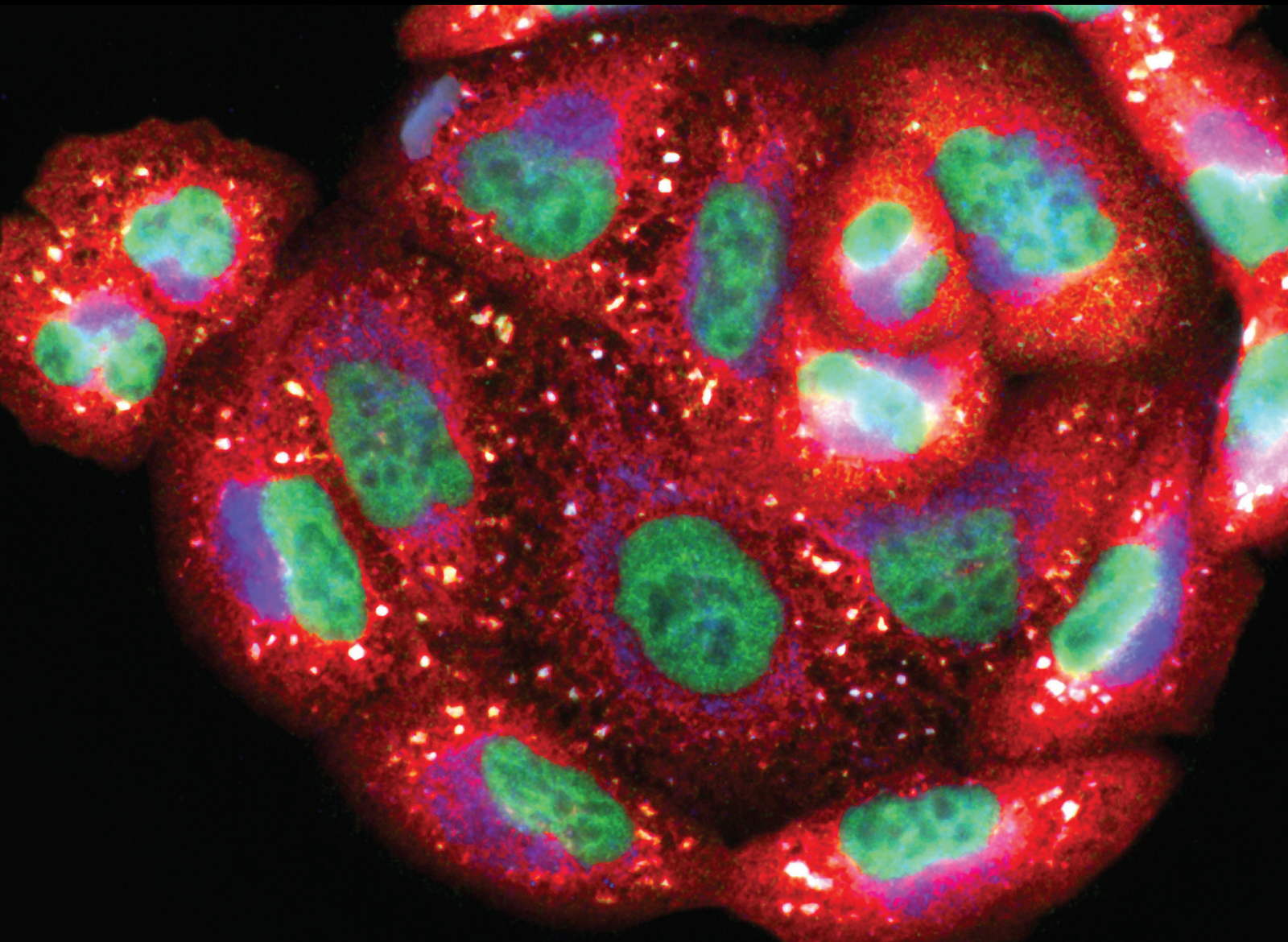# Oxidative Stress and Gut Microbiota in Major Complications after Hematopoietic Stem Cell Transplantation

Lead Guest Editor: Mingyi Zhao
Guest Editors: Teng Guan and Xiaowen Zhai

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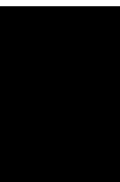Guest Editors: Teng Guan and Xiaowen Zhai

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# Contents

*Review Article*

# Role of Gut Microbiota and Oxidative Stress in the Progression of Transplant-Related Complications following Hematopoietic Stem Cell Transplantation

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Hematopoietic stem cell transplantation (HSCT), also known as bone marrow transplantation, has curative potential for various hematologic malignancies but is associated with risks such as graft-versus-host disease (GvHD), severe bloodstream infection, viral pneumonia, idiopathic pneumonia syndrome (IPS), lung fibrosis, and sinusoidal obstruction syndrome (SOS), which severely deteriorate clinical outcomes and limit the wide application of HSCT. Recent research has provided important insights into the effects of gut microbiota and oxidative stress (OS) on HSCT complications. Therefore, based on recent studies, we describe intestinal dysbiosis and OS in patients with HSCT and review recent molecular findings underlying the causal relationships of gut microbiota, OS, and transplant-related complications, focusing particularly on the involvement of gut microbiota-mediated OS in postengraftment complications. Also, we discuss the use of antioxidative and anti-inflammatory probiotics to manipulate gut microbiota and OS, which have been associated with promising effects in improving HSCT outcomes.

## 1. Introduction

Hematopoietic stem cell transplantation (HSCT) is a potentially life-saving procedure for a multitude of congenital and acquired diseases of the hematopoietic system, including malignancy, severe hematopoietic deficiency, and immune dysfunction [1]. Human hematopoietic stem cells (HSCs) with strong regenerative potential are uniquely implanted into the bone marrow of recipients, providing long-term multilineage hematopoiesis and reconstituting a complete hematopoietic system [2]. Complications after HSCT, including graft-versus-host disease (GvHD), severe

bloodstream infection, viral pneumonia, idiopathic pneumonia syndrome (IPS), and sinusoidal obstruction syndrome (SOS) are closely associated with peritransplant morbidity and mortality and severely limit the wide application of HSCT. Despite efforts made in improving transplant outcomes, such as the high resolution of human histocompatibility locus genotyping, prophylactic use of calcineurin inhibitors [3], and infection control using wide-spectrum antibiotics [4], the management of postengraftment complications remains the cornerstone of successful HSCT.

The gut microbiota benefits from the warm nutrient-rich environment of a healthy gut and serves as an important health regulator for hosts. Firmicutes including *Lactobacillus*, *Streptococcus*, *Mycoplasma*, *Clostridium*, and Bacteroidetes comprise 90% of the total gut microbiota. Healthy gut microbiota contributes to intestinal ecosystem homeostasis. Rapid shifts in the composition and function of intestinal microbial communities, known as intestinal dysbiosis, are associated with intestinal barrier disruption and lead to the development of inflammatory [5], cancer [6], metabolic diseases [7], and neurodegenerative diseases [8]. Patients undergoing HSCT display significant changes in the gut microbiota due to the underlying malignancy and exposures to extensive chemotherapy, immunosuppressants, and systemic antibiotics [9]. Due to the clinical significance of gut microbiota, significant interest has emerged to understand the interplay between gut microbiota and HSCT-related complications and reveal the therapeutic value of this interaction.

Reactive oxygen species (ROS) including hydroxyl radicals (OH), superoxide anions, and hydrogen peroxide ($H_2O_2$) are byproducts of oxidative phosphorylation and trigger the activation of cyclooxygenases, nitric oxide (NO) synthase, lipoxygenases, and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. HSCTs are known to increase the intracellular and extracellular accumulation of ROS, leading to an oxidative stress (OS) status for the occurrence of chemoradiotherapy conditioning and iron overload. Moreover, many recent studies have shown that both commensal and pathogenic bacteria can alter ROS production and promote the progression of neurodegeneration [10], fatty liver disease [11], and diabetes mellitus [12] (Table 1). OS impairs hematopoietic progenitor function and is potentially associated with posttransplant complications, leading to adverse clinical outcomes.

Targeting the OS and gut microbiota may represent an attractive therapeutic avenue for the management of transplant-related complications after HSCT. This review provides an in-depth examination of the crosstalk between OS, the gut microbiota, and transplant-related complications after HSCT. We first briefly reviewed the intestinal dysbiosis and OS in patients who underwent HSCT followed by comprehensive scrutiny of recent molecular findings underlying the causal relationships between gut microbiota, OS, and transplant-related complications, focusing on the gut microbiota-mediated OS involved in postengraftment complications. A better understanding of these relationships in patients with HSCT may allow unraveling the treatment for transplant-related complications by targeting OS and gut microbiota.

## 2. Intestinal Dysbiosis in Patients Undergoing HSCT: Adverse Effect of Conditioning Regimen and Prophylactic Antibiotics

Gut microbiota can promote intestinal homeostasis and protect intestinal integrity by supporting mucosal immunity maturation and preventing (invading) pathogen colonization [13]. Multiple factors can influence the compositional and functional dynamic balance of the intestinal microbiota, resulting in dysbiosis. HSCT recipients are particularly vulnerable to dysbiosis because of their underlying malignancies, long-term hospitalizations, prolonged application of antibiotics, and the use of preparative regimens prior to transplantation [14].

Dysbiosis in HSCT patients commonly manifests as a reduction in gut microbial diversity, diminished strictly anaerobic commensal bacteria, and expansion of pathogenic bacteria. Metagenomic analysis revealed the mean urinary indoxyl sulfate levels that can serve as an indirect marker of bacterial diversity in all patients receiving allo-HSCT dropped from $42.5 \pm 11$ mmol/L to $11.8 \pm 2.8$ mmol/L [15]. Intensive chemotherapy and/or radiation preparative regimens are responsible for the expansion of *Lactobacillales* and *Enterobacteriales* and the prominent loss of *Clostridiales* in mice [16]. Patients routinely consume antimicrobials prophylactically to diminish anaerobic bacteria and prevent opportunistic infections in the early posttransplantation period. However, metagenomic analysis of the stool microbiome revealed that microbial composition shifts and diversity loss were more pronounced after extensive antimicrobial exposure in HSCT patients [15]. The drastic loss of diversity in the microbiota is often accompanied by the expansion of a single taxon. *Enterococcus* predominance is more obvious under exposure to antibiotics such as ciprofloxacin and metronidazole [17] with a notable expansion of *E. faecium* and a complementary decrease in Firmicutes and other commensal phyla [15]. Rifaximin is a prophylactic antibiotic that effectively reduces intestinal infections and subsequent acute GvHD [18]. However, new research shows that rifaximin could contribute to microbiome disruption and favor an outbreak of life-threatening *Candida* spp. infections [19]. Microbial SCFAs, including acetate, butyrate, and propionate, are products of carbohydrate fermentation by the anaerobic commensal bacteria (*Clostridia* spp., for instance). SCFAs can preserve intestinal barrier integrity by supporting the functions of intestinal epithelial and goblet cells through coordinated regulation of tight junction proteins. Furthermore, SCFAs can induce tolerance and inhibit inflammatory cascade mediated by inhibiting nuclear factor kappa b (NF-$\kappa$B) activation in macrophages, inducing colonic regulatory T (Treg) cell expansion, and upregulating gut-homing molecules and forkhead box protein P3 (Foxp3) of Treg cells [20]. The post-HSCT abundance of butyrogenic bacteria (mainly *Clostridia*) in the intestinal microbiota is higher in patients with resistance to lower tract respiratory infections and lower in patients who are susceptible to acute GvHD (aGvHD) [21].

A low diversity of the intestinal microbiota from allo-HSCT recipients was associated with significantly increased mortality (52%) compared with a high diversity of the intestinal microbiota (8%). Microbiota disruption characterized

TABLE 1: Role of gut microbiota-derived oxidative stress in the progressions of different diseases.

| Intestinal microbiota | Mechanisms | Relative diseases | Reference |
|---|---|---|---|
| Enterococci faecalis ↑ | Increase the production of hydroxyl radicals, contribute to DNA breaks, point mutations, and protein-DNA crosslinking, and induce aneuploidy in colonic epithelial cells | Colorectal cancer | [110] |
| Proteobacteria ↑ Bifidobacteria ↓ | Contributes to the occurrence of dementia not only through the significant reduction of beneficial SCFAs but also through interfering with lipid metabolism | Alzheimer's disease | [111] |
| Gut-lung axis | Activating oxidative stress through TLR4/NF-kB pathway in the lung and mediating lung injury through the regulation of the gut barrier | Acute lung injury | [112] |
| Butyrate producers ↓: Fusobacterium Veillonella Atopobium parvulum | Dysbiosis dampen host $H_2S$ defense systems induce mitochondrial dysfunction likely resulting in ROS production, contributing to mucus degradation, opening the intestinal barrier to toxic compounds and pathobionts | Crohn's disease | [113] |
| Prevotella Clostridium | Produce endogenous $H_2$, which have antioxidant properties to neutralize toxic hydroxyl radicals, downregulate the expression of proinflammatory factors, and preserve cerebrovascular reactivity | Parkinson's disease | [114] |
| Escherichia coli ↑ | Increase production of uric acid, which contributes to the overproduction of oxygen free radicals, vascular endothelial dysfunction, and inflammation | Atherosclerosis | [115] |
| Eggerthella lenta ↑ Fusobacterium nucleatum ↑ | Increase serum uraemic toxins, which are relative to increased severity of oxidative stress, glomerulosclerosis, and renal fibrosis and increased serum levels of creatinine and/or urea in sham-fed rats | End-stage renal disease | [116] |
| H. pylori | Produce and induce the production of ROS by neutrophils and macrophages | — | [117] |
| Lactobacilli Bifidobacteria | High catalase and $\alpha,\alpha$-diphenyl-$\beta$-picrylhydrazyl free radical scavenging activity | Anticancer effect | [118] |
| Lactobacillus rhamnosus GG | Ameliorates alcohol-induced intestinal oxidative stress, intestinal hyperpermeability, and liver injury in rodent models of alcohol steatohepatitis | Alcoholic liver disease | [119] |

by loss of diversity and single taxa domination is particularly associated with negative outcomes in allo-HSCT recipients. The domination of enterococci in posttransplant stool specimens is positively related to the subsequent development of gastrointestinal GvHD, and the mean proportion of enterococci increased by 53% at the time of active GvHD [15, 22]. Also, intestinal dysbiosis in HSCT patients is correlated with multiple infections including bloodstream infection [23], diarrhea [9], multidrug-resistant organism (MDRO) infection [24], and pulmonary infections [25]. Moreover, a retrospective observational analysis of 541 patients undergoing allo-HSCT identified that the intestinal microbiota could be associated with relapse/progression of disease after allo-HSCT [26].

## 3. Oxidative Stress in Patients Undergoing HSCT

### 3.1. ROS Generation in HSCT Patients: Conditioning Regimens and Iron Overload. 
Sustained and high-quality transplantation of donor HSCs requires pretransplantation adaptation. Chemotherapy and total-body irradiation are widely used as myeloablative conditioning regimens in patients before HSCT and remove most of the hematopoietic

and immune systems of the host. Ionizing radiation can penetrate cells in living organisms and generate ROS via water radiolysis [27]. ROS react rapidly with macromolecules, including proteins, nucleic acids, and lipids, leading to cell damage and apoptotic cell death [28]. Damaged tissues release damage-associated molecular patterns (DAMPs) and initiate acute inflammatory responses through the activation of mitogen-activated protein kinase (MAPK) and nuclear factor kappa-B (NF-$\kappa$B) signaling cascades. Such pathways are also regarded as the major mediators or inducers of the propagation of radiation-induced bystander effects that induce ROS to increase and replicate irradiation-related DNA damage in nonirradiated cells [29].

