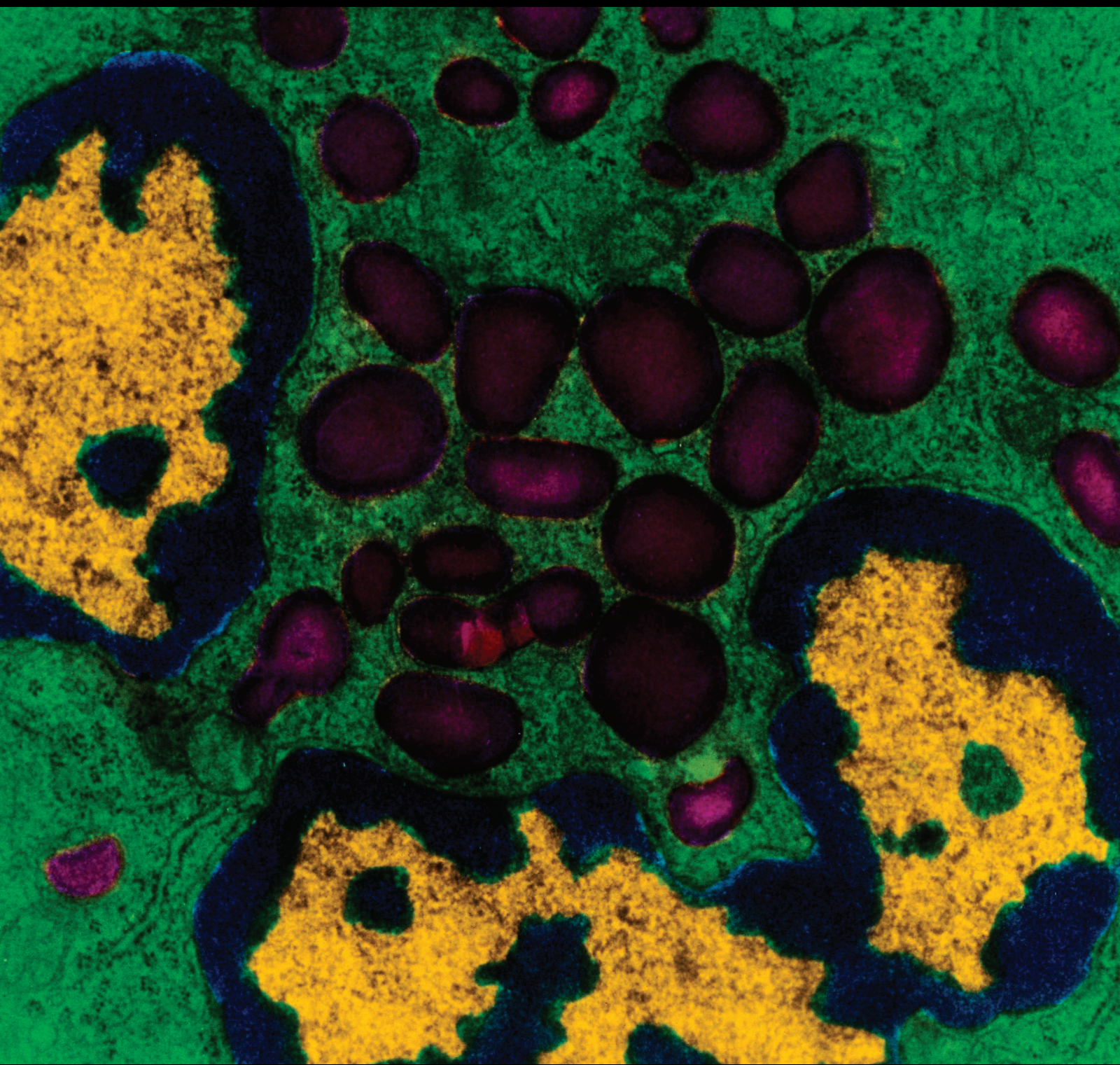
Mediators of Inflammation

Nutrients and Inflammatory Diseases

Guest Editors: Jie Yin, Michael Conlon, and Sung Woo Kim



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Editorial
Nutrients and Inflammatory Diseases

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Inflammation has been widely demonstrated to be involved in various stimuli, such as oxidative stress, bacterial and virus infection, and some physiological process, while a chronic and excessive inflammatory response is a significant risk factor for developing various human diseases [1].

An increasing number of compelling reports are recently published suggesting that some nutrients, like amino acids, oligosaccharides, and short-chain fatty acids, exhibit anti-inflammatory effect [2, 3], which will help the understanding of nutritional contributions in the treatment and control of certain inflammatory diseases. Meanwhile, nutrients show a close relationship with the gut microbiota, which further influences gastrointestinal inflammatory responses [3]. Little is known about how this relationship is affected by dietary nutrients that alleviate inflammation via mediating the composition and richness of the microbiota. Also, molecular mechanisms of selected nutrients functioning to alleviate inflammatory diseases have not been clearly investigated. Thus, there is an urgent need to advance scientific knowledge on nutrients alleviating inflammatory diseases.

The articles contained in this special issue include 4 review papers and 10 original research papers that are focused on characterizing the contribution and molecular mechanisms associated with nutrients and inflammation. A brief description of these 14 works is detailed below.

Amino acids and their metabolites have been widely demonstrated to exhibit anti-inflammatory effect on various inflammatory models, such as inflammatory bowel disease. X. Bao et al. provide a detailed review of the literature on the relationship between amino acids and inflammatory bowel disease in their paper titled "Roles of Dietary Amino Acids and Their Metabolites in Pathogenesis of Inflammatory Bowel Disease."

In the paper titled "Role of Uric Acid Metabolism-Related Inflammation in the Pathogenesis of Metabolic Syndrome Components Such as Atherosclerosis and Non-alcoholic Steatohepatitis," A. Kushiyama et al. outline the molecular mechanisms underlying inflammation occurrence in relation to uric acid metabolism.

Inflammation contributes to the development of various metabolic diseases, such as obesity and diabetes. S. Chen et al. review the anti-inflammatory properties of quercetin in relation to obesity and type 2 diabetes in the paper titled "Therapeutic Effects of Quercetin on Inflammation, Obesity, and Type 2 Diabetes."

Intestinal microbiota is highly involved in host physiology and pathology through activity of the microbiome and its metabolic products. "Diet-Intestinal Microbiota Axis in Osteoarthritis: A Possible Role" by Y. Li et al. concludes that intestinal microbiota is a major hidden risk factor for osteoarthritis and an important explanation for person-level risk factors.

Four groups from R. Garib et al., H. Xiao et al., H. Ni et al., and M. Li et al. investigated the anti-inflammatory effects of amino acids on different inflammatory models.

The papers include "Effect of Previous High Glutamine Infusion on Inflammatory Mediators and Mortality in an Acute Pancreatitis Model," "N-Acetyl-L-cysteine Protects the Enterocyte against Oxidative Damage by Modulation of Mitochondrial Function," "Effects of Glutamate and Aspartate on Serum Antioxidative Enzyme, Sex Hormones, and Genital Inflammation in Boars Challenged with Hydrogen Peroxide," and "High-Methionine Diet Attenuates Severity of Arthritis and Modulates IGF-I Related Gene Expressions in an Adjuvant Arthritis Rats Model."

In the article "In Vitro Anti-Inflammatory Effects of Three Fatty Acids from Royal Jelly," Y.-F. Chen et al. evaluate and compare the in vitro anti-inflammatory effects of three fatty acids on lipopolysaccharide-stimulated RAW 264.7 macrophages and find that MAPK and NF-κB signaling pathways involve in the mechanism of anti-inflammatory effects of fatty acids from royal jelly.

In rats infected with enterotoxigenic *Escherichia coli*, G. Liu et al. find that dietary chitosan markedly alleviated intestinal inflammation. The paper is titled "Chitosan Modulates Inflammatory Responses in Rats Infected with Enterotoxigenic *Escherichia coli*."

Traditional medical plants and plant extracts have been widely explored to treat inflammatory diseases. In this issue, two articles about curcumin and *Veronicastrum axillare* were collected: "Curcumin Alters Neural Plasticity and Viability of Intact Hippocampal Circuits and Attenuates Behavioral Despair and COX-2 Expression in Chronically Stressed Rats" by G.-Y. Choi et al. and "*Veronicastrum axillare* Alleviates Lipopolysaccharide-Induced Acute Lung Injury via Suppression of Proinflammatory Mediators and Downregulation of the NF-κB Signaling Pathway" by Q. Ma et al.

A clinical trial titled "The Effect of Immunonutrition on the Postoperative Complications in Thymoma with Myasthenia Gravis" by Y. Xin et al. concludes that preoperative immunonutrition support is effective in reducing postoperative complications in patients of thymoma with myasthenia gravis.

High-mobility group box 1 protein (HMGB1) and autophagy are vital to maintain cellular homeostasis and protect against inflammatory response. In the paper titled "Regulation of Autophagy-Related Protein and Cell Differentiation by High Mobility Group Box 1 Protein in Adipocytes" by H. Feng et al., it focuses on the relationship between HMGB1 and autophagy in adipocytes.

Acknowledgments

We would like to thank all authors and reviewers who contributed to this special issue. We hope that this collection aids the development of nutritional strategies for the treatment and/or prevention of inflammatory diseases.

Jie Yin
Michael Conlon
Sung Woo Kim

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Review Article

Roles of Dietary Amino Acids and Their Metabolites in Pathogenesis of Inflammatory Bowel Disease

Xianying Bao,[1,2,3,4] Zemeng Feng,[2,3,4,5] Jiming Yao,[6] Tiejun Li,[1,2,3,4,5] and Yulong Yin[1,2,3,4]

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Inflammatory Bowel Disease (IBD) is a kind of chronic inflammation, which has increasing incidence and prevalence in recent years. IBD mainly divides into Crohn's disease (CD) and ulcerative colitis (UC). It is hard to cure IBD completely, and novel therapies are urgently needed. Amino acids (AAs) and their metabolites are regarded as important nutrients for humans and animals and also play an important role in IBD amelioration. In the present study, the potential protective effects of AAs and their metabolites on IBD had been summarized with the objective to provide insights into IBD moderating using dietary AAs and their metabolites as a potential adjuvant therapy.

1. Inflammatory Bowel Disease Prevalence and Increasing Incidence

Inflammatory bowel disease (IBD) is a kind of chronically multifactorial inflammatory disorder affecting the gastrointestinal tract [1]. It is triggered as a consequence of excessive proinflammatory cytokines production, persistent macrophage activation, and cell death induced by subsequent bacterial or/and viral infections. IBD is the major intestinal health concerns causing severe diarrhea, abdominal pain, weight loss, metabolic disorder, and malabsorption, and it mainly comprises two major forms: Crohn's disease (CD) and ulcerative colitis (UC) [2, 3]. CD and UC have different inflammatory location in the gastrointestinal tract. CD affects all layers of the gastrointestinal tract and is associated with excess expression of IL-12/IL-23 and IFN-γ/IL-17, while UC mainly occurs in colon affecting the mucosa with primarily excess production of IL-13 [3, 4]. Currently, the incidence and prevalence of IBD are increasing with time around the world, especially the rate within elderly patients [5]. The developed countries (Europe and North America) present the highest occurrence [6]. In the West, the prevalence of IBD is 37 to 249 cases per 100,000 people for UC and from 26 to 319 cases per 100,000 people for CD [6, 7]. By contrast, Eastern Europe, Asia, and other developing countries have a lower IBD incidence [7–9]. The occurrence of UC in Asian countries is higher than that of CD [10].

