
<td>0.9% Saline versus balanced crystalloid</td>
<td>Major morbidity</td>
<td>Higher mortality, increased transfusion requirements, dialysis requirements, and increased buffer requirements in saline group</td>
</tr>
<tr>
<td>Maitland et al. [52] 2011</td>
<td>Multicentric randomized trial</td>
<td>3141</td>
<td>Albumin bolus and saline bolus</td>
<td>Mortality</td>
<td>Boluses resulted in increased mortality</td>
</tr>
</tbody>
</table>

Table summarizing studies evaluating crystalloids. aGlomerular Filtration Rate, bPostoperative Day 3.
Table 2: Summary of studies evaluating colloids.

<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>Study design</th>
<th>Sample size</th>
<th>Study fluid</th>
<th>Primary endpoint</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Schierhout and Roberts</td>
<td>1998</td>
<td>Meta-analysis</td>
<td>1315</td>
<td>All colloids</td>
<td>Mortality</td>
<td>Increased mortality</td>
</tr>
<tr>
<td>Laxenaire et al.</td>
<td>1994</td>
<td>Multicentre prospective</td>
<td>19593</td>
<td>All colloids</td>
<td>Adverse effects</td>
<td>Galactins and dextran-independent risk for anaphylactoid reactions</td>
</tr>
<tr>
<td>Myburgh et al.</td>
<td>2012</td>
<td>RCTa</td>
<td>7000</td>
<td>HESb versus 0.9% saline</td>
<td>90-day mortality</td>
<td>HESb associated with increased incidence of RRT</td>
</tr>
<tr>
<td>Perner et al.</td>
<td>2012</td>
<td>RCTa</td>
<td>804</td>
<td>HESb versus Ringer's acetate</td>
<td>Death/dialysis dependence at 90 days</td>
<td>Death and dialysis dependence more in HES</td>
</tr>
<tr>
<td>Zarychanski et al.</td>
<td>2013</td>
<td>Meta-analysis</td>
<td>10,290</td>
<td>HESb</td>
<td>Mortality and AKIc</td>
<td>Significant increase in risk of mortality and AKIc</td>
</tr>
<tr>
<td>Guidet et al.</td>
<td>2012</td>
<td>RCTa</td>
<td>196</td>
<td>HESb versus 0.9% saline</td>
<td>Hemodynamic efficacy and safety</td>
<td>HESb better hemodynamic efficacy and no difference in AKIc</td>
</tr>
<tr>
<td>Finfer et al.</td>
<td>2004</td>
<td>RCTa</td>
<td>6997</td>
<td>Albumin versus 0.9% saline</td>
<td>28-day mortality</td>
<td>No difference</td>
</tr>
<tr>
<td>Myburgh et al.</td>
<td>2007</td>
<td>Post hoc analysis of SAFE trial</td>
<td>460</td>
<td>Albumin versus 0.9% saline</td>
<td>Safety in TBId</td>
<td>Albumin unsafe for TBId</td>
</tr>
<tr>
<td>Delaney et al.</td>
<td>2011</td>
<td>Meta-analysis</td>
<td>1977</td>
<td>Albumin</td>
<td>Safety for resuscitation</td>
<td>Albumin associated with lower mortality</td>
</tr>
<tr>
<td>Annane et al.</td>
<td>2013</td>
<td>RCTa</td>
<td>2857</td>
<td>Colloids versus crystalloids</td>
<td>28-day mortality</td>
<td>No difference</td>
</tr>
</tbody>
</table>

Table summarizing studies evaluating colloids. aRandomized Controlled Trial, bHydroxyethyl starch, cAcute Kidney Injury, dTraumatic Brain Injury.

shock without sepsis or trauma). They used colloids (n = 1414; gelatins, dextrans, hydroxyethyl starches, 4% or 20% of albumin) or crystalloids (n = 1443; isotonics saline, hypertonic saline, or Ringers lactate) for fluid interventions, other than fluid maintenance throughout the ICU stay. There was no difference in 28-day mortality between the two groups (359 in colloid group versus 390 in crystalloid group, \( P = 0.26 \)). However, a secondary outcome, 90-day mortality, was lower in patients receiving colloids, but it is difficult to draw strong conclusions from this study due to the heterogeneity of fluid composition in the two groups.

From the many trials that have been carried out so far, it is clear that some synthetic colloids should be avoided in sepsis and that 0.9% saline may have disadvantages over balanced crystalloids. However, whether to select albumin over a crystalloid remains uncertain. Based on the SAFE study, one potential advantage of albumin is that less fluid is ultimately required to achieve hemodynamic end goals. This will only prove beneficial, if a more positive balance is associated with worse outcomes.

4. How Much Fluid?

Boyd and colleagues retrospectively reviewed the association of positive fluid balance at 12 hours and at 4 days in 778 patients of the Vasopressin in Septic Shock (VASST) study [70]. They found that the quartile that had the least positive balance at 12 hours [0.569 (0.405–0.799) for Quartile 1 and 0.581 (0.414–0.816) for Quartile 2] and at 4 days [0.466 (0.299–0.724) for Quartile 1 and 0.512 (0.339–0.775) for Quartile 2] had a lower hazard ratio relative to the quartile with the maximum positive balance. Furthermore we know that a fluid restrictive strategy is beneficial in patients with concomitant ARDS [71]. Although it is expected that 3 to 4 times the volume of crystalloids may be required to achieve the hemodynamic efficacy of colloids, the SAFE study found that the volume of saline used was only 40% more than albumin, perhaps because the clearance of crystalloids is decreased during the stress response of critical illness. Furthermore, there are settings where one fluid is clearly advantageous, such as sepsis patients with traumatic brain injury where albumin and hypotonic resuscitation fluids should be avoided [72]. Similarly, patients requiring fluid restrictive strategy, such as those with ARDS or concomitant abdominal compartment syndrome, might benefit from an albumin based strategy (Figure 1).

5. Conclusion

In conclusion, a perfect one-size-fits-all fluid strategy does not exist. In sepsis, clinicians should understand the limitations and potential benefits of each strategy. Each fluid should be considered a drug, with specific pharmacokinetic, pharmacodynamic, and adverse effect profiles, which can be carefully matched to the patient. Whichever fluid is chosen, resuscitation should be titrated to evidence based
Sepsis with hypotension

Preload responsive?
(PPV or SVV >15% in a mechanically ventilated patient or CVP variance in a spontaneously breathing patient)

Yes

Fluid resuscitation mandated

Considerations in fluid resuscitation

TBI

ARDS

0.9% saline preferred
Avoid albumin and hypotonic crystalloids

Abdominal sepsis with compartment
Syndrome or chronic liver disease

Avoid using only crystalloids, consider albumin

Consider restrictive fluid strategy
Consider albumin in addition to crystalloids

No

Consider early vasopressors/inotropes and confirm fluid status on bedside ECHO

All other sepsis

Balanced crystalloids preferred

Figure 1: Algorithm to guide fluid therapy in the septic patient.

targets, combining clinical assessment, such as signs of tissue perfusion with dynamic hemodynamic monitoring. Balanced crystalloids may be preferred first choice, followed by albumin, based on their comparative safety profiles. 0.9% saline should only be used after consideration of its potential to cause harm and current evidence would suggest starches (HES) should be avoided in sepsis.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgment

Work was in part funded by New Clinician Scientist Program, National University Health System Singapore (MEC).

References


Research Article

Role of AMPKα in Skeletal Muscle Glycometabolism Regulation and Adaptation in relation to Sepsis

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Received 30 January 2014; Accepted 16 June 2014; Published 29 June 2014

Academic Editor: Baoli Cheng

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Background. AMP-activated protein kinase (AMPK) and the translocation of glucose transporter 4 (GLUT4) protein always involve disturbance of carbohydrate metabolism. Objective. To determine whether the change of blood glucose in the early stage of septic rat is associated with the alteration of AMPKα protein expression and GLUT4 protein translocation expression.

Methods. Animal models of sepsis were induced by tail vein injection of LPS in Wistar rats. The dynamic values of blood glucose within 2 hours after injection of LPS were observed. AMPKα protein and GLUT4 protein translocation in different tissues (such as soleus muscle and extensor digitorum longus) were assessed by western blot.

Results. Blood glucose levels appeared to rise at 0.5h after injection of LPS, arrived the peak value at 1h, then fell at 1.5h and 2h Animals in LPS group experienced the increase of phos-AMPKα protein and GLUT4 protein translocation expression in soleus muscle and extensor digitorum longus.

Conclusion. The dynamic change of blood glucose, represented in a form of initiative increase and subsequent decrease in the early stage of sepsis, may be related to glycometabolism disorder in the skeletal muscle, coming down to enhancement of GLUT4 translocation expression promoted by activation of AMPKα.

1. Introduction

Sepsis is a serious medical condition that is characterized by a whole-body inflammatory state, resulting from the systemic response to bacterial infection. If the bacteria die, the endotoxin will be released into the bloodstream. Sepsis remains one of the leading causes of morbidity and mortality in critically ill intensive care unit patients [1]. The systemic administration of lipopolysaccharide (LPS), an outer component of the gram-negative bacterial wall, has been applied as an experimental model to mimic some of the clinical findings of human septic shock [2]. The kind of vicious stimulus, at the same time, leads to severe metabolic disorder. Baseline hyperglycemia, including stress-induced hyperglycemia, is common in patients with severe sepsis. Similarly, stress-induced hyperglycemia is associated with adverse outcomes in septic patients [3–7]. In a 2001 study of critically ill intensive care unit (ICU) patients, van den Berghe and associates demonstrated that aggressive insulin therapy to maintain blood glucose between 4.4 and 6.1 mmol/L reduced mortality from 8.0% with conventional treatment to 4.6%, a relative reduction of 42% [3]. Mortality reduction in the intensive insulin treatment group was attributed to lower rates of organ failure and bacteremia. Mackenzie and colleagues recently reported that when intensive glycemic control was managed by the bedside nurse, average morning glucose concentration was 7.0 ± 2.4 mmol/L, but 42% of patients suffered hypoglycemic episodes, defined as a serum glucose <2.2 mmol/L [8]; thus studies recommend a cautious approach to the control of glucose levels in acutely ill emergency department patients, with a target glucose of below 8 to 9 mmol/L [9]. In present, we only try to control blood glucose with insulin therapy. In fact these metabolic effects induced by 5’-adenosine monophosphate-activated protein kinase (AMPK) are associated with lowering blood glucose levels in hyperglycemic individuals [10]. AMPK is widely present in eukaryotic cells, sensing the changes of cellular energy metabolism, known as the “cellular energy regulator.” Impaired glucose metabolism regulated by activated AMPK is the response to cellular stress, such as exercise, hypoxic
stress, and ischemic stimulus [11–14]. It is well known that sepsis is a sophisticated morbid process and this unique model of sepsis induced by LPS always relates to the change of AMP/ATP ratio, ischemia, hypoxia, nutrition, and metabolic disorders. Therefore, as a new target for antidiabetic drugs, AMPK expression in sepsis should be noted.

Recent study showed that patients with type 2 diabetes were more prone to develop dysregulated glucose disposal, which was associated with altered AMPK phosphorylation in skeletal muscle [15]. GLUT4 is a glucose transport protein found in fat and striated muscle cells [16]. When carbohydrates are ingested, the major cellular mechanism that diminishes blood glucose is insulin-stimulated glucose transport into skeletal muscle. Skeletal muscle both stores glucose as glycogen and oxidizes it to produce energy following the transport step. The principal glucose transporter protein that mediates this uptake is GLUT4, which plays a key role in regulating whole body glucose homeostasis [17]. When insulin receptor is activated, it induces the GLUT4 protein to move from its reserves held inside cells. GLUT4 can also be recruited to the cell surface through muscle contraction. In the absence of insulin or muscle contraction, GLUT4 is stored in vesicles within the cell. In addition to insulin, skeletal muscle glucose transport is possible stimulated by other media or by other pathways. AMPK is really another known regulator of glucose metabolism in skeletal muscle [18]. Activation of AMPK in muscle leads to an increase in glucose transport, accompanied by increased translocation of GLUT4 to the plasma membrane [19]. Therefore, as the important targets which always involve disturbance of carbohydrate metabolism, whether AMPK and the translocation of GLUT4 protein expression appear to change to adapt the stress hyperglycemia in early stage of sepsis still needs to be paid attention to. Thus the present study is designed to explore whether the acute blood glucose dynamic changes are partly based on translocation of GLUT4 regulated by AMPK signal pathway in the early stage of sepsis.

2. Materials and Methods

2.1. Main Materials. Anti-Phos-AMPKα-Thr172 antibody and anti-AMPKα antibody were purchased from the U.S. Cell Signaling, Inc.; anti-GLUT4 antibody was obtained from Santa Cruz Biotechnology; anti-α-tubulin antibody was obtained from Merck Millipore, Billerica, MA; lipopolysaccharide (LPS, Escherichia coli O111: B4) was purchased from USA Sigma Company; insulin kit was purchased from the U.S. Adlitteram Diagnostic Laboratories Inc.; Membrane Protein Extraction Kit was purchased from the Fermentas International Inc.

2.2. Animal Model. 12 healthy male Wistar rats (8 weeks old, 200 to 250 g) were purchased from Experiment Animal Center of Chinese Academy of Sciences in Shanghai (SCXX (Shanghai) 2007-0005). The rats were divided into two groups: LPS group (received LPS 5 mg/kg (concentration of 2 mg/mL) by tail vein injection) and control group (given normal saline (NS) 2.5 mL/kg by tail vein injection) [20]. Body temperature of the rat was measured using the rectal probe. The procedures in our experiments were approved by the Animal Care and Use Committee of Zhejiang University, China.

2.3. The Determination of Blood Glucose and Insulin Levels. Blood glucose levels were determined at 0 h, 0.5 h, 1 h, 1.5 h, and 2 h after injection of LPS or NS with an Accu-chek glucometer (Roche, Mannheim, Germany) from tail-bled samples (made with a needle stick). At 2 hours, anesthesia was executed by 3% pentobarbital sodium (0.15 mL/100 g) intraperitoneal injection. 4–6 mL blood was taken from carotid artery; serum was segregated and stored at −20°C for measurement of insulin level. Insulin levels were determined using an Ultrasensitive Insulin ELISA kit according to the manufacturer’s instructions.

2.4. Western Blot. The samples of heart, liver, soleus muscle, and extensor digitorum longus were frozen into liquid nitrogen and stored. 100 mg of each tissue was homogenized in 1 mL modified lysis buffer (0.3 mol/L sucrose, 10 mmol/L imidazole, 10 mmol/L sodium metabisulfite, 1 mmol/L DTT, 0.3 mmol/L PMSF) [21]. The protein concentration was determined by the Bradford method.

Western blot analysis of AMPKα and Pho-AMPKα protein and α-tubulin were performed in heart, liver, soleus muscle, and extensor digitorum longus, while western blot analysis of GLUT4 was performed only in soleus muscle and extensor digitorum longus. Aliquots containing the protein for Phos-AMPKα-Thr172, AMPKα, GLUT4, and α-tubulin were loaded on the SDS-polyacrylamide gel with 10% acrylamide separating gel, respectively, and separated by electrophoresis for 30 min. The separated Phos-AMPKα-Thr172, AMPKα, GLUT4, and α-tubulin proteins were electrophotically transferred onto nitrocellulose membranes (Amersham Life Science). All of the membranes were incubated at 4°C overnight with anti-Phos-AMPKα-Thr172 antibody (1:1000), or anti-AMPKα antibody (1:1000) or anti-GLUT4 (1:3000), or anti-α-tubulin antibody (1:1000) in 5% Carnation instant milk/TBS. After incubating with a secondary antibody (1:500) (Beijing Zhongshan Biotechnology, China) in 5% Carnation instant milk-TBS-Tween 20, the blots were developed using enhanced chemiluminescence according to the manual (Biological Industries, Beit Haemerk LTD, Israel) and exposed to X-ray film [22]. Normalization of protein expression was carried out using α-tubulin as control.

2.5. GLUT4 Translocation Analysis. Preparation of plasma membrane fraction from the skeletal muscles was performed as described by Dombrowski et al. [23]. Briefly, three grams of the SOL or EDL muscles were homogenized in 10 mM sodium bicarbonate, 0.25 M sucrose, 5 mM sodium azide, and 100 μM PMSF. The homogenate was subjected to specific centrifugations for subcellular fractionation. The crude membrane was separated from homogenized tissue by use of triple centrifugation at 1200, 9000, and 19 000 × g, respectively. The plasma membrane fractions were further separated by sucrose density-gradient centrifugation (25%, 32%, and 35%)
at 150,000 × g for 16 h. The plasma membrane GLUT4 (m-GLUT4) protein was collected from the fraction of 25% sucrose solution, subjected to 190,000 × g for 60 min, and analyzed by Western blot. Immunoblotting of the tissue protein extracts was performed using anti-GLUT4 antiserum (1:3000). The blotted protein was quantified using quantity one software system [24–26].

2.6. Statistical Analysis. Data were reported as means plus or minus Standard Deviation (SD). The various kinds of indexes between control group and LPS-treated groups were compared using analysis of one-way ANOVA with SPSS 16 software. Values were considered significantly different when \( P < 0.05 \).