Cytostatic agents, including cyclophosphamide (CTX), busulfan, etoposide, melphalan, and carmustine (BCNU), are widely used in HSCT preconditioning to exert their antitumor action and reduce the recurrence rate. However, in recent years, the accumulation of free radicals has been implicated in the administration of cytostatic agents in various categories both in vitro and in vivo. Bone marrow stromal cells from patients receiving daunorubicin secreted higher levels of $H_2O_2$ than that of healthy control participants, leading to the accumulation of DNA damage in cocultured hematopoietic cells [30]. Chemotherapeutic agents
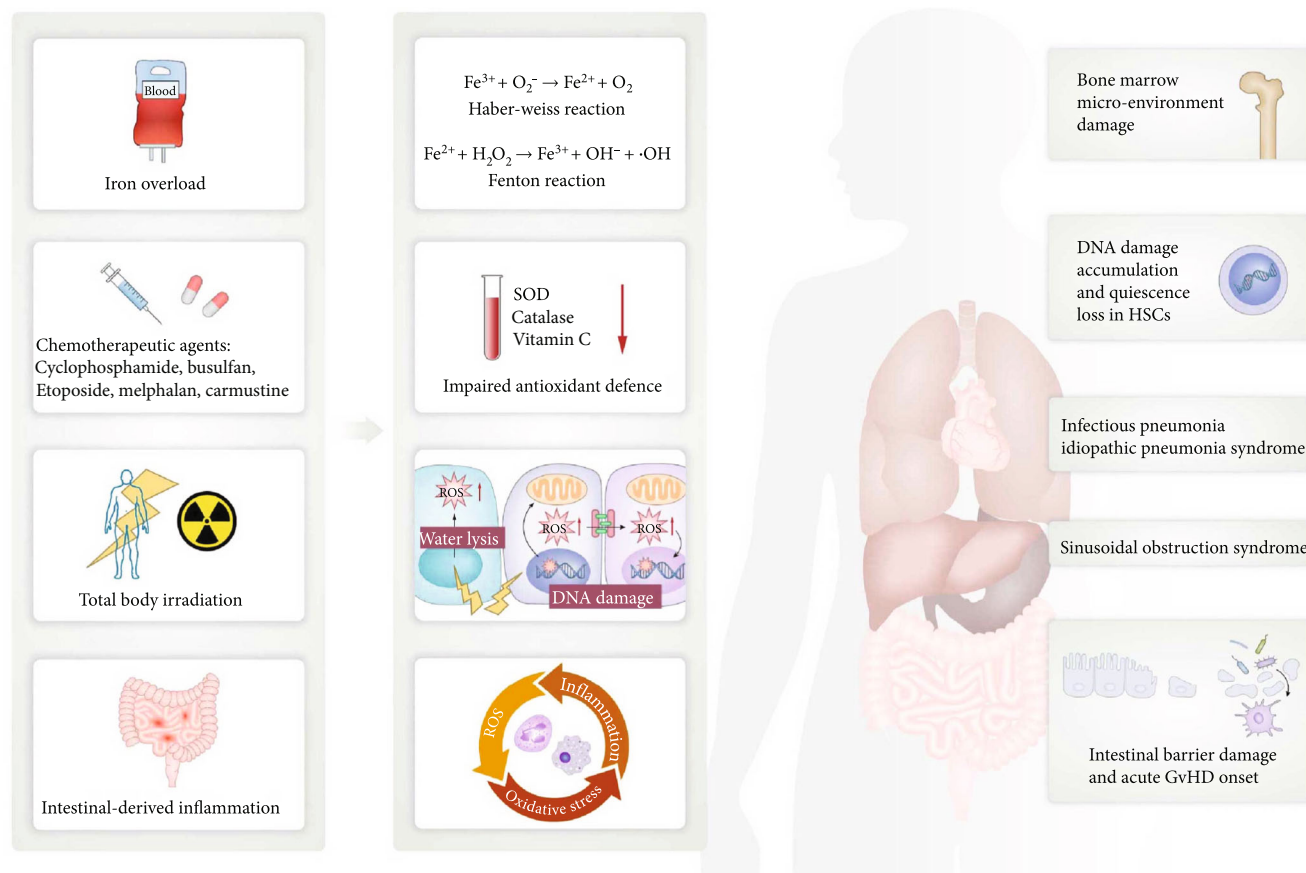
FIGURE 1: Mechanisms of oxidative stress during HSCT and its impact on the human body. Preengraftment conditioning with chemotherapy and total body irradiation are leading causes of disruption of redox balance and oxidative stress status in patients who underwent HSCT through increasing free radical production and diminishing host antioxidant defense. Iron overload increases ROS production via Haber-Weiss and Fenton reactions. Inflammatory cell infiltration during intestinal inflammation produces excessive oxidative intermediate ROS, which directly damages tissues and further promotes inflammatory response. Oxidative stress status in HSCT patients may contribute to subsequent transplant-related complications including bone marrow microenvironment damage, HSC dysfunction, intestinal barrier damage, and liver and lung injury. HSCT: hematopoietic stem cell transplantation; ROS: reactive oxygen species; HSCs: hematopoietic stem cells.

also disrupt redox balance by impairing antioxidant defense (e.g., superoxide dismutase and catalase) in human beings [31]. Plasma levels of vitamin C and catalase, which are powerful antioxidants for scavenging $O^{2-}$, $H_2O_2$, and $OH^-$, decline after the application of melphalan and CTX-BCNU-etoposide conditioning regimens [32]. The oxidant effects of CTX are associated with its active metabolites, such as phosphoramide mustard and acrolein, resulting in the accumulation of ROS, which can cause DNA damage and genetic instability, inducing bone marrow suppression [33]. Moreover, chemotherapy with busulfan, BCNU, and cisplatin can cause depletion of plasma glutathione, a nonenzymatic antioxidant [34], and thus amplify oxidative stress.

Iron is a critical cofactor for proteins in the respiratory chain and for cell growth and multiplication. It is potentially toxic to the host when excessive iron is deposited in the cells and tissues of some parenchymal organs. This condition is known as iron overload and is defined by elevated ferritin and liver iron content of approximately 30% and 32%–60%, respectively. Iron overload is a common event associated with HSCT due to the following possible reasons

(Figure 1): (1) patients with hematologic diseases usually receive multiple red blood cell transfusions before and after HSCT; (2) chemotherapeutic agents inhibit erythropoiesis, resulting in iron underutilization; (3) the bone marrow, tumor cells, and liver are damaged after high-dose preconditioning, resulting in the release of internal iron pools. The most damaging effect of iron overload is the cycling between $Fe^{2+}$ and $Fe^{3+}$ via Haber-Weiss and Fenton reactions, ultimately generating reactive and toxic free radicals, such as $OH^-$ and HO. Iron toxicity induces ROS and triggers inflammation, mediates oxidative and genotoxic stress of HSCs to damage the graft, and promotes recurrence, further damaging the already dysfunctional bone marrow microenvironment of HSCT recipients. Elevated ferritin levels have been associated with decreased overall survival, increased risk of infections, aGvHD, and sinusoidal occlusive disease [35].

### 3.2. Microbiome Changes May Affect ROS Levels in HSCT Patients.
OS in HSCT patients induced by pretransplant conditioning and iron overload has been reported in the

literature; however, research on the association between microbiota-derived OS and transplant-related complications and outcomes is limited. The clinical significance of gut microbiota-derived OS in a multitude of diseases, including inflammatory, cancer, metabolic, and neurodegenerative diseases, indicates that changes in intestinal homeostasis can extensively influence the OS status in different systems of the body. Specific commensal and pathogenic bacteria can stimulate OS in the intestinal system. Commensal bacteria induce superoxide production by NADPH oxidase-1 and increase cellular ROS by stimulating formyl-peptide receptors on macrophages and neutrophils, resulting in inflammation of the intestinal epithelium [36]. Gut *Lactobacilli* and *Bifidobacterium* can convert nitrate and nitrites into NO, making the gut epithelia a rich source of NO. NO at high concentrations results in a detrimental effect due to the production of ROS, such as superoxide and $H_2O_2$, which further form highly reactive hydroxyl radicals [10]. *E. faecalis* produces substantial extracellular superoxide and derivative reactive nitrogen and oxygen species, such as $H_2O_2$ and OH, through the autoxidation of membrane-associated demethylmenaquinone [37]. However, OS occurring during intestinal instability and inflammation is a risk factor for dysbiosis because it strongly decreases microbial diversity and promotes the expansion of specific bacterial taxa. Leukocyte infiltration accompanied by the generation of reactive oxygen and nitrogen species during intestinal inflammation kills strictly anaerobic bacteria that are susceptible to oxygen intoxication and also promotes the selective growth of bacterial groups including *Enterobacteriaceae* (*Salmonella* and *Citrobacter*) as well as *Escherichia coli* through nitrate and tetrathionate respiration [38, 39].

*3.3. The Adverse Effects of Excessive ROS and OS Status on HSCT Outcomes.* OS is commonly resulting from chronic inflammation and subsequent generation of ROS and nitrogen species that are capable of damaging cellular DNA, protein, and organelles, thus altering gene expression and cell phenotypic traits. OS is suspected to promote cancer and contribute to diverse degenerative neurological disorders, cardiac dysfunction, and aging. The biological characteristics of HSCs are tightly regulated by the OS, and the control of ROS levels is important to maintain their self-renewal capacity. At low concentrations, ROS and reactive nitrogen species control diverse cellular functions, such as stem cell differentiation, and are used in intercellular communication. Murine HSCs with low ROS levels are more quiescent and exhibit increased longitudinal self-renewal and pluripotent differentiation compared to HSCs with higher ROS levels [40]. Exceedingly high ROS levels, which occur during important OS conditions such as chronic inflammation or iron overload, can promote quiescence loss and subsequently limit the capacity for regeneration and reconstitution of the entire hematopoietic system after transplantation into recipients [41, 42]. Excess free radicals and ROS cause severe damage to biological macromolecules (especially DNA damage) and dysregulation of the cell cycle, leading to inflammation and injury to the intestinal epithelium as well as intestinal dysbiosis, which heralds adverse outcomes and is associated with deteriorated overall survival after HSCT [43].

## 4. Gastrointestinal Toxicities and Bloodstream Infection after HSCT

Patients undergoing HSCT and routinely receiving immunosuppressive therapy are at a high risk of catastrophic bloodstream infections (BSIs); such infections are associated with significant morbidity and mortality after HSCT. In a case-cohort study of 16,875 pediatric and adult patients who underwent HSCT, 13% developed BSI due to bacterial translocation across the compromised mucosal barrier [44].

Mucosal barrier injury is also a frequent complication of allo-HSCT and an independent risk factor for the invasion of the gut microbiota into the bloodstream. Healthy intestinal epithelial cells, including intestinal stem cells, goblet cells, and Paneth cells, are connected by tight junctions and assemble into the intestinal epithelium. The intestinal epithelium, with a mucus layer, provides a physical and biochemical barrier, limiting the penetration of microbes and intestinal luminal contents into the host tissues. Pretransplant conditioning with radiation and chemotherapy is associated with increased ROS levels. Excessive OS causes DNA damage, inflammation, and cell apoptosis, leading to shifts in the microbiota, intestinal leakage, and radiation-induced enteritis. Chemoradiation therapy-induced DNA damage promotes the production of epithelial-derived interleukin- (IL-) $1\beta$, which initiates intestinal barrier damage by compromising epithelial tight junctions [45]. Patients receiving pretransplant conditioning are not only susceptible to aggravated gastrointestinal epithelial cell damage but also to the elimination of circulating granulocytes and monocytes, markedly increasing susceptibility to subsequent bacterial translocations and disseminated infections [46, 47]. Iron overload is also related to OS status in HSCT patients and can cause tissue damage by protein oxidation, membrane lipid peroxidation, and nucleic acid modification, with the conversion of $H_2O_2$ to ROS [43]. Patients with high pretransplant serum ferritin, a surrogate indicator of tissue iron overload, have an increased incidence of BSI/death (60 vs. 44%, $P = 0.042$) than those with normal levels of pretransplant serum ferritin [35]. The severity of intestinal injury (also referred to as mucositis) after myeloablative conditioning is considered to be the most important determinant of the post-HSCT inflammatory response and is associated with the occurrence of inflammatory complications, including bacteremia, lung injury, and GvHD [48].

*E. coli* and *Klebsiella pneumoniae* BSIs with concomitant gut colonization by these organisms suggest that profound disturbances in the gut microbiota populations play an important role in BSI after HSCT [49]. Furthermore, the dominance of a single bacterial genus such as *Enterococcus* (vancomycin-resistant *Enterococcus* [23]), *Streptococcus* (viridian-group *Streptococcus* [50]), and various Proteobacteria [24] has been identified as the most common cause of bacteremia.

It is imperative to develop strategies to maintain the gut microbiota and gastrointestinal health to prevent subsequent enteric bacterial BSI and improve survival [51]. Prophylactic administration of fluoroquinolones, such as ciprofloxacin and levofloxacin, can reduce the risk of intestinal domination with Gram-negative microbes,

including *Proteobacteria* [52] (*Escherichia*, *Klebsiella*, and *Enterobacter*) [53], which are significantly associated with decreased bacteremia without increased risk of *Clostridium difficile*-associated diarrhea, aGVHD, or MDRO [54]. In addition to the prophylactic use of antimicrobial agents, gut decontamination with nonabsorbable antibiotics in the peri-HSCT period was reported to protect against gut-derived BSI by decreasing the microbial load of gut pathogens [55]. For intestinal barrier protection, the IL-1 receptor antagonist anakinra and anti-IL-1$\beta$ antibody canakinumab limit the inflammatory reaction and improve intestinal barrier integrity in HSCT patients and murine [45, 56].

## 5. Graft-Versus-Host Disease after HSCT

*5.1. Pathophysiology of Acute GvHD (aGvHD).* GvHD is a common secondary disease in patients undergoing HSCT, which has long limited the efficacy of HSCT. Before transplantation, the patient's tissues and immune system have been profoundly damaged due to underlying disease, treatment for the disease, infections, and the conditioning regimen. Allogenic T cells from a foreign donor activate and respond upon binding human leukocyte antigens that are expressed on host tissue. A compromised host immune system is incapable of rejecting the immunocompetent cells, leading to amplified CD4$^+$/CD8$^+$ T cell activation and subsequent GvHD initiation. Subsequently, cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells induce the target cells' apoptosis through the Fas/Fas ligand pathway and perforin/granzyme pathway. Furthermore, inflammatory cytokines synergize with CTLs, resulting in further tissue injury and possible target organ dysfunction (Figure 2). Active GvHD primarily targets the skin (81%), gastrointestinal tract (54%), and liver (50%) of the hosts [57], closely associated with nonrelapse mortality following HSCT.

*5.2. Intestinal Barrier, Microbial Dysbiosis, and the Onset of aGvHD.* Damage to host tissues, especially the intestinal mucosa, caused by the conditioning regimen, is the most important initial step in the pathophysiology of aGvHD. Pretransplant conditioning regimens and GvHD can directly impair gut epithelium, especially Paneth cells. Paneth cell damage contributes to the loss of antimicrobial peptides (e.g., $\alpha$-defensins) and growth factors (e.g., epidermal growth factor and transforming growth factor-$\alpha$), then accelerates the loss of microbial diversity, and compromises epithelial regeneration capacity in GvHD, which leads to a higher risk of nonrelapse mortality [58]. Early studies reported that the GvHD-related mortality was significantly reduced in germ-free mice or when intestinal decontamination was performed [17, 59]. A longitudinal study reported that the development of GvHD was preceded by remarkable shifts in the gut microbiota that can serve as an early predictor of GvHD and transplant-related mortality after HSCT [60], with a predominant role played by Gram-positive bacteria belonging to Firmicutes phylum [61]. The hypothesis that lymphocytes sensitized against microbial antigens cross-react with epithelial antigens in GvHD is the most widely accepted model of microbial interactions in the pathogenesis of GvHD. Microbial products like lipopolysaccharide (LPS) and other pathogen-associated molecular patterns (PAMPs) systemically translocate from the bowel lumen through a damaged intestinal mucosa to the systemic circulation and then stimulate mononuclear cells (monocytes/macrophages) via pathogen recognition receptor (PRR) family such as NOD-like receptors (NLRs) and Toll-like receptors (TLRs) [62]. The amplified activation of these antigen-presenting cells triggers a cytokine storm (tumor necrosis factor-$\alpha$ (TNF-$\alpha$) and IL-1) and a lower Treg/T helper (Th) 17 cell ratio, leading to amplification and propagation of a cytokine storm. These cytokines induce inflammatory damage and increase the expression of major histocompatibility complex (MHC) antigens and adhesion molecules in host tissues, enhancing the alloreactivity of mature donor T cells against host tissues, which are equivalent to GvHD [63, 64].

High-throughput metabolomic analysis revealed that GvHD development seems to be associated with major metabolomic changes in the intestinal microbiota compared with patients who did not develop GvHD. AhRs can modulate Th17 response and encourage tolerance by promoting Treg cells [65]. Microbially derived indole compounds are AhR ligands, which show a significant decrease, even undetectable in recipients with GvHD, and are associated with GvHD onset and severity. In addition, reduced plasmalogens, together with increased bile acids and polyunsaturated acids, are potential metabolomic pathways that could be involved in the early proinflammatory response during GvHD [66]. Mucosa-associated invariant T (MAIT) cells are a group of innate-like T cells that inhibit the proliferation of CD4$^+$ T cells. Poor reconstitution of MAIT cells after HSCT is significantly associated with the development and severity of GvHD [67]. Peripheral expansion of MAIT cells requires riboflavin (vitamin B2), the metabolite derived from healthy microbiota, which was observed to be significantly decreased in disrupted microbiota of HSCT patients [68]. Intestinal microbial metabolite plasmalogens produced by *Clostridium* strains and *Bifidobacterium longum* have many antioxidant effects in vitro and in vivo [69]. The level of microbiota-derived plasmalogens was dramatically low at the onset of aGvHD, leading to an imbalance between oxidation and antioxidation preceding GvHD. Increasing research on the crosstalk between the host and gut microbiota has provided opportunities to better understand the complex network of GvHD and optimize therapeutic strategies for decreasing HSCT-related morbidity and mortality.

*5.3. GvHD Treatments Based on Targeting the Gut Microbiota.* Prevention of GvHD mainly focuses on T cell depletion and regulation of T cell activation, proliferation, effector, and regulatory functions. Multimodal treatment is often used, but systemic corticosteroids are usually the mainstay of GvHD treatment. From the perspective of gut microbiota, restoring the intestinal epithelium and maintaining intestinal homeostasis represents the adjunct therapeutic strategies to standard immunosuppressive treatment of GvHD without compromising graft-versus-leukemia (GVL) effects. The GVL effect is a type of graft-versus-host
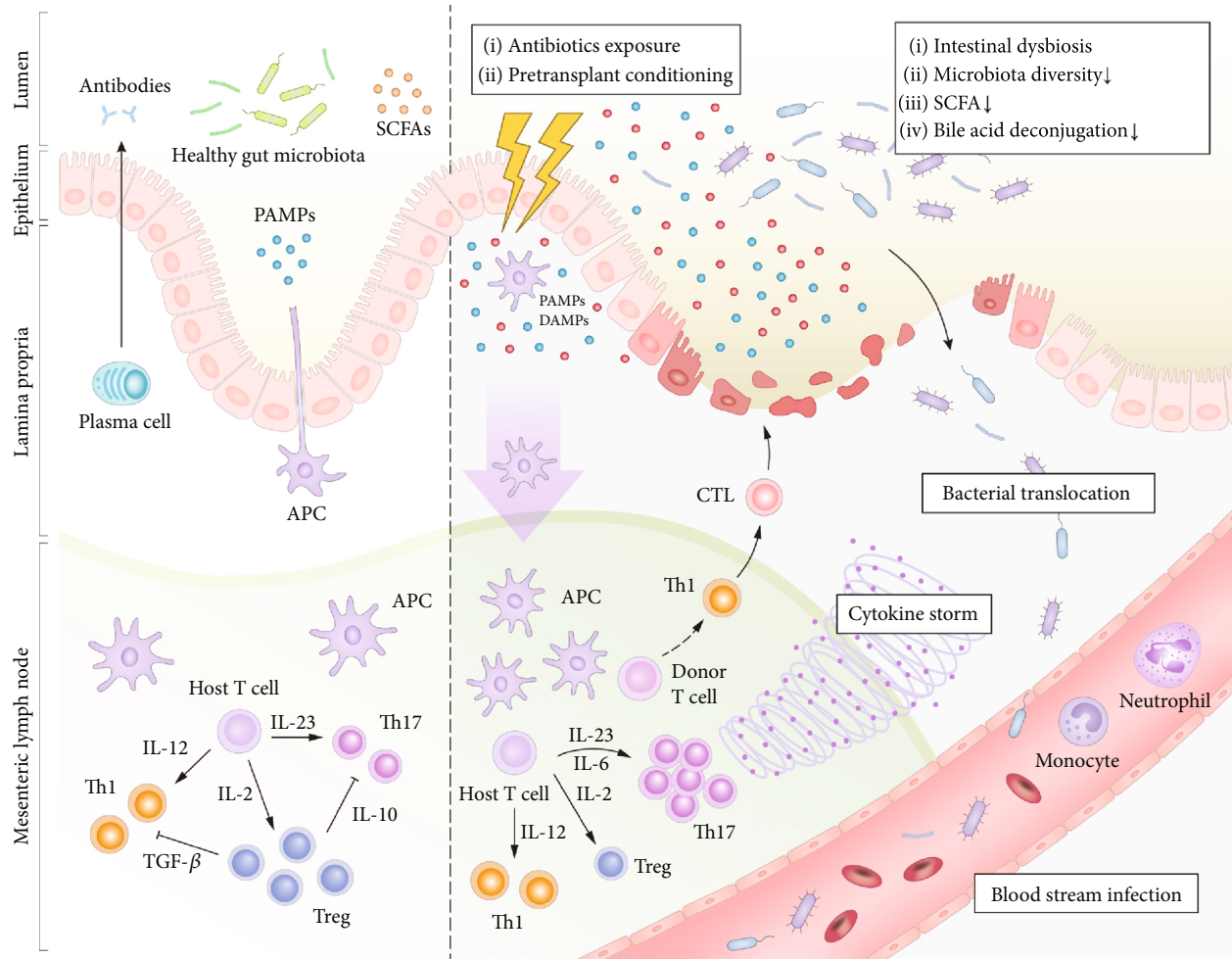
FIGURE 2: The intestinal damage and the pathogenesis of BSI and GvHD. A healthy intestinal system is important to maintain immune homeostasis. The intestinal homeostasis and epithelial cells are damaged by the cytotoxic conditioning regimen as well as by extensive antibiotic exposure, leading to disruption of the intestinal barrier, intestinal dysbiosis, and decreased production of beneficial metabolites (e.g., SCFAs). Bacteria translocate into circulation, leading to BSI and disseminated infections. DAMPs released by the dying intestinal epithelial cells as well as translocating bacteria and PAMPs activate host APCs resulting in a cytokine storm and donor T cell activation. T cells subsequently proliferate and differentiate into Th1 and Th17 types, which are involved in the activation of CTL that mediate tissue damage. Effector T cells together with cytokine storm attack the epithelial cells of the skin, liver, lung, and gastrointestinal tract, culminating in clinically GvHD. SCFA: short-chain fatty acid; BSI: bloodstream infection; DAMP: danger-associated molecular pattern; PAMP: pathogen-associated molecular pattern; APC: antigen-presenting cell; CTL: cytotoxic T lymphocytes; GVHD: graft-vs.-host disease.