The pathogenesis of IBD is complex and still unclear. Mounting evidences suggest that IBD is the consequence of abnormal immune regulation induced by genetic and/or environmental factors (e.g., diet, infection) [1, 11]. Various inflammation mediators including reactive oxygen species

and proinflammatory cytokines (NF-κB, cytochrome c, and tumor necrosis factor- (TNF-)-α) act as predisposing factors for chronic inflammatory diseases [12]. The typical clinical inflammation of the gastrointestinal tract leads to IBD with excessive production of proinflammatory mediators and loss of the intestinal mucosal barrier integrity. Since there is no complete cure for IBD, the therapies of IBD primarily focus on inducing or maintaining remission and promoting the expression of anti-inflammatory genes [3].

At the present, the conventional treatments of IBD such as biological therapies targeting specific cytokines or pathways and clinical medication have been improved in recent years. Mesalamine, antibiotics, and budesonide are used in patients with mild disease status, while patients with moderately severe IBD take systemic corticosteroids, immunemodulators (thiopurine analogues, azathioprine (AZA), 6-mercaptopurine (6-MP), and methotrexate), and anti-TNFα agents (infliximab, adalimumab, and certolizumab pegol) [13]. However, proper administration selection becomes a common clinical dilemma with those different kinds of medicines. The conventional treatments in severe IBD have a short-term favorable prognosis, while there is still a challenge in the development of alternative therapies character with low-risk and long-term outcomes [14]. Besides, treatments mentioned above are proved to have limited efficacy, adverse effects, drug interactions, and potential toxicity [15, 16]. The use of AZA and 6-MP in IBD patients leads to serious adverse drug reactions such as hepatotoxicity, pancreatitis, and gastrointestinal disturbances [17]. Immunosuppressive and anti-TNF therapy in IBD cause dermatological adverse effects, including skin infections, drug hypersensitivity, psoriasis, eczema, and nonmelanomatous skin cancer [18, 19].

In most cases, patients suffering IBD usually face long duration which becomes a considerable economic burden. With the development of medical and life science, a large number of new strategies are provided to treat IBD. Functional nutrients have aroused growing interest, which help prevent or remit malnutrition, moderate the mucosal immune response, and benefit intestinal homeostasis [20–22]. Essential nutrient has the potential to ameliorate restore redox balance and inflammation in the gastrointestinal tract showing the possibility of nutrients in IBD treatment [3]. Part of the nutrients that benefit the management of IBD had been listed in Table 1.

2. Amino Acids: Application in Inflammatory Bowel Disease Therapies

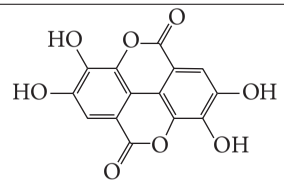
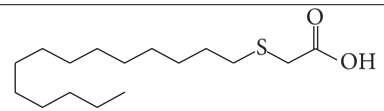
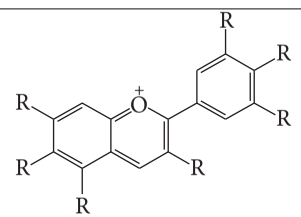
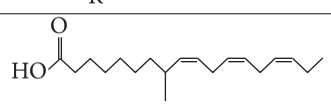
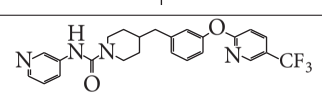
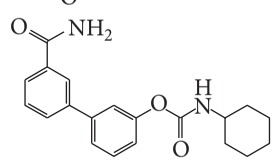
Among IBD benefit nutrients, amino acids (AAs) act as the key regulatory factor in metabolic pathways controlling and have important effects on keeping the intestinal health. AAs are considered as the building blocks for protein synthesis and also play major roles in other functions, such as cell signaling, gene expression, intracellular protein turnover, maintenance, reproduction, oxidative stress, and immunity [46, 47]. Systemic inflammation can cause malnutrition symptoms and general glutamine (Gln) deprivation which is associated with depression, muscle loss, and emotional fatigue [48]. Both UC and CD disturb AAs metabolism in serum and plasma by increasing levels of isoleucine (Ile) (and its first degradation product 3-methyl-2-oxovalerate), methionine (Met), lysine, glycine (Gly), arginine (Arg), and proline (Pro), while decreasing levels of valine, tyrosine, and serine [49]. Some of the increased AAs were also reported to be increased in fecal extracts [50], while Ile and leucine (Leu) have apparently low concentrations in colonic mucosa of active IBD [51]. Met is an essential amino acid and a precursor of homocysteine, a metabolite shown highly elevated in both plasma and colonic mucosa from IBD patients [52].

AAs also have trophic and cytoprotective effects on health in humans and animals [53, 54]. T cells are regarded as central effectors of the adaptive immune system. T helper (Th) cells (Th1, Th2, and Th 17 cells) differentiate from native $CD4^+$ T cells and are involved in the pathogenesis of several inflammatory immune-mediated disorders such as producing different cytokines in immune responses [55]. CD is a Th1-type T cell-mediated inflammation while UC is a Th2-type T cell-mediated inflammation [4]. AAs directly serve as a fuel source for T cells and are considered to have influence on shaping T cell-mediated immune responses [56]. Additionally, the signaling central integrator of environmental stimuli for the regulation of T cell activation and differentiation is mammalian target of rapamycin (mTOR) [57, 58]. mTOR have two distinct protein complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). In T cells, AAs work as signaling molecules with mTORC1 acting as a key mediator. AAs regulate the intracellular localization and activation of mTORC1 by the lysosome-based signaling system composed of Ras-related GTPases (Rags) and Regulator v-ATPase, GAP activity towards Rags, and folliculin complexes [59, 60]. AAs could protect whole body and muscle from protein loss *via* mTOR activation and downstream signaling to protein synthesis through mTORC1 in the acute phase of inflammation [61, 62]. Leu-enriched diet accelerated recovery from muscle damage by alleviating excessive expression of proinflammatory cytokine and preventing invasion of inflammatory cells into muscle [63]. Otherwise, general controlled nonrepressed (GCN2) kinase is a key orchestrator of the integrated stress response which senses AAs depletion. Acute AAs starvation in mice protects the symptoms of colitis, limits Th17 cells, and suppressed IBD via a GCN2-dependent mechanism, providing another mechanism of AAs in regulating IBD [64].

In conclusion, growing evidence shows that the anti-inflammatory activities of tryptophan (Trp), Gln, Met, cysteine (Cys), and Arg have been well established, suggesting a therapeutic role of AAs in IBD, which were listed in Table 2. It is necessary to illustrate the biological activity of specific immunomodulatory AAs in IBD.

2.1. Tryptophan. Trp is an essential AA for human and animals and plays an important role in inflammatory regulation beyond building block for proteins synthesis [65]. The serious Trp concentration shows a highly inverse correlation with disease activity in IBD patients [66]. Dietary supplementation of Trp can reduce IBD through its immune-regulatory metabolites [35, 36]. In a porcine model of dextran-sodium sulfate- (DSS-) induced colitis, Trp administration at 80% of

Table 1: Studies on dietary nutrients supplementation for the management of IBD.

Nutrients	Primary components	Chemical structure	Functions	Ref.
Corabion	A mixture of vitamin C, vitamin E, ω3-PUFAs (EPA and DHA), and Arg		Reduction of DAI, neutrophil recruitment, oxidative stress, proinflammatory cytokines, and E-cadherin internalization; attenuation of colon shortening and tissue damage	[23]
Pomegranate extract	Ellagic acid		Reduction of MPO activity, TNF-α levels, (COX-2), iNOS overexpression, and MAPKs phosphorylation; preventing NF-κB translocation	[24, 25]
Krill oil	ω3-PUFAs and phospholipids		Reduction of DAI, HCS, colon length, and protein oxidation markers; improvement of Pparglα and (PG)E3 expression	[26]
Tetradecylthioacetic acid	An artificial 16-carbon fatty acid with a sulphur-substitution in the β-position		Reduction of colonic oxidative damage and colon wall thickness; improvement of expression of Pparglα; inhibition of the production of inflammatory cytokines (TNF-α, IL-1β, and IL-6)	[27]
Fibre	Nonstarch polysaccharides, resistant oligosaccharides, analogous carbohydrates, and lignin		Maintain remission and reduce lesions of the intestinal mucosa	[28, 29]
Anthocyanins	Natural agents derived from strawberry, blueberry, barberry, and other plants		Cytoprotection; remission of oxidative stress and inflammatory cytokines; suppression of cellular signaling pathways of inflammatory processes	[30]
α-Linolenic acid	ω3-PUFA: plant-derived oil		Inhibition of the production of IL-6 and TNF-α; reduction of cell apoptosis, intestinal permeability, and bacterial translocation; improving histological repair	[31, 32]
FAAH inhibitors	PF-3845		Possess anti-inflammatory effect in TNBS-induced colitis in mice; alter the levels of endocannabinoids	[33]
	FAAH-II		Inhibition of inflammatory miRNAs and cytokines; reduction of the number of activated T cells, the frequency of macrophages, and neutrophils in the colon	[34]

EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; (COX-2), cyclooxygenase-2; iNOS, inducible nitric oxide synthase; MAPKs, mitogen-activated protein kinases; MPO, myeloperoxidase; NF-κB, nuclear transcription factor kappa B; PUFAs, polyunsaturated fatty acids; DAI, disease activity index; HCS, histological combined score; Ppargla, PPAR-g coactivator 1α; (PG)E3, prostaglandin-E3; IL-1β, interleukin-1β; PUFA, polyunsaturated fatty acids; FAAH, fatty acid amide hydrolase.

Table 2: Application of partial AAs in inflammatory bowel disease therapies.