3. Results

3.1. General State of the Rats. Rats in control group were still active as usual, with good state, while those in LPS group showed mental weaknesses, physical inactivity dull coat, breathing frequently, greedy overdrink, and abnormal body temperature. Body temperature represented in a form with a rapid decline after 0.5 h and then kept lower within 2 h. In an hour after treatment, there was statistically significant effect on half-hourly body temperature between LPS group and control group (35.86 ± 0.88 versus 37.07 ± 0.65 at 1 h, \( P < 0.05 \); 34.57 ± 0.86 versus 37.81 ± 0.36 at 1.5 h, \( P < 0.05 \); 34.32 ± 0.86 versus 37.75 ± 0.69 at 2 h, \( P < 0.05 \), separately) (see Figure 1).

3.2. Dynamic Change of Blood Glucose. Blood glucose levels appeared to rise at 0.5 h after injection of LPS, arrived the peak value at 1 h, then fell at 1.5 h and 2 h in LPS group. In half an hour after treatment, there was statistically significant effect on half-hourly blood glucose between LPS group and control group (3.69 ± 1.21 versus 5.42 ± 1.45 at 0.5 h, \( P < 0.05 \); 3.33 ± 0.45 versus 7.01 ± 2.65 at 1 h, \( P < 0.01 \); 4.30 ± 0.82 versus 6.91 ± 0.79 at 1.5 h, \( P < 0.01 \); 4.00 ± 0.79 versus 6.21 ± 1.40 at 2 h, \( P < 0.01 \), separately) (see Figure 2).

3.3. Changes of Plasma Insulin. At 2 hour, there was no significant different of serum insulin level between LPS and control group (1.85 ± 0.85 versus 1.89 ± 1.09, \( P > 0.05 \)) (see Figure 3).

3.4. Effects of LPS on Protein Expression of Phos-AMPK\(\alpha\) and AMPK\(\alpha\). LPS failed to alter the protein expression of AMPK\(\alpha\) in different tissues (soleus muscle 0.78 ± 0.55 versus 1.03 ± 0.52, \( P > 0.05 \); extensor digitorum longus 1.05 ± 0.26 versus 1.38 ± 0.12, \( P > 0.05 \)).

Figure 1: Change of body temperature induced by LPS. The change in body temperature of the rat was dynamically measured at 0 h, 0.5 h, 1 h, 1.5 h, and 2 h after injection of LPS or NS. Data are expressed as mean ± S.D. (\( n = 6 \) per group). \( * P < 0.05, ** P < 0.01 \) LPS group (LPS) versus control group (Con).

Figure 2: Change of blood glucose induced by LPS. Blood glucose levels were determined at 0 h, 0.5 h, 1 h, 1.5 h, and 2 h after injection of LPS or NS. Data are expressed as mean ± S.D. (\( n = 6 \) per group). \( * P < 0.05, ** P < 0.01 \) LPS group (LPS) versus control group (Con).

Figure 3: Change of blood insulin induced by LPS. At 2 hours after injection of LPS or NS, 4–6 mL blood was taken from carotid artery; serum was segregated for measurement of insulin level. Data are expressed as mean ± S.D. (\( n = 6 \) per group). \( * P < 0.05, ** P < 0.01 \) LPS group (LPS) versus control group (Con).
versus 1.28±0.32, P > 0.05; liver 1.28±0.24 versus 1.43±0.22, P > 0.05; and myocardium 2.52±1.26 versus 3.00±0.82, P > 0.05). No impact of LPS on abundance of Phos-AMPKα proteins of cardiac (2.77±0.80 versus 2.80±0.53, P > 0.05) and liver (1.03±0.70 versus 1.22±0.68, P > 0.05) was exhibited in this study. However, LPS induced significant increase of Phos-AMPKα proteins in soleus muscle (1.03±0.29 versus 0.52±0.29, P < 0.01) and extensor digitorum longus (1.20±0.21 versus 0.73±0.33, P < 0.01) (see Figure 4).

3.5. Effects of LPS on Expression of GLUT4 Protein Translocation in Skeletal Muscle. GLUT4 and m-GLUT4 expression levels in skeletal muscle by Western blot. As shown in Figures 5(a) and 5(b), no significant differences in total GLUT4 protein in soleus muscle (1.15±0.08 versus 1.10±0.12, P > 0.05) and extensor digitorum longus (1.17±0.23 versus 1.21±0.17, P > 0.05) were observed between LPS and control group. However, LPS induced the increase in the expression of GLUT4 protein translocation of soleus muscle (0.84±0.06 versus 0.67±0.08, P < 0.01) and extensor digitorum longus (0.74±0.12 versus 0.57±0.13, P < 0.05).

4. Discussion

Sepsis is a kind of severe illness, caused by infection in the body. When inflammation overpowers the host, simple infections will develop into sepsis. Sepsis is associated with various metabolic and endocrine disorders that can be confusing [27]. On the one hand, metabolic disorders in sepsis express high catabolic state with increased energy consumption. These patients often exhibit a well-defined endocrine and metabolic adaptive response to stressor agents, partly characterized by incremented resting energy expenditure (hypermetabolism, which is believed to signify increased energy requirements) [28]. That is to say, a cardinal manifestation is hyperglycemia [27]. On the other hand, some metabolic pathways were demolished in sepsis. For example, prolonged sepsis and exposure to an inflammatory milieu decreases muscle protein synthesis and reduces muscle mass [29]. Hyperglycemia is frequently easily observed during bacterial infection and it is a marker of a poor clinical outcome in critically ill patients. Lipopolysaccharides (LPS) of the cell wall of Gram (−) bacteria trigger inflammation, which is associated with marked changes in glucose metabolism; thus recently more and more attention has been paid to LPS-induced glucose metabolism disorder, which is a prominent pathological problem [30, 31].

Our experiment showed that, blood glucose levels were elevated in 0.5 h after injection of LPS, and there was statistically significant effect on half-hourly blood glucose between LPS group and control group from 0.5 h to 2 h. In fact, physical trauma, surgical-site infection, and many forms of severe stress can temporarily increase glucose levels [32–34]. Even only hypothermia can have the “perverse result.” For example, adverse events may develop when a patient is treated with hypothermia [35]. One of the adverse events associated with hypothermic therapy is a decrease in insulin sensitivity and insulin secretion, which can lead to hyperglycemia [35]. In our experiment, body temperature represented in a form with rapidly decline after 0.5 h induced by LPS, then kept lower within 2 hours. In fact, sepsis is a complex pathological process, and multiple factors are involved in abnormally high blood sugar. So far the mechanism of stress hyperglycemia in early stage of sepsis still leaves a puzzle. Stress in early stage of sepsis can increase sympathetic nerve activity, then the autonomic nervous system regulated adrenocortical function, and catecholamines facilitated the action of glucocorticoids. In addition to pituitary adrenocorticotropin, there are other extrapituitary factors regulating adrenal steroidogenesis in septic shock [36]. Similarly, glucagon was triggered to increase the level of glucose [36, 37]. In present, even plasma glucose >120 mg/dL in the absence of diabetes is a clinical sign of sepsis. Of course, hyperglycemia may be associated with increased mortality, while strict regulation of glucose levels has been found to decrease mortality and length of stay in the ICU. Now we only try to control blood glucose with insulin therapy. However, in our experiment, no significant changes in insulin levels were observed after 2 h of LPS injection, similar with the results of D. T. Yates et al. [38]. It is speculated that only 2 hours after LPS injection were too little time to finish the desired changes of insulin levels. However, in 2 hours after LPS injection, blood glucose levels significantly fell a long way from their peak simultaneously. Thus, rather than insulin action, another way, such as 5'-adenosine monophosphate-activated protein kinase (AMPK), maybe become the pathway to affect the self-regulation of blood glucose after IV bolus of LPS.

It is well known that as a highly conserved serine/threonine protein kinase, AMPK can become the important metabolic stress protein kinases, constituted by α, β, and γ 3 subunit. Once activated, AMPK phosphorylates several downstream substrates, the overall effect of which is to switch off ATP-consuming pathways (e.g., fatty acid synthesis and cholesterol synthesis) and to switch on ATP-generating pathways (e.g., fatty acid oxidation and glycolysis) [39]; thus the phosphorylation of AMPK become a central link of cellular energy regulation. And AMPK on the regulation of carbohydrate metabolism is mainly reflected the promotion of glucose uptake, glycolysis, inhibit gluconeogenesis, and glycogen synthesis. Even in recent years AMPK has become an attractive pharmacological target for the treatment of insulin resistance and type 2 diabetes-associated dyslipidaemia [40].

Patients with sepsis have a hypermetabolic and hypercatabolic state, which can be represented by increased oxygen demand on the body tissue and reduced oxygen consumption because of microcirculatory disturbance, thus ATP generation is decreased, the AMP to ATP ratio is increased, and AMPK is activated at last. It is well known that AMPK activation favors carbohydrate metabolism under some certain conditions. For example, activation of AMPK is thought to mediate, at least partially, the increases in skeletal muscle fatty acid oxidation and glucose transport that occur during acute exercise [41]. AMPK activation is a complex and elaborates the regulating process. As the action sites, α-subunit 172 threonine can provide the key role on AMPK activation. Therefore, activation of AMPK requires its phosphorylation.
Figure 4: The effects of LPS on the protein expression of phos-AMPKα and AMPKα in different tissues: heart (a), liver (b), soleus muscle (c), and extensor digitorum longus (d). Equal amounts of protein were subjected to electrophoresis and immunoblotted, as described. Data were represented as mean ± S.D. (n = 6, per group) * P < 0.05, ** P < 0.01 LPS group (LPS) versus control group (Con).
Our experiment showed that AMPKα and Phos-AMPKα in myocardium and liver tissue of septic rats had no significant difference, compared with those in control group, after 2 h of LPS injection. However, the levels of Phos-AMPKα in the soleus muscle and extensor digitorum longus were significantly increased, although the expression of AMPKα was not impaired. In association with the alteration of blood glucose, it was speculated AMPK activation in exercising muscles could take part in the glycometabolism process in early stage of sepsis, while the metabolic capacity of blood glucose was not relate to AMPK activation in myocardial and liver tissue.

The signaling mechanism, downstream of AMPK, which regulates muscle glucose transport, is unclear in septic rat. Previous studies showed that, in skeletal muscle, AMPK was activated by exercise/contraction, metformin, and thiazolidinediones resulting in an increase in glucose uptake [43]. The skeletal muscle is the main peripheral tissue of glucose metabolism. The rate-limiting step of glucose metabolism is the pathway of glucose into skeletal muscle cells, which requires direct involvement of GLUT4 on the cell membrane. In cell culture, Edward O. Ojuka et al. [44] found AICAR (5-amino-4-ammonia ribonucleotide formyl imidazole), as AMPK activator, could activate AMPK to divert GLUT4 within the cell toward cytomembrane. And Bergeron et al. [45] showed that, in the quiet state, AICAR could activate AMPK, promoting GLUT4 protein translocation in cell membrane, which would increase glucose transport and uptake in skeletal muscle.

The adjustment mechanism of AMPK has been confirmed in state of exercise. On the one hand, islet β-cell insulin receptor, insulin-like growth factor receptor and peripheral insulin receptors mRNA expression, and protein expression can be adjusted by activation of AMPK [46]. On the other hand, AMPK can be activated by noninsulin signals in skeletal cells, so that GLUT4 within cytoplasm will shift to Cytolemma and various plasma membrane, enhancing the capacity of glucose transport [47]. In the experiment, LPS induced the increase in the expression of GLUT4 protein translocation of soleus muscle and extensor digitorum longus. Prompt decline in blood glucose at this time may be related to activation of AMPK regulation of skeletal muscle glucose metabolism [44, 48]. Because the result in this study showed that the level of insulin in LPS group did not alter; thus, in the early stage of sepsis, GLUT4 protein translocation by noninsulin dependent pathway can be actually a mechanism for glucose metabolism in skeletal muscle.

Generally skeletal muscle fibers are a mixture of 3 types of muscle fibers: type I (red fibers, slow-twitch, and slow oxidative), type II a (red fibers, fast-twitch, and fast oxidative), and type II b (white fibers, fast-twitch, fast glycolytic). Soleus muscle fibers mainly belong to type I, while extensor digitorum longus muscle fiber belongs to type II. To the different muscle fiber types, AMPK response is various. AMPK may be involved in the signal transduction pathway induced by fast muscle movement, while AMPK is not related to the slow-twitch fibers [49–51]. But in this experiment,
AMPK on the regulation of glucose metabolism in skeletal muscle

Promote skeletal muscle glucose metabolism. Activation of AMPK increases the expression of GLUT4 protein translocation to promote skeletal muscle glucose metabolism. The changes of blood glucose have no bearing on glucose metabolism in cardiac muscle and liver tissue. Non-insulin-dependent AMPK signaling pathway can increase the expression of GLUT4 protein translocation to promote skeletal muscle glucose metabolism. Activation of AMPK on the regulation of glucose metabolism in skeletal muscle has no relation to muscle fiber type.

In conclusion, the dynamic changes of blood glucose appeared to be an increase at first and then a drop in early stage of acute sepsis. The changes of blood glucose have no bearing on glucose metabolism in cardiac muscle and liver tissue. Non-insulin-dependent AMPK signaling pathway can increase the expression of GLUT4 protein translocation to promote skeletal muscle glucose metabolism. Activation of AMPK on the regulation of glucose metabolism in skeletal muscle has no relation to muscle fiber type.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

This project is supported by National Science Foundation of China (Grant no. 81101445) and by Medical and Health Projects of Zhejiang Province, China (Grant no. 2013KYA063).

References


Review Article

Intensive Insulin Therapy for Septic Patients: A Meta-Analysis of Randomized Controlled Trials

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Received 7 January 2014; Accepted 18 March 2014; Published 18 June 2014

Academic Editor: Baoli Cheng

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Background. Studies on the effect of intensive insulin therapy (IIT) in septic patients with hyperglycemia have given inconsistent results. The primary purpose of this meta-analysis was to evaluate whether it is effective in reducing mortality.

Methods. We searched PubMed, Embase, the Cochrane Library, clinicaltrials.gov, and relevant reference lists up to September 2013 and including randomized controlled trials that compared IIT with conventional glucose management in septic patients. Study quality was assessed using the Cochrane Risk of Bias Tool. And our primary outcome measure was pooled in the random effects model.

Results. We identified twelve randomized controlled trials involving 4100 patients. Meta-analysis showed that IIT did not reduce any of the outcomes: overall mortality (risk ratio [RR] = 0.98, 95% CI [0.85, 1.15], \( P = 0.84 \)), 28-day mortality (RR = 0.66, 95% CI [0.40, 1.10], \( P = 0.11 \)), 90-day mortality (RR = 1.10, 95% CI [0.97, 1.26], \( P = 0.13 \)), ICU mortality (RR = 0.94, 95% CI [0.77, 1.14], \( P = 0.52 \)), hospital mortality (RR = 0.98, 95% CI [0.86, 1.11], \( P = 0.71 \)), severity of illness, and length of ICU stay. Conversely, the incidence of hypoglycemia was markedly higher in the IIT (RR = 2.93, 95% CI [1.69, 5.06], \( P = 0.0001 \)).

Conclusions. For patients with sepsis, IIT and conservative glucose management show similar efficacy, but IIT is associated with a higher incidence of hypoglycemia.

1. Introduction

Sepsis has been a long-standing issue in modern medicine that in too many instances leads to mortality. Every year in the United States there are approximately 750,000 documented cases, of which at least 225,000 are fatal [1]. Though there have been advances in intensive care, the mortality rate of patients with sepsis has remained between 20% and 30% over the past three decades [1, 2]. One pathophysiological component in septic patients is hypermetabolism, including perturbations of glucose metabolism resulting in hyperglycemia [3].

Hyperglycemia is prevalent in ICU patients, especially those with sepsis [4–6]. Hyperglycemia is associated with many adverse outcomes, including immune disorder, oxidative stress, susceptibility to infection, and endothelial dysfunction [7, 8]. Its impact is believed by research that has found hyperglycemia to be independently associated with increased mortality in patients with sepsis because it enhances the inflammatory response [6, 9]. Some randomized controlled clinical trials have attempted to determine whether intensive insulin therapy targeted on establishing normoglycemia could benefit septic patients [10–14].

In 2001, a randomized controlled trial showed that intensive treatment with insulin (80–110 mg/dL) resulted in a lower hospital mortality in the surgical ICU, which was attributed to a reduction of mortality in patients with sepsis [15]. Conversely, the VISEP study, the first to specifically
investigate intensive insulin therapy for septic patients, found no significant reduction in mortality [10]. Then, further randomized controlled trials failed to replicate the mortality benefit in septic patients [16, 17]. Despite this continuing debate, the Surviving Sepsis Campaign included an upper limit for blood glucose of 180 mg/dL in their guidelines based upon systematic reviews of studies involving critically ill patients [18]. Although sepsis is the chief cause of death in ICUs, whether the impact and safety of intensive insulin therapy in septic patients are the same as those in critically ill patients is uncertain.

In order to clarify this matter, we conducted a meta-analysis to assess the use of intensive insulin therapy in managing glycemic control for septic patients. The primary purpose was to evaluate the effects of tight glycemic control on mortality stratified into four subgroups (90-day and 28-day mortality and hospital and ICU mortality).