reaction targeting leukemic cells in recipients, leading to reduced recurrence and superior survival [70]. Enteral nutrition as first-line nutritional support in patients who undergo HSCT can maintain the intestinal microecology and effectively inhibit GvHD onset [71]. Pretransplant administration of IL-25, a growth factor for goblet cells, allowed the conservation of goblet cells, prevented bacterial translocation, reduced plasma concentrations of interferon-$\gamma$ (IFN-$\gamma$) and IL-6, and ameliorated GvHD [72]. The glucagon-like peptide 2 promotes the regeneration of Paneth cells and intestinal stem cells, which reduces aGvHD and steroid-refractory GvHD without compromising GVL effects in multiple mouse models [73]. A clinical trial (NCT02641236) revealed a decrease in the incidence of aGvHD in patients who underwent gut decontamination with oral vancomycin and polymyxin B; however, these investigations need to be sig-

nificantly expanded [55]. Prophylactic administration of antimicrobials is a controversial topic because systemic antibiotic exposure not only suppresses anaerobic bacterial growth but also causes microbial diversity loss, decreases the production of anti-inflammatory SCFAs, and increases the incidence and severity of GvHD [74]. On the other hand, fecal microbiota transplantation (FMT) and probiotic supplementation have been analyzed in clinical trials, with a promising therapeutic value of restoring the intestinal microbiota, diminishing OS, reducing the incidence and severity of GvHD, and preventing drug-resistant bacterial colonization and virus infections [75–77]. The microbe-derived SCFA butyrate and propionate can effectively expand Foxp3^{+} Tregs through upregulation of GPRs expression, thus effectively inhibiting the occurrence of GvHD and promoting immune remodeling [20, 78]. Oral administration of *Bacteroides fragilis* has a

beneficial effect on the preservation of intestinal integrity and reduces inflammatory cytokine levels by increasing SCFAs, IL-22, and Treg cells [79].

5.4. Oxidative Stress and the Development of aGvHD. Inflammation is a key driver of GvHD; longstanding inflammatory conditions could result in increased oxidative stress. Leukocyte filtration induced by intestinal inflammation results in superoxide production by NADPH oxidase-1, increasing cellular ROS [36]. During an allogeneic immune response, the translocating intestinal flora activates neutrophils, the largest human leukocyte population. The neutrophil infiltration could amplify the tissue damage and contribute to GvHD in the manner of producing ROS. Selective NOX2 deficiency in neutrophils impairing ROS production led to lower levels of tissue damage, GvHD-related mortality, and effector phenotype T cells. Enterococcus faecalis is a commensal microorganism of the human intestinal tract that produces substantial extracellular superoxide ($O_2^-$) and derivative ROS such as $H_2O_2$ and hydroxyl radical, through autoxidation of membrane-associated demethylmenaquinone. The predominance of Enterococcus faecalis in GvHD patients was confirmed in metagenomic analysis of fecal microbiome [15]. Excessive ROS produced by Enterococcus faecalis could increase DNA damage in colonic epithelial cells and thus may contribute to active GvHD [37, 80].

The levels of NO and its metabolites increase in mice with GvHD, which may play a role in the pathogenetic mechanism of GvHD. Treatment with NO synthesis inhibitor significantly reduces the levels of NO production and bacterial translocation across the intestine, abrogates GvHD-associated enteropathy, and reduces lymphocytic infiltration in the intestinal epithelium, as a result, prolonging the survival of rats with GvHD [81, 82]. As we mentioned above, intestinal injury plays a pivotal role in the development of acute GvHD by providing a portal of entry for Gram-negative bacteria and LPS to enter the host tissues. Ellison et al. reported that LPS injection can consistently induce intestinal epithelial cell apoptosis in graft-versus-host mice triggering mucosal macrophages to release NO, and macrophage-derived NO is the principal mediator of intestinal injury in GvHD [83]. The released NO compromises the integrity of the intestinal epithelium and makes it more permeable to endotoxin. As this occurs, a vicious cycle of intestinal epithelial injury is established in which more endotoxin triggers the release of more NO, and so on [84].

On the other side, oxidative stress can intensify inflammatory responses. Damage of oxidative stress results in oxidized proteins, glycated products, and lipid peroxidation and then turns into the release of inflammatory signal molecules and peroxiredoxin 2 (PRDX2), a ubiquitous redox-active intracellular enzyme [85]. PRDX2 from LPS-stimulated macrophages can alter the redox status of cell surface receptors and allow the induction of inflammatory cascade in chronic inflammatory diseases [85]. Therefore, overproduction of oxidative stress can activate a variety of inflammatory mediators that involve in amplifying the inflammation and form a vicious circle that contributes to the GvHD development in HSCT patients. The strategies to limit oxidative stress in GvHD are highly desirable. Sofi et al. [86] reported that Trx1 is a common antioxidant enzyme that can reduce ROS accumulation in donor T cells and decrease downstream molecules including NF-$\kappa$B and T-bet, which restrained the ability of T cells to activate, expand, and migrate to the target organs in response to alloantigens in vivo. The administration of human recombinant Trx1 can decrease the pathogenicity of T cells and severity of GvHD and preserve the GVL effect, which has a great translational potential in patients with hematological malignancies undergoing allo-HCT.

# 6. Pulmonary Complications after HSCT

Pulmonary complications (PCs) are reported in up to 70% of HSCT recipients and account for significant morbidity and mortality [25]. HSCT patients are immunocompromised after engraftment as a consequence of chemotherapy, irradiation, acute/chronic GvHD, and maturing recipient marrow. In the postengraftment period, patients are at risk of opportunistic infections by Pneumocystis jirovecii and cytomegalovirus. Further, patients represent increased susceptibility to infectious pneumonitis, commonly associated with respiratory viruses, including influenza, respiratory syncytial, and adenoviruses [87]. In addition, chronic GvHD (cGvHD) can also occur later in the postengraftment period where the lung involvement results in chronic obstructive or restrictive pulmonary diseases.

Several studies have reported the relationship between intestinal dysbiosis and many pulmonary diseases, such as allergic airway diseases [88], obstructive pulmonary diseases [89], lung cancer [90], and pneumonia [91]. Therefore, it is pertinent to explore the influence of gut-lung crosstalk on the occurrence of PCs in HSCT recipients. Harris et al. performed a single-center observational study on 94 patients who underwent HSCT and were previously enrolled in a protocol for 16S ribosomal RNA sequencing of the fecal microbiota. They found that low diversity and γ-proteobacteria dominance in the fecal microbiota (which included common respiratory pathogens) were the independent predictors for the occurrence of PC postengraftment and overall mortality [25]. One possible mechanism is that the impaired gut barrier may facilitate microbial translocation to the lungs through circulation or indirect lung injury by a microbiota-induced systemic inflammatory response, provoking alveolar inflammation and pulmonary dysfunction. Another study analyzing post-HSCT lung microbiota in humans reported that increased relative abundance of Proteobacteria in the lung was correlated with impaired lung function after engraftment [92]. These evidences indicate toward a disordered gut-lung axis underlying postengraftment PCs. LPS is a structural component of Gram-negative bacteria and was shown to cause innate immune activation, accumulation of alloreactive T cells, and histologic damage by interacting with TLR4 in allo-HSCT models. Treatment with a TLR4 antagonist could protest against transplant-related lung injuries after HSCT [93]. This research confirmed the role of LPS in promoting the development of alloimmune lung injury after HSCT independent from systemic GvHD in
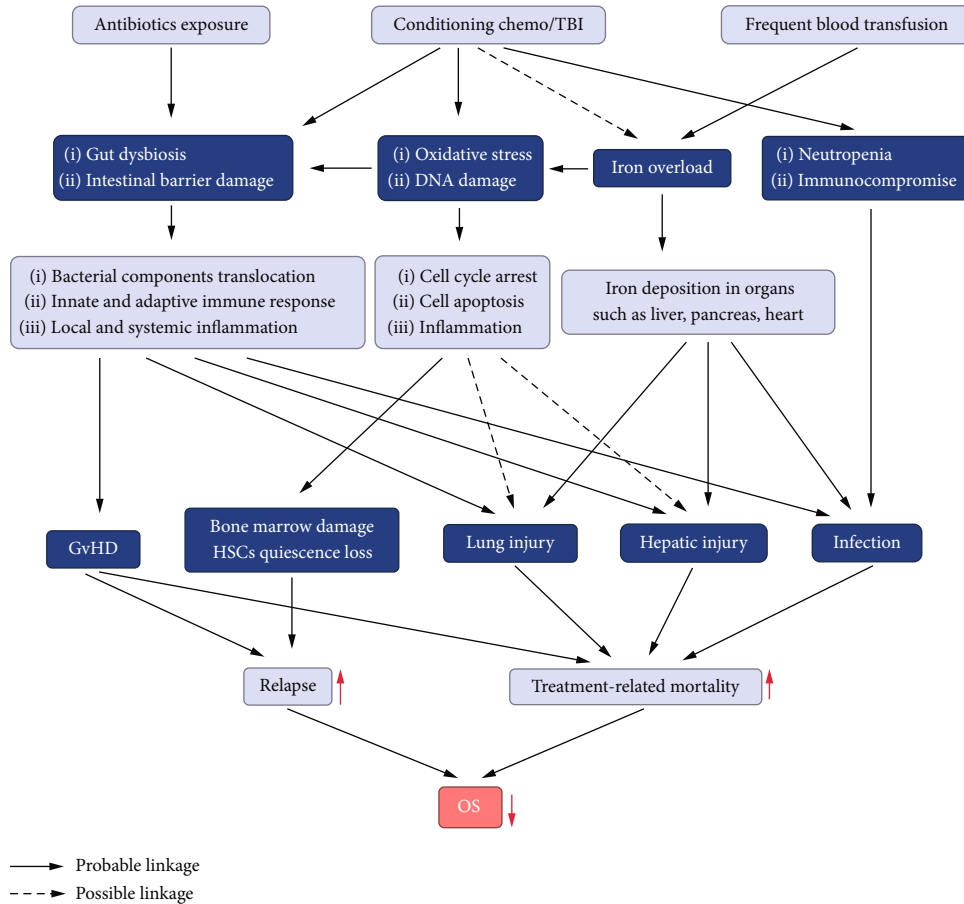
FIGURE 3: The interplay between intestinal dysbiosis and oxidative stress as well as the transplant-related complications. The interaction between intestinal dysbiosis and oxidative stress caused by preengraftment conditioning and prophylactic antibiotics exposure in this picture. These pathogeneses contribute to different HSCT-related complications such as GvHD, BSI, HSC dysfunction, pulmonary/hepatic injury, and infections which are leading cause of relapse and treatment-related mortality that affect the OS of HSCT. HSCT: hematopoietic stem cell transplantation; GVHD: graft-vs.-host disease; BSI: bloodstream infection; HSCs: hematopoietic stem cells; OS: overall survival.

the allo-HSCT model without systemic GvHD. On the other side, the microbial-derived metabolites of SCFAs have the ability to modulate host inflammation and promote immune tolerance against various bacterial and viral infections. HSCT patients with higher levels of SCFA-producing microbial communities were fivefold less likely to develop the pulmonary virus infection with lower respiratory tract infection, independent of other factors (adjusted HR = 0.22, 95% CI 0.04-0.69) [94]. Restoring the balance of endogenous gut microflora may play a role in the treatment of postengraftment PCs by elevating SCFA production.

OS may also play an important role in the pathogenesis of lung injuries, such as IPS and lung fibrosis, following HSCT. The lung is especially susceptible to oxidative damage because it has the largest endothelial surface area in the body, making it vulnerable to circulating toxins. Gut *Lactobacilli* and *Bifidobacterium* possess the ability to convert nitrate and nitrites into NO, making the gut epithelia a rich source of NO [95]. Similarly, *Streptococcus* and *Bacillus* pro-

duce NO from L-arginine using nitric oxide synthase. A higher pulmonary concentration of NO combined with superoxide results in the formation of peroxynitrite, a strong oxidant that can oxidize a number of biomolecules including tyrosine-containing proteins, resulting in nitrotyrosine formation. An increased concentration of exhaled NO in the lower respiratory tract and increased nitrotyrosine formation in the alveolar fluid following HSCT were identified as potential markers of IPS [96]. IPS is characterized by noninfectious diffuse lung injury associated with a high-dose chemotherapy regimen (BCNU, cyclophosphamide, and cisplatin) and the incidence of GvHD after HSCT. Murine models of IPS have shown that the conditioning regimen causes lung injury beginning with substantial OS, which further promotes intense monocytic cellular infiltration and macrophage activation. An increased alveolar macrophage population in the epithelial lining fluid has a significantly higher oxidative burst, which may further exacerbate lung inflammation and widespread alveolar injury [97]. In addition, increased ROS and cellular DNA damage in pulmonary

fibroblasts are key events in the progression of pulmonary fibrosis, which is frequent post-HSCT [98].

## 7. Sinusoidal Obstruction Syndrome

Hepatic SOS, also known as venoocclusive disease, is a potentially life-threatening complication that occurs in 13% of HSCT patients, belonging to a group of diseases increasingly identified as transplant-related, systemic endothelial diseases [99]. Severe SOS results in multiorgan dysfunction with a mortality rate > 80%. The SOS primarily insults both sinusoidal endothelial cells and hepatocytes in zone 3 of the hepatic acinus, which can be triggered by multiple factors including the toxicity of the conditioning regimens [100], cytokine cascade, microbial endotoxins, immune and alloreactivity.

Elevated oxidative stress in HSCT patients may be involved in the development of SOS. (-)-Epicatechin is a natural flavonol that was found to obviously enhance liver GSH levels and reduce the increased ROS amounts, thus reversing liver oxidative injury and attenuating SOS by activating nuclear translocation of nuclear factor erythroid 2-related factor 2 (Nrf2) antioxidant pathway [101]. As mentioned above, iron accumulation promotes the production of ROS via the catalytic activity of free iron. As the liver is one of the organs in which iron preferentially accumulates, oxidative stress promoted by iron overload in livers after conditioning regimens might be attributed to triggering and exacerbating hepatic injury including SOS in HSCT patients [102]. Yeom et al. demonstrated that ROS levels of the murine liver increased according to cumulative iron dose and correlations with pathologic score for SOS, including sinusoidal hemorrhage and endothelial damage, in HSCT mice with no significant differences between the syngeneic and allogeneic groups [103]. Mitigating oxidative stress with antioxidants has shown protective effects on SOS-related liver injury in many studies [101, 104, 105]. Sesame oil has antioxidant properties that offer better protection against increased blood pressure, hyperlipidemia, and lipid peroxidation by increasing enzymatic and nonenzymatic antioxidants. Inhibiting OS with prophylactic sesame oil prevents the rounding up of sinusoidal endothelial cells and thus attenuates SOS in murine [105].

Preclinical studies suggest that microbial products translocated across impaired intestinal barriers may participate in the pathogenesis of endothelial damage, interfering with procoagulant and fibrinolytic endothelial responses [106]. LPS in especially activates various signaling mechanisms in endothelial cells, ultimately leading to cellular dysfunction and injury [107]. A retrospective case-control study in allo-HSCT pediatric patients conducted by Masetti et al. reported that having healthy gut microbiota characterized by a high diversity and richness of beneficial microorganisms in the pretransplant period is associated with a reduced occurrence of SOS. The disrupted intestinal barrier with depleted beneficial taxa and low production of beneficial SCFAs could lead to greater translocation of microbial molecules. The microbial endotoxin, particularly

LPS, translocates across impaired intestinal barriers, reaches the liver sinusoid through the portal vein, and participates in endothelial damage by activating various signaling mechanisms, including NF-$\kappa$B and p38 MAPK [107, 108]. LPS-induced nitrooxidative stress may also participate in damaging liver microcirculation. The iNOS expression was increased in livers of the LPS-injected mouse group, evidenced by increased liver dihydroethidium staining and increased liver protein nitrotyrosination which can be blunted by the effect of iNOS inhibition [109]. These endothelial changes lead to the narrowing of the central vein lumen and obstruction of the blood flow. This is followed by the organization of subintimal edema and deposition of additional collagen. Thickened collagen cuffs surrounding the central veins characterize chronic SOS.