AAs	Dose	Administration method	Models	Major functions and effects	Ref.
Tryptophan	88 mg/kg·BW·day	Intragastric infusion	Piglets	Amelioration of clinical symptoms; reduction of gut permeability and cell apoptosis; inhibition of the production of inflammatory cytokines (TNF-α, IL-6, IFN-γ, IL-12p40, IL-1β, and ICAM-1)	[35]
Melatonin	20 mg/kg·BW·day	Intragastric infusion	Rats	Antioxidant; inhibition of the production of inflammatory cytokines (TNF-α, COX-2, SOD, and NF-κB); accelerating healing of gastric ulcer	[36]
Glutamine	25% of the total nitrogen	Dietary	Mice	Anti-inflammation; reduction of expression of PSGL-1, LFA-1, and CCR9 by Th cells	[37]
Glutamate	12 μg Glu/0.3 μL saline	Microinjection	Rats	Neurotransmitter; inhibition of T-cell response and inflammation	[38]
Methionine	0.12% L-Met	Dietary	Piglets	Protection of the small-intestinal mucosa	[39]
Cysteine	144 mg/kg·BW·day	Intragastric infusion	Piglets	Reduction of intestinal permeability and cell apoptosis; inhibition of the local expression of inflammatory mediators (IL-6, TNF-α, IL-12p40, and IL-1β)	[40]
Histidine	Not mentioned	Dietary	Mice	Reduction of histologic damage, colon weight, IL-6, and TNF-α production; inhibition of NF-κB	[41]
Arginine	1% (wt/vol) solution	Drinking water	Mice	Improvement of the clinical parameters and body weight loss; reduction of the colonic permeability; reduction of the proinflammatory cytokine and chemokine expression	[42]
Glycine	5% Gly	Dietary	Rats	Diarrhea amelioration; prevention of the increases of IL-1β and TNF-α	[43]
Taurine	30 mM	Rectal administration	Rats	Anti-inflammation; inhibiting NF-κB activity	[44]
	2%	Drinking water	Mice	Inhibitory effects on the secretion of MIP-2; cytoprotective functions on the epithelial barrier	[45]

TNF-α, tumor necrosis factor-α; IL, interleukin; IFN-γ, interferon-γ; ICAM-1, intracellular adhesion molecule-1; PSGL-1, P-selectin glycoprotein ligand-1; LFA-1, leukocyte function-associated antigen-1; CCR9, C-C chemokine receptor type 9; MIP-2, macrophage inflammatory protein 2.

the daily recommended intake could ameliorate colitis clinical symptoms, improve histological parameters and intestinal permeability, reduce the expression of proinflammatory cytokines, and increase the expression of proapoptotic genes, which is important for maintaining gut homeostasis [35]. Trp presents strong anti-inflammation activity by triggering calcium-sensing receptor (CaSR) activation in intestine [67], which is a sensing receptor for dietary nutrients in the gastrointestinal tract to maintain mucosal immune homeostasis. Trp treatment allosterically activate CaSR which significantly reduces TNF-α-induced interleukin- (IL-) 8 secretion indicating a novel therapy in intestinal inflammation [67].

In addition, Trp exerts anti-inflammatory function via the metabolites including serotonin (5-hydroxytryptamine, 5-HT) and melatonin (MT). 5-HT is an important compound derived from Trp which serves as a neurotransmitter and inhibiter of the production of inflammatory cytokines and superoxide [46]. MT is abundant in gastrointestinal tract [68, 69] and has a positive impact on IBD with no or negligible side effects due to regulation of macrophage activity, reduction of cell migration and myeloperoxidase activity, and inhibition of NF-κB and TNF-α expression [36, 68, 70]. MT added to omeprazole treatment obviously accelerates chronic gastroduodenal ulcers over the obtained with omeprazole alone [71]. Additionally, in mice with DSS-induced colitis, MT exerts anti-inflammatory effects by alleviating the severity of mucosal injury and recovering the expression of IL-6, IL-17, and adiponectin [72].

2.2. Glutamine and Glutamate. As an abundant AA in the blood and tissues, Gln is mostly used as nitrogen source and/or alternative energy fuel [73]. In various clinical situations, appropriate exogenous Gln supply is safe and can beneficially contribute to diminishing risks of high-dose chemotherapy and radiation [74]. It has been additionally implicated as an immunomodulatory nutrient [75] and has pharmacological function in the IBD treatment [76]. Gln plays a key role in maintaining the integrity of the intestinal mucosa and has been shown to reduce inflammation and relieve CD [77]. In an UC mouse model, dietary Gln supplementation combined with dietary fiber and oligosaccharide has suppressive effects on mucosal damage [78]. A Gln diet replacing 25% of the total nitrogen decreases the expression of chemokine and endothelial adhesion molecules via suppression of T cell migration in mice [37]. These experimental data suggest Gln as a potential nutrient in protecting intestinal integrity and modulating immunity.

Glutamate (Glu) is produced from Gln with the catalysis of glutaminase and generally plays roles in protein synthesis and energy metabolism. Dietary Glu can also function as a signal to regulate the gastrointestinal tract via the gut-brain axis [9]. As a precursor of glutathione (GSH), Arg and Pro, Glu is of critical importance in intestinal metabolism and physiology [53]. Microinjection of Glu into the hypothalamic paraventricular nucleus in UC rats significantly increases the cell proliferation and antioxidant levels and decreases apoptosis and the expression of proinflammatory factors in the colonic mucosa [38]. Poly-γ-glutamate (P-Glu) significantly reduced histopathological evidence of injury, attenuated DSS-induced blood vessel densities, and attenuated DSS-induced expression of VEGF-A and its receptor in C57BL/6 mouse colitis model. P-Glu has potential application in conditions marked by inflammatory-driven angiogenesis and mucosal inflammation [79]. These findings above indicate that dietary supplementation of Gln and Glu is of functional and nutritional importance in intestinal mucosal growth and gut inflammation.

2.3. Sulfur Amino Acids. Sulfur amino acids (SAAs) mainly contain Met, Cys, and cystine. SAAs metabolism mainly takes place in the gastrointestinal tract. Dietary deficiency of SAAs will suppress intestinal mucosal growth, reduce intestinal epithelial cell proliferation, and increase intestinal oxidant stress in piglets [80].

As essential AA, Met has been considered as the first and second or third limiting AA in poultry and nursery pigs, respectively. Piglets fed the diet supplemented with Met present increased growth performance and exhibited improved integrity and barrier function of the small-intestinal mucosa [39]. Dietary supplementation with Met metabolites also can affect the susceptibility to colitis, reduce inflammation and tissue injury, and decrease the expression of multiple inflammatory genes in mice [81]. It is also interesting that Met (twice NRC recommendation) combined with fish oil (2.5%) can enhance immune response in IBD challenged broiler chickens which may be a novel treatment in IBD therapy for poultry [82].

Cys is a nonessential AA playing roles in protein metabolism and is regarded as the key factor in the synthesis of GSH. Dietary supplementation with *N*-acetyl-L-cysteine improves the clinical symptoms and decreases the chemokines without any side effects [83]. Cys administration can attenuate DSS-induced weight loss and intestinal permeability, decrease the expression of proinflammatory cytokines, and restore susceptibility of activated immune cells to apoptosis, indicating Cys supplementation as a novel therapy for IBD [40]. In addition, γ-glutamyl cysteine treatment can ameliorate DSS-induced clinical signs and histological damage *via* activating CaSR [84].

Besides, GSH, taurine (Tau), and hydrogen sulfide (H_2S), the products of catabolism of SAAs, play major roles in anti-inflammation and antioxidant system [46]. Tau is regarded as an antioxidant and membrane stabilizer against oxidative stress and inflammation by inhibiting chemokine secretion from intestinal cells [85, 86]. Moreover, the cellular metabolite taurine chloramine of Tau in the rectal shows anti-inflammatory property on IBD via inhibition on NF-κB activity [44, 87]. Tau treatment exerts beneficial effects in rats with 2,4,6-trinitrobenzene sulphonic acid- (TNBS-) induced colitis with decreasing inflammatory reactions and apoptosis [88]. H_2S is a signaling molecule and a gaseous mediator that exhibits several anti-inflammatory activities and contributes to mucosal protection [89–91]. One of the possible mechanisms of H_2S in the resolution of IBD is proved to be mediated via stabilization of hypoxia-inducible factor-1α [92].

2.4. Arginine. Arg is a semiessential AA that has protective effects against oxidative stress. As the substrate for nitric oxide (NO) synthesis, amino acid Arg is reported to be therapeutic in wound healing and has potent anti-inflammatory properties as a mediator of autoimmune diseases [93–96]. Otherwise, exogenous Arg is associated with antiapoptotic effects on the rat intestine and useful in the treatment of intestinal ischemia/reperfusion injury [97]. Dietary supplementation with Arg can improve the immune status of humans and animals and has the potential to be used to supplement current treatments for IBD [12]. Serum Arg concentration is a useful biomarker of UC disease severity [98]. Further, in a DSS-induced fulminant colitis murine model, treatment with hepatocyte growth factor and Arg can decline associated symptoms such as pain and diarrhea [99]. Arg is absorbed and transferred by cationic AA transporters (CAT) in intestine. DSS-induced inflammation reduced the expression of CAT2 in colonic and Arg uptake with body weight loss, reducing colonic permeability. Supplementation with Arg markedly attenuates the clinical parameters above and reduces the expression of proinflammatory cytokine and chemokine [42]. Arg might reduce the inflammation associated with AA-induced colitis through the NF-κB/nitric oxide pathway [100]. NO participates in nutrient metabolism and exerts protective effects against IBD including inhibition of macrophage activation and proinflammatory cytokine levels [101].

2.5. Other AAs. Besides the functional AAs above, other AAs have been reported to possess anti-inflammation functions to some extent. In addition to acting as an important precursor for the biosynthesis of GSH, Gly is proved to ameliorate diarrhea and body weight loss in TNBS induced colitis in the rats, indicating that Gly may be a useful immunomodulating nutrient for the treatment of IBD [43]. Histidine has proven to be a novel therapeutic agent for CD by inhibition of NF-κB activation, downregulating proinflammatory cytokine production in IL10$^{-/-}$ mice [41]. Ergothioneine (EGT) is a natural water-soluble amino acid which can be derived from mushroom or synthesized by nonyeast fungi [102, 103]. In ultraviolet-B-irradiated mice, the administration of EGT inhibited the UV-B-induced inflammatory responses and DNA halogenation, showing the modulatory effects of EGT in inflammation [103].

3. Future Perspectives and Challenge

Lacking of effective medical therapies for IBD makes it of utmost importance to find alternative therapeutic strategies

[3]. AAs can relieve intestinal inflammation through regulation of proinflammatory cytokines suggesting a possible approach to IBD treatment. However, further investigations and clinical studies are needed to fully understand the therapeutic mechanism and potential of AAs in preventing inflammation in both humans and animals.

Disclosure

The funding sources played no role in the study design, data collection and interpretation, or the decision to submit the work for publication.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Curcumin Alters Neural Plasticity and Viability of Intact Hippocampal Circuits and Attenuates Behavioral Despair and COX-2 Expression in Chronically Stressed Rats

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Curcumin is a major diarylheptanoid component of *Curcuma longa* with traditional usage for anxiety and depression. It has been known for the anti-inflammatory, antistress, and neurotropic effects. Here we examined curcumin effect in neural plasticity and cell viability. 60-channel multielectrode array was applied on organotypic hippocampal slice cultures (OHSCs) to monitor the effect of 10 μM curcumin in long-term depression (LTD) through low-frequency stimulation (LFS) to the Schaffer collaterals and commissural pathways. Cell viability was assayed by propidium iodide uptake test in OHSCs. In addition, the influence of oral curcumin administration on rat behavior was assessed with the forced swim test (FST). Finally, protein expression levels of brain-derived neurotrophic factor (BDNF) and cyclooxygenase-2 (COX-2) were measured by Western blot in chronically stressed rats. Our results demonstrated that 10 μM curcumin attenuated LTD and reduced cell death. It also recovered the behavior immobility of FST, rescued the attenuated BDNF expression, and inhibited the enhancement of COX-2 expression in stressed animals. These findings indicate that curcumin can enhance postsynaptic electrical reactivity and cell viability in intact neural circuits with antidepressant-like effects, possibly through the upregulation of BDNF and reduction of inflammatory factors in the brain.