2. Methods

2.1. Search Strategy. We searched for randomized controlled trials of intensive insulin therapy targeting euglycemia among septic patients in PubMed, Embase, the Cochrane Library, and clinicaltrials.gov dating up to September 2013 without language restriction. We used the exploded Medical Subject Heading (MeSH) terms “insulin,” “blood glucose,” and “hypoglycemic agents,” with the text words “hyperglycemia,” “insulin,” “blood glucose,” and “glycemic control,” for the intensive insulin filter. The MeSH term “sepsis” with the text words “sepsis,” “severe sepsis,” “septic shock,” and “septicemia” for the sepsis filter. Additionally, a highly sensitive search strategy described in the Cochrane Handbook was utilized for the randomized controlled trials filter [19]. The MeSH terms and text words were combined with the Boolean operator OR, and then the three filters were combined with the Boolean AND operator. We also checked the reference lists of retrieved reviews and clinical trials to identify additional studies.

2.2. Study Selection. Two investigators independently reviewed all of the titles and abstracts. Included articles met the following criteria: (1) trials had a randomized controlled clinical design, with or without blinding; (2) patients were adults with sepsis; (3) the intensive insulin therapy group had a targeted glucose concentration of ≤150 mg/dL, and the control group had a higher glucose level; and (4) the outcome measures included at least one of the following: mortality, severity of illness, length of ICU stay, and hypoglycemia (≤40 mg/dL). We also included data from trials in critically ill patients if the data concerning sepsis could be extracted.

2.3. Data Abstraction and Quality Assessment. Independently, the same two investigators abstracted the data and assessed the methodological quality of eligible trials. If there was any disagreement, a third investigator participated in a group discussion and made the final decision. The abstracted data was as follows: first author, year of publication, region or country, number of study site, sample size, population, patient age, history of diabetes mellitus, initial glucose level, targeted glucose level, and achieved mean glucose value. The primary outcome was mortality with a preference for 90-day mortality. If this was not reported in the outcome, we used hospital mortality, 28-day mortality, or ICU mortality, in that order. The secondary outcomes were severity of illness, length of ICU stay, and hypoglycemia. We also stratified mortality by 90-day and 28-day mortality, as well as hospital and ICU mortality in subgroup analyses.

The methodological quality was formally evaluated using the Cochrane risk of bias assessment tool, which incorporates random sequence generation, allocation concealment, blinding of participants and personnel, blinding of outcome assessment, incomplete outcome data, selective outcome reporting, and other potential sources of bias [19]. Each item was stratified into one of three categories: (1) high risk, which represented low quality, (2) low risk, which represented high quality, or (3) unclear, in which there was insufficient information to judge or the study did not involve this outcome.

2.4. Statistical Analysis. We used the Review Manager software to conduct the statistical analyses [20]. For each outcome measure, we used the relative risk (RR) for dichotomous data and the standardized mean difference (SMD) for continuous data. We used a random-effect model for all analyses which provides a more conservative pooled estimate than a fixed-effect model, considering the anticipated clinical heterogeneity among eligible articles [21]. Some data were presented with means and 95% confidence intervals, necessitating that we calculate standard deviations from the data provided. We assessed the heterogeneity among studies using

![Figure 1: Flow diagram of study selection.](image-url)
### Table 1: Characteristics of included randomized controlled trials.

<table>
<thead>
<tr>
<th>First author, year (country)</th>
<th>Number of study sites</th>
<th>Sample size</th>
<th>Population, %</th>
<th>Mean age, (years)</th>
<th>Diabetic, %</th>
<th>Initial glucose, mg/dL</th>
<th>Glucose goal, mg/dL</th>
<th>Glucose achieved, mean (SD), mg/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cappi 2012 (Brazil) [14]</td>
<td>1</td>
<td>63</td>
<td>Severe sepsis</td>
<td>53</td>
<td>24</td>
<td>IIT 144 [97–182]</td>
<td>IIT 80–110</td>
<td>IIT 99 (18) (39.6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Septic shock</td>
<td></td>
<td></td>
<td>Control 141 [101–160]</td>
<td>Control 140–180</td>
<td>Control 155</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>COITITSS 2010 (France) [16]</td>
<td>11</td>
<td>509</td>
<td>Septic shock</td>
<td>64</td>
<td>NA</td>
<td>IIT 216 (NA)</td>
<td>IIT 80–110</td>
<td>IIT 120–140 (160)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>100</td>
<td></td>
<td></td>
<td>Control 204 (NA)</td>
<td>Control 180–200</td>
<td>Control 159 (31)</td>
</tr>
<tr>
<td>Savioli 2009 (Italy) [12]</td>
<td>3</td>
<td>90</td>
<td>Severe sepsis</td>
<td>61</td>
<td>13.3</td>
<td>IIT 175 (101)</td>
<td>IIT 80–110</td>
<td>IIT 112 (23) (74)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>65.6</td>
<td></td>
<td></td>
<td>Control 160 (74)</td>
<td>Control 180–200</td>
<td>Control 159 (31)</td>
</tr>
<tr>
<td>Iapichino 2008 (Italy) [11]</td>
<td>3</td>
<td>72</td>
<td>Severe sepsis</td>
<td>62.3</td>
<td>17</td>
<td>IIT 137 (45)</td>
<td>IIT 80–110</td>
<td>IIT 110 (17) (29)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>19.4</td>
<td></td>
<td></td>
<td>Control 151.7 (36.6)</td>
<td>Control 180–200</td>
<td>Control 163 (29)</td>
</tr>
<tr>
<td>Brunkhorst 2008 (Germany) [10]</td>
<td>18</td>
<td>537</td>
<td>Septic shock</td>
<td>64.6</td>
<td>30</td>
<td>IIT 130 [108–167]</td>
<td>IIT 80–110</td>
<td>IIT 112 (NA) (118)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>100</td>
<td></td>
<td></td>
<td>Control 138 [111–184]</td>
<td>Control 180–200</td>
<td>Control 151 (NA)</td>
</tr>
<tr>
<td>Ellger 2008 (Belgium) [25]</td>
<td>1</td>
<td>950</td>
<td>Severe sepsis</td>
<td>62</td>
<td>14</td>
<td>IIT 163 (73)</td>
<td>IIT 80–110</td>
<td>IIT 106 (26) (30)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>51.4</td>
<td></td>
<td></td>
<td>Control 161 (70)</td>
<td>Control 180–200</td>
<td>Control 150 (30)</td>
</tr>
<tr>
<td>Yu 2005 (China) [13]</td>
<td>1</td>
<td>55</td>
<td>Sepsis</td>
<td>46</td>
<td>NA</td>
<td>IIT 153 (61)</td>
<td>IIT 80–110</td>
<td>IIT 103 (22) (29)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Control 151 (65)</td>
<td>Control 180–200</td>
<td>Control 198 (29)</td>
</tr>
<tr>
<td>Dong 2009 (China) [27]</td>
<td>1</td>
<td>27</td>
<td>Septic shock</td>
<td>44</td>
<td>0</td>
<td>IIT 157 (45)</td>
<td>IIT 74–110</td>
<td>IIT 108 (27) (34)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>100</td>
<td></td>
<td></td>
<td>Control 159 (39.6)</td>
<td>Control 112–150</td>
<td>Control 148 (34)</td>
</tr>
<tr>
<td>NICE-SUGAR 2009 (Australia and New Zealand—Canada) [17]</td>
<td>42</td>
<td>1299</td>
<td>Severe sepsis Septic shock</td>
<td>60.2</td>
<td>20.1</td>
<td>IIT 146 (52.3)</td>
<td>IIT 81–108</td>
<td>IIT 115 (18) (23)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>60.2</td>
<td></td>
<td></td>
<td>Control 144 (49.1)</td>
<td>Control 180 or less</td>
<td>Control 144 (23)</td>
</tr>
<tr>
<td>Arabi 2008 (Saudi Arabia) [24]</td>
<td>1</td>
<td>122</td>
<td>Severe sepsis</td>
<td>52.4</td>
<td>40.0</td>
<td>IIT 195 (75)</td>
<td>IIT 80–110</td>
<td>IIT 115 (18) (34)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Septic shock</td>
<td></td>
<td></td>
<td>Control 211 (81)</td>
<td>Control 180–200</td>
<td>Control 171 (34)</td>
</tr>
<tr>
<td>Zhang 2008 (China) [26]</td>
<td>1</td>
<td>22</td>
<td>Sepsis</td>
<td>66.3</td>
<td>27.5</td>
<td>IIT 165 (55)</td>
<td>IIT 80–110</td>
<td>IIT 119 (7.6) (79)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Control 200 (100)</td>
<td>Control 130–150</td>
<td>Control 141 (79)</td>
</tr>
<tr>
<td>Jin 2009# (China) [23]</td>
<td>14</td>
<td>356</td>
<td>Severe sepsis</td>
<td>65.7</td>
<td>NA</td>
<td>IIT NA Control NA</td>
<td>IIT 80–110; 120–150</td>
<td>IIT 80–110 (31); 120–150; 133 (34)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Septic shock</td>
<td></td>
<td></td>
<td>Control 120–150</td>
<td>Control 180–200</td>
<td>Control 189 (40)</td>
</tr>
</tbody>
</table>

IIT: intensive insulin therapy; NA: not available from article or author.

* Median (interquartile range).

# Abstract only.

Cochran’s Q-test ($P < 0.10$ for statistical significance) and the $I^2$ statistic ($I^2$ value $>50\%$ for substantial heterogeneity). To eliminate the heterogeneity, we conducted either a sensitivity analysis or subgroup analysis.

### 3. Results

#### 3.1. Literature Search

Our predefined search strategy yielded a total of 1,842 abstracts (Figure 1). After reviewing the titles and abstracts, we excluded 1,816 studies because they
Table 2: Risk of bias in included randomized controlled trials.

<table>
<thead>
<tr>
<th>Study</th>
<th>Random sequence generation</th>
<th>Allocation concealment</th>
<th>Blinding of participants and personnel</th>
<th>Blinding of outcome assessment</th>
<th>Incomplete outcome data</th>
<th>Selective reporting</th>
<th>Other sources of bias</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cappi 2012 [14]</td>
<td>Low risk</td>
<td>Low risk</td>
<td>High risk</td>
<td>Low risk</td>
<td>Low risk</td>
<td>Low risk</td>
<td>Low risk</td>
</tr>
<tr>
<td>COITSS 2010 (France) [16]</td>
<td>Low risk</td>
<td>Low risk</td>
<td>High risk</td>
<td>Low risk</td>
<td>Low risk</td>
<td>Low risk</td>
<td>High risk&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Savioli 2009 [12]</td>
<td>Low risk</td>
<td>Unclear</td>
<td>High risk</td>
<td>Low risk</td>
<td>Low risk</td>
<td>Low risk</td>
<td>Low risk</td>
</tr>
<tr>
<td>Brunkhorst 2008 [10]</td>
<td>Low risk</td>
<td>Low risk</td>
<td>High risk</td>
<td>High risk</td>
<td>Low risk</td>
<td>Low risk</td>
<td>Low risk</td>
</tr>
<tr>
<td>Ellger 2008 [25]</td>
<td>Low risk</td>
<td>Low risk</td>
<td>High risk</td>
<td>Low risk</td>
<td>Low risk</td>
<td>Low risk</td>
<td>Low risk</td>
</tr>
<tr>
<td>Dong 2009 [27]</td>
<td>Unclear</td>
<td>Unclear</td>
<td>High risk</td>
<td>Low risk</td>
<td>Low risk</td>
<td>Unclear</td>
<td>Low risk</td>
</tr>
<tr>
<td>NICE-SUGAR 2009 (Australia and New Zealand—Canada) [17]</td>
<td>Low risk</td>
<td>Low risk</td>
<td>High risk</td>
<td>Low risk</td>
<td>Low risk</td>
<td>Low risk</td>
<td>High risk&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Arabi 2008 [24]</td>
<td>Low risk</td>
<td>Low risk</td>
<td>High risk</td>
<td>Low risk</td>
<td>Low risk</td>
<td>Low risk</td>
<td>High risk&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Zhang 2008 [26]</td>
<td>Unclear</td>
<td>Unclear</td>
<td>High risk</td>
<td>Low risk</td>
<td>Low risk</td>
<td>Unclear</td>
<td>Low risk</td>
</tr>
<tr>
<td>Jin 2009&lt;sup&gt;*&lt;/sup&gt; [23]</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

<sup>a</sup>Abstract only.
<sup>b</sup>NA: not available from article or author.
<sup>a</sup>The two treatment groups differ in the use of medications other than insulin.
<sup>b</sup>Intensive insulin therapy was terminated early because of increasing hypoglycemic events.
<sup>c</sup>Inclusion used subjective criteria.
<sup>d</sup>It had a different baseline.

were nonrandomized controlled trials, not specific to septic patients or pertained to interventions other than intensive insulin therapy. We checked the full text of the remaining 26 articles. One trial that met our inclusion criteria was excluded because the data were presented in diagrams from which we were unable to abstract values [22]. No additional studies were found in the screened reference lists. Finally, 12 randomized controlled trials were included in our meta-analysis [10–14, 16, 17, 23–27]. One trial was only an abstract, despite contacting the authors to request a copy of the full article [23].

### 3.2. Study Characteristics

The 12 randomized controlled trials included 4,100 patients in all, of whom 2,094 were assigned to the intensive insulin group and 2,006 to the control group. The details of the included studies are listed in Table 1. For the intervention, all the trials used tight glycemic control (80–110 mg/dL) except for one trial used that used two glycemic subcategories: 80–110 mg/dL or 120–150 mg/dL [23]. Because our inclusion criterion was an intensive insulin therapy group targeting a glucose concentration of ≤150 mg/dL, we combined the data of the two glycemic subcategories for mortality analysis. The mean glucose concentration of included patients varied significantly among eligible trials, from 130 mg/dL to 216 mg/dL. The proportion of patients with septic shock ranged widely (32.7–100%). In 3 trials, the baseline parameters of the septic patients were undocumented in the intensive and the control groups [17, 23, 24]. Most of the included trials used a specific method of random sequence generation (Table 2). None of the trials met the "blinding of participants and personnel" bias criterion and thus were rated as high-risk. On the contrary, all studies were identified as low risk because they did not have "incomplete outcome data."

### 3.3. Primary Outcome: Mortality

The all-cause mortality was reported in the 12 randomized controlled trials [10–14, 16, 17,
23–27]. There were 681/2,094 (32.5%) deaths in the intensive insulin intervention group and 661/2,006 (33%) in the control group. Meta-analysis showed that the rate of death did not differ significantly between the two groups (RR = 0.98, 95% CI [0.85, 1.15], P = 0.84) (Figure 2).

In the subgroup analysis, there was no significant difference in 28-day mortality (RR = 0.96, 95% CI [0.69, 1.10], P = 0.11), 90-day mortality (RR = 1.10, 95% CI [0.97, 1.26], P = 0.13), ICU mortality (RR = 0.94, 95% CI [0.77, 1.14], P = 0.52), or hospital mortality (RR = 0.98, 95% CI [0.86, 1.11], P = 0.71) (Figure 3).

The statistical heterogeneity was substantial for all-cause mortality (I² = 51%; P = 0.03) and for 28-day mortality (I² = 74%; P = 0.02). Because we could not acquire all the data, we could not determine whether they had a normal distribution [19].

Therefore, we excluded this trial and the heterogeneity for all-cause mortality was resolved (I² = 0%; P = 0.51). The heterogeneity was still significant for 28-day mortality. We noted that the Cappi trial showed a wide confidence interval size (0.95, 95% CI [0.71, 1.27], P = 0.74) did not change significantly.

3.4. Secondary Outcomes: Severity of Illness, Length of ICU Stay, and Hypoglycemic Events. The included trials used the SOFA (Sequential Organ Failure Assessment) score, APACHE II (Acute Physiology and Chronic Health Evaluation II) score, SAPS II (Simplified Acute Physiology Score II), and MODS (Multiple Organ Dysfunction Score) to evaluate severity of illness after intensive insulin therapy. Five trials reported SOFA score [10–13, 16], two studies reported APACHE II score, and only the MODS [23] and SAPS II [27] scores were reported in only one study each. The data about SOFA score could only be extracted from two trials that included 578 participants [10, II]. The pooled estimate in the intensive insulin group was similar to that in the control group (SMD = 0.05, 95% CI [–0.12, 0.21], P = 0.57) with no statistical heterogeneity (I² = 0%; P = 1.00) (Figure 4). Similarly, there was no significant difference in APACHE II score (P = 0.46) and SAPS II (P = 1.00). The MODS was lower in the intensive insulin group, but its methodological quality was low. This suggests that intensive insulin therapy does not reduce the severity of sepsis.

Five trials reported the length of ICU stay as an outcome [10, II, 16, 23, 26]. It should be noted that in four of the trials the data were presented as the median (interquartile range) [10, II, 16, 26], each with no statistically significant difference between the intensive insulin and control groups. The remaining trial had an unclear methodological quality and found that intensive insulin therapy was associated with a shorter ICU stay, but the result was dubious [23]. We did not merge the medians (interquartile range), because we could not determine whether they had a normal distribution [19].