## 8. Conclusion

Intestinal dysbiosis and OS caused by preengraftment conditioning and prophylactic antibiotics result in different HSCT-related complications such as BSI, GvHD, pulmonary injury, and hepatic injury, which are the leading causes of adverse outcomes after HSCT. Disturbance in intestinal microbiota is due to the conditioning regimen, antimicrobial administration, and iatrogenic immunocompromisation in patients undergoing HSCT. Preengraftment conditioning affects the intestinal mucosa due to increased OS and DNA damage in the intestinal epithelial cells. Translocation of commensal and pathogenic bacteria into the bloodstream through impaired intestinal barriers may induce BSIs and host immune responses. Excessive translocation of microbial components leads to allogeneic donor T cell activation and a series of cytokine storms, greatly enhancing the immune response to the recipient antigen and launching cytotoxic attacks on the recipient target cells, which are positively related to the GvHD occurrence. Gut bacteria and their endotoxins can cause pulmonary and liver inflammation and infection through hematogenous dissemination and are also related to pulmonary infections, IPS, and SOS posttransplantation. Several studies have reported antibiotic-mediated decrease in gut bacterial diversity. Further, strategies are also described for restoring the intestinal flora using fecal microbial transfer and probiotics in an aim to manage transplant-related complications and improve clinical outcomes (Figure 3). The immunoregulatory effects of microbial metabolites on SCFAs have also been confirmed in GvHD. Removing the disturbance of redox balance to antioxidant supplements and OS depletion by reducing preconditioning intensity and decreasing iron accumulation has beneficial effects in the management of GvHD, infections, and organ injury in HSCT patients. However, further studies are needed to elucidate the role of intestinal flora-mediated OS in the pathology and treatment of HSCT-related complications, which may provide additional understanding of the pathways employed by gut microbiota in mediating the process of HSCT-related complications.

## Abbreviations

HSCT:     Hematopoietic stem cell transplantation

HSCs: Hematopoietic stem cells
GvHD: Graft-versus-host disease
IPS: Idiopathic pneumonia syndrome
SOS: Sinusoidal obstruction syndrome
ROS: Reactive oxygen species
OH: Hydroxyl radical
$H_2O_2$: Hydrogen peroxide
NO: Nitric oxide
OS: Oxidative stress
SCFAs: Short-chain fatty acids
AhR: Aryl hydrocarbon receptor
Treg: Regulatory T
Foxp3: Forkhead box protein P3
MDRO: Multidrug-resistant organisms
DAMPs: Damage-associated molecular patterns
MAPK: Mitogen-activated protein kinase
NF-$\kappa$B: Nuclear factor kappa-B
CTX: Cyclophosphamide
BCNU: Carmustine
BSI: Bloodstream infection
IL-1$\beta$: Interleukin-1$\beta$
PAMPs: Pathogen-associated molecular patterns
GPRs: G protein-coupled receptors
TNF: Tumor necrosis factor
Th: T helper
MHC: Major histocompatibility complex
MAIT: Mucosa-associated invariant T
GVL: Graft-versus-leukemia
FMT: Fecal microbiota transplantation
PCs: Pulmonary complications
LPS: Lipopolysaccharide.

## Ethical Approval

This article does not contain any studies with human participants/animals performed by any of the authors.

## Conflicts of Interest

All the authors declare that they have no conflict of interest.

## Authors' Contributions

Mingxuan Chi, Tao Jiang, and Xing He were involved in writing the article. Haoyu Peng, Yunlong Li, Qing Nian, Jiong Zhang, Li Wang, Kuai Ma, and Chi Liu critically revised the manuscript. All authors read and approved the final manuscript. Mingxuan Chi, Tao Jiang, and Xing He contributed equally to this work.

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*Research Article*

# A Novel Scientometrics Research on the Interaction between Oxidative Stress and Hematopoietic Stem Cell Transplantation Complications: From Graft-versus-Host Disease to Sepsis

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As major and serious complications after hematopoietic stem cell transplantation (HSCT), graft-versus-host disease (GVHD) and sepsis are the chief causes of low survival rates as well as mortality and for HSCT recipients. Although the overall treatment outcomes of HSCT have improved significantly in recent years, there is still an increased incidence rate of complications and mortality after transplantation. In the immediate past, with a deeper understanding of oxidative stress, more and more shreds of evidence have shown that it is closely related to transplantation-related sepsis. However, there is currently a precious little research on the interaction between oxidative stress and complications after HSCT, and the major mechanism has not yet been clarified. The objective of this study was to assess the internal connection between and potential mechanisms as well as visualized the scientometrics results of all important literature related to the topic. Through exhaustive scientometrics analysis, we searched and carefully screened 286 related publications from the Web of Science Core Collection (WoSCC) with "((HSCT) OR (hematopoietic stem cell transplantation)) AND (oxidative stress)" as the search strategy. Then, detailed visualization of the overall information analysis was made by scientific and rigorous bibliometrics software or website. Next, we analyzed retrieved articles extensively and then 59 publications that are relevant to this topic were selected for nuanced analysis and summary. The assessment of these studies proved the validity of the interaction between oxidative stress and complications after HSCT objectively and directly.

## 1. Introduction

Hematopoietic stem cell transplantation (HSCT) refers to a treatment method that pretreats the transplant recipient through high-dose radiotherapy, chemotherapy, or other immunosuppressants, clears the tumor cells and abnormal clonal cells in the recipient, blocks the pathogenesis, and then transfuses autologous or allogeneic hematopoietic stem cells to the recipient so that the recipient can reestablish normal hematopoietic and immune functions in the body, so as to achieve the purpose of treatment [1–3]. Hematopoietic stem cell transplantation is widely used in the treatment of malignant hematological diseases, nonmalignant refractory hematological diseases, some solid tumors, genetic diseases, epidemic diseases, and congenital metabolic diseases and

has achieved good curative effects. Although HSCT has a better curative effect and more and more applications, it also has many serious complications. It includes infection, transplant failure, graft-versus-host disease (GVHD), and hepatic vein occlusion disease (VOD) [4, 5]. One of the most common factors that affect the long-term survival of transplant individuals is infection and its related sepsis [6].

Sepsis is a clinical syndrome caused by excessive inflammation after infection, including immune abnormalities, coagulation abnormalities, and systemic multiple organ dysfunction [7]. The early manifestation of sepsis is a systemic inflammatory reaction. After receiving hematopoietic stem cell transplantation, patients have a significantly increased chance of infection-related sepsis due to the suppression of immune function. Once sepsis occurs, it gradually turns into

(a)



(b)
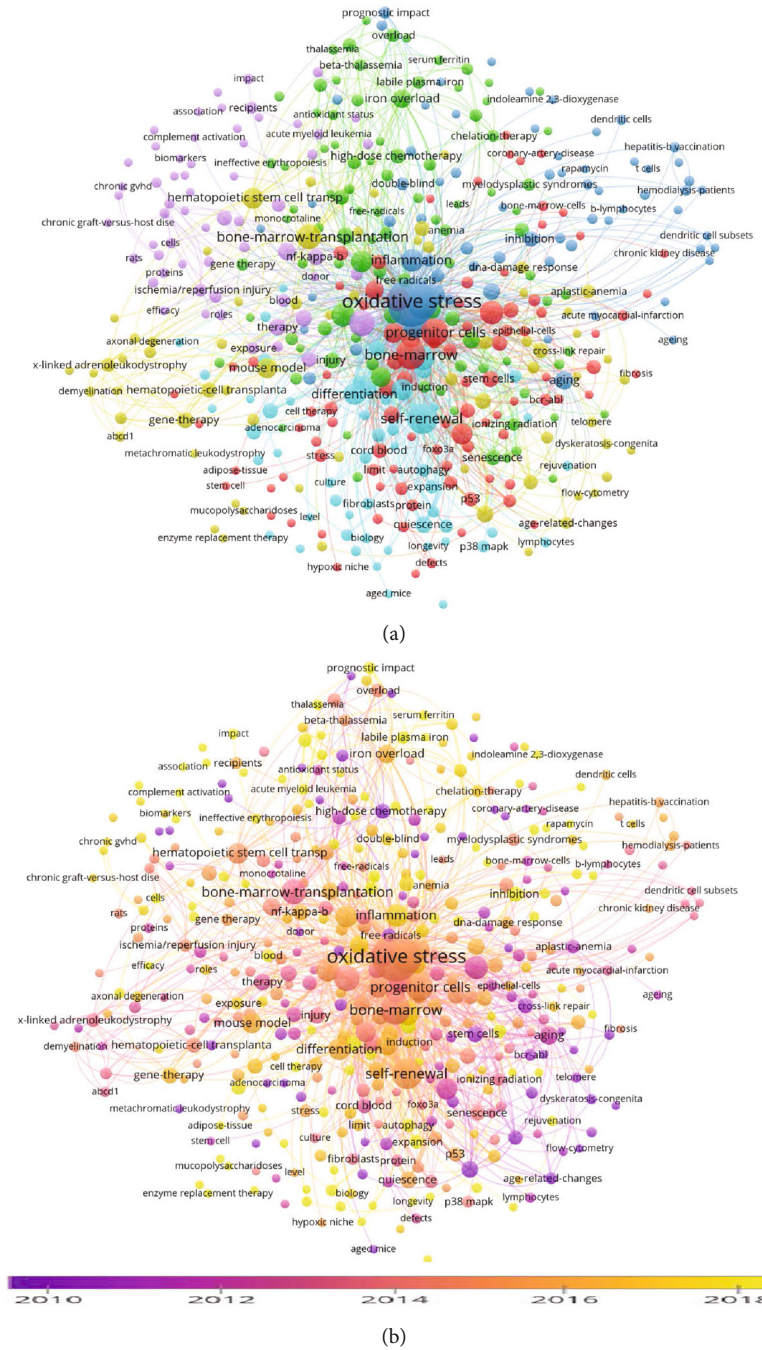
Figure 1: Keyword analysis based on initial search results. (a) Cluster analysis was performed on all keywords based on the cooccurrence relationship. Keywords of the same color come from the same cluster. The size of bubbles represents the frequency of keywords, and the connection between bubbles reflects the cooccurrence relationship between them. (b) Hot spot time analysis of keyword occurrence. Through analysis, the time point with the highest frequency of each keyword is obtained and this information is visualized. The corresponding relationship between color and different times can be seen in the legend.

shock, multiple organ failure, and eventually death with the development of the disease [8, 9]. The disease progresses rapidly, the prognosis is dangerous, and the clinical treatment is complex. It has become one of the main causes of treatment failure and death after hematopoietic stem cell transplantation. Some studies have pointed out that studies have shown that the increase of proinflammatory cytokines in the body and the process of oxidative stress caused by

them are two of the basic pathological changes of sepsis, while patients with hematopoietic stem cell transplantation often have problems such as intestinal flora disorder, intestinal barrier function decline, and systemic defense function damage in the process of diagnosis and treatment, which will lead to an increased risk of infection from wide range pathogens, and its severity is closely related to mortality [6]. At the same time, although the international community has

provided guidelines for the diagnosis and treatment of severe sepsis, the mortality of severe sepsis is still high, and clinical work is still facing great challenges. Although a series of studies have proved that oxidative stress (OS) plays a very important role in the pathological procedures of both sepsis and HSCT-related complications [10–12], solving sepsis related to hematopoietic stem cell transplantation is still a major problem that puzzles clinical and basic research, especially the in-depth study between oxidative stress and intestinal flora and the related scientometrics research and visual presentation, which also urgently needs to be studied in depth.

Scientometrics is a novel discipline that mainly focuses on the research of the quantitative angle of the scientific process as an important communication system [13]. Scientometrics focuses on the quantitative characteristics and characteristics of science and scientific research. The key and difficult point of it is how to conduct in-depth research and investigation on scientific development and mechanism through statistical mathematical methods. In recent years, scientometrics has played an important role in the performance evaluation and measurement of various scientific researches [14]. Scientometrics focuses on but is not limited to citation analysis in academic literature. It can also conduct qualitative and quantitative analyses of literature from multiple dimensions, such as cooccurrence analysis, cocitation analysis, coauthor analysis, and bibliographic coupling. Moreover, scientometrics usually involves various in-depth clustering analyses. In basic scientific metrology research, the most basic process and research foundation are to construct the citation map, which can show the detailed network of citations between different publications in charts [15–17]. In addition, visualization is also an important attribute and feature of scientometrics as well as bibliometrics [18]. By integrating complex and difficult-to-understand data and presenting them in the form of images, researchers can clearly and quickly obtain the latest research hot spots and directions, thus providing a theoretical basis for further research. Scientific metrology has become a popular research method in many fields based on large-scale literature databases. In this study, we searched and carefully screened all relevant publications in the science core collection network for comprehensive and accurate analysis and scientific measurement for further processing and scientific metrology research.

Therefore, the purpose of this study is to conduct real-time scientometrics analysis of existing relevant studies and present the latest trend of the interaction between oxidative stress in HSCT and its related sepsis, so as to find the current research hot spots, lay a theoretical foundation for subsequent clinical research, and further provide the relevant research foundation for the application of evidence-based medicine and translational medicine, with the aim of providing new insights for OS- and HSCT-related complications.

## 2. Materials and Methods

### 2.1. Search Strategies.
A publication search was conducted in the Web of Science Core Collection (WoSCC) of the Web of Science database on July 19, 2022. Firstly, we used ((HSCT) OR (hematopoietic stem cell transplantation)) (All Fields)

TABLE 1: The keywords with the top 20 number of occurrences.

| Keyword | Occurrences | Total link strength |
| --- | --- | --- |
| oxidative stress | 189 | 839 |
| self-renewal | 53 | 327 |
| transplantation | 52 | 248 |
| bone-marrow | 50 | 242 |
| progenitor cells | 38 | 188 |
| mice | 33 | 154 |
| bone-marrow-transplantation | 30 | 128 |
| hematopoietic stem cells | 28 | 164 |
| expression | 26 | 139 |
| hematopoietic stem-cells | 26 | 86 |
| stem-cells | 26 | 118 |
| in-vivo | 24 | 137 |
| inflammation | 24 | 80 |
| differentiation | 20 | 106 |
| stem-cell transplantation | 20 | 91 |
| activation | 19 | 91 |
| life-span | 19 | 109 |
| hematopoietic stem cell transplantation | 18 | 73 |
| proliferation | 18 | 105 |
| mouse model | 17 | 73 |
| apoptosis | 16 | 90 |
| DNA-damage | 15 | 83 |
| disease | 14 | 69 |
| hematopoietic stem | 14 | 69 |
| in-vitro | 14 | 83 |
| iron overload | 14 | 80 |
| aging | 13 | 74 |
| versus-host-disease | 12 | 58 |
| quiescence | 11 | 64 |
| therapy | 11 | 55 |

AND (oxidative stress) (All Fields) as the search strategy, and then, 286 publications were obtained. Next, we read all the search results extensively and excluded 227 publications that were not closely related to postoperative complications. Finally, 59 publications were obtained. Then, we exported all the full records and cited references with the data style of TXT.

### 2.2. Data Processing.
In the process of data processing, VOSviewer (version 1.6.17) and Citespace (version 6.1.R2) and an online website of bibliometrics (https://bibliometric.com/) were used to conduct the following analysis and visualizations based on the scientometrics and bibliometric principle:

(1) Cluster analysis and hot time presentation of all keywords from 286 publications based on the cooccurrence relationship

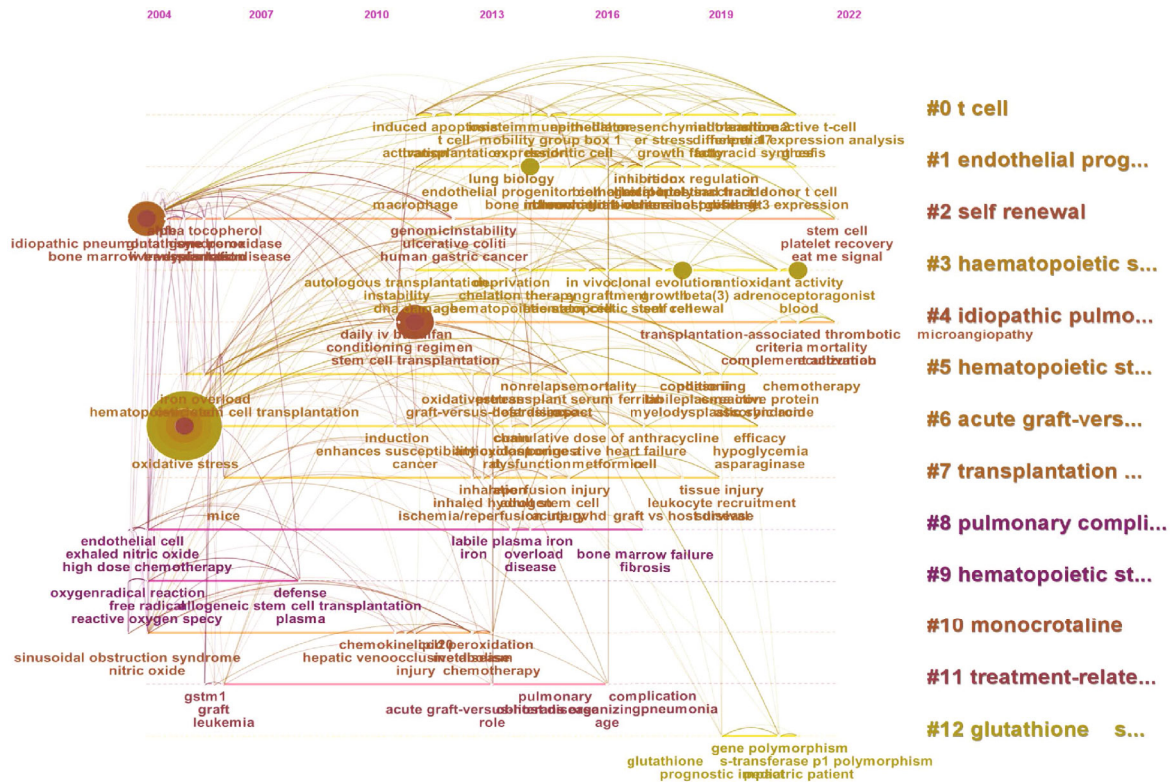(2) The timeline visualization of the keywords from the 59 screened publications

FIGURE 2: The timeline view of cluster analysis of keywords from screened publications.