1. Introduction

Excessive stress can cause anxiety, tension, and depression and have an adverse effect on normal life. Chronic stress can alter nervous, endocrine, and immune systems, thus affecting the overall homeostatic mechanisms in the body [1]. Stress influences have been widely recognized and studied in numerous research projects [2, 3]. Depression under stressful situations evokes a variety of cognitive symptoms, as well as physical changes such as lack of motivation, leading to chronic melancholy. A recent brain imaging study revealed changes in neurotransmitters in the brains of patients under depression, implying an etiology associated with depression [4].

Continuous progress has been achieved in the pursuit of effective antidepressants. Various classes of antidepressants have been developed based on the neurotransmitter system and used in clinical settings, including selective serotonin reuptake inhibitor (SSRI), norepinephrine-dopamine reuptake inhibitor (NDRI), and serotonin-norepinephrine reuptake inhibitor (SNRI). However, these antidepressants demonstrate gradual effects with slow onset and take least

4 to 6 weeks to achieve their effect. Also, there are issues of side effects including dry mouth, constipation and orthostatic hypotension, which have been frequently reported during the early phase of the treatment period. Therefore, there is a high demand for antidepressants with fewer side effects [5].

Recently, tension relievers such as L-theanine and anxiolytics such as alprazolam (Xanax, Pfizer Inc.) have been used to alleviate stress [6]; however, these drugs cannot become standard treatments because of their significant side effects. Therefore, botanical extracts are under investigation as alternatives for relieving stress since they are considered to have fewer side effects than chemical drugs.

Curcumin is a major diarylheptanoid and polyphenol component of *Curcuma longa* with both medical and nutritional values. It is extracted from the dry rhizome of *Curcuma longa* Linn (Zingiberaceae), a perennial herb that is widely cultivated in tropical regions of Asia [7]. It has been used for centuries in indigenous medicine for the treatment of a variety of inflammatory conditions and other diseases. Curcumin was also shown to significantly reverse chronic stress-induced behavioral and cognitive alterations in stressed rats [8, 9].

Previous research findings demonstrated that BDNF and COX-2 play an important role in the pathogenesis of depression and inflammation. Curcumin has already been shown to stimulate BDNF and inhibit COX-2 in chronically stressed rat models; however, the neuroprotective effect of curcumin in the chronically stressed rat model remains unknown. Therefore, we investigated the function of curcumin in modulating depressant-like behaviors in chronically stressed rats. This study reveals the neuroprotective effects of curcumin associated with antidepression activity through long-term depression- (LTD-) associated neural plasticity.

2. Materials and Methods

2.1. Materials. Curcumin, dimethyl sulfoxide (DMSO), lipopolysaccharides from *Escherichia coli* (L2637), HEPES (H4034), L-glutamine (G-8540), D-glucose (G-7528), and kainic acid were purchased from Sigma (St. Louis, MO, USA). Minimum essential medium (MEM, LM 007-01), Hank's balanced salt solution (HBSS, LB 003-01), and horse serum (S 104-01) were purchased from JBI (Daegu, South Korea). Penicillin streptomycin was obtained from Gibco BRL (LS 202–02, USA).

2.2. Animal Models. Sixteen 6-week-old male Sprague-Dawley (SD) rats with an average weight of 71 ± 8 g were used for in vivo experiments. SD rats were purchased from Orient Bio Inc. (Gyeonggi, Republic of Korea) and were fed on a standard pellet diet supplied by Orient Bio Inc. They were housed two per cage at the animal facility of the Kyung Hee University under maintained conditions of temperature 26 ± 1°C and relative humidity 60 ± 5% with a 12 h light/dark cycle (lights on 8:00 a.m., lights off 8:00 p.m.). All rats were allowed to acclimatize to the laboratory conditions for at least 1 week prior to the experiments. They were given tap water and a standard diet ad libitum during the experiments except when the chronic stress procedure required deprivation.

2.3. Drug Administration and Experimental Groups. Curcumin was dissolved in DMSO at a concentration of 10 mg/mL and the concentration of DMSO did not exceed 0.1% of the total volume.

Sixteen rats at the age of 6 weeks were randomly assigned to four groups (n = 4/group): Group 1 received 0.1% DMSO (10 mL/kg) and served as the sham; Group 2 was exposed to chronic stress and received 0.1% DMSO (10 mL/kg) as the control; Group 3 was exposed to chronic stress and received curcumin at a concentration of 50 mg/kg; Group 4 was exposed to chronic stress and received curcumin at a concentration of 100 mg/kg. The curcumin was injected at a dose of 1 mL per 100 g body weight. All treatments consisted of an oral injection between 10:00 and 11:00 a.m. administered once a day for 18 consecutive days. All the animals under test had been survived throughout the procedures, which were analyzed for statistical difference using ANOVA. All animal procedures complied with the Institutional Care and Use Committee (KHUASP(SE)-15-024) of Kyung Hee University and were performed in accordance with the guiding principles for the care and use of animals approved by the Council of the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Figure 1).

2.4. Chronic Stress Procedure. The procedure of chronic stress induction was conducted as previously described [10], with slight modifications. Stress was administered once a day over a period of 18 days between 2:00 p.m. and 5:00 p.m. The chronic stress protocol consisted of a variety of stressors: restraint (3 h), water deprivation (24 h), food deprivation (24 h), foot shock for 45 min (1 mA, 1 s duration, average 1 shock/min), or isolation (24 h). These stressors were randomly scheduled for 18 days. The control group was housed in a separate room without contact with the stressed group (Figure 1).

2.5. Forced Swim Test. The forced swim test (FST) was performed to measure behavioral despair of the rats. The test was performed as described previously with minor modifications [11]. The forced swim test was performed on the 18th day of curcumin administration. In each trial two rats were placed in two acrylic cylinders (height: 49.7 cm, diameter: 24.4 cm) containing water at 23°C with a depth of 30 cm. There was a white barrier between the cylinders to induce visual isolation. Rats were allowed a 15-minute preswim as an induction procedure. After the preswim, the animals were wiped with a towel and returned to their home cages. The cylinders were cleaned after each preswim trial with 75% alcohol to remove olfactory cues and refilled with fresh water. After 24 h the rats were put in a cylinder again for a 6 min forced swim test with a recording camera. After 6 min of swimming, the cylinder was cleaned and the water was replaced. Immobility was defined as the total time that the animal remained afloat without moving its limbs. Rodents adopt immobile positions when they lose hope of exiting the cylinder. In this circumstance, behavioral despair was defined as the immobility of a rat that has ceased any effort to escape the cylinder. Immobility was measured with a multichannel stopwatch program.

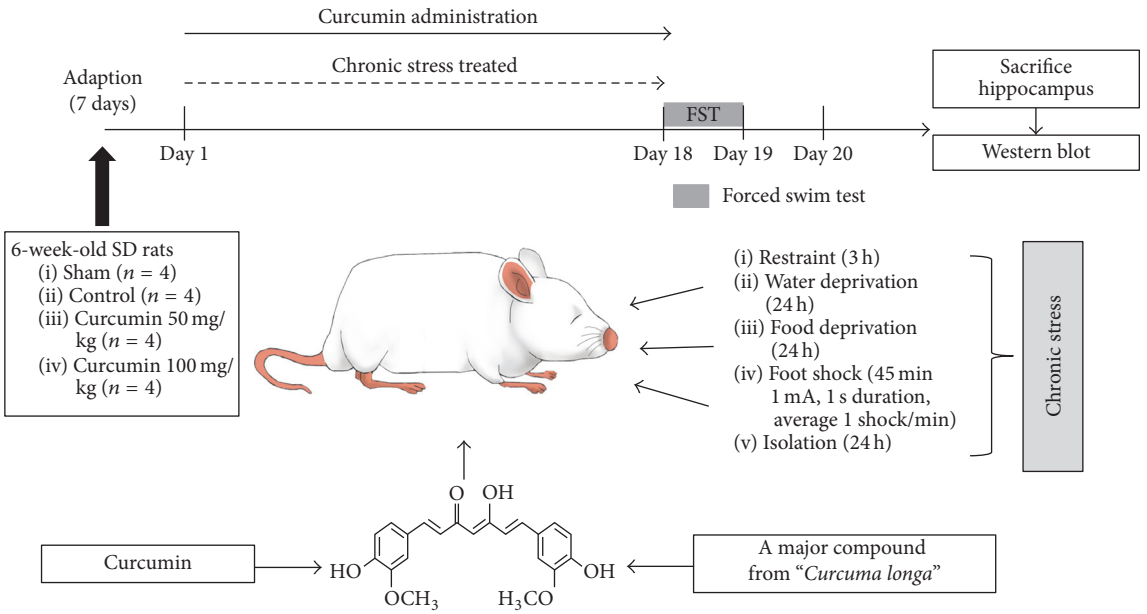

Figure 1: Experimental schedules for curcumin administration to produce an antidepressant effect in chronic stress-induced rats. The experiment was designed to explore the efficacy of curcumin against chronic stress-induced depression in an animal model using behavioral and biological methodologies. All animals were given distilled water for one week after arrival to habituate them to laboratory conditions. Starting on the first day, sham ($n = 4$) SD rats and control ($n = 4$) SD rats were fed 10 mL/kg distilled water until the end of the experiment. For the treatment groups, curcumin 50 ($n = 4$) SD rats were fed 50 mg/kg curcumin and curcumin 100 ($n = 4$) SD rats were fed 100 mg/kg curcumin. Curcumin was dissolved in DMSO. Stress was administered once a day over a period of 18 days in the control and curcumin groups. Behavioral testing commenced 60 min after the last curcumin treatment. On the last day, the rats were killed for biological experiments.

2.6. Tissue Preparation. After 3 weeks of treatment the rats were sacrificed. On the last day of the experiment, all rats were deprived of food or water for 24 h. The next day, the rats were anesthetized with isoflurane and blood samples from the right ventricle were collected by cardiac puncture. These samples were centrifuged at 2500 ×g for 20 min at 4°C to obtain plasma, which was divided into aliquots and stored at −80°C. The brains were rapidly excised and dissected. Tissues were stored at −80°C for later Western blot analysis.