For the occurrence of hypoglycemia, we extracted data from seven trials including 2,213 participants [10, II, 13, 14, 16, 25, 27] (Figure 5). There were 196/1,093 (17.9%) hypoglycemic events in the intensive insulin group and 55/1,120 (4.9%) in the control group. The intensive insulin group had a higher rate of hypoglycemia than the control group (RR = 2.93,
3.3.1 28-day mortality

<table>
<thead>
<tr>
<th>Study or subgroup</th>
<th>IIT Events</th>
<th>IIT Total</th>
<th>Control Events</th>
<th>Control Total</th>
<th>Weight</th>
<th>Risk ratio M-H, random, 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brunthorpe et al. 2008</td>
<td>61</td>
<td>247</td>
<td>3</td>
<td>10</td>
<td>75</td>
<td>289</td>
</tr>
<tr>
<td>Cappi et al. 2012</td>
<td>3</td>
<td>28</td>
<td>10</td>
<td>35</td>
<td>10</td>
<td>38</td>
</tr>
<tr>
<td>Jin and Guolong 2009</td>
<td>47</td>
<td>225</td>
<td>35</td>
<td>131</td>
<td>51</td>
<td>455</td>
</tr>
<tr>
<td><strong>Subtotal (95% CI)</strong></td>
<td><strong>500</strong></td>
<td><strong>550</strong></td>
<td><strong>455</strong></td>
<td><strong>100.0%</strong></td>
<td><strong>455</strong></td>
<td><strong>0.66 [0.40, 1.10]</strong></td>
</tr>
</tbody>
</table>

Total events 111 136

Heterogeneity: $r^2 = 0.13; \chi^2 = 7.65, df = 2 (P = 0.02); I^2 = 74%$

Test for overall effect: $Z = 1.61 (P = 0.11)$

3.3.2 90-day mortality

<table>
<thead>
<tr>
<th>Study or subgroup</th>
<th>IIT Events</th>
<th>IIT Total</th>
<th>Control Events</th>
<th>Control Total</th>
<th>Weight</th>
<th>Risk ratio M-H, random, 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brunthorpe et al. 2008</td>
<td>98</td>
<td>247</td>
<td>102</td>
<td>288</td>
<td>35.0%</td>
<td>1.12 [0.90, 1.39]</td>
</tr>
<tr>
<td>Iapichino et al. 2008</td>
<td>13</td>
<td>36</td>
<td>11</td>
<td>36</td>
<td>3.9%</td>
<td>1.18 [0.61, 2.28]</td>
</tr>
<tr>
<td>The NICE-SUGAR Study Investigators 2009</td>
<td>202</td>
<td>673</td>
<td>172</td>
<td>626</td>
<td>56.9%</td>
<td>1.09 [0.92, 1.30]</td>
</tr>
<tr>
<td>Savioli et al. 2009</td>
<td>14</td>
<td>45</td>
<td>13</td>
<td>45</td>
<td>4.2%</td>
<td>1.07 [0.57, 2.03]</td>
</tr>
<tr>
<td><strong>Subtotal (95% CI)</strong></td>
<td><strong>1001</strong></td>
<td><strong>1000</strong></td>
<td><strong>995</strong></td>
<td><strong>100.0%</strong></td>
<td><strong>995</strong></td>
<td><strong>1.10 [0.97, 1.26]</strong></td>
</tr>
</tbody>
</table>

Total events 327 298

Heterogeneity: $r^2 = 0.00; \chi^2 = 0.08, df = 3 (P = 0.99); I^2 = 0%$

Test for overall effect: $Z = 1.51 (P = 0.13)$

3.3.3 ICU mortality

<table>
<thead>
<tr>
<th>Study or subgroup</th>
<th>IIT Events</th>
<th>IIT Total</th>
<th>Control Events</th>
<th>Control Total</th>
<th>Weight</th>
<th>Risk ratio M-H, random, 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arabi et al. 2008</td>
<td>18</td>
<td>55</td>
<td>15</td>
<td>67</td>
<td>11.0%</td>
<td>1.46 [0.81, 2.62]</td>
</tr>
<tr>
<td>Ellger et al. 2008</td>
<td>112</td>
<td>479</td>
<td>128</td>
<td>467</td>
<td>77.9%</td>
<td>0.86 [0.69, 1.07]</td>
</tr>
<tr>
<td>Iapichino et al. 2008</td>
<td>8</td>
<td>36</td>
<td>6</td>
<td>36</td>
<td>4.1%</td>
<td>1.33 [0.51, 3.46]</td>
</tr>
<tr>
<td>Savioli et al. 2009</td>
<td>9</td>
<td>45</td>
<td>8</td>
<td>45</td>
<td>5.1%</td>
<td>1.13 [0.48, 2.65]</td>
</tr>
<tr>
<td>Yu et al. 2005</td>
<td>3</td>
<td>28</td>
<td>4</td>
<td>27</td>
<td>1.9%</td>
<td>0.72 [0.18, 2.93]</td>
</tr>
<tr>
<td><strong>Subtotal (95% CI)</strong></td>
<td><strong>643</strong></td>
<td><strong>642</strong></td>
<td><strong>646</strong></td>
<td><strong>100.0%</strong></td>
<td><strong>646</strong></td>
<td><strong>0.94 [0.77, 1.14]</strong></td>
</tr>
</tbody>
</table>

Total events 150 161

Heterogeneity: $r^2 = 0.00; \chi^2 = 3.63, df = 4 (P = 0.46); I^2 = 0%$

Test for overall effect: $Z = 0.65 (P = 0.52)$

3.3.4 hospital mortality

<table>
<thead>
<tr>
<th>Study or subgroup</th>
<th>IIT Events</th>
<th>IIT Total</th>
<th>Control Events</th>
<th>Control Total</th>
<th>Weight</th>
<th>Risk ratio M-H, random, 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cappi et al. 2012</td>
<td>5</td>
<td>28</td>
<td>10</td>
<td>35</td>
<td>1.8%</td>
<td>0.63 [0.24, 1.62]</td>
</tr>
<tr>
<td>COIITSS Study Investigators 2010</td>
<td>117</td>
<td>255</td>
<td>109</td>
<td>254</td>
<td>42.8%</td>
<td>1.07 [0.88, 1.30]</td>
</tr>
<tr>
<td>Dong et al. 2009</td>
<td>0</td>
<td>13</td>
<td>0</td>
<td>14</td>
<td>Not estimable</td>
<td></td>
</tr>
<tr>
<td>Ellger et al. 2008</td>
<td>160</td>
<td>479</td>
<td>172</td>
<td>471</td>
<td>53.8%</td>
<td>0.91 [0.77, 1.09]</td>
</tr>
<tr>
<td>Yu et al. 2005</td>
<td>4</td>
<td>28</td>
<td>4</td>
<td>27</td>
<td>1.0%</td>
<td>0.96 [0.27, 3.47]</td>
</tr>
<tr>
<td>Zhang et al. 2008</td>
<td>3</td>
<td>10</td>
<td>2</td>
<td>12</td>
<td>0.6%</td>
<td>1.80 [0.37, 8.74]</td>
</tr>
<tr>
<td><strong>Subtotal (95% CI)</strong></td>
<td><strong>813</strong></td>
<td><strong>813</strong></td>
<td><strong>813</strong></td>
<td><strong>100.0%</strong></td>
<td><strong>813</strong></td>
<td><strong>0.98 [0.86, 1.11]</strong></td>
</tr>
</tbody>
</table>

Total events 289 297

Heterogeneity: $r^2 = 0.00; \chi^2 = 2.81, df = 4 (P = 0.59); I^2 = 0%$

Test for overall effect: $Z = 0.37 (P = 0.71)$

4. Discussion

We conducted a meta-analysis of randomized controlled trials of intensive insulin therapy for septic patients, which showed no reduction of mortality overall nor in any of the subgroups (28-day and 90-day mortality; ICU and hospital mortality). Likewise, in terms of the severity of illness, the intensive insulin group did not show a statistically significant difference from the control. Though one trial found that the intensive insulin therapy group had a shorter ICU stay than the control group, its methodological quality was vague. Due to the data format, we could not merge the other studies with the length of ICU stay. Intensive insulin therapy, however, notably increased the episodes of hypoglycemia. We found substantial heterogeneity in the pooled analysis of mortality and hypoglycemia, but the results remained the same when the heterogeneity was removed using sensitivity analysis.

During the past 30 years, no effective new therapies appeared, despite that our understanding of the pathophysologic features of sepsis has advanced [28]. A number of...
observational studies indicate that hyperglycemia is associated with a higher mortality rate, in particular, sepsis-induced hyperglycemia rather than mortality due to preexisting diabetes mellitus [6]. The landmark randomized controlled trial showed that intensive insulin therapy (targeting 80–110 mg/dL) reduced hospital mortality in septic patients [15], so clinicians enthusiastically received it as an effective therapy for these patients. Unfortunately, subsequent randomized controlled trials failed to confirm this beneficial effect.

Several prior reviews investigated the effect of intensive insulin therapy (IIT) for a general population of ICU patients [29–31]. Though we obtained similar results regarding the effect of IIT on mortality and risk of hypoglycemia, there are some differences in our findings compared with the prior reviews. The previous reviews concentrated on a general population of ICU patients. Solymez Wiener et al. [29], Kansagara et al. [30], and Griesdale et al.'s [31] trials were grouped by type of ICU (medical ICU, surgical ICU, and mixed ICU). Kansagara et al.'s [30] trials were also grouped by type of patients (myocardial infarction and stroke); however, we focused on patients with sepsis. Even though septic patients are intermixed with other ICU patients, they possess distinct treatment and prognostic and clinical outcomes as compared with other ICU patients. Therefore, the effect of intensive insulin therapy for ICU patients is not necessarily congruent with the effect for septic patients. In addition, we classified the outcome of mortality into four subgroups (90-day, 28-day, hospital, and ICU mortality) and added the outcome of severity of illness and the length of ICU stay that were not evaluated in the prior reviews.

We included one specific study [25] that contained a database of two randomized controlled trials [15, 32], which used identical protocols and were implemented at the same center but one year apart. Thus, we treated these studies as a consecutive study. This study [25] showed that intensive insulin therapy reduced the ICU mortality in septic patients who stayed in the ICU for at least three days, but there was no statistically significant difference in septic patients who stayed for less than three days. They performed this subgroup analysis to specifically consider patients whose intensive care was limited or who were withdrawn from intensive care within 3 days of admission to the ICU. Another trial, the Brunkhorst et al. trial [10], showed no effect of intensive insulin on mortality in septic patients who stayed in the ICU at least three or five days. The remaining trials did not stratify by the length of stay in the ICU.
the authentic clinical practice environment into consideration, we pooled the overall ICU stay and found no significant difference.

Perhaps the failure to find a benefit with intensive insulin therapy can be attributed to several factors. It remains unclear whether hyperglycemia is a cause of increased mortality or is just a marker of an increased risk of death; it may even be a normal response [6]. Finfer’s [33] latest review states that “until quite recently stress hyperglycemia was seen as a normal and possibly beneficial physiological response to promote cellular glucose uptake” (pp: 1–6).

In agreement with previous studies, our meta-analysis demonstrated that intensive insulin therapy carries a markedly increased risk of hypoglycemia. Hypoglycemia has been reported to have an independent association with increased mortality in patients with sepsis [34, 35]. Considering the increased risk of death due to increased hypoglycemia and the findings that intensive insulin therapy does not reduce mortality, the use of IIT as a strategy to maintain normoglycemia remains unclear.

Septic patients are apt to suffer from a striking increase in blood glucose variability, which causes endothelial dysfunction, oxidative stress, and organ dysfunction [6, 9, 36, 37]. Though the mean blood glucose concentration is similar among trials, the degree of glucose variability may be quite different. Several observational studies have shown that blood glucose variability is independently associated with an increased mortality rate, even more than continuous hyperglycemia [9, 35]. The glycemic lability index, which is calculated from continuous glucose monitoring, reveals the inherent variability better than the standard deviation of the mean blood glucose value [35]. The included trials mainly used sampling at a predefined time, rather than monitoring 24-hour continuous glucose levels, resulting in insufficient data to determine the relationship between glucose variability and mortality rate.

### Table 3: Sensitivity analysis.

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Number of studies</th>
<th>Number of patients</th>
<th>RR (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low risk of random sequence generation and allocation concealment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mortality</td>
<td>6</td>
<td>3478</td>
<td>1.04 (0.95–1.15)</td>
<td>0.40</td>
</tr>
<tr>
<td>Hypoglycemia</td>
<td>4</td>
<td>2059</td>
<td>3.27 (1.62–6.57)</td>
<td>0.0009</td>
</tr>
<tr>
<td>Trials containing &gt;500 patients</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mortality</td>
<td>4</td>
<td>3293</td>
<td>1.04 (0.94–1.14)</td>
<td>0.44</td>
</tr>
<tr>
<td>Hypoglycemia</td>
<td>3</td>
<td>1996</td>
<td>3.81 (1.89–7.69)</td>
<td>0.0002</td>
</tr>
<tr>
<td>Patients with similar baseline</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mortality</td>
<td>9</td>
<td>1910</td>
<td>1.00 (0.89–1.13)</td>
<td>0.97</td>
</tr>
<tr>
<td>Hypoglycemia</td>
<td>7</td>
<td>2213</td>
<td>2.93 (1.69–5.06)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Patients with septic shock</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mortality</td>
<td>4</td>
<td>1533</td>
<td>1.03 (0.89–1.18)</td>
<td>0.69</td>
</tr>
<tr>
<td>Hypoglycemia</td>
<td>4</td>
<td>1535</td>
<td>2.97 (1.72–5.12)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

RR: risk ratio; CI: confidence interval.
There are several limitations to our meta-analysis. Septic patients who survive to hospital discharge still have a high risk of death in the following months and years, which has been suggested in many studies [38]. The NICE SUGAR trial found that intensive insulin therapy increased mortality compared with the control group at 90 days, but not at 28 days [17]. Furthermore, the VISEP study, which found that intensive insulin therapy did not reduce mortality in septic patients, was discontinued early due to an excess risk for hypoglycemia [10]. Trials included in our meta-analysis mainly contained data gathered before hospital discharge and follow-up was limited. Therefore, our results may not be appropriate for long-term prognosis.

The glucose target range of the most trials in this meta-analysis was 80–110 mg/dL. Perhaps, a result favoring intensive insulin therapy could have been found to occur if a higher concentration range was used in the intensive insulin group, in comparison with uncontrolled hyperglycemia. Restricted by the lack of adequate data, we were not able to stratify trials based on the type of ICU and the proportion of calories provided parenterally or to evaluate secondary outcome measures, such as severity of illness, length of ICU stay, and cost. Also, there is the possibility of publication bias in our review.

Unfortunately, none of the included randomized controlled trials used blinding of participants and personnel. Several trials had few mortality events, so we could not detect small differences between groups. Furthermore, the patient characteristics, blood glucose control, and coexisting interventions varied across the included studies.

5. Conclusion

Overall, the results of our meta-analysis suggest that intensive insulin therapy provides no benefit for septic patients. The highly sensitive search strategy, searching multiple databases and clinicaltrials.gov, searching publications written in any language, and performing subgroup analyses and sensitivity analyses, provides strength and rigor for our meta-analysis. Future reviews of septic patients may require individual patient data and more data that captures outcome measures.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

References


Review Article

Myeloid-Derived Suppressor Cells in Sepsis

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Received 6 March 2014; Accepted 3 May 2014; Published 3 June 2014

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Sepsis is a systemic, deleterious host response to widespread infection. Patients with sepsis will have documented or suspected infection which can progress to a state of septic shock or acute organ dysfunction. Since sepsis is responsible for nearly 3 million cases per year in China and severe sepsis is a common, expensive fatal condition in America, developing new therapies becomes a significant and worthwhile challenge. Clinical research has shown that sepsis-associated immunosuppression plays a central role in patient mortality, and targeted immune-enhancing therapy may be an effective treatment approach in these patients. As part of the inflammatory response during sepsis, there are elevations in the number of myeloid-derived suppressor cells (MDSCs). MDSCs are a heterogeneous population of immature myeloid cells that possess immunosuppressive activities via suppressing T-cell proliferation and activation. The role of MDSCs in sepsis remains uncertain. Some believe activated MDSCs are beneficial to the sepsis host by increasing innate immune responses and antimicrobial activities, while others think expansion of MDSCs leads to adaptive immune suppression and secondary infection. Herein, we discuss the complex role of MDSCs in immune regulation during sepsis, as well as the potential to target these cells for therapeutic benefit.