(3) Coauthor relationship between countries and organizations and their relative number of publications

(4) Statistical analysis of basic information of journals with the largest number of publications

(5) The cluster analysis of the cited journals is based on the cocitation relationship

(6) Visualization of citation relationship and cluster analysis between journals of the screened publications and their references

## 3. Results

*3.1. Keyword Clustering Analysis of the First Search Results.* After cluster analysis of 286 publications in the field of HSCT and oxidative stress using VOSviewer software, the cooccurrence relationship and occurrence frequency of keywords are presented (Figure 1(a)). Among the total 1638 keywords, 111 keywords have a frequency of occurrence equal to or more than 5 times. We present the occurrence and link strength of keywords with times of occurrence greater than 10 in Table 1. In addition, based on cluster analysis, we also analyzed the time nodes with a high frequency of occurrence of each keyword (Figure 1(b)).

*3.2. In-Depth Keyword Clustering Analysis of Screened Publications.* After excluding studies unrelated to complications or prognosis of hematopoietic stem cell transplantation, we obtained 59 publications. After that, an in-depth

analysis of the keywords of these publications using Citespace software (Figure 2) was also performed. Cluster analysis based on cooccurrence relationship is used here and 13 clusters are obtained. In addition, each cluster is marked with a label, and the keywords under the label are closely related to it. The temporal hot spots of the keywords are analyzed and presented in the figure, and the cooccurrence relationship between them is presented in the form of lines.

All the keywords were clustered based on the cooccurrence relationship, and 13 clusters were obtained. The labels of each cluster are displayed on the right side, and the time when the keywords mainly appear is also indicated on the horizontal axis. In addition, the connection between keywords also reflects their cooccurrence relationship.

*3.3. Analysis of Coauthorship between Organizations and Countries.* Here, we continue to conduct an in-depth analysis of the cooperation relationship and research situation among the units of 59 selected publications so as to find out the research units with deep research in this field. The online website of bibliometrics (https://bibliometric.com/). It is used for cooperation between countries (Figure 3(a)). Among them, the United States and China have the largest number of publications, and their cooperation is close. The cooperation relationship of the organization is completed by using VOSviewer software through clustering analysis based on the coauthor relationship (Figure 3(b)). We can intuitively see from the figure that Soochow University has a large number of publications, and the University of

(a)



(b)
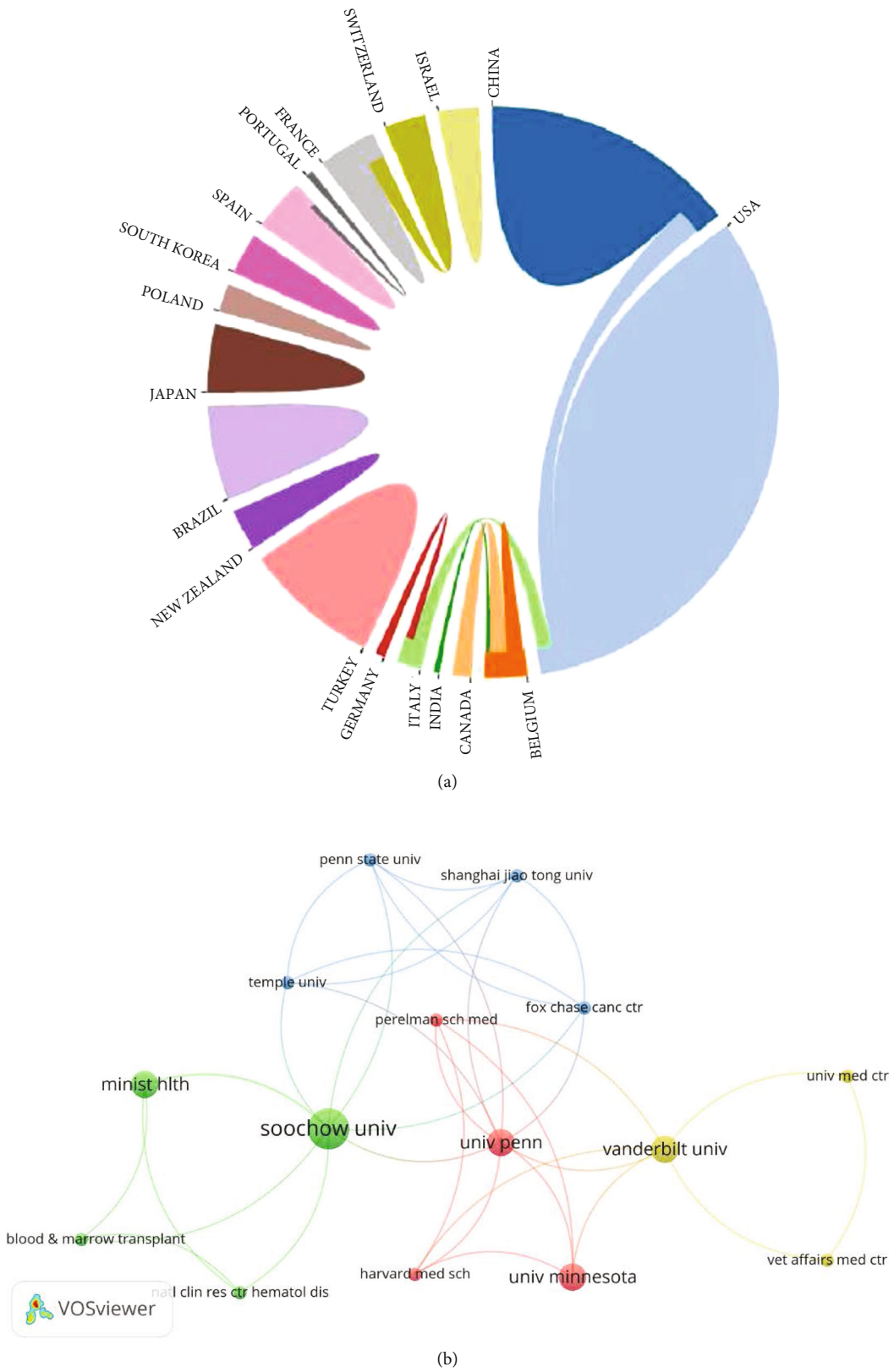
FIGURE 3: The coauthor relationship in screened publications. (a) Cooperation between countries. In the chord diagram, different color blocks are used to correspond to countries, and the area reflects the number of national publications. The connection between the color patches reflects the cooperative relationship between countries. (b) Cooperation between organizations. The bubble size reflects the number of publications, and the connection between them reflects the coauthor relationship between organizations. In addition, the clustering situation is also shown in color in the figure.
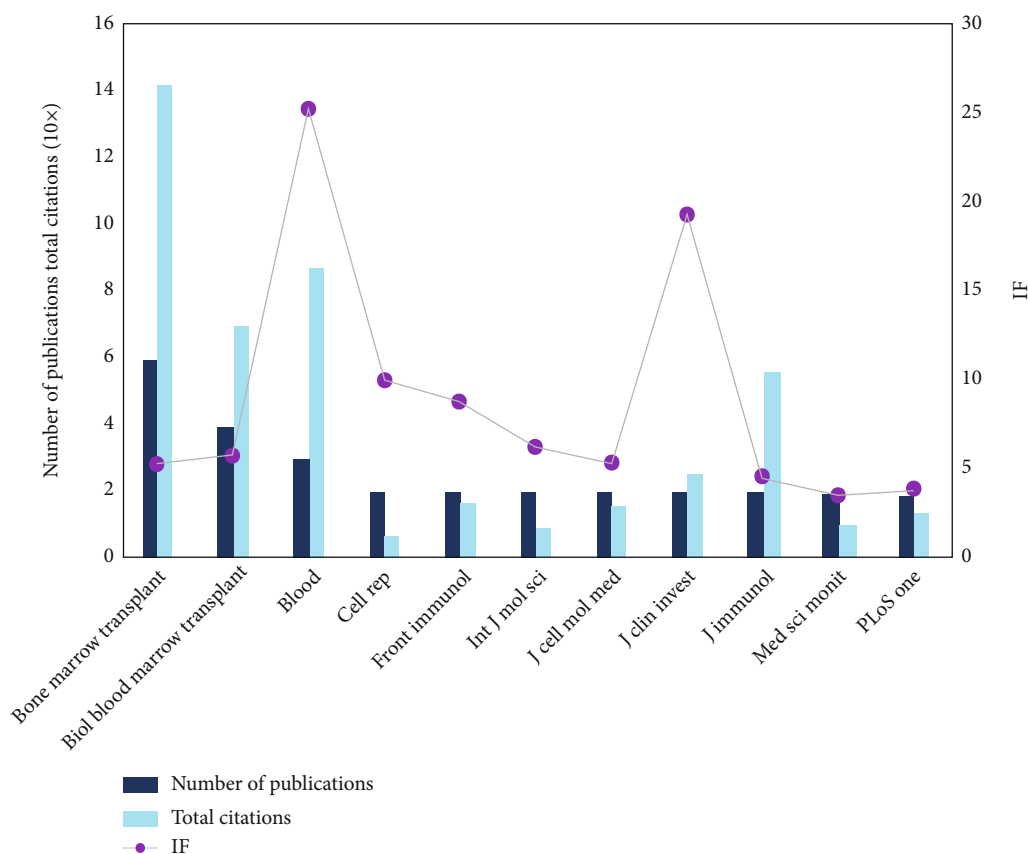
FIGURE 4: Basic information of journals with a top number of publications.

Pennsylvania has the largest number of cooperation projects with other institutions.

*3.4. Association Analysis between Journals Based on Citation Relationships.* Firstly, we statistically processed the journals with more screened publications and visually presented the total number of publications, the total number of citations, and the impact factor (Figure 4). The number of publications, the total citation times, and the impact factor of the journals are presented. After that, for the references of these 59 publications, we conducted a cluster analysis based on the cocitation relationship for the journals from which they came (Figure 5). The connection reflects that they are cited by a publication at the same time, and the bubble size reflects the number of publications. In these two analyses, blood, bone marrow translation, and biology blood marrow translation all have high occurrence times and high if, which means they have a high influence in this field. Finally, the citation relationship between the screened publications and the journals from the reference sources is presented, and all the journals are clustered into multiple clusters, where the topic relevance is presented.

And then, the visualization of citation relationship and cluster analysis between journals have been made (Figure 6). The left part is the journal of our screened publication, and the right part is the journal of the reference of the publication. The journals on the left and right sides have done cluster analysis according to the citation situa-

tion (they have been clustered into several clusters, and the specific situation has been presented in the figure with labels). The middle line shows the main reference situation.

## 4. Discussions

One of the most common complications after hematopoietic stem cell transplantation is infection and its related sepsis because as long as large doses of radiotherapy and chemotherapy are carried out, there is a period of bone marrow suppression, which will cause patients to cause a variety of infections due to granulocyte deficiency, including bacterial infection, fungal infection, and viral infection. If poorly controlled, it is easy to lead to sepsis, which will lead to a worse prognosis and irretrievable outcome.

The oxidative stress after hematopoietic stem cell transplantation is in a delicate balance, and its specific mechanism remains to be elucidated. Based on this, we conducted a literature search and a novel scientometrics research on this hot topic. The initial search was based on the topic of hematopoietic stem cell transplantation and oxidative stress. For the retrieved publications, we conducted cluster analysis on all the keywords and mapped the cooccurrence network. The purpose was to preliminarily get the relationship between the research hot spots under the relevant subjects, so as to select the hot information or topics related to them. In this research, in addition to "oxidative stress" as the search term, the words "progenitor cells", "self-renewal", "iron overload",
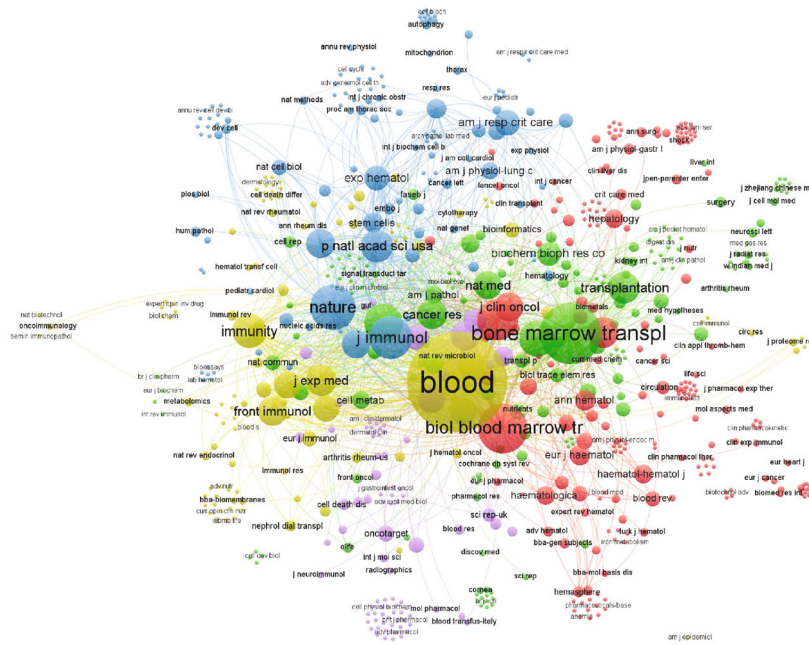
FIGURE 5: Cluster analysis of cited references of the screened publications based on the cocited relationship.
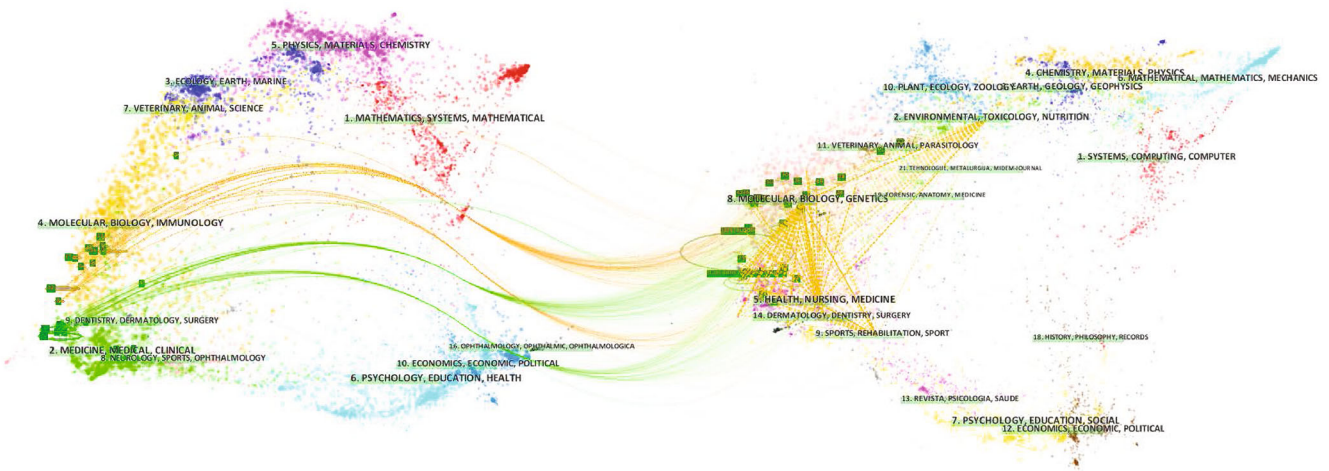


FIGURE 6: Visualization of citation relationship and cluster analysis between journals.

and so on appear more frequently. In addition, there are some words that have appeared in recent years although they are less frequent, such as diagnostic impact, ineffectiveness, indoleamine 2,3-dioxygenase, and labile plasma iron. These words may become new hot spots in future applications or research. Readers can choose the keywords they are interested in or further explore the keywords that have a strong cooccurrence relationship with them and have a relatively recent appearance.

Next, in order to obtain more accurate results, we excluded studies unrelated to complications or prognosis, and only 59 publications were retained. Timeline view is used here to present the clustering results of keywords and their cooccurrence relationships. We also divided the keywords into 13 clusters, and each cluster is labeled with a tag. This is a summary of the keywords in this cluster. Through such tags, readers can quickly identify the hot spots they are interested in. For example, the tag of the first cluster is T cell, and the keywords related to immune cells or immunity are mainly presented in this cluster. In addition, hot spots and cooccurrence networks are also shown in the figure. The cooperation relationship between the countries/ organizations of the screened publications is shown in the study. The observation of visual images can help readers

Table 2: The multidimensional presentation and intensive reading of recently published literatures.

| Author | Journal/IF/year | Study object | Treatment | Result | Conclusion |
|---|---|---|---|---|---|
| Patterson et al. | *Stem Cell Rev Rep* IF:6.692/2021 | Patients with multiple myeloma | Patients received meloxicam with filgrastim before apheresis. | The number of CD34+ cells collected decreased significantly, the expression of CXCR4 on CD34+ cells decreased, and the proportion of CD4 +/CD8+ T cells increased. RNA sequencing showed downregulation of oxidative phosphorylation related genes. | Mitigate hematopoietic stem and progenitor cells oxidative stress. Lessen stem cell exhaustion and enhance graft quality. |
| Rodionov et al. | *Bone Marrow Transplant* IF:5.174/2022 | G-CSF mobilized human peripheral blood cells and nonobese diabetic-severe combined immune deficiency (NOD-SCID) IL2Rγnull (NSG) mice. | A treatment of MPBCs with Fas ligand (FasL, CD95L). | Selectively induce apoptosis of CD3+ T cells, B cells, and antigen presenting cells, but CD34+ hematopoietic stem cells and progenitors. Reduce IFN-γ secreted by cells. | Increase the possibility of graft survival and function, reduce GVHD, and reduce the proinflammatory capacity of macrophages. |
| Wang et al. | *Int Immunopharmacol* IF:5.714/2020 | BALB/c (H-2d) mice induced GVHD. | Treated with the combination of BBR and CsA. | Reduce weight loss and GVHD index scores. Reduce intestinal and liver damage, inflammation, and oxidative stress. Suppress NF-κB signaling in liver and intestine. Reduce the number of Th1 cells. | Reduce oxidative stress and alleviate inflammatory response induced by acute GVHD. Improve the survival rate of GVHD mice. |
| Rezende et al. | *J Immunol Res* IF:4.493/2019 | C57BL/6 and B6D2F1 mice induced GVHD. | Intraperitoneally inject apocynin during the experiments. | The treated mice reduced mortality and disease progression, reduced oxidative stress, reduced liver and intestinal damage, and inhibited inflammation. | Regulate the inflammatory response related to GVHD without impairing the engraftment which is associated with controlling oxidative stress. |

choose which countries or organizations have more in-depth research in this field. In-depth analyses of the journals from which the publications are sourced were done. We first conducted a multidimensional statistical analysis of the data of the journals from which the selected publications came, including the statistics of the total number of publications, the number of citations of all publications, and the presentation of the value of different journals. We found that journals such as *Blood*, *Bone Marrow Translation*, and *Biology of Blood and Marrow Transplantation* have a high number of publications and citations and have considerable influence factors; readers can selectively read the publications from these journals based on the information when making inquiries. We also conducted cluster analysis based on the cocitation relationship for the source journals of the cited references. We believe that the cocitation relationship can effectively reflect the relevance and theme consistency between journals. For example, *Blood*, *Frontiers in Immunology*, and *Nature Reviews Endocrinology* appear in the same cluster. Perhaps this relationship can be used to select journals and help readers to contribute. In addition, we also presented the citation relationship between the publication source journals and their reference source journals and also perform simple clustering based on this. The specific situation has been marked with different labels. Meanwhile, an intensive reading part of recently published literatures was shown in Table 2 with appropriate and detailed descriptions [19–22].