2.7. Body Weight. Rats were weighed weekly from day 1 to day 20 during the procedure.

2.8. Western Blot Analysis. Tissues from animals in sham, control, curcumin 50 mg/kg, and curcumin 100 mg/kg groups were analyzed by Western blotting. Briefly, previously sectioned brains were removed from −80°C storage and a region of the hippocampus was dissected out on dry ice. The dissected hippocampus was homogenized by sonication in cold cell lysis buffer containing phosphatase inhibitors and a complete protease inhibitor cocktail. Hippocampus extracts were then incubated on ice for 30 min and centrifuged at 14,000 ×g for 10 min at 4°C. Protein concentrations of supernatants were measured by the Bradford protein assay [12] and equal amounts of protein were separated on a 10% SDS-PAGE gel and transferred to PVDF membranes. The membranes were blocked in TBS with 0.1% Tween 20 containing 5% dry skim milk for 1 h and incubated in 5% skim milk with primary antibodies overnight at 4°C. Antibodies used were polyclonal antibody against brain-derived neurotrophic factor (sc-33904, Santa Cruz Biotechnology), polyclonal antibody against cyclooxygenase-2 (sc-1747, Santa Cruz Biotechnology), and mouse monoclonal antibody against beta actin (sc-47778, Santa Cruz Biotechnology). Membranes were washed and further incubated for 1 h at room temperature with secondary antibodies (goat anti-mouse and donkey anti-goat IgG conjugated to horseradish peroxidase; sc-2005, sc-2056, Santa Cruz Biotechnology). After a final wash the bands were developed using a horseradish peroxidase-conjugated secondary antibody and visualized with an ECL Western Blotting Detection System (ATTO system). All experiments were repeated at least three times with different batches of tissue samples and the results were fully reproducible.

2.9. Organotypic Hippocampal Cultures. Organotypic hippocampal slice cultures (OHSCs) were prepared as previously reported [13]. All procedures were carried out in a sterile environment. In brief, 7-day-old Sprague-Dawley rats were decapitated and their brains were quickly removed. The brains were immediately immersed in ice-cold HBSS medium (LB 003-01, Sigma) with 20 mM HEPES (H-4034, Sigma). The frontal cortex and the cerebellum were delicately removed. The hippocampus was harvested and sectioned transversely at 350 μm using a tissue chopper (Mickle Laboratory Engineering Co., Surrey, UK). Four to five slices were plated on each 0.4 μm culture insert (Millicell-CM; Millipore, Bedford, MA, USA), which were set into wells of a 6-well

plate filled with 1 mL of culture medium (MEM, supplemented with 20 mM HEPES, 25% (v/v) Hank's balanced salt solution, 6 g/L D-glucose, 1 mM L-glutamine, 25% (v/v) heat-inactivated horse serum, and 1% penicillin streptomycin, pH 7.1). The culture slices were maintained at 36°C in a 5% CO_2 and 95% O_2 humidified incubator. The culture medium was changed three times a week and the sections were cultured for 14 days before experimental treatments.

2.10. Measurement of Propidium Iodide (PI) Uptake. Cell death was assessed by fluorescent image analysis of propidium iodide (PI; Sigma) staining. At 2 weeks after OHSC, the PI stock (5 μg/mL) was added to serum-free culture medium and the slices were incubated in the dark at 36°C. After 2 h incubation, the tissues were washed with culture medium and cell death in the hippocampal layers was detected by lack of PI uptake.

Curcumin was dissolved in DMSO and stored at −20°C until use. Aliquots were diluted in culture media. OHSCs were treated with 5 μM kainic acid (KA) and 10 μM curcumin [14]. The effects of KA and curcumin treatment for 24 and 48 h on cell death were observed by PI staining.

PI stained images were captured using a laser scanning microscope (LSM510, Carl Zeiss, Mannheim, Germany). Areas of PI uptake were measured with LSM 510 software (Carl Zeiss) and the image intensity was analyzed using the Image J program.

2.11. Preparation of Organotypic Hippocampal Slice Tissue on Microelectrode Array Probes. A single stabilized hippocampal slice was carefully removed from a membrane insert with a needle and placed on an 8 × 8 microelectrode array (MEA) of 10 μm diameter electrodes spaced 100 μm apart (Multi Channel Systems, Reutlingen, Germany) that was precoated with 0.01% polyethylenimine. MEAs consist of a high-density electrode array with stimulator, amplifier, temperature control unit, and computer for data acquisition. The slice was stabilized in artificial cerebrospinal fluid (aCSF: 114 mM NaCl, 26 mM $NaHCO_3$, 10 mM glucose, 3 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM HEPES, pH 7.4) for 30 min at 33°C with 95% O_2 and 5% CO_2 gas aeration. Extraneous aCSF was then removed using a pipette. The MEA containing the hippocampal slice was transferred to an MEA1060 amplifier interface. The solution in the array was grounded using an Ag/AgCl pellet. Data were sampled from each channel at a speed of 25 kHz and recorded using Recorder-Rack software (MEA systems, MCS software). The stimulating channel was disconnected from the sampling device during stimulation. After the experiment, the array was cleaned with 2% ultrasonol 7 (Carl Roth GmbH, Karlsruhe, Germany) in distilled water for approximately 30 min, rinsed with distilled water, and then kept in distilled water at room temperature.

2.12. Induction of LTD in Hippocampal Slices. The MEA system is composed of a 64-channel array with four stimulating and 60 recording electrodes (STG1004; Multi Channel Systems GmbH, Germany), an amplifier (MEA1060; Multi Channel Systems GmbH, Germany), temperature control units (Multi Channel Systems GmbH, Germany), and data acquisition software (http://www.multichannel-systems.com/) [15]. The amplifier was placed in a Faraday cage. Bipolar electrical stimulation was applied to the CA2 stratum radiatum region to stimulate the Schaffer collateral (SC) and commissural pathways. The intensity of bipolar test pulse stimulation was set at 100 mA; this value was optimized to provide 40–65% of the maximum tissue response and was delivered once every 60 sec. Baseline responses were evoked for at least 30 min, of which the last 40 minutes were recorded, before application of the low-frequency stimulation (1 Hz for 15 minutes; 900 total pulses; Figure 2(a)) to induce LTD. After the conditioning stimulation, field excitatory postsynaptic potentials (fEPSPs) were recorded every 60 sec for another 50 min from 59 microelectrodes spanning the hippocampus. During experiments the slices were continuously perfused with fresh aCSF solution or aCSF with drugs (dissolved with 95% O_2, 5% CO_2) at a rate of 3 mL/min and carbogen consumption of 161/h.

2.13. Electrophysiology Data Processing. MC_Rack (v.3.2.1.0, Multi Channel Systems) was used to digitize the analog MEA signal and isolate EPSPs from triggering amplitudes greater than 40 mV, and a custom MATLAB (v.7.0.1, Mathworks, Inc.) program was used to remove stimulus artifacts and integrate the evoked field potential trajectory, as reported previously [15, 16].

2.14. Statistical Analysis. The results in the figures are expressed as the mean ± standard error of the mean (SEM). Statistical comparisons were conducted using one-way and two-way analysis of variance (ANOVA) followed by Duncan's post hoc multiple comparison test using SPSS 20.0 for windows (SPSS Inc., Chicago, IL, USA). Differences were considered statistically significant if the p value was less than 0.05.

3. Results

3.1. Effects of Curcumin on Body Weight. Body weight measurements are shown in Figure 5. Before the experiment, all rats had an average body weight of 71 ± 2 g. At the end of 3 weeks, the body weight of sham and control groups were different; the sham group showed a significant increase in body weight whereas the control stressed group did not. The body weight of rats in the sham group increased by 85 ± 7.4 g compared to 66 ± 4.9 g for control rats. A valid difference was not detected with GLMM or Kruskal-Wallis test.

3.2. Effects of Curcumin on Forced Swim Test. The FST was applied on day 18. As shown in Figure 6, statistical analysis of the FST data showed differences in the immobility time among the four groups. The chronically stressed control group spent a longer time immobile than the sham group; the immobility time of the control group was 15.9% higher than that of the sham group. Treatment with 50 mg/kg curcumin decreased the immobility time by 8.6% compared to the control group. Moreover, treatment with 100 mg/kg curcumin

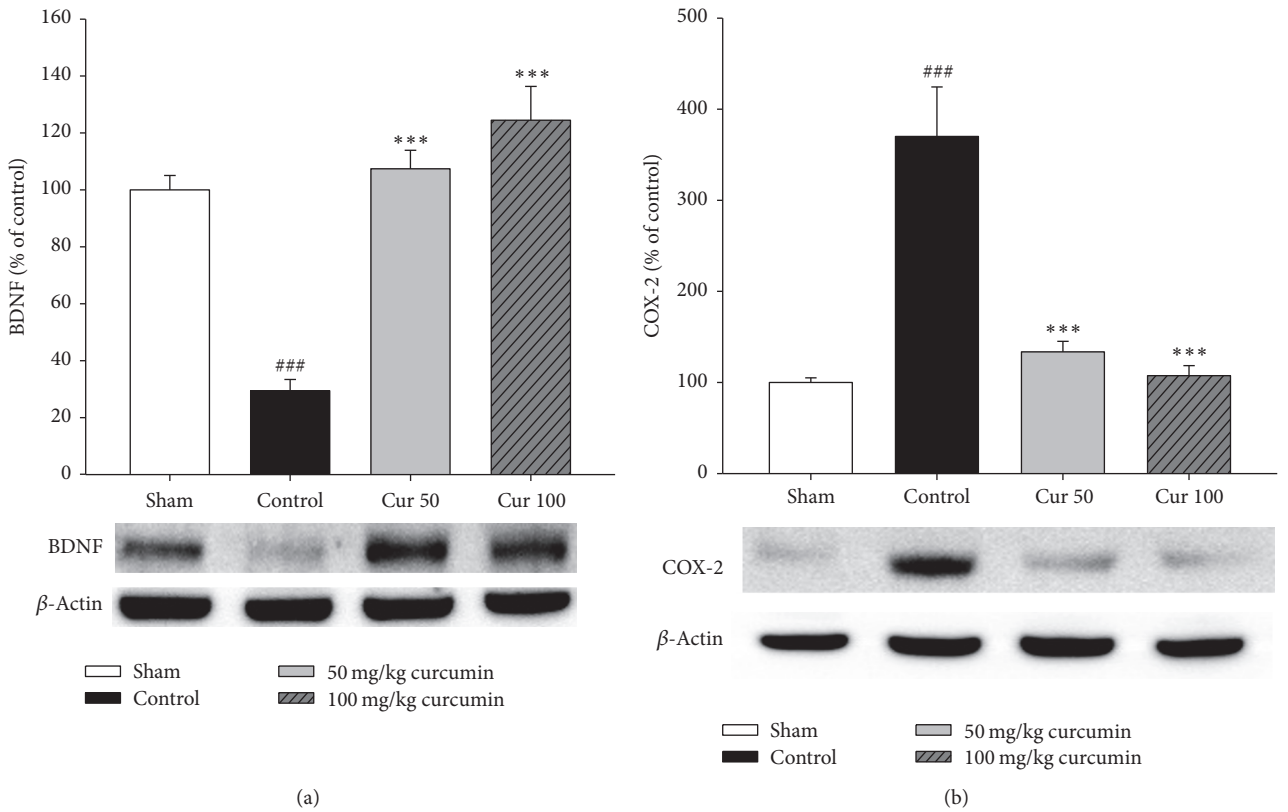

Figure 2: Effect of curcumin on the protein expression of brain-derived neurotrophic factor (BDNF) and cyclooxygenase-2 (COX-2) in rats with chronic stress-induced hippocampal impairment ($n = 4$/group). (a) The protein level of BDNF in rat hippocampus was measured by Western blot analysis using anti-BDNF specific antibody. (b) The protein level of COX-2 in rat hippocampus was measured by Western blot analysis using anti-COX-2 specific antibody. The data were normalized against β-actin levels and expressed as percentage of control values. $^{###}p < 0.001$ versus sham group. $^{***}p < 0.001$ versus control group.

significantly reduced immobility time by 48.8% compared to the control group ($p < 0.05$). These results showed that curcumin has antidepressant effects in the FST as evidenced by decreased immobility.