1. Introduction

Over the last decade, during the emergence of the “host theory,” it was first assumed that the clinical features of sepsis were the result of an uncontrolled inflammatory response and an increase in inflammatory cytokines, commonly referred to as a “cytokine storm” [1]. Patients with sepsis may present with hypothermia, shock, elevated heart rate, altered mental status, tachycardia, an elevated white-cell count, and acute organ dysfunction [2, 3]. Recently, researchers have advanced this theory and suggested that infection triggers a much more complex, variable, and prolonged host response in which both proinflammatory and anti-inflammatory cytokines grow rapidly in number. Additionally, some patients with sepsis rapidly produce both categories of cytokines, whereas others have either a predominance of anti-inflammatory cytokines or globally depressed cytokine production [4]. Many investigative agents have been examined in an effort to downregulate cytokine release, which led to the survival of the majority of patients in the early period of this syndrome. Unfortunately, the patients who survive are at risk for developing nosocomial infections with organisms not typically pathogenic in immunocompetent hosts and can have reactivation of latent viruses [5, 6]. The failure of several clinical trials in sepsis has led researchers to be firmly convinced that future research needs to take a new direction [7–9]. Experts have delineated reasons for the failures of new investigative drugs and have presented some advice in the design and conduct of sepsis trials. For example, previous clinical drug studies tested drugs in young and healthy animal models, but patients frequently present with coexisting illness. The outcomes in patients are poorer than those in animals, so animal models of aging and preexisting disease are needed. Torgersen and colleagues have highlighted key immunological defects that impair host immunity including impairment of splenocyte function and depletion of immune effector cells [10]. Additionally, their clinical studies have found that ICU patients whose deaths are sepsis-related have biochemical, flow cytometric, and immunohistochemical findings consistent with
MDSCs possess the ability to suppress antigen-specific CD8+ and CD4+ T-cell activation, and several studies have found that MDSCs dramatically increase with sepsis [12]. The role of MDSCs in sepsis is not fully understood. It has been proposed that the overall role of MDSCs involves much more than simply being an immunosuppressive population. Rather, MDSCs expansion is a common response in all inflammatory processes and the expansion of MDSCs population may be protective for the host by increasing immune surveillance and innate immune responses [13]. Exploring the relationship between sepsis and MDSCs will provide insight into how these cells function and guide the development of future treatments for sepsis in clinical trials.

2. Sepsis Etiology: A Central Role of Host Immunity

Sepsis is one of the oldest and most elusive syndromes in medical history. In China, the number of patients with sepsis may exceed 3,000,000 per year with the true incidence being presumably higher, and our group reported an 8.68% occurrence rate of severe sepsis in surgical ICUs in China, with a hospital mortality rate of 48.7% [14]. Studies from the United States show a similar rate of sepsis in the general population [15]. Although many treatments have been applied and many of the treatment successes are owed to antibiotic therapy, the mortality of sepsis still remains at about 20% to 30%. High mortality rates have drawn attention to the incidence of sepsis within populations. The incidence of sepsis depends on both the characteristics of the invasive pathogen and the state of the host immune system. Epidemiologic studies have shown that pneumonia is the most common etiology, followed by intra-abdominal and urinary tract infections [15, 16]. Typically, blood cultures are positive in only one third of cases [15, 17, 18]. *Staphylococcus aureus* and *Streptococcus pneumonia* are the two most common gram-positive isolates, whereas *Escherichia coli*, *Klebsiella* species, and *Pseudomonas aeruginosa* predominate among gram-negative isolates, with a greater total incidence of gram-positive infections [17, 19]. In a recent study, gram-negative bacteria were isolated in 62% of patients who had positive cultures, 47% with gram-positive bacteria and 19% with fungi [20].

When pathogens invade the host, they will activate immune cells through an interaction with pattern-recognition receptors. These receptors recognize structures that are conserved among microbial species, which leads to the upregulation of inflammatory gene transcription and the initiation of innate immunity. Phagocytes like monocytes, macrophages, neutrophil granulocytes, and MDSCs can migrate to the infectious tissues and secrete anti-inflammatory cytokines. They phagocytize the pathogens, promote tissue repair, induce regulatory T-cells, and reduce inflammation. When sepsis initiates, the abilities of immune effector cells are strongly impaired, along with antigen-specific primary antibody production [21]. Some patients may become infected with a pathogen due to a weakened immune system. Researchers have shown that patients who survive early sepsis, but remain dependent on intensive care, have immunosuppression, which is evidenced by a reduced expression of HLA-DR on myeloid cells [II]. These patients have been found to have ongoing infectious foci or reactivation of latent viral infections, despite antimicrobial therapy [6, 10]. Other scholars have found that patients who died of sepsis in the ICU encountered strong functional impairments of splenocytes and the lungs also had increased expression of MDSCs phenotypes from sepsis versus control tissue (47.9% versus 15.7%) [II].

3. The Derivation and Subsets of MDSCs

In the early 1900s, these cells gained increasing appreciation in extramedullary haematopoiesis (EMH) and neutrophilia in tumors, which were later shown to possess abnormal myeloid-cell differentiation. Originally, these abnormal myeloid cells were described as myeloid progenitor cells, which could inhibit lymphocyte numbers and cytotoxic T lymphocyte (CTL) activity [22]. In 1987, hematopoiesis and suppressor bone marrow cells were first observed in patients with Lewis lung carcinoma [23]. In 1996, these cells became known as myeloid-derived suppressor cells. At present, it was clear that these cells lacked membrane markers which express on the surface of mature T-cells, B-cells, and natural killer (NK) cells, as well as macrophages [24]. For several decades, the understanding of MDSCs ranged from “the abnormal myeloid cell” to “immature myeloid cells” to “myeloid-derived suppressor cells.” From recent studies, we now know that MDSCs are a heterogeneous population of cells that have an immature state and a remarkable ability to suppress T-cell responses [12]. They are an intrinsic part of the myeloid-cell lineage comprising myeloid-cell progenitors and precursors of myeloid cells. The functional importance of MDSCs in the immune system has received attention over the last decade, and recent spotlights can be attributed to MDSCs’s role in the negative regulation of immune responses during cancer, other chronic diseases, and sepsis [13].

Relevant in vivo and in vitro studies have shown that approximately 1–5% of MDSCs form myeloid-cell colonies, and about one-third of this population have the ability to differentiate into mature macrophages and DCs in the presence of appropriate cytokines [25, 26]. In mice, MDSCs are characterized by the coexpression of the myeloid lineage differentiation antigens, Gr-1 and CD11b [27]. Normal murine bone marrow contains 20–30% of cells with this phenotype, but only a small proportion (2–4%) is present in the spleen cells. In sepsis, approximately 40% of the splenocyte population, approximately 90% of cells in bone marrow, and 3–5% cells in peripheral lymph nodes coexpress Gr-1 and CD11b [13]. Conversely, MDSCs in humans do not express this phenotype, rather the cells are most commonly defined as CD14+CD11b+ cells. Human MDSCs may express the common myeloid marker CD15 or CD33 but lack the expression of markers of mature myeloid and lymphoid cells and the MHC-class-II molecule HLA-DR [28–30]. The cells
described as MDSCs in human studies comprise only 0 to 0.5% of peripheral blood mononuclear cells.

Recently, many studies indicate that MDSCs can be delineated into two types: granulocytic MDSCs and monocytic MDSCs. Granulocytic-MDSCs have a CD11b⁺Ly6G⁺Ly6C<sub>low</sub> phenotype, whereas monocytic MDSCs are CD11b⁺Ly6G⁻Ly6C<sub>high</sub> [31]. These two different phenotypes possess different functions in the pathophysiology of sepsis. Firstly, only monocytic MDSCs have the ability to differentiate into mature DCs and macrophages in vitro. Furthermore, the granulocytic subset of MDSCs was found to express high levels of reactive oxygen species (ROS) and low levels of nitric oxide (NO), whereas the monocytic subset expressed the opposite pattern. Both subsets expressed arginase I (Arg-1) [31]. The abilities of the two cell types to suppress T-cell activity are also different. Monocytic MDSCs produced NO and strongly inhibited T-cell proliferation, while granulocytic-MDSCs produced low levels of NO and did not inhibit T-cell proliferation [32]. The specific difference between the two MDSCs subsets remains to be elucidated.

### 4. The Activation and Mechanisms of MDSCs

In healthy individuals, immature myeloid cells (IMCs) are generated in bone marrow and quickly differentiate into mature granulocytes, macrophages, or dendritic cells (DCs). In septic conditions, inflammatory factors such as IL-6, IL-10, IL-12, G-CSF, dsRNA, IFN-γ, VEGF, and GM-CSF are elevated, which prevents IMCs from differentiating into mature myeloid cells [33] (Figure 1). In sepsis, MDSC expansion is regulated by many factors, and these factors trigger several different signaling pathways. GM-CSF and IFN-γ have the potential to induce toll-like receptor (TLR) mediated myeloid differentiation primary response gene 88 (MyD88) signaling. Granulocyte-colony stimulating factor (G-CSF) and its receptor initiate the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway [34–36] (Figure 1). These factors not only improve the accumulation of MDSCs, but also initiate their activation.

The most important function of MDSCs is to inhibit immune response via suppressing T-cell proliferation and activation [37]. It has been shown that MDSCs mediate their effect on T lymphocytes in cancer through direct contact and/or through a combination of multiple major mediators such as inducible nitric oxide synthase (iNOS), arginase-1 (Arg1), reactive oxygen species (ROS), transforming growth factor-β (TGF-β), IL-10, regulatory T-cells (Treg), and macrophages [38]. The following is a summary of the mechanisms of action of these mediators.

Arg1 and iNOS are expressed highly in monocytic-MDSCs and utilize L-arginine to produce urea and NO, respectively. Monocytic-MDSCs inhibit T-cell responses through the depletion of L-arginine via the two enzymes. The activation of either of these enzymes inhibits T-cell proliferation by interfering with the transduction of intracellular signals and by inducing T-cell apoptosis [39]. In vitro, iNOS inhibitors (L-NMMA) alone and in combination with Arg1 inhibitors block inhibition of T-cells by MDSCs. Similarly, phosphodiesterase-5 inhibitors delay tumor progression by decreasing Arg1 and iNOS expression and by regulating the suppressive machinery of MDSCs [40]. ROS production has been shown to be a major regulator of the suppressive activity of the granulocytic-MDSCs in both murine models and human cancers [41, 42]. In three different studies, inhibition of ROS production was associated with complete elimination of the suppressive activities of the MDSCs that were isolated from mice and human cancers [28, 41]. In addition, the combination of NO and ROS was associated with the production of peroxynitrite. Peroxynitrite causes protein dysfunctions in target cells and nitration of the T-cell receptor, which in turn, leads to suppression of CD8<sup>+</sup> T-cell responses [43].

TGF-β is an immunosuppressive cytokine that has been firmly associated with MDSCs function and with the regulation of tumor induction and expansion [44]. In a study of squamous cell carcinoma of the head and neck, the CD14<sup>+</sup>HLA-DR<sup>−</sup> MDSC subset was noted to be the most predominant and produced higher levels of TGF-β compared with other MDSCs subsets [45]. TGF-β antibody partially restored T-cell proliferation and IFN-γ production. This evidence indicates that MDSCs are likely to be a major source for TGF-β production and their immunosuppressive effect is mediated by factors including TGF-β [45]. In a separate study, Lu et al. reported that TGF-β production promoted tumor cell invasion and metastasis [46]. Yang et al. found that the deletion of TGF-β receptor type II resulted in the infiltration of MDSCs in breast cancer and the production of large quantities of TGF-β that led to the promotion of tumor invasion and metastasis [47].

MDSCs may also inhibit T-cell proliferation indirectly by promoting the development of inducible CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg. The development of Treg is linked to IL-10 plus TGF-β production [48]. Delano and coworkers have shown that MDSCs can still express several cytokines and chemokines, such as interleukin 10, TNF-α, RANTES, and MIP-1β [34]. High levels of CD80 expression by MDSCs were observed in many cancer tissues. Genetic knockout of CD80 expression in MDSCs alleviated the suppression of antigen-specific immune responses. CD80 itself suppressed antigen-specific immunity via Treg [49]. Another study analyzed the interaction of MDSCs with macrophages in a mouse cancer model and showed that, through IL-10 secretion, MDSCs also induced a type-2 polarization of macrophages which is characterized by a decrease of IL-12 secretion and that promotes tumor growth [50].

### 5. The Role of MDSCs in Sepsis

Although most of the current information about the function of MDSCs in immune responses has come from studies in the cancer field, there are increasingly more studies that directly investigate the roles of MDSCs in sepsis. Some researchers believe MDSCs are deleterious to the sepsis host. Delano et al. first demonstrated that MDSCs contribute to sepsis-induced T-cell suppression and preferential Th2 polarization [34]. They reported that GR-1<sup>−</sup>CD11b<sup>+</sup> MDSCs population was
Figure 1: The origin and signaling pathways involved in MDSCs in sepsis. Haematopoietic stem cells (HSCs) differentiate into immature myeloid cells (IMCs) and then quickly differentiate into mature granulocytes, macrophages, or dendritic cells (DCs). In septic conditions, inflammatory factors such as IL-6, IL-10, IL-12, G-CSF, ds RNA, VEGF, and GM-CSF are elevated. They prevent IMCs from differentiating into mature myeloid cells. MDSCs expansion and activation is regulated by many signaling pathways, such as a toll-like receptor (TLR) mediated myeloid differentiation primary response gene 88 (MyD88) signaling and the granulocyte-colony stimulating factor receptor (G-CSFR) mediated the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway. They contribute to the increased production of reactive oxygen species (ROS), inducible nitric oxide synthase (iNOS), and arginase 1 (ARG1). MDSCs in sepsis can reduce the capacity of septic monocytes, macrophages, and neutrophils to respond to bacterial toxins, inhibit the activation of T-cells, and promote Th2 polarization. In addition, MDSCs can secrete several cytokines and chemokines, such as interleukin 10, TNF-α, RANTES, and MIP-1β.
able to inhibit T-cell proliferation. Furthermore, adoptive transfer of day 10 MDSCs to septic mice attenuated peritoneal cytokine production, increased bacterial clearance, and dramatically improved survival rates [55]. In the same animal model, another research group had similar findings in terms of the duration needed for MDSCs to acquire their protective effect. Adoptive transfer of early (day 3) MDSCs from septic mice into naive mice after caecal ligation and puncture increased proinflammatory cytokine production and early mortality. Conversely, transfer of late (day 12) MDSCs from septic mice had the opposite effect. Early and late MDSCs studied ex vivo also differed in their inflammatory phenotypes. Early MDSCs expressed nitric oxide and proinflammatory cytokines, whereas late MDSCs expressed arginase activity and anti-inflammatory IL-10. They concluded that as the septic inflammatory process progresses, the heterogeneous MDSCs shift from being proinflammatory to anti-inflammatory [56]. Taken together, the role of MDSCs in sepsis is still controversial.

6. Therapy Targeting MDSCs in Sepsis

The treatment of sepsis is challenging and complex, thus, sepsis-related mortality remains high. The principles of the initial management approach are to provide cardiorespiratory resuscitation and to mitigate the immediate threats of uncontrolled infection. Many therapies could be applied to a patient with sepsis, including the use of intravenous fluids and vasopressors, oxygen therapy and mechanical ventilation, and organ function support and other intensive life support. New research has shown that most patients admitted to intensive care units for treatment of sepsis had unresolved septic foci postmortem, suggesting that patients were unable to eradicate invading etiologic pathogens and were highly susceptible to nosocomial organisms, or both [II]. Several clinical trials of drugs that boosted immunity suggested that therapies that improve host immunity might increase survival because immunosuppression has a central role in sepsis-related deaths. Sepsis can be thought of as a battle between the invading microbes and the host's immune response, with each side seeking success. In addition to studying current management approaches, immunotherapies for sepsis must also be examined, especially those focused on the regulation of MDSCs.

Many efforts have been made to target MDSCs in cancer. Both sepsis and cancer share many immunological defects, therefore, some investigators postulate that the recent success of several immunomodulatory drugs in cancer may provide potential immunostimulatory therapies for sepsis [57]. To mitigate the immunosuppressive activities of MDSCs, one of the effective strategies is to differentiate MDSCs into mature cells. All-trans retinoic acid (ATRA) at therapeutic levels has been shown to reduce MDSCs and induce MDSCs differentiation into dendritic cells and macrophages in cancer patients and mice [58]. Nefedova et al. suggested that an upregulation of glutathione synthesis and a reduction in ROS levels were the main mechanisms involved in ATRA-mediated MDSCs differentiation [59]. In a recent randomized clinical trial involving patients with small cell lung cancer ($n = 41$), systemic depletion of MDSCs using ATRA in combination with cancer vaccination led to a statistically significant improvement in the immune response to p53 vaccination in comparison with the vaccination-only group ($20\%, P = 0.02$) [60]. In another clinical study, Mirza et al. reported that ATRA administration in patients with metastatic renal cell carcinoma markedly reduced the number of MDSCs (Lin- HLA-DR CD33$^+$). ATRA also improved the myeloid/dendritic cell ratio and the ability of patients' mononuclear cells to stimulate allogeneic T-cells, increased the dendritic cells/MDSCs ratio in the peripheral blood, and improved the T-cell immune response [61]. In addition, Martire-Greco and colleagues demonstrated that ATRA improves immunocompetence in a murine model of lipopolysaccharide-induced immunosuppression by decreasing the number of viable MDSCs [51].