As a negative effect of free radicals in the body, the process of oxidative stress is often and considered to be an important factor leading to diseases [23]. In particular, uncontrolled oxidative stress is one of the important culprits of HSCT and its related sepsis. Patients receiving HSCT may experience significant changes in the process of oxidative stress due to their potential malignant tumors and exposure to a wide range of chemotherapy and systemic antibiotics and further trigger chain reactions, such as cell and tissue damage, imbalance of gut microbiota, and production of harmful metabolites [24–26]. All of them can have direct or indirect effects on the post-HSCT operation. However, the precise mechanism of oxidative stress in HSCT complications and its related sepsis has not been fully elucidated. The occurrence of oxidative stress may be related to graft-versus-host disease or infection after HSCT, especially the systemic immune response. The main mechanisms are the respiratory burst of macrophages caused by infection or rejection, or the release of a large number of reactive oxygen species from mitochondria and endoplasmic reticulum after endothelial cell injury. Then, excessive reactive oxygen species will further stimulate macrophages and endothelial cells and form positive feedback. In addition, the depletion of the antioxidant system is also an important factor leading to the imbalance of oxidative stress. Many problems, such as how macrophages produce ROS during this process and the specific roles of different cytokines as well as the finding of biomarkers related to oxidative stress to predict prognosis, still need to be further explored. Meanwhile, the limitation of this research such as lacking more data on clinical trials as well as a comprehensive evaluation of the importance of clinical nursing and other objective factors on the successful treatment of HSCT still needs further research.

## 5. Conclusions

In this study, we conducted a novel scientometrics analysis on the publications on the topic of oxidative stress and HSCT. As far as keywords, countries, research institutions, and journals, we present their potential relationships, including cooccurrence relationships, coauthor relationships, and cocited relationships, in a variety of analytical ways. The multidimensional visual analysis helps readers to select the hot spot information they are interested in according to the chart, so as to carry out further research, clarify the mechanism, and optimize the clinical diagnosis and treatment measures.

## Data Availability

The original contributions presented in the study are included in the article; further inquiries can be directed to the corresponding authors.

## Conflicts of Interest

The authors declare that they have no conflicts of interest.

## Authors' Contributions

All authors contributed to the study conception and design. JS and SX collected the literature and drafted the initial manuscript. SX and MC assisted in the preparation of the figures and tables. MC revised the manuscript and edited the language. All authors approved the final manuscript as submitted and are accountable for all aspects of the work.

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Hindawi

*Research Article*

# Homoharringtonine combined with cladribine and aclarubicin (HCA) in acute myeloid leukemia: A new regimen of conventional drugs and its mechanism

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*Objective*. The prognosis of children with refractory acute myeloid leukemia (AML) is poor. Complete remission (CR) is not always achieved with current salvage chemotherapy regimens before transplantation, and some patients have no chance of transplantation. Here, we aimed to describe a new regimen of conventional chemotherapy drugs (homoharringtonine, cladribine , and aclarubicin (HCA)) for refractory AML and its mechanism in vitro. *Methods*. We retrospectively collected the clinical data of 5 children with primary refractory AML using HCA as reinduction chemotherapy, and CR rates, adverse reactions, and disease-free survival (DFS) were analyzed. The effects of homoharringtonine, cladribine, and aclarubicin alone or in combination on the proliferation of HL60 and THP1 cells were analyzed by CCK-8 assay. Furthermore, CCK-8 was used to determine the effects of HCA, alone or in combination with apoptosis inhibitors, necroptosis inhibitors, ferroptosis inhibitors, or autophagy inhibitors, on the proliferation of HL60 and THP1 cells and to screen for possible HCA-mediated death pathways in AML cells. The pathway of HCA-mediated AML cell death was further verified by Hoechst/PI staining, flow cytometry, and Western blotting. *Results*. After 2 cycles of conventional chemotherapy, none of the 5 children with AML achieved CR and were then treated with the HCA regimen for two cycles, 4 of 5 achieved CR, and another child achieved CR with incomplete hematological recovery (CRi). After CR, 3 children underwent hematopoietic stem cell transplantation (HSCT), and only 2 of them received consolidation therapy. As of the last follow-up, all 5 patients had been in DFS for a range of 23 to 28 months. The inhibition rate of homoharringtonine, cladribine, and aclarubicin in combination on HL60 and THP1 cells was significantly greater than that of a single drug or a combination of two drugs. We found that inhibitors of apoptosis and necroptosis were able to inhibit HCA-mediated cell death but not ferroptosis or autophagy inhibitors. Compared with the control group, the number of apoptotic cells in the HCA group was significantly increased and could be reduced by an apoptosis inhibitor. Western blot results showed that PARP, caspase-3, and caspase-8 proteins were activated and cleaved in the HCA group, the expression of Bax was upregulated and that of Bcl-2 was downregulated. The expression of apoptosis-related proteins could be reversed by apoptosis inhibition. Compared with the control group, the expression levels of the necroptosis-related proteins RIP1, RIP3, and MLKL were downregulated in the HCA group but were not phosphorylated. The necroptosis inhibitor increased the expression of RIP1 but caused no significant changes in RIP3 and MLKL, and none were phosphorylated. *Conclusions*. HCA, as a new regimen of conventional drugs, was a safe and efficacious reinduction salvage strategy in children with refractory AML before HSCT. HCA exhibits the synergistic growth inhibition of AML cells and induces cell death mainly through apoptosis.

# 1. Introduction

The outcomes of children with acute myeloid leukemia (AML) have been significantly improved in recent years because of the application of hematopoietic stem cell transplantation (HSCT), the combination of multiple drugs with chemotherapy, and the rise of novel targeted drugs. However, children with refractory AML still have poor outcomes. The main reason for the formation of refractory AML is the resistance of leukemia cells to chemotherapeutic drugs. The treatment options for refractory leukemia mainly include the use of drugs without cross-resistance, medium and high doses of cytarabine (Ara-C), HSCT, new targeted therapy drugs or biological therapy. Among these treatment regimens, HSCT may be the best choice for refractory patients. It is important to achieve complete remission (CR) before HSCT and longer disease-free survival (DFS) for patients who have no chance of transplantation.

Although there has been significant development of targeted drugs, traditional chemotherapy drugs were mature, stable, and economical. We should not give up the optimization of traditional chemotherapy regimens. Common chemotherapeutic agents for AML include purine analogues, Ara-C, homoharringtonine, and anthracyclines. However, among these chemotherapeutic agents, increasing anthracycline and Ara-C doses may not improve response rates in AML with relapse or nonresponse [1]. Regimens containing homoharringtonine or cladribine with different CR rates of reinduction that range from 0% to 75% have been confirmed to be effective and safe in children with AML [2–8], and they have been reported in a few studies on children with refractory or relapsed AML (RR-AML) [3, 4]. Therefore, it is important to find an optimal combination of traditional chemotherapeutic drugs without increasing the severe damage to the patient's body and achieve effective reduction of minimal residual disease (MRD) and CR.

In this study, we described a new regimen of conventional chemotherapy drugs (homoharringtonine, cladribine, and aclarubicin (HCA)) for refractory AML. The patients were followed up for 23-28 months, and the CR rate and DFS were 100%. Moreover, we examined the effects of HCA on AML cell line proliferation and identified the cell death pathway of these drugs.

# 2. Materials and Methods

## 2.1. Ethics Statement.
After the study was approved by the IRB of the Third Xiangya Hospital of Central South University, we retrospectively collected the basic data, clinical indicators, and laboratory indicators of the children treated with the HCA regimen in the Xiangya Hospital or in the Third Xiangya Hospital. All five children enrolled in this study were treated at Xiangya Hospital (Changsha, China).

## 2.2. Treatment.
The HCA regimen was as follows: cladribine at $5 \, mg/m^2$/day as a continuous infusion for 24 hours on days 1–4, aclarubicin at $14 \, mg/m^2$/day as an intravenous infusion over 2 hours on days 1–4, homoharringtonine at $1 \, mg/m^2$/day as an intravenous infusion over 3 hours on days 1–7.

Bone marrow (BM) aspirates were collected before each course of HCA and posttreatment on day 28 (±7 days) after HCA to assess the response. Response was assessed by bone marrow aspirates and MRD. Cytogenetic analysis can also be used to assess response status. Discontinue treatment in the event of a serious adverse reaction. Provide supportive care according to local institutional guidelines.

## 2.3. Definitions.
CR was defined as less than 5% blasts on BM morphology examination and satisfactory hematological recovery (neutrophil count $> 1.0 \times 10^9$/L, platelet count $> 80 \times 10^9$/L). Reaching CR with incomplete hematological recovery was defined as a CRi. Refractory acute myelogenous leukemia was defined as patients who had not achieved CR after at least two courses of previous-line induction chemotherapy [9, 10].

## 2.4. Cell Lines and Cell Cultures.
HL60, THP1, and Panc-1 cells were purchased from the Xiangya School of Medicine Type Culture Collection (Changsha, China). RPMI-1640 medium was used to culture HL60 and THP1 cells. DMEM was used to culture Panc-1 cells. All cells were cultured at 37°C with 5% $CO_2$ in a cell incubator. Complete medium contained culture medium, 10% FBS and 1% antibiotics.

## 2.5. Antibodies and Reagents.
Antibodies against GAPDH (#97166S, 1 : 1000), PARP (#9542S, 1 : 1000), caspase-3 (#9662S, 1 : 1000), cleaved caspase-3 (#9664S, 1 : 1000), caspase-8 (#9746S, 1 : 1000), cleaved caspase-8 (#9496S, 1 : 1000), Bax (#5023S, 1 : 1000), Bcl-2 (#15071S, 1 : 1000), p-RIP1 (Ser166; #65746S, 1 : 1000), RIP3 (#95702S, 1 : 1000), anti-rabbit IgG, HRP-linked antibody (#7074S, 1 : 3000), and anti-mouse IgG, HRP-linked antibody (#7076S, 1 : 3000) were obtained from Cell Signaling Technology (Danvers, MA, USA). Antibodies against MLKL (#ab184718, 1 : 1000), p-MLKL (Ser358; #ab187091, 1 : 1000), RIP1 (#ab72139, 1 : 1000), and p-RIP3 (Ser227; #ab209384, 1 : 1000) were purchased from Abcam (Cambridge, UK). Cladribine, homoharringtonine, Z-VAD-FMK, cycloheximide, chloroquine, ferrostatin-1, and necrostatin-1 were purchased from TargetMol (Shanghai, China). Aclarubicin was obtained from APExBIO (Houston, TX). A stock solution containing cladribine, homoharringtonine, and aclarubicin was prepared in DMSO and kept at -80°C. TNF-alpha was purchased from ABclonal (Wuhan, China).

## 2.6. Growth Inhibition Assay.
The inhibition of HL60 or THP1 cell growth was assessed with CCK-8 (Dojindo, Japan). Cells ($8 \times 10^5$/mL) were seeded in 96-well plates with different concentrations of homoharringtonine, cladribine, and aclarubicin. $10 \, \mu$L of CCK-8 solution was added to each well after 24 or 48 hours in culture. The 96-well plates were incubated in a cell incubator in the dark for 4-6 hours, and then, the absorbance at 450 nm was measured using a microplate reader (Thermo Multiskan MK3, USA). The cell line experiments were repeated at least three times with three replicates for each trial.

TABLE 1: Patient baseline characteristics.

| Characteristics | Patient | | | | |
| --- | --- | --- | --- | --- | --- |
| | Case 1 | Case 2 | Case 3 | Case 4 | Case 5 |
| Age (yrs) | 6.3 | 1.6 | 1 | 8.3 | 13 |
| Gender | F | M | M | F | F |
| WBC at diagnosis ($\times 10^9$/L) | 2.9 | 55.5 | 3.2 | 0.7 | 4.4 |
| Hb at diagnosis (g/L) | 98 | 94 | 106 | 68 | 95 |
| Plt at diagnosis ($\times 10^9$/L) | 317 | 178 | 300 | 36 | 29 |
| FAB subtype | AML-M2 with extramedullary infiltration | AML-M2 | AML-M5 with extramedullary infiltration | AML-M5 | AML-M2 |
| Risk stratification[†] | Intermediate | Favorable | Intermediate | Intermediate | Intermediate |
| Primary induction chemotherapy | DAE [1], IAE [1] | DAE [2], HA [1] | MAG [2], HA [1] | MAG [2] | DAE [2] |
| Stage before HCA | Primary induction failure[‡] | Primary induction failure | Primary induction failure | Primary induction failure | Primary induction failure |
| Bone marrow blast pre-HCA (%) | 32.5 | 16 | 12.5 | 20 | 20.5 |
| Bone marrow blast post-HCA (%) | 2.5 | 2 | 1 | 0 | 2 |
| Karyotype analysis of AML | Normal | Normal | 48, XY, +19, +21 | Normal | Normal |
| Translocations/fusion gene analysis | Normal | AML1-ETO+ | Normal | Normal | Normal |

[†]According to the 2017 risk stratification by the European Leukemia Net (ELN) [11]. [‡]As defined by lack of response (complete or partial remission) after one course of induction chemotherapy or persistent leukemia after at least two courses of induction chemotherapy. WBC: white blood cell count; Hb: hemoglobin; Plt: platelets; IAE: regimen of idarubicin, etoposide, and Ara-C; HA: regimen of homoharringtonine and Ara-C. The number in "[]" refers to the number of the treatment cycle.

*2.7. Evaluation of Apoptosis.* Cells were treated with different combinations of reagents for 48 hours, collected in EP tubes, washed twice with PBS, and resuspended by adding binding buffer. Apoptotic cells were stained using an Annexin V-FITC/PI Apoptosis Detecton kit (GEnView, Beijing, China). Then, $5\,\mu$L of Annexin V-FITC and $5\,\mu$L of PI were successively added to the cells according to the instructions. Apoptotic cells were detected using a CytoFLEX flow cytometer (A00-1-1102, Beckman Coulter) after 15-minutes of incubation at room temperature in the dark. Early apoptotic cells showed Annexin V-FITC$^+$/PI$^-$, and late apoptotic cells showed Annexin V-FITC$^+$/PI$^+$.

*2.8. Hoechst/PI Double Staining.* HL60 and THP1 cells were treated with different combinations of reagents for 24 hours, Hoechst 33342 was added to the culture medium at a final concentration of $2\,\mu$g/mL, incubated at 37°C for 10 minutes, harvested, and washed with PBS. Then, the cells were resuspended in PBS containing PI ($10\,\mu$g/mL), incubated at 4°C in the dark for 15 minutes, and washed with PBS. The cell death rate was calculated by determining the Hoechst$^+$/PI$^+$ ratio.

*2.9. Western Blot Analysis.* Cells were collected into EP tubes, washed twice with PBS, lysed using RIPA buffer containing protease and phosphatase inhibitors, incubated on ice for 30 minutes, subjected to high-speed centrifugation and collected from the protein solution. The protein concen-

tration was measured with a BCA (Thermo Fisher Scientific). The calculated protein solution was mixed with 4× SDS sample buffer, boiled at 100 degrees for 10 minutes, and then separated by electrophoresis. After electrophoresis, proteins were transferred to PVDF membranes, blocked with 5% nonfat milk for 1 hour on a shaker, and incubated with primary antibodies overnight at 4°C. Primary antibodies were washed with TBST and then incubated with secondary antibodies for 2 hours at room temperature (RT) on a shaker. Finally, the membranes were washed with TBST, and the proteins were visualized using an ECL kit (GEnView, Beijing, China).The membranes were scanned and analyzed using a gel imager (Bio-Rad-1708195, USA).

## 3. Results

*3.1. Patient Baseline Characteristics.* Between June 2019 and December 2019, a total of 5 AML patients treated with the HCA regimen were reviewed. Patient baseline characteristics are summarized in Table 1. Among the 5 patients, 2 were boys and 3 were girls. The median age at initial diagnosis was 6.3 years (range, 1-13 years). Two patients received the mitoxantrone, Ara-C, and G-CSF (MAG) regimen, and 3 patients received the daunorubicin, Ara-C, and etoposide (DAE) regimen as their first-line induction therapy. These children with AML failed CR after 2-3 cycles of conventional chemotherapy and then received the HCA regimen.

TABLE 2: Response and outcomes.

| Patient | Response | Subsequent therapy (number of cycles) | Outcome | DFS (mon.) |
|---------|----------|---------------------------------------|---------|------------|
| Case 1 | CR | HA [1], EA [1] | Alive | 28 |
| Case 2 | CR | EA [1] | Alive | 27 |
| Case 3 | CR | HAE [1], Allo-SCT | Alive | 25 |
| Case 4 | CR | Allo-SCT | Alive | 24 |
| Case 5 | CRi | Allo-SCT | Alive | 23 |

DFS: disease-free survival; HA: regimen of homoharringtonine and Ara-C; EA: regimen of Ara-C and etoposide; HAE: regimen of homoharringtonine, Ara-C, and etoposide; Allo-SCT: allogeneic stem cell transplantation. The number in "[]" refers to the number of the treatment cycle.