3.3. Effects of Curcumin on BDNF Expression. The Western blot results demonstrated that BDNF protein levels were decreased by 70.6% in the hippocampus of the stressed control group ($p < 0.001$ versus nonstressed sham group, Figure 2). However, treatment with curcumin significantly increased the BDNF protein levels in the hippocampus of stressed rats compared to control ($p < 0.001$, Figure 2). Specifically, curcumin at doses of 50 and 100 mg/kg (p.o.) increased BDNF protein levels by 78.0% and 95.1%, respectively, compared to the control group. These results indicate that curcumin enhanced the BDNF level in hippocampus of chronic stressed rats. Treatment with several doses of curcumin treatment showed that 100 mg/kg (p.o.) was most effective. However, both doses of curcumin tested had a significant effect on the expression ratio of BDNF/β-actin.

3.4. Effects of Curcumin on COX-2 Expression. We also measured the expression level of the inflammation maker COX-2 by Western blot analysis. COX-2 protein levels were significantly increased in the hippocampus of the stressed group ($p < 0.001$ versus nonstressed sham group). As shown in Figure 2, COX-2 levels after treatment with 50 mg/kg and 100 mg/kg curcumin treatment were reduced by 236.6% and 262.7%, respectively, compared to the control group ($p < 0.001$). These results indicate that curcumin decreased the COX-2 level in the hippocampus of chronic stressed rats. Both doses of curcumin had a significant effect on COX-2 expression, but curcumin 100 mg/kg was so effective that it showed no significant difference from the sham group.

3.5. Effects of Curcumin on Neuronal Cell Death. Average PI update in each group was measured after 2 days of treatment with KA alone and KA with curcumin. As shown in Figure 3(b), 5 μM KA caused neuronal cell death in the hippocampus at 24 and 48 hours after application. When the value of the control group was set as 100% cell death, the death rate of slices incubated with KA (5 μM) with curcumin (10 μM) was 128.3% (24 h) and 117.7% (48 h). Treatment with 10 μM curcumin significantly reduced the PI fluorescence (Figure 3). Therefore, curcumin treatment counteracted the effect of KA resulting in a similar death rate between the sham and KA + curcumin groups.

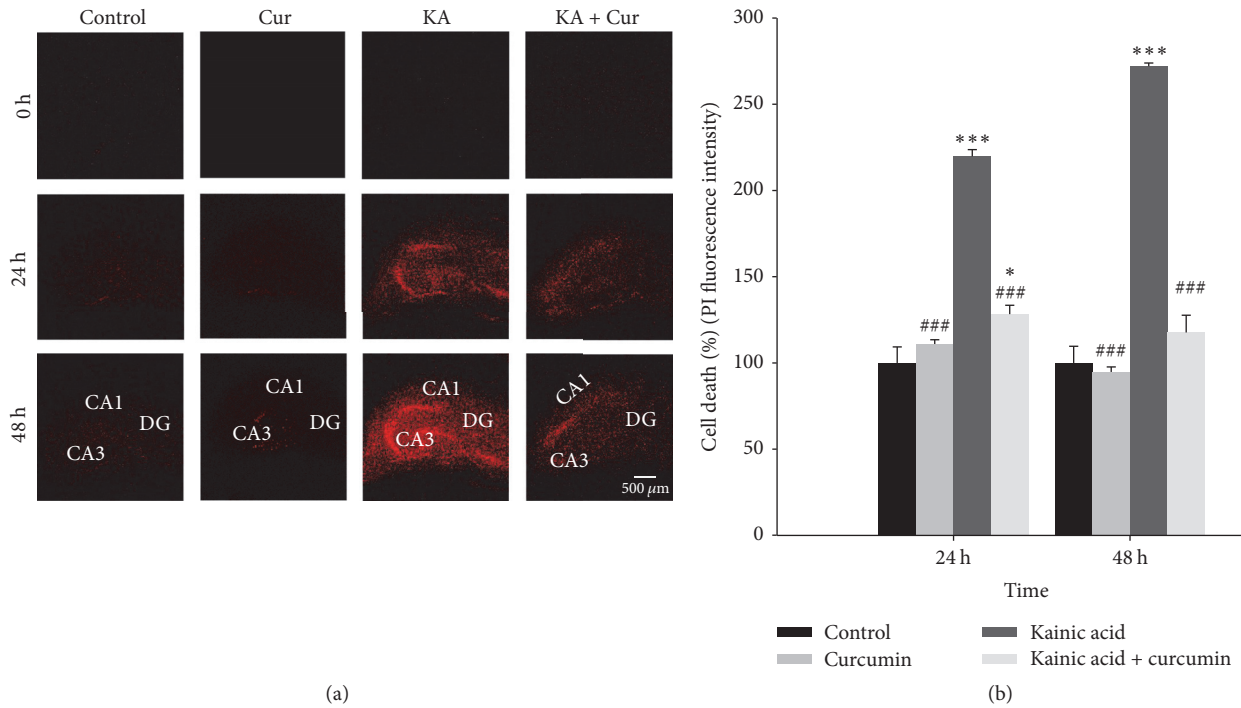

Figure 3: Detection of neuronal cell death in organotypic hippocampal slice cultures (OHSCs) with kainic acid (KA) and curcumin treatment by propidium iodide (PI) staining. OHSCs were treated with 10 μM curcumin and 5 μM KA. (a) Representative images of PI uptake in OHSCs. Red represents PI fluorescence, which indicates cell membrane damage. Scale bar = 500 μm. (b) Quantification of hippocampal cell death. Data are shown as the percentage cell death. The fluorescence intensity of the control group was designated as 100%. Differences in PI fluorescence intensities among control, control + curcumin only, KA-only-treated group, and KA with curcumin groups were observed by fluorescence microscopy. The PI value is shown as mean ± SEM. Four hippocampal slices were used in each group. $^{*}p < 0.05$, $^{***}p < 0.001$ versus control group. $^{\#\#\#}p < 0.001$ versus the KA-only-treated group.

3.6. The Effects of Curcumin on LTD in Hippocampal Tissue. LTD was recorded over 100 min and the average of fEPSPs was analyzed when applying LFS for approximately 30–40 min. According to the total activity of fEPSP calculated from the experimental results, slices perfused with aCSF containing curcumin showed an increase in LTD (Figure 4). In control slices from the rat hippocampus, fEPSP was potentiated to 83.25 ± 1.92% (n = 3-4). In curcumin (10 μM)-treated slices the fEPSP was potentiated to 97.25 ± 2.69% (n = 3-4, $p < 0.05$ versus control).

4. Discussion

The effect of curcumin on neural plasticity and cell viability has been investigated in in vitro and in vivo experimental models. There have been controversies concerning the effect of stress on body weight [17, 18]: one study reported that body weight was decreased in the stressed animal model [18], whereas another suggested that there was no significant difference in body weight in the stressed model compared to the placebo [17, 19]. In this experiment, the control group showed a decrease in body weight compared to the sham group that was not affected by stress (Figure 5). These contradictory findings between studies could be due to differences in the stressor used or the level of stress.

The antidepressant activity of curcumin was previously evaluated in forced swim tests using chronic stress-induced rat models [20]. Oral treatment with 50 and 100 mg/kg/day curcumin for 18 days showed an 8.6% and 48.8% reduction in immobility, respectively, compared to the control group. Curcumin-treated groups showed reduced immobility in a dose-dependent manner, indicating that curcumin may possess antidepressant properties. BDNF is a major neurotrophic factor that plays an important role in the maintenance and the survival of neurons [21–23]. Upon binding to TrkB receptor, BDNF induces glutamate release from presynaptic region following MAPK/ERK and CaMKII signaling activation in postsynaptic neuron. It results in elevation of neural plasticity [2]. The curcumin-treated group showed an increase in BDNF protein expression in the hippocampus region suggesting a strong link between antidepressant effects and BDNF expression. This also suggests that curcumin might partially exert its antidepressant activities by attenuating or reversing abnormalities in BDNF expression induced by chronic stress [24] (Figure 2).

The neuroplastic changes in depression can be mediated through neuroimmune processes, including humoral and cellular neuroimmunological factors. Inflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor (TNF) increase COX-2 protein expression in various

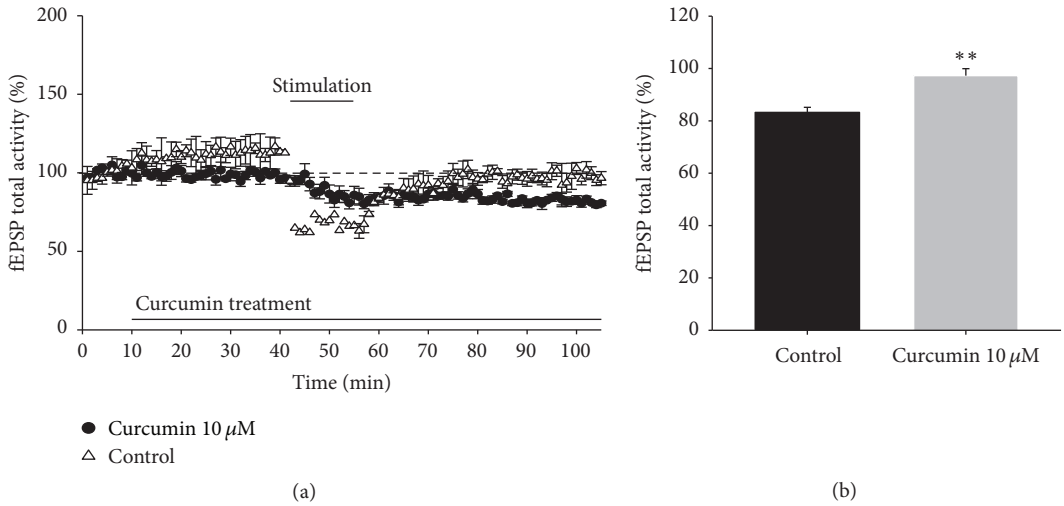

Figure 4: Effects of curcumin on long-term depression (LTD) in rat hippocampal tissue (n = 3-4/group). (a) Time course of LTD from all recordings made from control or curcumin-treated (10 μM) hippocampal tissue; (b) average LTD amplitude measured 30–40 min after LFS; $^{**}p < 0.01$ versus the control group.