Inhibition of the signal pathways that regulate the production of the suppressive factors of MDSCs is another promising approach. Sildenafil, a phosphodiesterase-5 inhibitor, reduced arginase 1 and nitric oxide synthase-2 expression in a mouse tumor model. It enhanced intratumoral T-cell infiltration and activation, reduced tumor outgrowth, and improved the antitumor efficacy of adoptive T-cell therapy. Furthermore, sildenafil restored T-cell proliferation of peripheral blood mononuclear cells from multiple myeloma and head and neck cancer patients in vitro [62]. It is not clear whether this favorable effect will be observed clinically in cancer patients. Nitroaspirin is a classic aspirin molecule covalently linked to an NO donor group and is able to release NO. Nitroaspirin does not possess direct antitumor activity. However, by interfering with the inhibitory enzymatic activities of MDSCs, orally administered nitroaspirin normalized the immune status of tumor-bearing hosts, increased the number and function of tumor-antigen-specific T lymphocytes, and enhanced the preventive and therapeutic effectiveness of the antitumor immunity elicited by cancer vaccination [63]. Recently, cimetidine, a histamine type-2 receptor antagonist, was shown to reduce NO production and arginase 1 expression of MDSCs. MDSCs were prone to apoptosis due to cimetidine treatment resulting in a reversal of MDSCs-mediated T-cell suppression and improved IFN-γ production [64].

Several studies in mice tested a "cell-based therapy" approach using MDSCs or MDSCs-like cells in the treatment of diabetes [65], immunological hepatic injury (IMH) [66], and graft-versus-host disease (GVHD) [39]. Yin et al. found that administration of MDSCs can prolong the survival of diabetic mice transplanted with allogeneic pancreatic cells [65]. Highfill et al. reported that the adoptive transfer of MDSCs significantly improved survival in a model of graft-versus-host disease [67]. In both of these studies, MDSCs were generated ex vivo by culturing BM cells with a combination of colony stimulating factors and interleukins.

Presently, there is no clinical trial targeting MDSCs in sepsis. Some experts believe that the absence of activated MDSCs in patients with sepsis might be the reason why these patients succumb to nosocomial infection [68]. Therefore, pharmacologic agents known to regulate the production of
the suppressive factors of MDSCs or promote the expansion of MDSCs such as growth factors, chemokines, and sildenafil require further study in an effort to improve patient outcomes in sepsis.

7. Conclusions

In recent years, it has become clear that most septic patients do not die from an overwhelming proinflammatory immune response, but rather succumb to their illness in an immunosuppressive state. Although control of the infection and supportive therapies will remain the mainstay for treatment in the early phase of sepsis, there is a developing trend towards immunostimulation for patients in immunosuppressive states. MDSCs are a heterogeneous population of cells that have an immature state and the ability to suppress T-cell responses. The roles of MDSCs in sepsis remain uncertain. Some believe MDSCs are beneficial to the septic host and that the absence of activated MDSCs is the reason why some patients subsequently succumb to nosocomial infection. Others believe that MDSCs are deleterious and the expansion of MDSCs in the host following sepsis leads to global adaptive immune suppression and secondary infection. The roles and mechanisms of MDSCs warrant further exploration and MDSCs could serve as a viable target for sepsis treatment.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

This study was supported by the National Natural Science Foundation of China (81072416; 81272139; 81130036) and the National Science and Technology Support Program (2012BAI04B05).

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Clinical Study
Cortisol Is an Associated-Risk Factor of Brain Dysfunction in Patients with Severe Sepsis and Septic Shock

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Received 1 February 2014; Accepted 6 April 2014; Published 28 April 2014

Academic Editor: Baoli Cheng

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Objectives. To investigate cortisol levels in brain dysfunction in patients with severe sepsis and septic shock. Methods. In 128 septic and sedated patients, we studied brain dysfunction including delirium and coma by the evaluation of Richmond Agitation Sedation Scale (RASS), the Confusion Method Assessment in the ICU (CAM-ICU) after sedation withdrawal and the measurement of serum S100B biomarker of brain injury. Serum cortisol and S100B were measured within 12 hours after ICU admission and daily over the next four days. Results. Brain dysfunction was observed in 50% (64/128) before but in 84% (107/128) of patients after sedation withdrawal, and was more common in the patients older than 57 years ($P = 0.009$). Both cortisol ($P = 0.007$) and S100B levels ($P = 0.028$) were higher in patients with than patients without brain dysfunction. Cortisol levels were associated with ICU mortality (hazard ratio = 1.17, $P = 0.024$). Multivariate logistic regression showed that cortisol (odds ratio (OR): 2.34, 95% CI (2.01, 3.22), $P = 0.02$) and the combination effect of cortisol with age (OR: 1.004, 95% CI (1.002, 1.93), $P = 0.038$) but not S100B were associated with brain dysfunction. Conclusions. Cortisol was an associated-risk factor of brain dysfunction in patients with severe sepsis and septic shock.

1. Introduction

Cortisol release from the hypothalamic-pituitary-adrenal axis (HPA) is vital for the host survival in stress [1]. In sepsis, high cortisol release could result not only from stress but also from HPA dysfunction and following a metabolism reduction of this hormone at the organs [2–4]. However, an excessive release or chronic exposure to high cortisol levels could be harmful for the host brain, especially at the hippocampus and the frontal cortex where corticoid receptors are highly concentrated. It has been shown in rodents’ experimental studies that cortisol reduced the neurons viability to toxic insults (glutamate, hypoxia, ischemia, or hypoglycemia) by ATP energy deprivation in these parts of the brain [5, 6].

Cortisol has been suggested as biomarker for the diagnosis of delirium [7]. High cortisol release associated with delirium was previously reported in stroke [8], in postcardiac surgery [9], in the elderly patients with hip fracture [10], in psychological depression, and in Cushing syndrome [11]. In clinical severe sepsis, high cortisol levels have been reported in patients who developed fatal brain dysfunction [12] but it remained unclear if this could be a sole indicator of severe inflammation and disease severity or was due to brain dysfunction. Also, these results were only reported in a small
number of patients with severe sepsis and remain to be confirmed in a larger study sample.

In this observational prospective study, we investigated the effect of cortisol on the development of brain dysfunction in patients with severe sepsis and septic shock. Brain dysfunction including coma and delirium was evaluated by the Glasgow Coma Score (GCS) or the Richmond Agitation Sedation Scale (RASS) combined with the Confusion Assessment Method in the ICU (CAM-ICU) and with the measurement of serum biomarker of brain injury S100B protein [13].

We also compared the predictive value of cortisol with S100B on the development of brain dysfunction as the measurements of serum biomarkers (S100B protein, neuron-specific enolase, and glial fibrillary acidic protein) have been proposed to help diagnose brain dysfunction regardless of sedation [14].

2. Patients and Methods

We studied 140 patients with severe sepsis and septic shock who were consecutively included in the study from October 2009 to January 2011. The Ethical Committee of our hospital approved the study and the informed consent was obtained from the patient’s relatives.

Severe sepsis and septic shock was considered before ICU admission and the inclusion criteria were defined following the international consensus guidelines of sepsis [15]. Exclusion criteria included patients younger than 18 years old, pregnancy, acute cerebral disorder (trauma, stroke, hemorrhage, post-neurosurgery, cardiopulmonary arrest, and meningitis), concomitant treatment with corticoid or etomidate in the previous 24 hours, drugs or alcohol withdrawal, severe psychiatric disorder, dementia with disable neuro-muscular disorders, severe chronic liver or renal failure, and nonsurvivors in the first 24 hours from sepsis.

Early goal-directed therapy for sepsis resuscitation and lung protective ventilation strategy were conducted following the international consensus sepsis guidelines. Resuscitation was targeted to obtain a mean arterial pressure ≥ 65 mm Hg and a urine output > 0.5 mL/kg/min with both colloid and crystalloid infusion, combined with norepinephrine (up to 1 μg/kg/min) and dobutamine (up to 10 μg/kg/min). Mechanical ventilation targeted a tidal volume of 6-7 mL/kg ideal body and a plateau pressure < 30 cm H₂O.

Sedation in the ICU was achieved by midazolam (up to 0.05 mg/kg/h) or propofol (up to 10 mg/kg/h) and analgesia was achieved by fentanyl (up to 0.05 mg/kg/min) or remifentanil (up to 0.75 μg/kg/h). Sedation and analgesia doses were daily adapted to obtain a Richmond Agitation Sedation Scale (RASS) score between 0 and −3. Neuromuscular blocking agent administration was interrupted within the first 48 hours after ICU admission.

Organ failure and the critical illness severity were evaluated by the daily Sequential Organ Failure Assessment (SOFA) and the APACHE III scores, respectively.

2.1. Brain Dysfunction Assessment. Before sedation, brain dysfunction was assessed by either GCS or CAM-ICU and it was considered when the GCS ≤ 13 or CAM-ICU was positive. After 24 hours of complete sedation withdrawal and up to ICU discharge, CAM-ICU was assessed together with the RASS score twice per day by the nurse or the physician in charge of the patient.

Delirium was confirmed by the physician in charge of the patient who was not aware about the cortisol and S100B results when RASS score > −3 and CAM-ICU was positive for at least two consecutive days. Hyperactive delirium was defined as the RASS score of 1 to 5 with agitation, irritation, confusion, and emotional labiality. Hypoactive delirium was defined as the RASS of −2 to −1 with apathy, decreased responsiveness or movement, and low consciousness. Coma was defined as the RASS score ≤ −4 throughout the ICU stay. Acquired critical illness neuromyopathy (CINM) was confirmed by electromyography.

2.2. Cortisol and S100B Protein Measurement. After ICU admission and sepsis confirmation, serum cortisol and S100B were simultaneously collected not at fixed time between 6 and 12 hours after hemodynamics stabilization and then once daily in the morning for four consecutive days.

Total serum cortisol concentration was measured in all patients by radioimmunooassay (Diasorin, Stillwater, USA). Normal values in the morning range (7–10 a.m.) were 171–535 nmol/L without gender difference. The within run coefficient was <5% and the between run coefficients of variation were <10% (range: 27 to 1650 nmol/L).

Serum S100B protein was obtained in 75 patients by radioimmunooassay (immunoradiometric assay, Roche Diagnostics GmbH, Germany) and the normal value provided by the commercial kit was ≤ 0.105 μg/L.

2.3. Statistical Analysis. The parametric statistical methods were used with SPSS version 20.0 (SPSS, Chicago, IL) and SAS version 9.3 (SAS Institute, North Carolina, USA) for analysis.

Chi-square test or Fischer’s exact test was used when appropriate for comparisons of categorical variables. Logarithm transformation was used to assume a normal distribution for continuous variables. Student’s t-test was used for comparisons of cortisol between groups. Linear mixed models for repeated measures (including a random intercept effect and adjusting with age and gender) were used to compare the global difference of biomarkers over 4 days of measurement between groups. Nonparametric Mann-Whitney U test was used for comparisons of skewed values of S100B between groups.

The Pearson or Spearman correlation test was used to evaluate the correlation between continuous variable when appropriate.

Kaplan-Meier analysis with log-rank test was used to analyze the ICU survival time. The Cox proportional hazards regression model adjusted with stepwise selection of covariates (SOFA score at the first 4 days, age, cortisol levels at admission, S100B levels at day 2, and 2 binary variables: occurrence of brain dysfunction or nosocomial infection)
was used to determine the risk factors associated with ICU mortality.

Multivariate binary logistic regression model adjusted with stepwise selection of covariates (cortisol levels at admission, age, SO100B levels at day 2, the SOFA score at the first 4 days, gender, the length of sedation, and one interaction term (age x cortisol levels at admission)) was used to determine the associated-risk factors for the development of brain dysfunction. Variables with univariate chi-square value <0.2 were added and retained at P ≤ 0.05. The receiver operating characteristic (ROC) curve and the area under the curve (AUC) were calculated for the logistic regression model or to determine for the cut-off value of cortisol and age associated with delirium. Smaller Akaike criteria value was used for the selection between different logistic regression models.

Statistical significance was considered at two-sided P value <0.05.

### 3. Results

Of the 140 patients with severe sepsis and septic shock, 12 (9%) who died under sedation without proper neurological evaluation were excluded from analysis.

Brain dysfunction developed in 50% (64/128) of patients before sedation but up to 84% (107/128) after sedation withdrawal. Delirium developed in 85/107 patients (80%): 41 hypoactive (48%) and 44 hyperactive (52%). Twenty-two patients (20%) remained comatose: brain CT scan showed a hemorrhage in five, a stroke in three and septic emboli in one.

In delirium patients, brain CT scan was negative in 15/44 hyperactive delirium patients but showed a stroke in two and a hemorrhage in one out of 21/41 hypoactive delirium patients.

#### 3.1. The Patients’ Characteristics and Comorbidities at ICU Admission (Table 1).

Only difference in age was found with regard to brain dysfunction after sedation withdrawal. However, age was not different in regard to brain dysfunction occurring before starting of sedation. Patients who developed brain dysfunction were older than the other patients (67 ± 13 versus 58 ± 18 years, \( P = 0.009 \)). Also comatose patients were older than delirium patients (72 ± 9 versus 65 ± 13 years, \( P = 0.007 \)). As expected, ICU nonsurvivors were older than survivors (68 ± 13 versus 62 ± 14 years, \( P = 0.035 \)).

#### 3.2. Brain Dysfunction and ICU Evolution (Table 2).

When compared with non-brain dysfunction, patients who developed brain dysfunction had higher degree of SOFA score at the first four days, higher incidence of nosocomial infection and septic shock recurrence, and higher prevalence of acquired critical illness neuromyopathy (CINM) but a trend of prolonged ICU length of stay (15 ± 10 versus 23 ± 18 days, \( P = 0.06 \)).

Brain dysfunction was associated with higher ICU mortality: death occurred in 41% (18/44) hyperactive delirium, 68% (28/41) hypoactive delirium, and all (100%) of 22 comatose patients (\( P < 0.001 \)) but 14% (3/21) in patients without brain dysfunction. The median (interquartile) ICU survival time was 36 (14, 48) days for delirium and 8 (7, 15) day for comatose patients (all log-rank tests, \( P < 0.01 \)).

The Cox proportional hazards regression model identified three risk factors independently associated with ICU mortality: SOFA score at the first 4 days (hazard ratio (HR) = 1.22, \( P = 0.05 \)), cortisol levels at admission (HR = 1.17, \( P = 0.024 \)), and occurrence of brain dysfunction (HR = 4.89, \( P = 0.001 \)).

#### 3.3. Cortisol and S100B Protein Levels in Brain Dysfunction.

Cortisol levels at admission (448 ± 813 versus 211 ± 122 nmol/L, \( P = 0.013 \)) and over four days (\( P = 0.007 \), Figure 1) were higher in patients who developed brain dysfunction than non-brain dysfunction after adjusting with age and gender. Cortisol levels were higher in ICU nonsurvivors than survivors (510 ± 352 versus 248 ± 187 nmol/L, \( P = 0.05 \)) but were not different between delirium and coma.

![Figure 1: Patients with brain dysfunction released higher cortisol levels than non-brain dysfunction.](image-url)
Table 2: Biomarkers levels, ICU clinical evolution, and outcome.

<table>
<thead>
<tr>
<th></th>
<th>All patients (n = 128)</th>
<th>Non-brain dysfunction (n = 21)</th>
<th>Brain dysfunction (n = 107)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortisol admission (nmol/L)</td>
<td>413 ± 757</td>
<td>211 ± 122</td>
<td>448 ± 813</td>
<td>0.013</td>
</tr>
<tr>
<td>Cortisol day 4 (nmol/L)</td>
<td>279 ± 256</td>
<td>314 ± 352</td>
<td>273 ± 238</td>
<td>0.441</td>
</tr>
<tr>
<td>S100B admission (µg/L)</td>
<td>0.13 (0.07, 0.28)</td>
<td>0.97 (0.51, 0.18)</td>
<td>0.13 (0.06, 0.49)</td>
<td>0.188</td>
</tr>
<tr>
<td>S100B day 4 (µg/L)</td>
<td>0.13 (0.06, 0.2)</td>
<td>0.08 (0.04, 0.13)</td>
<td>0.12 (0.08, 0.24)</td>
<td>0.038</td>
</tr>
<tr>
<td>Mechanical ventilation, days</td>
<td>16 ± 14</td>
<td>12 ± 11</td>
<td>15 ± 14</td>
<td>0.109</td>
</tr>
<tr>
<td>Inotropics length, days</td>
<td>6 ± 5</td>
<td>4 ± 2</td>
<td>6 ± 5</td>
<td>0.191</td>
</tr>
<tr>
<td>SOFA score for the first 4 days</td>
<td>8 ± 4</td>
<td>6 ± 4</td>
<td>9 ± 4</td>
<td>0.006</td>
</tr>
<tr>
<td>Sedation, days</td>
<td>9 ± 7</td>
<td>8 ± 6</td>
<td>10 ± 8</td>
<td>0.315</td>
</tr>
<tr>
<td>Midazolam, n (%)</td>
<td>80 (62)</td>
<td>12 (57)</td>
<td>68 (64)</td>
<td>0.579</td>
</tr>
<tr>
<td>Fentanyl, n (%)</td>
<td>75 (59)</td>
<td>13 (62)</td>
<td>62 (58)</td>
<td>0.736</td>
</tr>
<tr>
<td>Propofol, n (%)</td>
<td>48 (37)</td>
<td>9 (43)</td>
<td>41 (38)</td>
<td>0.696</td>
</tr>
<tr>
<td>Nosocomial infection, n (%)</td>
<td>67 (52)</td>
<td>5 (24)</td>
<td>62 (58)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Shock recurrence, n (%)</td>
<td>37 (29)</td>
<td>1 (5)</td>
<td>36 (34)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>CINM, n (%)</td>
<td>64 (50)</td>
<td>4 (19)</td>
<td>60 (56)</td>
<td>0.003</td>
</tr>
<tr>
<td>ICU mortality, n (%)</td>
<td>69 (54)</td>
<td>3 (14)</td>
<td>66 (62)</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Continuous variables were represented as mean values ± standard deviation. S100B levels were represented as median (interquartile) due to skewed values.