TABLE 3: Safety and toxicity.

| Adverse event | Number of events | % |
|---------------|------------------|---|
| Hematological toxicities | | |
| Neutropenia (IV) | 10 | 100 |
| Thrombocytopenia (IV) | 8 | 80 |
| Nonhematological toxicities | | |
| Infections | | |
| Febrile neutropenia | 6 | 60 |
| Sepsis | 2 | 20 |
| Pneumonia | 2 | 20 |
| Soft-tissue infection | 1 | 10 |
| Intestinal infection | 5 | 50 |
| Mucositis | 2 | 20 |
| Elevated bilirubin | 3 | 30 |
| Elevated ALT | 4 | 40 |
| Decreased cardiac function | 0 | 0 |

### 3.2. Treatment Administration, Response, and Outcome.

Five children with AML who failed to achieve CR after two to three courses of conventional chemotherapy received the HCA regimen for two cycles. The regimen for HCA treatment was as follows: 5 mg/m$^2$ cladribine on days 1-4, 1 mg/m$^2$ homoharringtonine on days 1-7, and 14 mg/m$^2$ aclarubicin on days 1-4.

All patients received the HCA regimen, and the details of the responses and evaluation of hematological can be seen in Table 2. All AML patients achieved an overall response after treatment with the HCA regimen: 4/5 AML patients achieved CR and 1 achieved CRi.

Among the five patients, one child received 2 courses, and one child received 1 course of consolidation chemotherapy without HSCT and was followed up without other treatment. One child received 1 course of consolidation chemotherapy and underwent allogeneic stem cell transplantation (Allo-SCT) 3 months later. The remaining 2 children did not receive consolidation chemotherapy and underwent Allo-SCT at the 6th and 2nd months after achieving CR using the HCA regimen. As of the last follow-up, all 5 patients were in DFS, ranging from 23 to 28 months.

### 3.3. Safety and Toxicity.

Five patients received a total of 10 cycles of the HCA regimen. All patients developed severe bone marrow suppression due to their disease and chemotherapy. The median time for neutrophil recovery $> 0.5 \times 10^9$/L was 15 days, and that for platelet recovery $> 20 \times 10^9$/L was 6 days. The most common grade 3 to 4 nonhematologic toxicities were febrile neutropenia, pneumonia, sepsis, and intestinal infection. There were treatment-related deaths in this study. The toxicity of the HCA regimen in the children with AML is shown in Table 3.

### 3.4. HCA Synergistically Inhibited HL60 and THP1 Cell Growth.

Homoharringtonine, cladribine, and aclarubicin are commonly used chemotherapeutic drugs for the treatment of leukemia, and they can inhibit proliferation and induce cell death. To examine whether HCA produces synergistic antitumor activity, HL60 and THP1 cells were treated with various concentrations of the three drugs for 24 or 48 hours. The following concentrations were used: cladribine (100, 200, 400, and 800 nmol/L), homoharringtonine (4.5, 9, 18, and 36 nmol/L), and aclarubicin (45, 90, 180, and 360 nmol/L). The half-maximal inhibitor concentration (IC50) values of cladribine, homoharringtonine, and aclarubicin for HL60 at 24 and 48 hours were 1.18 μmol/L and 0.17 μmol/L, 69.59 nmol/L and 11.8 nmol/L, and 1.69 μmol/L and 0.61 μmol/L, respectively ($n = 3$). The IC50 values of cladribine, homoharringtonine, and aclarubicin for THP1 at 24 and 48 hours were 3.57 μmol/L and 0.22 μmol/L, 110.2 nmol/L and 18.22 nmol/L, and 0.41 μmol/L and 0.33 μmol/L, respectively ($n = 3$). The cytotoxicity of the three drugs to HL60 and THP1 cells was measured with CCK-8 assays. The inhibition of HL60 or THP1 cell growth was concentration dependent. Our data show that HCA has a significantly higher inhibitory effect on AML cells than any monotherapy or two-drug combination (Figures 1(a) and 2(a)) ($P < 0.01$). The combination index (CI) values when HCA was applied to AML cells were analyzed using CompuSyn software, and the results showed a synergistic effect (Figures 1(b)–1(e) and 2(b)–2(e)). Thus, HCA had a synergistic inhibitory effect on the growth of AML cell lines.

### 3.5. HCA Induced Apoptosis in HL60 and THP1 Cells.

According to the cell inhibition rate, cladribine, homoharringtonine, and aclarubicin had the strongest inhibitory effect on AML cells at concentrations of 800, 36, and 360 nmol/L, respectively, and these concentrations were used in the next experiment. It has been reported that homoharringtonine, cladribine, and aclarubicin all individually induce apoptosis in leukemia cells. In this study, we investigated whether the combination of these three drugs also promotes apoptotic death in AML cells. We found that HCA-induced cell death was suppressed after the addition of the apoptosis inhibitor Z-VAD-FMK (Figure 3(a)). FACS analyses and Hoechst/propidium iodide (PI) staining indicated that HCA could induce apoptosis in AML cells (Figures 3(b) and 3(c)).

Western blotting was used to evaluate the expression of apoptosis-related proteins including Bcl-2, Bax, and caspase family members in AML cells after treatment with HCA for

(a)



(b)



(c)



(d)



(e)

FIGURE 1: The growth inhibition and CI in HL60 cells treated with different combinations. The rate of growth inhibition induced by cladribine (2-CdA), homoharringtonine (HHT), aclarubicin (ACR), 2-CdA+HHT, 2-CdA+ACR, HHT+ACR, and 2-CdA+HHT+ACR (HCA) in HL60 cells (a) for 24 hours. CI values for 2-CdA+HHT combination treatments at a molar ratio of 1:0.045 in HL60 cells (b), 2-CdA+ACR (1:0.45) (c), HHT+ACR (10:1) (d), and 2-CdA+HHT+ACR (1:0.045:0.45) (e). $^{**}P < 0.01$, 2-CdA+HHT+ACR vs. 2-CdA+HHT. $^{\#\#}P < 0.01$, 2-CdA+HHT+ACR vs. 2-CdA + ACR. $^{\&\&}P < 0.01$, 2-CdA+HHT+ACR vs. HHT+ACR.

(a)

(b)

(c)

(d)

(e)
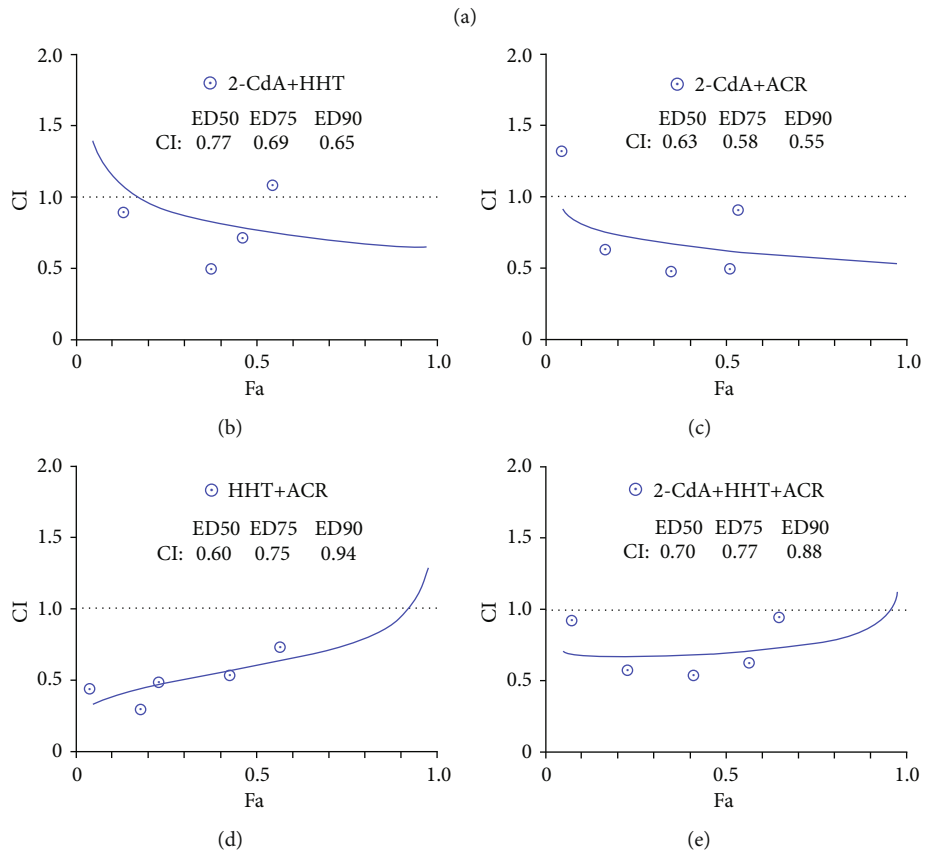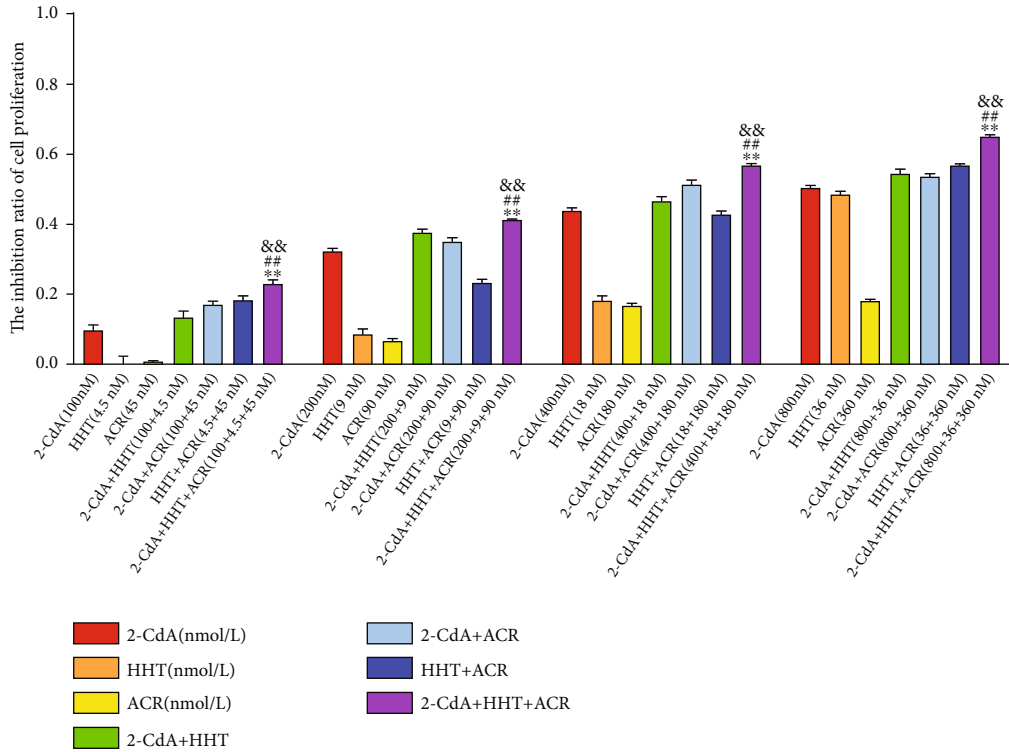
FIGURE 2: The growth inhibition and CI in THP1 cells treated with different combinations. The rate of growth inhibition induced by 2-CdA, HHT, ACR, 2-CdA+HHT, 2-CdA+ACR, HHT+ACR, and HCA in THP1 cells (a) for 24 hours. CI values for 2-CdA+HHT combination treatments at a molar ratio of 1 : 0.045 in THP1 cells (b), 2-CdA + ACR (1 : 0.45) (c), HHT+ACR (10 : 1) (d), and 2-CdA+HHT+ACR (1 : 0.045 : 0.45) (e). $^{**}P < 0.01$, 2-CdA+HHT+ACR vs. 2-CdA+HHT. $^{##}P < 0.01$, 2-CdA+HHT+ACR vs. 2-CdA+ACR. $^{\&\&}P < 0.01$, 2-CdA+HHT+ACR vs. HHT+ACR.
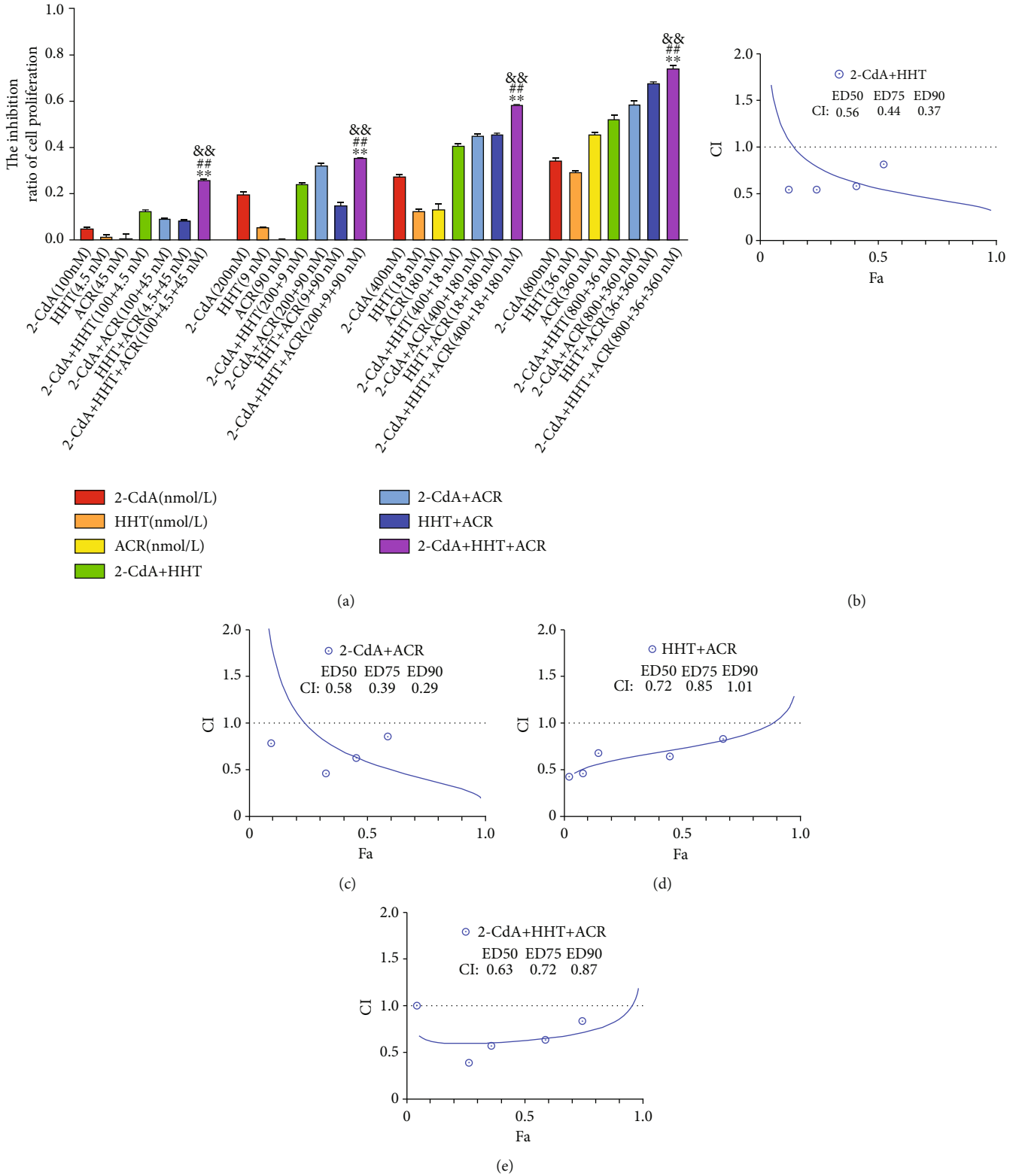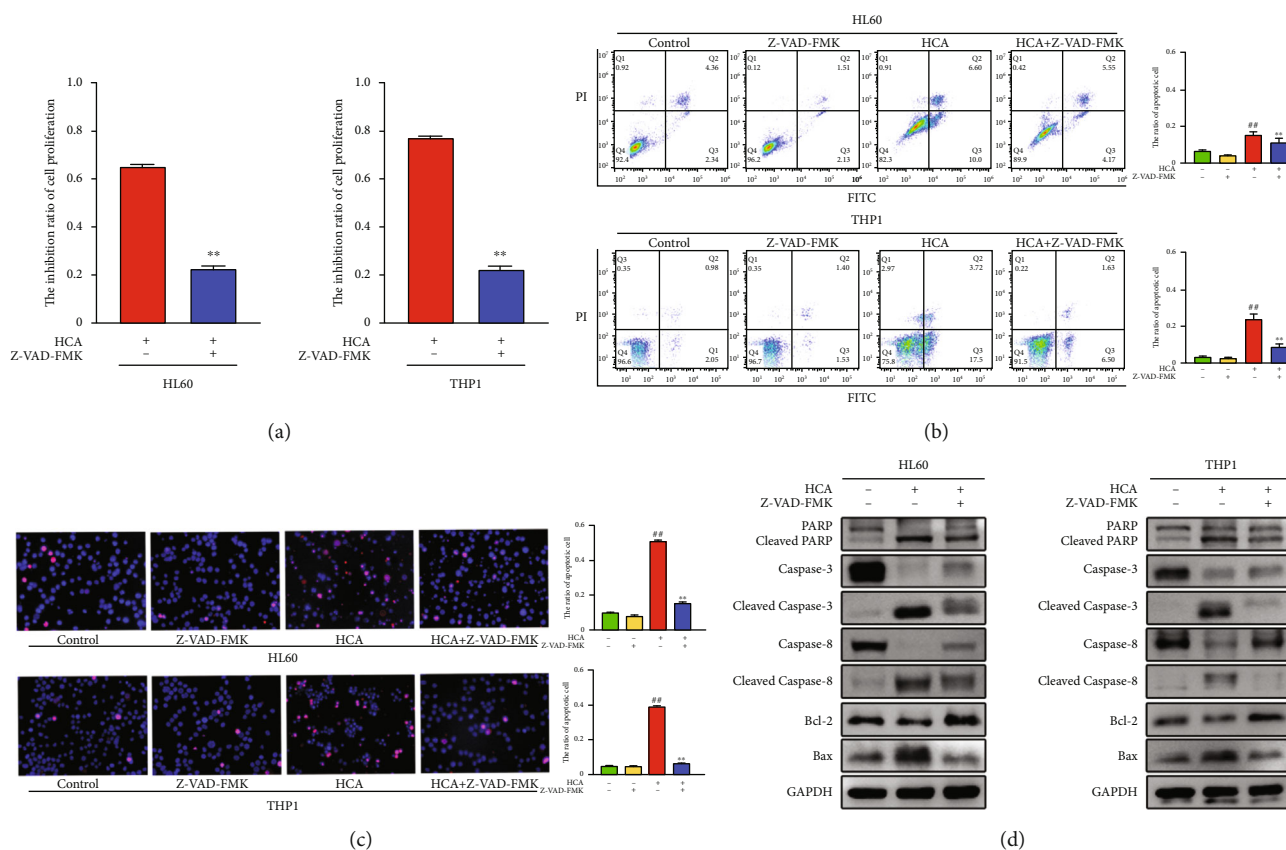
(a)



(b)



(c)



(d)

FIGURE 3: HCA induced apoptosis. HL60 cells and THP1 cells were treated with 2-CdA (800 nM), HHT (36 nM), and ACR (360 nM) for 24 hours. The rate of growth inhibition induced by HCA, with or without 60 $\mu$mol/L Z-VAD-FMK in HL60 cells and THP1 cells (a). The rate of HL60 and THP1 apoptotic cells was measured by flow cytometry (b). Hoechst/PI staining showed that HL60 and THP1 cells in the HCA group had the highest number of condensed and/or fragmented nuclei, and it was lower in the Z-VAD-FMK groups (c). Western blot analysis of apoptotic protein expression after 24 hours (d). $^{##}P < 0.01$, HCA vs. control. $^{**}P < 0.01$, HCA+Z-VAD-FMK vs. HCA.