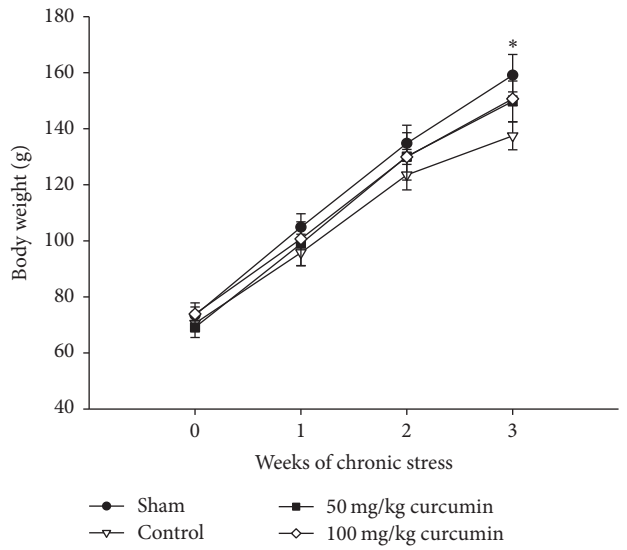

Figure 5: Effects of chronic stress and curcumin treatment on body weight in SD rats. Data represent mean ± SEM (n = 4/group). $^{*}p < 0.05$ by GLMM or Kruskal-Wallis test.

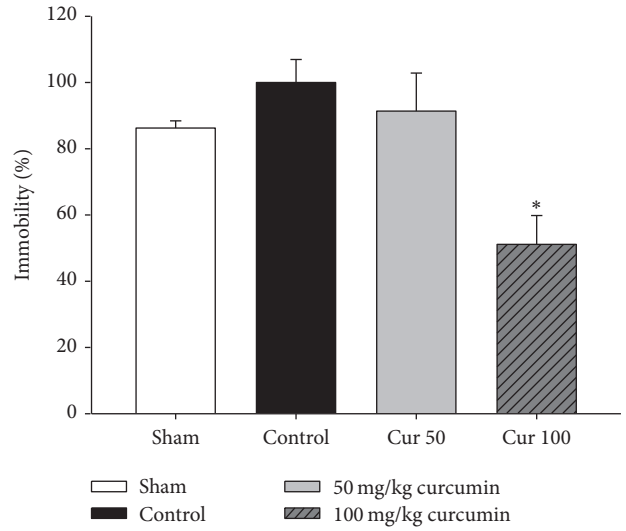

Figure 6: Effects of curcumin administration on behavior immobility in forced swim test. Curcumin was administered for 18 days before the FST. The immobility time was measured during a 6 min experimental session. Data are shown as mean ± SEM. According to Kurtosis figure or Kurtosis/Kurtosis error, each group had normal distribution. $^{*}p < 0.05$ versus control by ANOVA. In addition, $^{*}p < 0.05$ by Kruskal-Wallis test.

human cell types [25]. COX-2 is an enzyme to covert arachidonic acid into prostaglandins such as PGD, PGI2, and PGE2 in response to neuroinflammatory factors [1]. Therefore, its expression level is widely used as an indicator to represent degree of inflammation. In line with it, COX-2 inhibitors have demonstrated neuroprotective effects in numerous CNS-related disorders [26]. Curcumin has also shown antidepressant-like effects in a LPS-induced depression model, which could be attributed to reduced levels of proinflammatory cytokines like iNOS and COX-2 via the NF-κB signaling pathway. Our results imply that immobilization by stress could cause enhanced COX-2 protein expression in the cortex, particularly the hippocampus regions [27] (Figure 2). In the present study, curcumin reduced COX-2 protein expression in the hippocampus of stress-induced rats. These findings suggest that curcumin partially induces antidepressant activities by attenuating or reversing chronic stress-induced abnormalities resulting from COX-2 protein expression. Likewise, curcumin pretreated rat models showed a reduced NO level under KA treatment, implicating a protective effect of curcumin against KA-induced neuronal loss [28]. It has been reported that seizure induced by KA results

in hippocampal cell death and upregulation of caspase-3, GFAP, eNOS, and HO-1. In contrast, curcumin prevented neuronal cell death and attenuated the upregulation of these proteins in astrocytes [29].

Currently, the effects of curcumin on LTD are not fully understood. This study demonstrated the neuroprotective effects of curcumin. Long-term potentiation (LTP) is defined as a strong depolarization that occurs when the excitatory neurotransmitter glutamate in presynaptic terminals is recognized by the postsynaptic glutamate receptor (NMDA receptor). LTP increases the synaptic efficacy in the mammalian brain and is also associated with learning and memory [30]. However, storage of information requires mechanisms for weakening, as well as strengthening. LTD is the functional inverse of LTP and a candidate mechanism for learning and memory development in the hippocampus [31]. We demonstrated that hippocampal field potential LTD was prevented by curcumin. The results of this study might advance our understanding of the neuroprotective effects of curcumin for memory storage and restoration (Figure 4).

In this study we demonstrated the anti-inflammatory and antidepressant properties of curcumin. We also observed the neuroprotective effects of curcumin in a LTD model. Based on these findings, we believe that curcumin might be a potential candidate for anxiolytic or antidepressant therapy.

Competing Interests

The authors have no competing interests to declare.

Authors' Contributions

Ga-Young Choi and Hyun-Bum Kim contributed equally to this work as co-first authors.

Acknowledgments

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Research Article

Chitosan Modulates Inflammatory Responses in Rats Infected with Enterotoxigenic *Escherichia coli*

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This study aims to investigate the effects of dietary chitosan (COS) on gastrointestinal pathogen resistance in mice model. For two weeks, a control group of ICR mice received a basal diet whilst the intervention group received the basal diet supplemented with 300 mg/kg COS. After two weeks, the mice fed the supplemented diet had a lower body weight. Then enterotoxigenic *Escherichia coli* (*E. coli*) was administered to the mice through oral gavage, with each mouse receiving 10^8 CFU. At day 7 after infection, the bacterial load in the jejunum and faeces was significantly lower in the COS group than that in the control group. Moreover, the mRNA and protein levels of IL-1β, IL-6, IL-17, IL-18, and TNF-α were significantly lower in the group of mice receiving the COS diet; also the jejunal production of toll-like receptor-4 (TLR-4) was suppressed in the COS group. These results indicate the intervention influenced inflammation and controlled *E. coli* infection.

1. Introduction

For most people, enterotoxigenic *Escherichia coli* (ETEC) infection is merely the unpleasant and inconvenient cause of traveller's diarrhoea in many instances. ETEC is responsible for the majority of diarrhoeal disease in developing countries; according to WHO it claims approximately 380,000 lives each year [1] with children under the age of 5 years being especially vulnerable. ETEC is also prevalent amongst newborn farm animals, such as piglets [2].

ETEC infections usually emerge 1–3 days following pathogen exposure and manifest as acute watery, diarrhoea together with fever, headache, and vomiting. These symptoms typically last for 3-4 days though some people may experience diarrhoea for a week or longer [3]. ETEC colonizes the small intestine and releases enterotoxins that cause intestinal epithelial cells (IEC) to secrete fluids into the gut lumen, resulting in diarrhoea [4]. To minimise the effects of ETEC in weaning piglets, antibiotics are frequently added to their diet, with the intention of minimising infectious disease and promoting growth. However, this practice is likely to contribute to drug-resistance in pathogens by creating a reservoir of drug-resistant bacteria. These bacteria may transfer resistance genes to other pathogenic bacteria in the gut of animals and humans [5]. Also, the presence of drug residues is a concern for many consumers; therefore, the routine antibiotics in livestock diets is already banned or restricted in many countries.

Chitosan is an abundant nitrogenated polysaccharide; it is a component of fungal cell walls and exoskeletons of insects and diverse sea creatures, such as crustaceans, squid, and clams. Chitosan is derived from chitin. The immune

responses of pigs [6], mice [7], rats [8], and fish [9] have been modulated by chitosan. T cells and other immune cells produce cytokines, which are essential to the immune response. In vitro studies indicate that the degree of deacetylation and molecular weight of COS are positively correlated with the extent of the anti-inflammatory effect [10]. Whilst some studies have endeavoured to evaluate the effects of chitosan on the gut bacteria of pigs and chickens [11] the effects of chitosan on inflammatory and bacterial responses during and after infection remain limited. One possible mechanism by which chitosan may operate is to disrupt *E. coli* adhesion in the jejunum.

The hypothesis that underlies this study is that dietary chitosan supplements may directly clear enterotoxigenic *E. coli* and reduce proinflammatory signals and/or increase the anti-inflammatory response during infection. We tested the hypothesis in enterotoxigenic *E. coli* infection model; the changes in the bacterial count and proinflammatory molecules were evaluated.

2. Mice and Diet

This experiment used 20 ICR (Institute for Cancer Research) mice aged 6 weeks; they were bred and kept at Hunan Agricultural University. Approval for all experimental procedures with the mice was granted by the Animal Care and Use Committees of Hunan Agricultural University. The mice were housed separately in pathogen-free accommodation under appropriate environmental conditions (25°C; 53% relative humidity; 12-hour light/dark cycle). They had ad lib access to an appropriate rodent diet [12] and water. Mice were randomly allocated to two groups (control $n = 10$ and chitosan intervention $n = 10$). Endotoxin free chitosan (average molecular weight < 1 kDa; degree of deacetylation > 95%) was donated by Dalian Chemical and Physical Institute (Chinese Academy of Sciences, Dalian, China). For two weeks, the control group received the basal diet and the intervention group was given a basal diet supplemented with 300 mg/kg of chitosan. The dosage and duration were determined based upon previous study [6]. Throughout the experimental period, the intake of feed and water, together with body weight gain, was monitored and recorded.

3. Enterotoxigenic *E. coli* Infection and Enumeration

After two weeks of being on a basal or supplemented diet, mice were then infected with 10^8 CFU ETEC SEC470 [13]. At 7 day after infection, all the mice were killed by CO_2 asphyxiation. Jejunal tissues were homogenised, serially diluted, and then plated onto MacConkey agar with the antibiotic gentamicin (40 μg/mL) and tetracycline (50 μg/mL). The jejunal contents and faeces were weighted and then suspended in PBS buffer. Then serial dilutions were placed on MacConkey agar treated with gentamicin (40 μg/mL) and tetracycline (50 μg/mL). After 24 hours, the bacterial colonies were tallied. PCR was conducted to verify the identity of the bacteria isolated. The primers used (5′-CTGTATACGTGGCAG-3′) and (5′-ACTATGGTGAATGCTCAC-3′) were obtained from ETEC *fedF* gene (GenBank accession number Z26520). The other jejunal tissues, jejunal contents, and faecal samples were collected and stored at −80°C until required.

4. RT-PCR and ELISA Analysis of Cytokines

After mRNA was extracted using TRIZOL reagent (Invitrogen, USA), cDNA was reverse transcribed. RT-PCR was performed according to Xiao et al. [14]. The mRNA levels of *IL-1β, IL-6, IL-17, IL-18,* and *TNF-α* were analysed with GAPDH as the reference gene. The protein levels of *IL-1β, IL-6, IL-17, IL-18,* and *TNF-α* were analysed according to Ren et al. [15] with ELISA kit from eBioscience, CA, USA.

5. Immunoblotting of TLR-4

To measure the level of TLR-4, an appropriate TLR-4 assay kit was used (Cayman Chemical Company, MI, USA). Equal quantities of proteins collected from jejunal tissues were separated using SDS-PAGE. Then the samples were analysed according to the method described by a previous report [16]. Using β-actin protein as a reference, the intensity of the signal was measured digitally.