CNIM: critical illness neuromyopathy.

Figure 2: Patients with brain dysfunction released higher S100B levels than non-brain dysfunction.

A cortisol cut-off value of 232 nmol/L determined brain dysfunction after sedation withdrawal with a sensitivity 59% and a specificity 63%. An age cut-off value of 57 years determined brain dysfunction with sensitivity 83% and specificity 63%.

S100B was elevated in brain dysfunction but not in non-brain dysfunction patients (P = 0.028, Figure 2). Comatose patients had higher S100B levels than delirium patients (median (interquartile): 0.14 (0.11, 0.48) versus 0.1 (0.05, 0.37) µg/L, P = 0.007). Cortisol correlated with S100B levels at ICU admission (r = 0.32, P < 0.01) and with the SOFA score at the first 4 days (r = 0.55, P < 0.01). Neither cortisol nor S100B was correlated with age, serum creatinine, APACHE III or SOFA scores, and C-reactive protein (CRP), (all P value >0.05).

Two risk factors were associated with the development of brain dysfunction in sepsis: cortisol levels at ICU admission (OR: 2.34, 95% CI (2.01, 3.22), P = 0.02) and the combination effect of cortisol with age (OR: 1.004, 95% CI (1.002, 1.93), P = 0.038). The AUC of the ROC curve of this global model to discriminate brain dysfunction was 89% (95% CI: 82, 95%) and remained unchanged when only delirium patients were included in the model.

4. Discussion

In our series of septic patients, brain dysfunction was present in one-half of the patients before sedation was started but increased to 84% of the patients after sedation withdrawal. Hence, sedation and/or other factors aggravated brain dysfunction at a certain time point in a number of patients during the sedation period. Benzodiazepines and opiates administration, although aggravating delirium in the critically ill patients, likely did not significantly influence the results as the number of patients who were sedated with midazolam and fentanyl and the length of sedation were not different in regard to brain dysfunction [16].

Our results suggested that cortisol was an associated-risk factor of brain dysfunction in sepsis. Pfister et al. previously showed that high cortisol levels were associated with S100B elevation and fatal brain dysfunction in a small number of patients with severe sepsis and septic shock [12]. Our results confirmed these observations in a larger cohort of septic patients, as cortisol levels were higher in patients with than patients without brain dysfunction. This could not result from a sole reaction to stress and illness severity because no association was found with the APACHE and SOFA scores or the degree of inflammatory response (as reflected by blood CRP concentrations). Also, cortisol levels decreased over time probably result from deficiency or exhaustion of the stimulating effect of adrenocorticotropic hormone (ACTH) occurring in these patients [17].

It has been reported that cortisol could be harmful for the brain, especially at the hippocampus [18]. In rodents,
cortisol impaired the neurons viability to insults (hypoxia, ischemia, or hypoglycemia) by inhibiting glucose uptake and utilization, exacerbating energy ATP depletion, and accumulated neurotoxic products over the course of few days [5, 6].

A cortisol cut-off value of 353–550 nmol/L was previously reported to be associated with delirium in stroke [8] and after cardiac surgery [9, 19]. However, we observed a lower cortisol cut-off value of 232 nmol/L associated with brain dysfunction in severe sepsis and this value was still remaining in our normal laboratory reference value (170–535 nmol/L). Hence, other factors should predispose the host brain to be more sensitive to the effect of cortisol. Sepsis with multiple organ dysfunction and aging could contribute to this process as these factors remained the main fatal ICU risk factors.

Sepsis aggravates ongoing brain dysfunction as a component of multiple organ dysfunctions which was reflected by S100B elevation and higher organ dysfunction SOFA scores, respectively. Different regions of the brain were affected in sepsis but the hippocampus was particularly sensitive to hypoxia and inflammation [20]. Moreover, the hippocampus regulates a negative feedback effect of cortisol release from the HPA [6, 21]. The dysfunction and damage of this part of the brain induced aberrant cortisol release, cognitive dysfunction, delirium and was implicated in the development of long term cognitive impairment in the sepsis survivors [17, 22–24]. In addition, the BBB breakdown in sepsis could worsen brain dysfunction by facilitating a crossing of cortisol, toxic amino acids and other proinflammatory mediators from the blood into the brain [25–27]. Using S100B as biomarker of brain injury and the blood-brain barrier (BBB) breakdown [28] we found that S100B elevation correlated with cortisol levels to support this hypothesis. Also, S100B was associated with brain dysfunction severity as higher levels were observed in fatal comatose than other patients.

Sepsis and brain dysfunction commonly targeted the aging patients [29, 30]. Patients who developed brain dysfunction were older and had higher cortisol levels than other patients. Elderly patients with preexisting brain disorder (dementia, atherosclerosis, and degenerative diseases) were more vulnerable to ischemia and to the harmful effect of proinflammatory cytokines or neurotoxic products than younger subjects [17, 31]. However, in our study, sepsis had more impact than preexisting brain disorders as it induced brain dysfunction in more than half of patients at ICU admission even before sedation was starting.

The logistic regression model also confirmed that cortisol levels associated with brain dysfunction were more pronounced with age (older than 57 years old).

In addition to the above, the aging patients were chronically exposed to higher cortisol levels following an impairment of the negative feedback at the hippocampus with aging [32]. In these patients, cortisol could exacerbate neuronal dysfunction by energy deprivation, particularly at the hippocampus and the cortex, the parts of the brain where corticoid receptors are highly concentrated and sensitive to sepsis [6]. These observations raise concerns if the cortisol treatment should be selected in the aging patients with septic shock to prevent brain dysfunction, especially since it has been reported that a reduction of brain activity and cognitive dysfunction were observed after hydrocortisone administration in healthy volunteers [33].

In contrast with Pfister et al., we found that cortisol was more powerful than S100B to predict brain dysfunction and global ICU mortality. We previously showed that, due the short half-life time, S100B could only predict the early ICU nonsurvivors of sepsis [34]. Unlike S100B, cortisol was not solely a biomarker of brain dysfunction but was related with underlying life-threatening homeostasis disturbance of the host: abnormal HPA stress hormones response, glucose intolerance, immunodepression, and adrenal insufficiency [35–37].

S100B was sensitive to predict coma and severe brain injury (stroke, postanoxic encephalopathy, and head trauma) but more frequent measurements over a longer period than four days were probably necessary to diagnose delirium, which fluctuated over time [38–40]. Lacking of the statistical power with reduction of the predictive value of S100B to determine brain dysfunction could be another explanation as this biomarker was measured in half of patients.

Our study showed some limitations: we did not measure free serum cortisol which reflected its real biological activity although its advantage on total cortisol in sepsis was not confirmed by all authors [41]. Cortisol was not measured in the cerebral-spinal fluid although it was elevated in delirium patients [42]. Also, no direct harmful effect of cortisol on the brain or hippocampus could be demonstrated neither by neurophysiological studies nor brain imaging. We measured cortisol within a time interval but not at fixed time. However, as the circadian cortisol secretion dysfunction frequently occurred in sepsis this did not significantly influence our results [43].

S100B was not brain specific and could be released from sole stress or by other organs than the brain in shock [13, 44]. However, these factors likely did not significantly influence our results as no correlation was found with serum creatinine, APACHE, or SOFA scores. Moreover, it has been recently reported that the extracerebral source of S100B did not affect the levels of this biomarker in the serum after excluding major trauma or severe surgery [45].

5. Conclusion

Cortisol was an associated-risk factor of brain dysfunction in patients with severe sepsis and septic shock.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

References


Review Article

Hydrogen Gas Presents a Promising Therapeutic Strategy for Sepsis

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Received 7 February 2014; Accepted 1 April 2014; Published 16 April 2014

Academic Editor: Baoli Cheng

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Sepsis is characterized by a severe inflammatory response to infection. It remains a major cause of morbidity and mortality in critically ill patients despite developments in monitoring devices, diagnostic tools, and new therapeutic options. Recently, some studies have found that molecular hydrogen is a new therapeutic gas. Our studies have found that hydrogen gas can improve the survival and organ damage in mice and rats with cecal ligation and puncture, zymosan, and lipopolysaccharide-induced sepsis. The mechanisms are associated with the regulation of oxidative stress, inflammatory response, and apoptosis, which might be through NF-κB and Nrf2/HO-1 signaling pathway. In this paper, we summarized the progress of hydrogen treatment in sepsis.

1. Introduction

Sepsis is defined as the presence (probable or documented) of infection together with systemic manifestations of infection [1]. Severe sepsis is defined as sepsis plus sepsis-induced organ dysfunction or tissue hypoperfusion [1]. Sepsis-induced tissue hypoperfusion is defined as infection-induced hypotension, elevated lactate, or oliguria [1]. Septic shock is defined as sepsis-induced hypotension persisting despite adequate fluid resuscitation [1]. Sepsis and its various adverse sequelae, such as septic shock, acute respiratory distress syndrome (ARDS), and multiple organ dysfunction syndrome (MODS), continue to be a leading cause of mortality in intensive care unit (ICU) and a major public health burden throughout the world [2]. With recent dramatic advances in powerful antibiotics and monitoring devices, the mortality rate has been decreased over the past half century [3]. However, the number of people dying from sepsis continues to rise annually owing to the increasing morbidity. It is estimated that about 18 million cases of severe sepsis occur annually worldwide and the incidence rate of sepsis is increased by 1.5%–8% annually [4, 5]. At present, there are more than 1,000,000 cases of severe sepsis among hospitalized patients each year in the USA with a total annual cost of $16.7 billion [6, 7]. In China, the occurrence rate of severe sepsis in surgical ICU is 8.68% with a hospital mortality rate of 48.7% [8, 9].

The pathogenesis and mechanisms of sepsis are complex and not fully understood, which include the excessive release of inflammatory cytokines, the action of oxidative stress (excessive release of reactive oxygen species, ROS), intestinal bacteria and endotoxin translocation, neutrophil dysfunction, microcirculatory impairment, mitochondrial dysfunction, the imbalance between oxygen supply and oxygen consumption, immune and metabolic disorders, and coagulation disorders [10–15]. Our previous studies have found that the uncontrolled inflammatory response and oxidative stress are crucial to the pathogenesis of sepsis, MODS, and ultimately death [16–18]. In addition, it should be noted that genetic variations partially determine individual susceptibility to sepsis. An increasing number of candidate genes have been implicated in sepsis susceptibility, such as macrophage migration inhibitory factor, plasminogen activator inhibitor 1, protein C, and miRNA [19].

However, even when sepsis is timely recognized, there is no effective therapy to sepsis, except antibiotics, fluids, and vasopressors [9, 20]. Disappointedly, undeniable successes in numerous animal studies are not consistent with that
in clinical trials, such as direct anti-inflammatory strategies including anti-TNF-α, IL-1-based therapies, high-dose corticosteroids, and administration of activated protein C, which make the researches about pathogenesis and clinical treatment of sepsis troubled [10, 21–23]. The novel interventional strategies of sepsis should be invented to reduce mortality in sepsis.

Molecular hydrogen (H₂), the smallest, lightest, and most element in the universe, is colorless, odorless, and certain antioxidant. Many years ago, H₂ was regarded as physiological inert gas without more attention from scientists because of relatively low solubility and the difficulty to be absorbed. In 1975, Dole et al. found that exposure to a mixture of 2.5 percent oxygen and 97.5 percent hydrogen at a total pressure of 8 atmospheres for periods up to 2 weeks would cause a regression of squamous cell carcinoma via antioxidant effect [24]. In 1997, Shirahata et al. [25] reported that electrolyzed-reduced water, which dissolved large amounts of H₂, had the ability to protect DNA from oxidative damage, suggesting that it could reduce the risk of life style-related diseases and cancer. H₂ has also been used in medical applications to prevent decompression sickness in deep-sea divers for safety profiles [26]. In 2001, Gharib et al. [27] reported that treatment with 0.7 MPa hydrogen in a hyperbaric chamber for 2 weeks had significantly protective effects towards schistosomiasis-associated chronic liver inflammation, which was associated with antioxidant and anti-inflammatory properties of H₂. It is also proved that molecular hydrogen would directly react with the hydroxyl radical, a highly cytotoxic reactive oxygen species (ROS). In 2007, Ohsawa et al. [28] found that H₂ could exert a therapeutic antioxidant activity by selectively reducing hydroxyl radical and peroxynitrite (another cytotoxic ROS) in vitro, making researches about molecular hydrogen become hot around the world. In recent years, many researchers have found that molecular hydrogen can attenuate multiple organ damage, such as brain, spinal cord, heart, lung, liver, kidney, pancreas, and intestine [28–34]. Besides, it is widely proved that H₂ or H₂-rich saline exerts an effective therapeutic role in many diseases including sepsis, ischemia-reperfusion injury, organ transplantation, stroke, MODS, type 2 diabetes, atherosclerosis, neurodegenerative diseases, and oxygen toxicity [28, 30, 31, 35–40]. However, the mechanisms by which molecular hydrogen provides beneficial effects on many disorders remain unclear, which would be associated with reduction of oxidative stress, inflammation, and apoptosis, as well as regulation of several important signaling pathways.

2. Advances in Hydrogen Treatment of Sepsis

We have made several studies about H₂ treatment in animal models of sepsis. It is well known that cecal ligation and puncture (CLP) causes lethal peritonitis and sepsis due to a polymicrobial infection that is accompanied by multiple organ damage. We firstly investigate the possible therapeutic effects of H₂ on sepsis in a murine model of moderate or severe CLP. For severe CLP (100% lethality), we ligate the distal three-quarters of the cecum and make a single puncture with a 20-gauge needle; for moderate CLP (30–40% survival), we ligate the distal one-half of the cecum and make a single puncture with a 21-gauge needle. We find that H₂ inhalation starting at 1 and 6 h after CLP operation significantly improved the survival rate of septic mice with moderate or severe CLP in a concentration- and time-dependent manner [30]. Moreover, H₂ inhalation at a therapeutic dose (2% and 4%) has no adverse effects on the saturation level of arterial oxygen and hemodynamic parameters. We further find that H₂ treatment provides the beneficial effects on sepsis and sepsis-associated organ damage, including lung, liver, kidney, and brain [30]. Zymosan, a substance derived from the cell wall of the yeast S. cerevisiae, can lead to systemic inflammation by inducing a wide range of inflammatory mediators. The zymosan-induced generalized inflammation model has been widely used in many experimental studies for MODS. We also find that H₂ improves survival rate and organ damage in zymosan-induced generalized inflammation model [16]. 2% H₂ inhalation for 1 hour beginning at 1 and 6 hours after zymosan injection significantly improves the 14-day survival rate of zymosan-challenged mice from 10% to 70%. H₂ treatment significantly mitigates the impairments of liver and kidney function in the zymosan-challenged mice [17]. Intratracheal administration of lipopolysaccharide (LPS), the major component of the outer membrane of Gram-negative bacteria, is a well-established model of acute lung injury. Using this model, we find 2% H₂ or hydrogen-rich saline can exert protective effects in a mouse model of acute lung injury [29]. Meanwhile, combination therapy with H₂ (2%) and hyperoxia (98%) increases the 14-day survival rate of moderate sepsis mice to 100% and the 7-day survival rate of severe sepsis mice from 0% to 70% and alleviates injuries of lung, liver, and kidney in moderate and severe sepsis [41]. Similarly, we find that 2% H₂ inhalation significantly ameliorates short- and long-time cognitive function in sepsis survivors (unpublished data). In addition, we find that hydrogen-rich saline can significantly improve the outcome and cardiac function in a rat model of septic shock (unpublished data). Thus, H₂ or hydrogen-rich saline may be an effective therapeutic strategy for patients with sepsis (Table 1).

3. Mechanisms about Hydrogen Treatment of Sepsis

3.1. Anti-Inflammatory Effects. Sepsis is associated with a systemic inflammatory response, mediated by vascular endothelial cells and innate immune cells, including neutrophils, macrophages, and monocytes [48]. The release of proinflammatory cytokines and chemokines, including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-6, high-mobility group box 1 (HMGB-1), and monocyte chemoattractant protein-1 (MCP-1), as the most important cytokines mediating the acute phase of inflammatory response, normally triggers beneficial host innate immune response to confine the infection and tissue damage. However, in sepsis, the excessive and prolonged production of these cytokines can produce overwhelming inflammatory response, which is even more deadly than the original infection. These inflammatory
corticosteroids can result in a variety of pathologic phenomena, including priming of the vascular endothelium by synthesis of adhesion molecules, activation of neutrophils, synthesis of cyclooxygenase products, generation of nitrous oxide, ROS, apoptosis, and induction of hypotension and shock-like state [49–52]. It is well known that the excessive production of proinflammatory cytokines can result in a variety of pathologic phenomena, including priming of the vascular endothelium by synthesis of adhesion molecules, activation of neutrophils, synthesis of cyclooxygenase products, generation of nitrous oxide, ROS, apoptosis, and induction of hypotension and shock-like state [49–52]. It is well known that the excessive production of proinflammatory cytokines causes capillary leakage, tissue injury, and lethal multiple organ failure in severe sepsis [10, 49, 50]. It is also reported that elevated proinflammatory cytokine levels directly correlate with severity and mortality in human sepsis [53, 54]. The proinflammatory cytokines also lead to activation of the complement and coagulation cascades [57]. Furthermore, proinflammatory cytokines can upregulate the expression of inflammatory mediators via positive feedback loop and, consequently, induce further detrimental phenomena [58].