24 hours. As expected, PARP, caspase-3, and caspase-8 were cleaved in HL60 and THP1 cells after treatment with HCA, indicating that HCA could induce apoptosis in these cell lines (Figure 3(d)). Furthermore, a pronounced increase in Bax and a decrease in Bcl-2 were induced by the combination treatment (Figure 3(d)). The expression patterns of these apoptosis-inducing proteins were reversed after adding Z-VAD-FMK.

To determine whether the combination therapy with HCA also affects other modes of cell death in AML cells, we added necrostatin-1, a necroptosis inhibitor, chloroquine, an autophagy inhibitor, or ferrostatin-1, a ferroptosis inhibitor. As shown, HCA-mediated cell death was partially suppressed only after the addition of necrostatin-1 (Figure 4(a)). Then, Western blotting was used to observe the expression and activation of RIP1, RIP3, and MLKL as biomarkers of necroptosis. Interestingly, the target proteins were not activated but instead were cleaved in HL60 cells and THP1 cells treated with HCA or HCA+necrostatin-1 for 24 hours (Figure 4(b)). To explore whether necroptosis occurs when cell apoptosis is compromised, an apoptosis inhibitor was added when cell death was induced with HCA. We found that the expression level of RIP1 was increased, but there were no changes in the phosphorylation of RIP1, RIP3, and MLKL after the addition of Z-VAD-FMK

(Figure 4(c)). Altogether, these results demonstrated that combination therapy with HCA induced apoptotic cell death in AML cells.

## 4. Discussion

Despite enhanced therapeutic management of children with RR-AML, the prognosis remains poor, and their management remains a substantial clinical challenge with few available therapeutic options. It is widely accepted that HSCT is the best treatment option and the only hope to cure them. The mortality rate after HSCT remains high in children who receive repeated intensive chemotherapy. The remission status before transplantation is one of the risk factors associated with the long-term survival rate after transplantation [12–14]. It has been stated that the 5-year overall survival (OS) probabilities of patients undergoing transplantation after second CR, relapse, and primary induction failure are 45%, 20%, and 12%, respectively [15]. The final prognosis of children with refractory AML receiving HSCT without achieving a CR was much worse than that of children with relapsed AML [13]. Intensive salvage second-line therapy can cause increased infection-related mortality in RR-AML, and associated toxicities as well as anticipated future adverse effects must be considered [16].
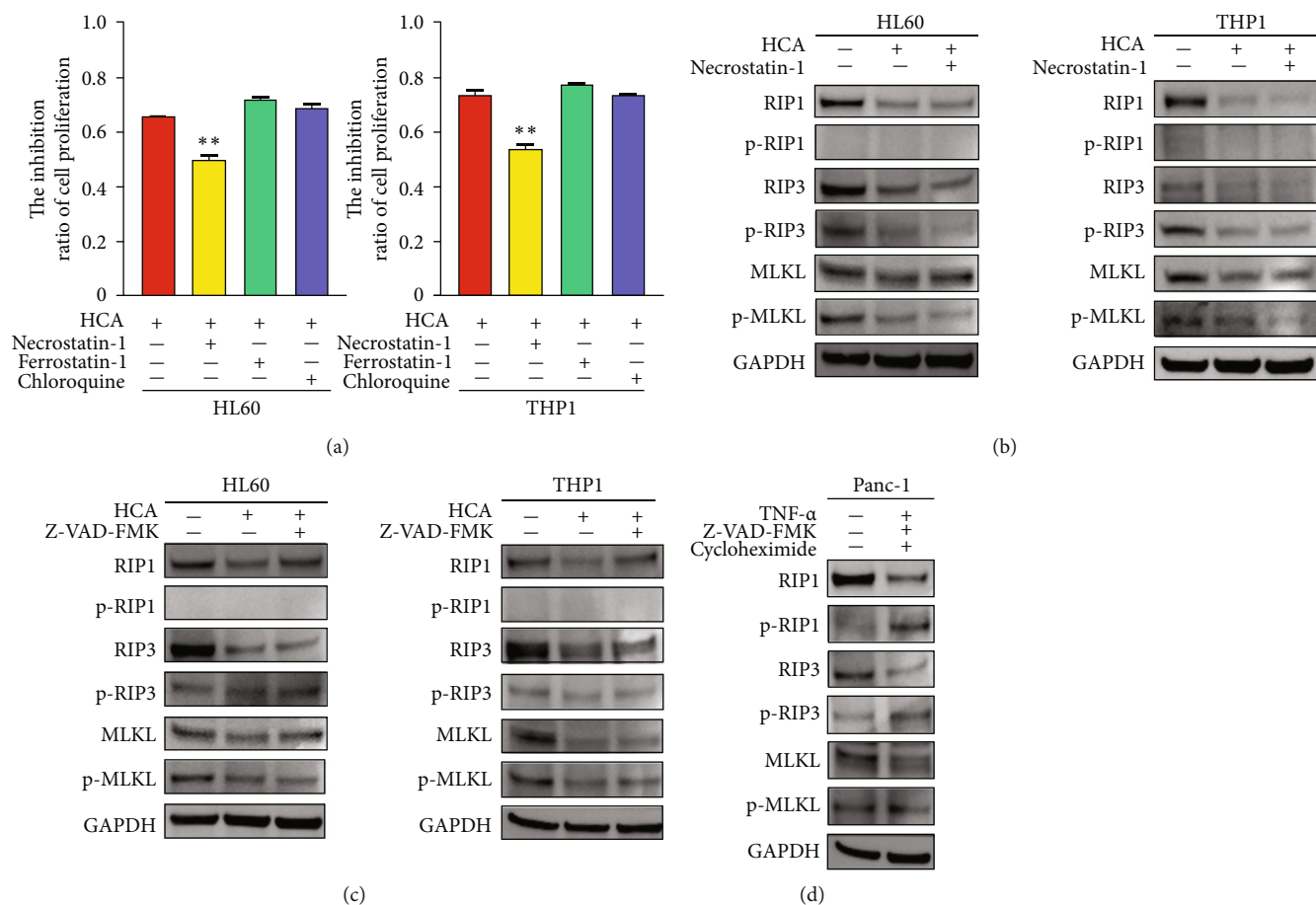
FIGURE 4: HCA induced other death modes of AML cells. The rate of growth inhibition induced by 2-CdA+HHT+ACR, with or without different inhibitors in HL60 and THP1 cells (a). Western blot analysis of necroptosis protein expression after 24 h (b). Western blot analysis of necroptosis protein expression after 24 hours (c). Western blot analysis of necroptosis protein expression in Panc-1 cells following treatment with or without TNF-alpha (20 ng/mL), cycloheximide (10 ng/mL), and Z-VAD-FMK (20 $\mu$M) for 12 hours as positive control (d). $^{**}P < 0.01$, HCA+necrostatin-1 vs. HCA.

Investigating optimal reinduction therapies to achieve as many CR as possible and bridge to HSCT thereafter is crucial.

Some reported chemotherapeutic regimens (administered as monotherapies or in combination), such as nucleoside analogs (clofarabine, cladribine, and fludarabine), tyrosine kinase inhibitors (sorafenib), proteasome inhibitors (bortezomib), epigenetic therapies (decitabine), and immunotherapies (gemtuzumab ozogamicin), have been used in children with RR-AML [17]. The fludarabine, Ara-C, and G-CSF (FLAG) combined with anthracycline regimens in children and young adults with RR-AML were reported to produce a CR rate of 69%-74% [18, 19], but the profound myelosuppression, high treatment-related death rate, and final low long-term survival rate have limited the use of this regimen as a standard reinduction chemotherapy protocol for pediatric RR-AML [20, 21].

Cladribine, the first-generation deoxyadenosine analog, has unique antileukemic properties. In the treatment of adult AML, the addition of cladribine to the standard regimen has a better efficacy [22]. For AML patients with relapse or poor risk stratification, the cladribine, Ara-C, and G-CSF (CLAG) regimen could effectively improve CR and prolong

OS compared with the FLAG regimen [23]. Cladribine has been found to have activity against AML in children when combined with Ara-C or topotecan [4]. Studies on the CR rate of reinduction therapy with cladribine in pediatric RR-AML are rare, and the results significantly differ. Clinical studies have reported that Ara-C combined with the cladribine regimen did not achieve CR after 2 cycles of treatment in 9 adolescent AML patients [24], while the CLAG regimen achieved a 75% CR rate in 12 children with RR-AML [3]. Although cladribine combined with anthracycline salvage chemotherapy in adult relapsed AML was reported to achieve a relatively high CR rate [25, 26], cladribine plus idarubicin in children with AML experiencing their first relapse achieved a CR rate of only 46% [27].

Aclarubicin is an anthracycline antibiotic isolated from *Streptomyces galilaeus* that exhibits major chemical differences from the conventional anthracyclines daunorubicin (DNR) and adriamycin (ADM). Aclarubicin can not only inhibit the replication and repair of DNA and the synthesis of RNA and proteins [28] but it can also lead to mitochondrial dysfunction [29]. In addition, aclarubicin induces oxidative DNA damage and apoptosis [30]. Aclarubicin-based chemotherapy protocols, Ara-C, aclarubicin, and G-CSF

(CAG) regimen, have better efficacy and safety in initially diagnosed or RR-AML [31–33], which is based mainly on observations in adult AML. Data on aclarubicin in pediatric AML are rare. Chen et al. reported that a CAG regimen in children with AML achieved a 38.4% CR rate without severe adverse effects [34].

Homoharringtonine is a plant alkaloid that is widely used to treat adults with initially diagnosed or RR-AML. Homoharringtonine exerts antitumor effects by inhibiting the synthesis of target proteins, such as phospho-eIF4E, SP1/TET1/5hmC, Smad3, and TGF-$\beta$ pathway components, and NF-$\kappa$B repressing factor [35–38], and promotes apoptosis in leukemia cells. Homoharringtonine-based protocols, such as homoharringtonine, Ara-C, and G-CSF (HAG) and homoharringtonine, Ara-C, and aclarubicin (HAA), appear to be more effective and better tolerated than intensive chemotherapy in the treatment of adult RR-AML [39–43]. Ma et al. reported that HAG+aclarubicin (GHAA), and HAG+pirarubicin (GHTA) are more efficient than conventional HAG without anthracycline, and the efficiency of GHAA/GHTA is positively correlated with B7.1 expression [44]. Satisfactory results can also be obtained when homoharringtonine is used in combination with orafenib or the CAG regimen in the treatment of AML patients [45, 46]. These data reveal the promising potential of HHT as an antileukemic agent. Even children under two years of age with AML can benefit from HHT-containing chemotherapy regimens with tolerable toxicities, such as nausea, vomiting, diarrhea, and mucositis [6, 8]. Few studies have investigated HHT in the treatment of pediatric RR-AML. In a previous study, children with refractory leukemia experienced death after treatment with HHT alone, the reason might be associated with single-drug administration [47].

Identifying new salvage regimens that produce a relatively high CR rate and low toxicity in children with RR-AML is still an area of investigation. For the selection of a treatment regimen for RR-AML, resistance to individual prior therapies and their associated toxicity must be considered. An increased dose of a conventional chemotherapeutic agent, such as Ara-C or daunomycin, is not optimal for pediatric RR-AML because of dose-related toxicities and non-obvious improvements [48]. Based on the above-described results, the efficacy and safety of the CLAG and HAA regimens, and observations for refractory patients at our center who previously received a DAE regimen, an idarubicin, Ara-C, and G-CSF (IAG) regimen or a mitoxantrone, Ara-C, and G-CSF (MAG) regimen as induction chemotherapy, we first administered the HCA regimen, which avoids the use of Ara-C and replaces daunomycin, idarubicin or mitoxantrone with aclarubicin, to children with primary refractory AML. We referred to the CLAG, HAA, and GHAA regimens in adult AML and our previous experiences in the treatment of AML and myelodysplastic syndrome (MDS) and chose 5 mg/m$^2$ cladribine on days 1-4 [49], 1 mg/m$^2$ homoharringtonine on days 1-7, and 14 mg/m$^2$ aclarubicin on days 1-4 as the reinduction protocol for children with primary refractory AML. All five children who did not respond to conventional chemotherapy achieved CR or CRi after two cycles of the HCA regimen, and the CR/CRi

rate was 100%. The most common events were severe myelosuppression, febrile neutropenia, and intestinal infection. All of the toxicities were tolerable and acceptable. No neurological or cardiac events or treatment-related deaths occurred during treatment. The median time for platelet recovery > $20 \times 10^9$/L was 6 days, which was shorter than that reported with the CLAG regimen (13 days), and the median time for neutrophil recovery > $0.5 \times 10^9$/L was 15 days, which was comparable to that reported in a previous study [3]. This suggests that this is a very good combination of traditional drugs.

In vitro, our study showed that homoharringtonine, cladribine, or aclarubicin treatment alone resulted in inhibition of the cell growth of HL60 and THP1 cells in a dose-dependent manner. These findings support the notion that homoharringtonine, cladribine and aclarubicin can inhibit the growth of AML cells [50–53]. To expand our analysis of the synergistic interaction among homoharringtonine, cladribine and aclarubicin, we used HCA to treat the two AML cell lines. Compared with the monotherapies and two-drug combination therapies, HCA exerted a synergistic antiproliferative effect on AML cells (Figures 1 and 2). This study provides persuasive evidence that HCA has a high CR rate in the treatment of refractory AML patients.

Apoptosis is a programmed cell death process and an important mechanism by which chemotherapeutic drugs regulate tumor development [54, 55]. Clinically, chemotherapeutic drugs often achieve therapeutic effects by inducing tumor cell death, such as apoptosis.Cladribine induces apoptosis in human leukemia cells by acting on mitochondria [50], and homoharringtonine regulates leukemia cell differentiation, proliferation, and apoptosis by inhibiting protein synthesis [52, 53]. In our study, HCA significantly induced apoptosis in HL60 and THP1 cells, as confirmed by Annexin V-FITC/PI staining and Western blot analysis (Figure 3).

Necroptosis, a form of cell death defined in recent years, can be inhibited by necrostatin-1. We found that the combined administration of necrostatin-1 or Z-VAD-FMK could reverse HCA-mediated cell death. However, the specific molecular biomarkers evaluated including phosphorylated RIP1, RIP3, and MLKL, which were used to detect the activation of RIP1, RIP3, and MLKL in necroptosis, were not increased after adding Z-VAD-FMK or necrostatin-1 alone to the HCA regimen (Figure 4). This indicated that necroptosis did not occur after HCA administration. In some research, it has been reported that when apoptosis is inhibited, necroptosis is activated as a "fail-safe" mechanism to prevent tumor development [56, 57]. However, in our research, we did not find that necroptosis occurred after apoptosis was inhibited. Furthermore, RIP1, RIP3, and MLKL were found to be degraded after HCA treatment, and RIP1 was able to recover after adding Z-VAD-FMK. The reduced expression of RIP1 and RIP3 confirms the notion that active caspase-8 proteolytically cleaves both RIP1 and RIP3, resulting in their inactivation, which leads to induction of the apoptosis pathway [58]. However, why HCA can affect MLKL expression and why the expression of RIP3 does not recover after the addition of an apoptosis

inhibitor requires further investigation. RIP1 dimerization can phosphorylate RIP1, and phosphorylated RIP1 composes complex IIb with FADD and caspase-8 while RIP3 forms complex IIc [59, 60]. Caspase-8 in complex IIb requires activated RIP1 activation, which then mediates RIP1-dependent apoptosis [61, 62]. Complex IIc mediates necroptosis. RIP1-dependent apoptosis may be involved in HCA-induced AML cell death. This may be why necrostatin-1 can inhibit AML cell death.

## 5. Conclusion

In summary, our study suggests that HCA is a very good combination of traditional drugs. HCA synergistically inhibits AML cells and triggers apoptosis. This is the first evaluation of reinduction therapy with the HCA regimen in pediatric primary refractory AML, and the results show that the HCA regimen can be considered a reinduction salvage strategy in children with refractory AML before HSCT or consolidation therapy.

## Data Availability

The datasets supporting the results of the current study are available from the corresponding author.

## Ethical Approval

The project was approved by the institutional review board (IRB) of Central South University Third Xiangya Hospital (ethics approval no.: 21133, approval date: 3 November 2021).

## Conflicts of Interest

The authors claim no conflict of interest.

## Authors' Contributions

Fenglin Wang, Min Xie, and Minghua Yang conceived and designed the experiments. Fenglin Wang and Min Xie performed the experiments. Minghua Yang supervised the study. Fenglin Wang, Min Xie, Minghua Yang, Pan Chen, and Dan Wang interpreted the results. Fenglin Wang wrote the manuscript. Fenglin Wang and Min Xie contributed equally to this work.

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