HMGB1 is a member of the high-mobility group protein superfamily that has been widely studied as nuclear proteins including sepsis, arthritis, cancer, autoimmunity diseases, and diabetes [60]. HMGB1 can interact with various receptors including RAGE, Toll-like receptor- (TLR-) 2, and TLR-4 to mediate chemotaxis and release of proinflammatory cytokines in monocytes/macrophages and delayed endotoxin lethality, which is required for the full expression of inflammation in animal models of endotoxemia, sepsis, and arthritis [59, 61]. Furthermore, targeting of HMGB1 with antibodies or specific antagonists has been found to have protective effects in established preclinical inflammatory disease models, including lethal endotoxemia and sepsis [62]. As the late inflammatory cytokine, HMGB1 plays a central role in the inflammatory response, becoming a key therapy to resolve inflammation [63, 64].

Our studies show that H2 inhalation can decrease the early proinflammatory cytokines (TNF-α, IL-1β, and IL-6) and late proinflammatory cytokine (HMGB1) in serum and tissues (lung, liver, and kidney) of preclinical animal models of sepsis [16, 29, 30, 41]. Furthermore, H2 treatment reduces the levels of chemokines (KC, MIP-1α, MIP-2/MCP-1, and MPO) and NF-κB signaling pathway inhibition of apoptosis (TUNEL, caspase-3). As the late inflammatory cytokine, HMGB1 plays a central role in the inflammatory response, becoming a key therapy to resolve inflammation [63, 64].

Our studies show that H2 inhalation can decrease the early proinflammatory cytokines (TNF-α, IL-1β, and IL-6) and late proinflammatory cytokine (HMGB1) in serum and tissues (lung, liver, and kidney) of preclinical animal models of sepsis [16, 29, 30, 41]. Furthermore, H2 treatment reduces the levels of chemokines (KC, MIP-1α, MIP-2, and MCP-1) in the bronchoalveolar lavage fluid of LPS-induced ALI mice. In addition, H2 treatment decreases LPS-induced neutrophils recruitment into the lungs [29]. All results demonstrate that H2 treatment downregulates the cytokines and chemokines

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in the different mouse models of sepsis. Therefore, it has been suggested to use molecular hydrogen as a new anti-inflammatory strategy.

Recently, some studies have shown that neuroinflammation in the central nervous system can cause brain damage in sepsis. The vast release of cytokines, such as TNF-α, IL-1β, and HMGB1, leads to alterations of cell function, the blood-brain barrier disruption, and brain dysfunction [65–67]. In particular, HMGB1, released from necrotic neurons via a NR2B-mediated mechanism, promotes cerebral edema via activation of microglial TLR4 and the subsequent expression of the astrocytic water channel, aquaporin-4 in traumatic activation of microglial TLR4 and the subsequent expression of NR2B-mediated mechanism, promotes cerebral edema via inflammatory mediators are proved to have a capacity to investant, can mediate the downregulation of inflammatory IL-10, as an important anti-inflammatory and immunosuppressant, can mediate the downregulation of inflammatory response by a variety of mechanisms. Importantly, IL-10 can inhibit the generation and release of a variety of cytokines in monocytes/macrophages, such as TNF-α, IL-1, IL-8, GM-CSF, and G-CSF [69]. IL-10 plays a protective role in the systemic inflammatory response. Combination therapy with H2 treatment, which is associated with the decrease of the levels of proinflammatory cytokines (TNF-α, IL-1β, and HMGB1) in the cerebral tissue (unpublished data).

With the accumulation of knowledge regarding proinflammatory cytokines produced by the immune system, investigators focus on the physiological mechanisms that maintain homeostasis. So far, various endogenous anti-inflammatory mediators are proved to have a capacity to prevent proinflammatory cytokine-mediated diseases [48]. IL-10, as an important anti-inflammatory and immunosuppressant, can mediate the downregulation of inflammatory response by a variety of mechanisms. Importantly, IL-10 can inhibit the generation and release of a variety of cytokines in monocytes/macrophages, such as TNF-α, IL-1, IL-8, GM-CSF, and G-CSF [69]. IL-10 plays a protective role in the systemic inflammatory response. Combination therapy with H2 (2%) and hyperoxia (98%) significantly increases the IL-10 level in serum and tissues (lung, liver, and kidney) of septic mice with moderate or severe CLP [41].

### 3.2. Antioxidant Effects

Oxidative stress defines disequilibrium between the levels of produced ROS and the ability of a biological system to detoxify the reactive intermediates [70]. ROS can be generated through several pathways such as direct interactions between redox-active metals and oxygen species via reactions including the Fenton and Haber-Weiss reactions, or by indirect pathways involving the activation of enzymes such as nitric oxide synthase (NOS) or NADPH oxidases. Intracellular accumulation of ROS, such as superoxide anion, hydrogen peroxide, singlet oxygen, hydroxyl radical, and peroxy radical, can arise from toxic insults or normal metabolic processes. These species may perturb the cell's natural antioxidant defense systems, resulting in damage to all of the major classes of biological macromolecules, including nucleic acids, proteins, carbohydrates, and lipids [70]. Furthermore, hydroxyl radical is one of the strongest oxidant species and reacts indiscriminately with nucleic acids, lipids, and proteins [71]. One type of ROS can be converted into another type via antioxidant enzymes in vivo. For example, superoxide dismutase (SOD) converts superoxide anion radical into H2O2, which is detoxified into H2O by either glutathione peroxidase or catalase (CAT) [72]. Besides ROS, reactive nitrogen species (RNS) can mediate nitrosative stress. RNS are generated by the quick reaction of superoxide with nitric oxide (NO), which results in the production of large amount of peroxynitrite [73, 74].

In excess, ROS and their by-products that are capable of causing oxidative damage may be detrimental to tissues and organs [75]. Recently, some studies demonstrated that H2 would exert a therapeutic antioxidant activity by selectively reducing hydroxyl radicals (the most cytotoxic ROS) and effectively reverse tissue damage such as transient cerebral ischemia, neonatal cerebral hypoxia-ischemia, liver injury, lung injury, and myocardial injury induced by ischemia and reperfusion [31, 32, 76–78]. A growing number of studies have found that excessive production of ROS and RNS plays important roles in the pathogenesis of sepsis [79–81]. Therefore, scavenging ROS, RNS, and their by-products is a critical antioxidant process, which may be a good and critical measure for treating sepsis. We report that H2 treatment significantly decreases the levels of 8-iso-prostaglandin-F2α (8-iso-PGF2α) in serum, lung, liver, and kidney tissue, which could exactly reflect the level of oxidative stress [16, 29, 30]. The levels of 8-iso-PGF2α in serum and tissues are also reduced by combination therapy with H2 and O2 [41]. In addition, our researches find that H2 treatment can significantly improve the activities of antioxidant enzymes (SOD and CAT) in serum and organ tissues of mice of moderate and severe sepsis models [16, 30, 41]. These outcomes suggest that H2 treatment provides beneficial effects on sepsis and sepsis-associated organ damage, which are associated with downregulation of oxidative stress.

### 3.3. Antiapoptosis Effects

Apoptosis, the regulated destruction of a cell, is a complicated process [82]. Many pathways can lead to activation of cell death. Death proteases are homologous to each other and are part of a large protein family known as the caspases, and blocking caspases can rescue condemned cells from their apoptotic fate. Besides the caspases, mitochondria sequester is a potent cocktail of proapoptotic proteins. Most prominent among these is cytochrome C, the humble electron carrier. Cytochrome C is one of the components required for activation of caspase-9 in the cytosol. Bcl-2 family is intimately involved in the regulation of cytochrome C crossing the mitochondria.

In addition, a role for oxidative stress in apoptosis has been shaped by several independent observations. For many years, direct treatment of cells with oxidants like hydrogen peroxide or redox-active quinones was thought to exclusively cause necrosis, but more recent studies have shown that lower doses of these agents can trigger apoptosis [83].

Apoptosis is a common pathological basis of many diseases, which plays an important role in the development of various diseases. Xiang et al. [84] detect that 2% H2 inhalation markedly attenuates morphological liver injury and apoptosis by reducing lipid peroxidation such as MDA. Cai et al. [77] find that 2% H2 therapy in a duration-dependent manner significantly reduces the number of positive TUNEL cells and suppresses caspase-3 and caspase-12 activities in neonatal hypoxia-ischemia rat model. We find that H2 inhalation markedly inhibits pulmonary cell
apoptosis by TUNEL staining in LPS-challenged mice. Similarly, the caspase-3 activity is significantly increased in the lungs of LPS-challenged animals, which is prevented by H₂ treatment [29]. Moreover, 1.3% H₂ can reduce the number of apoptotic positive cells and infarct sizes due to opening of mitochondrial K_{ATP} channels followed by inhibition of mPTP in the acute myocardial infarction and reperfusion model [85]. Besides, H₂-rich saline may effectively decrease the degree of necrosis, apoptosis, and cell autophagy in rats with acute CO poisoning, which could be related to decrease in the content of Fe and increase in the content of serum Cu associated with free radical metabolism [86].

3.4. Signaling Pathways. NF-κB transcription factors also regulate the expression of hundreds of genes that are involved in regulating cell growth, differentiation, development, inflammation, and apoptosis [87]. In quiescent cells, NF-κB activity is principally regulated by the IkB proteins, which possess ankyrin repeats and are generally inhibitory to DNA binding. The activity of the typical IkBs is controlled through phosphorylation by upstream IkB kinases (IKKs). The canonical NF-κB pathway is activated mostly by the stimulation of proinflammatory receptors, such as the TNF receptor superfamily, the Toll-like receptor family (TLRs), and cytokine receptors for the interleukins [88]. It is also activated by genotoxic agents as well. Phosphorylation of IkBα on serines 32 and 36 by the IKK complex (primarily IKKβ) targets it for ubiquitination. Subsequently the ubiquitinated IkBα is degraded by the proteosome and this unmasks the DNA-binding activity of the p50/RelA heterodimer and also allows it to translocate to the nucleus where it can bind to κB sites and activate gene transcription. It is well known that NF-κB regulates gene expression of cytokines, chemokines, and adhesion molecules. Therefore, NF-κB is increasingly recognized as a crucial player in many steps of regulation of inflammatory responses. Noncanonical NF-κB activation is stimulated by specific TNF receptor family members that signal through the recruitment of TRAF2 and TRAF3 [88]. In addition, different target genes are differentially induced by distinct NF-κB dimers. Furthermore, NF-κB subunits also contain sites for phosphorylations and other posttranslational modifications which are important for activation and crosstalk with other signaling pathways [87]. Heme oxygenase-1 (HO-1) and apoptosis-associated factors, including TRAF-1 and Bcl-XL, are also mediated by NF-κB [89]. A previous study indicates that H₂ inhalation reduces epithelial apoptosis in ventilator-induced lung injury via NF-κB activation [90]. H₂ inhibits TNF-α-induced lectin-like oxidized LDL receptor-1 expression by inhibiting the phosphorylation of IkB-α and activation of NF-κB in endothelial cells [91]. Moreover, H₂ can indirectly activate the NF-κB signaling through reducing oxygen free radical [28]. However, in our study, H₂ treatment inhibits the lung NF-κB p65 nuclear translocation and DNA-binding activity in LPS-challenged mice [29]. Nuclear factor erythroid 2-related factor 2 (Nrf2) is an important cytoprotective transcription factor [92]. Nrf2 controls the coordinated expression of important antioxidant and detoxification genes (Phase II genes) through a promotor sequence termed the antioxidant response element (ARE). Phase II genes, including heme oxygenase-1 (HO-1), glutathione S-transferases (GSTs), and NAD(P)H quinine oxidoreductase, work in synergy to constitute a pleiotropic cellular defense that scavenges reactive oxygen/nitrogen species (ROS/RNS), detoxifies electrophiles and xenobiotics, and maintains intracellular reducing potential. HO-1 is an ubiquitous and redox-sensitive inducible stress protein that degrades heme to CO, iron, and biliverdin [93]. Some studies have found that Nrf2 is a novel regulator of the innate immune response that dramatically improves survival during experimental sepsis by protecting against dysregulated inflammation [94]. Heme oxygenase-1 (HO-1) and the product of its enzymatic reaction, CO, not only have beneficial anti-inflammatory properties, but also enhance bacterial clearance by increasing phagocytosis and the endogenous antimicrobial response [95]. Some researchers found that hydrogen treatment during exposure to hyperoxia significantly improved blood oxygenation, reduced inflammatory events, and induced HO-1 expression, which did not mitigate hyperoxic lung injury or induce HO-1 in Nrf2-deficient mice [96]. Previous studies investigate that hydrogen gas inhalation significantly promotes the expression of Nrf2 in septic organs including lung, liver, and kidney. However, hydrogen gas did not improve the survival rate in Nrf2-deficient mice [46, 97]. Moreover, H₂ treatment dose-dependently attenuates the increased levels of proinflammatory cytokines and further increases the level of anti-inflammatory cytokine IL-10 with the increase of HO-1 protein expression and activity in LPS-stimulated RAW 264.7 macrophages [43]. Therefore, downstream molecules of Nrf2 signaling pathway play an important role in the pathophysiological process of sepsis.

4. Hydrogen-Rich Saline Exerts the Same Therapeutic Effect with H₂

Hydrogen-rich saline, in which the concentration of hydrogen is more than 0.6 mmol/L, is easily and safely manufactured [29]. Hydrogen-rich saline can alleviate inflammatory response, inhibit cell apoptosis, and reverse oxidative stress to reduce organ injuries, which would be a good method for clinical application. Currently, it is generally accepted that hydrogen-rich saline exerts an effective therapeutic role in many disorders including sepsis, ischemia-reperfusion injury, allergy, and degenerative diseases [29, 98–100].

In our study, we find that hydrogen-rich saline has similar beneficial effects on LPS-induced lung injury as hydrogen inhalation, which are also associated with inhibition of infiltration of inflammatory cell and activation of NF-κB [29]. In addition, hydrogen-rich saline effectively ameliorates hemodynamics, vascular reactivity in a dose-dependent manner in rat model of peritonitis-induced septic shock. Meanwhile, vital organ dysfunction, such as heart, lung, liver, and kidney, is significantly mitigated via resolving inflammatory responses and decreasing the iNOS expression [44]. Similarly, hydrogen-rich saline has potential protective effects against
sepsis by decreasing proinflammatory responses, oxidative stress, and apoptosis in a rat model of polymicrobial sepsis [45]. In addition, hydrogen-rich saline markedly reverses cognitive impairment and mortality in a dose-dependent manner in rats submitted to sepsis by cecal ligation and puncture, which are relative to the suppression of oxidative stress and cell apoptosis [101].

5. Advantages of H₂ or Hydrogen-Rich Saline Treatment of Sepsis

It is obvious that hydrogen is electronically neutral and has favorable distribution characteristics: it can penetrate biomembranes and diffuse into the cytosol, mitochondria, and nucleus [102]. Despite the moderate reduction activity of H₂, its rapid gaseous diffusion might make it highly effective for reducing cytotoxic radicals. Besides, it stands to reason that H₂ will react with only the strongest oxidants. H₂ is mild enough not to disturb metabolic oxidation reduction reactions or to disrupt ROS involved in cell signaling—unlike some antioxidant supplements with strong reductive reactivity, which increase mortality, possibly by affecting essential defensive mechanisms. Thus, H₂ treatment is advantageous for medical procedures without serious unwanted side effects [28]. Furthermore, H₂ is neither inflammable nor explosive at low concentrations (<4.6% in air and 4.1% in pure oxygen) [28]. Moreover, only 2% hydrogen gas can have obvious protective effects on sepsis. Meanwhile, hydrogen-rich saline is also available and safe for medical applications.

6. Conclusion

Although recent treatment modalities and interventions have contributed to the improvement for sepsis patients, the high mortality rate of severe sepsis suggests the necessity for additional therapies. Recently, vigorous experimental studies have been undergone to identify effective therapy of molecular hydrogen for sepsis. What is more, this novel therapy may be tested in clinical situation in the future. However, we should deeply proceed to more experimental researches to investigate the plausible and comprehensive mechanisms of hydrogen to treat sepsis.

Conflict of Interests

The authors have declared that no conflict of interests exists.

Authors’ Contribution

Keliang Xie and Lingling Liu contributed equally to this work.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (Nos. 81071533 to Yonghao Yu and 81101409 to Keliang Xie), the Natural Science Foundation of the Tianjin Science Committee (Nos. 11JCYBJC12900 to Yonghao Yu and 13JCQNJC1400 to Keliang Xie), and the Foundation of Tianjin Bureau of Public Health (2011KZ108 to Keliang Xie).

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