6. Statistical Analyses

The data are presented as means ± standard error of the mean (SEM). All the statistical analyses were undertaken using SPSS 22.0 software (Chicago, IL, USA). The Student's t-test was used to analyse data differences between the control and intervention groups. Difference level at $P < 0.05$ is considered significant.

7. Results

Over the two-week period, the average feed and water intake was higher in the in chitosan-supplemented mice than in control mice ($P < 0.05$) (Figures 1(a) and 1(b)). Despite this, the body weight of mice receiving the chitosan supplement was significantly lower than that of control group ($P < 0.05$) (Figure 1(c)). During the experiment, no diarrhoea was observed. At 7 days after infection, in the COS group, the ETEC loads in the jejunal contents, faeces, and jejunal tissues were significantly lower ($P < 0.05$) than that in the control group (Figure 2).

Seven days after infection, the expression of *IL-1β, IL-6, IL-17*, *IL-18,* and *TNF-α* mRNA was significantly lower in the jejunum of COS-fed mice compared to those of controls (Figure 3). The ELISA results confirmed the trend with IL-1β, IL-6, IL-17, IL-18, and TNF-α being significantly lower in COS mice (Table 1).

As Figure 4 indicates in the COS group the TLR4 protein level in the jejunum was significantly lower ($P < 0.05$) than that of the control group.

8. Discussion

Numerous studies have suggested that dietary supplements of chitosan can reduce body weight and may have applications

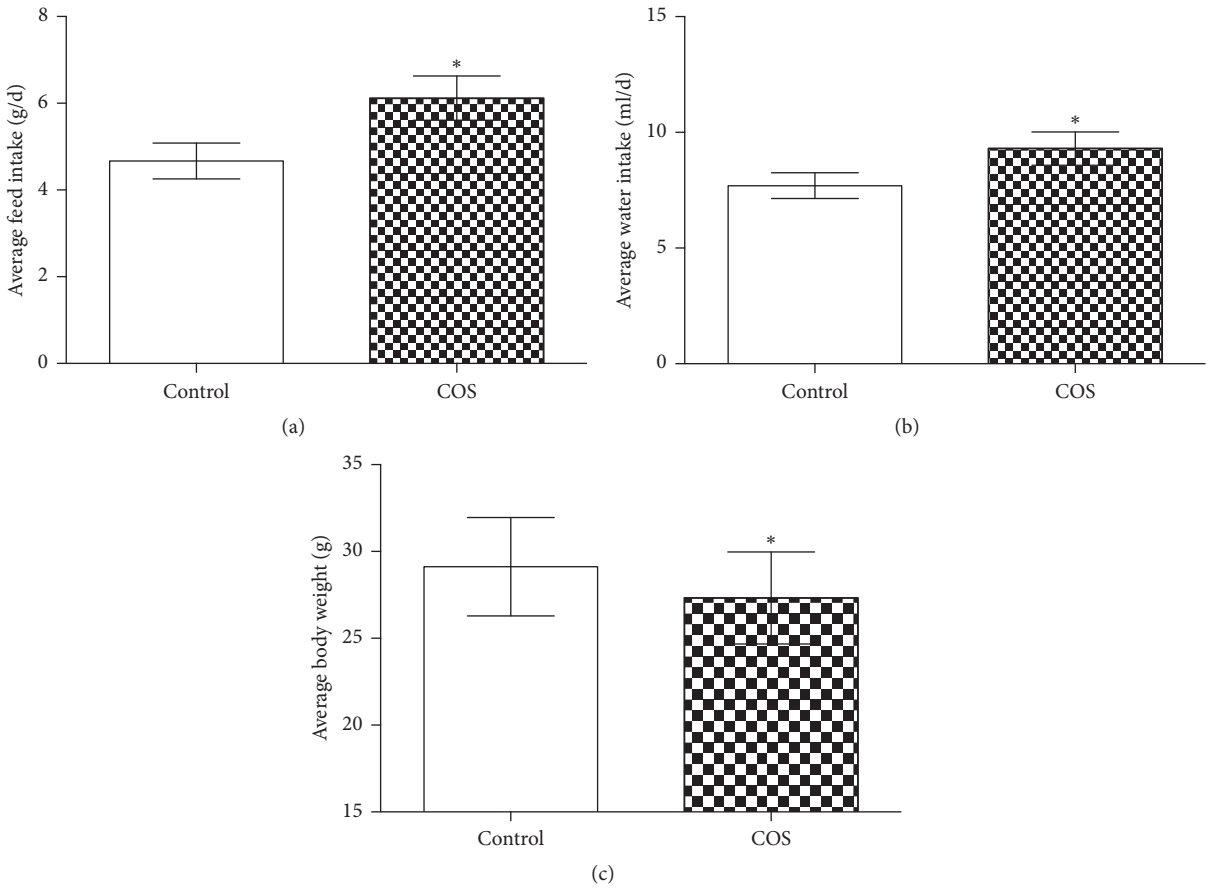

Figure 1: Chitosan supplementation lowers mouse body weight. (a) Average feed intake in the control group and COS group. (b) Average water intake in the control group and COS group. (c) Average body weight in the control group and COS group. ∗ indicates a significant difference between the control and COS groups ($P < 0.05$) ($n = 10$).

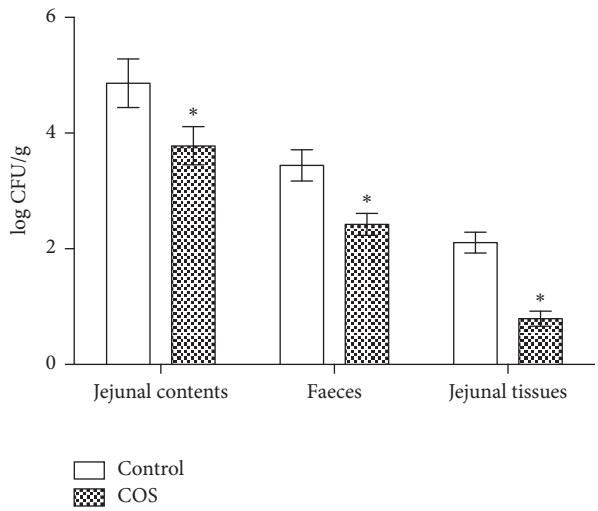

Figure 2: ETEC load at d 7 after infection from jejunal tissues, jejunal contents, and faeces of infected ICR mice ($n = 10$). ∗ indicates a significant difference between the control and COS groups ($P < 0.05$).

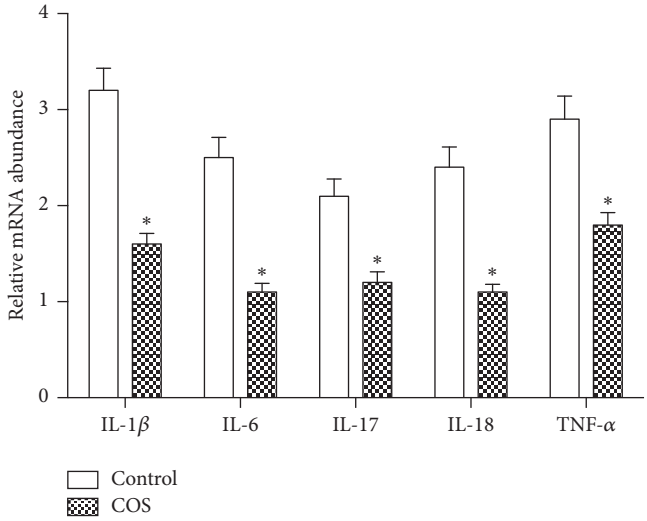

Figure 3: Mucosal inflammatory responses in the jejunum from the control group and COS group ($n = 6$); mRNA level of *IL-1β*, *IL-6*, *IL-17*, *IL-18*, and *TNF-α* as determined by RT-PCR. $*$ indicates a significant difference between the control and COS groups ($P < 0.05$).

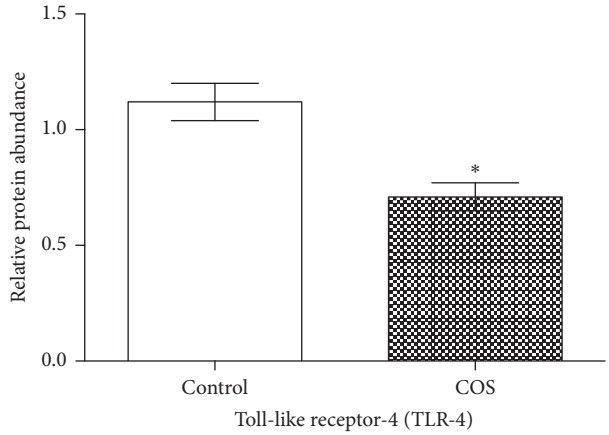

Figure 4: TLR4 protein level in jejunal mucosa in the control group and COS group ($n = 6$). $*$ indicates a significant difference between the control and COS groups ($P < 0.05$).

for weight management and obesity [7, 17]. During the experimental period, the mice receiving 10^8 CFU ETEC SEC470 did not suffer any diarrhoea. Many researches showed that the mice challenged by ETEC would not suffer from diarrhoea; however, ETEC could colonize the intestine and promote the inflammatory responses [18]. Thus we further investigated the body weight of the mice. The results demonstrate that mice fed a chitosan-supplemented diet experienced a reduction in body weight. One mechanism that has been proposed for this is that chitosan reduces the postprandial ratios of apolipoprotein B (apoB) isoforms, low-density lipoprotein cholesterol, and high-density lipoprotein. This is based on the findings of a study of the effects on lipid metabolism in glucose-tolerant rats receiving a high-sucrose diet [19]. The chitosan particles bind with cholesterol and fatty acids to form clusters in the gastrointestinal tract, thereby reducing lipid absorption [20]. In addition to its effect on lipid absorption, chitosan has been described as reducing blood glucose levels and being capable of inhibiting the carbohydrate hydrolysing enzymes maltase, sucrose, and sucrose-isomaltase in the gut [21, 22].

The findings from this study revealed that, compared to the control group, the COS mice had a significantly lower load of ETEC bacteria. According to the report of Tayel, chitosan may inhibit the growth of bacteria such as *E. coli*, Enterobacteriaceae, and *Staphylococcus aureus* [23]. As COS is a D-glucosamine oligomer, it is resistant to digestive enzyme degradation. COS may survive digestive enzymes to reach the jejunum where it interferes with the adhesion of ETEC to intestinal epithelial cells.

In this study the mRNA and protein levels of jejunal IL-1*β*, IL-6, TNF-*α*, and TLR-4 were observed in response to a chitosan-supplemented diet in mice. Intestinal epithelial cells express cytokine and chemokine receptors as well as toll-like receptors, such as TLR-4 [16]. TLR4 recruits the adaptor protein, MyD88, and TIRAP to activate NF-*κ*B, which in turn induces expression of signalling genes such as TNF-*α*, IL-1,

Table 1: Jejunal inflammatory responses in jejunum tissues from the control group and COS group ($n = 6$) and protein levels of IL-1β, IL-6, IL-17, IL-18, and TNF-α were determined by ELISA.

	Control	COS
IL-1β (pg/ml)	110.48 ± 8.31	67.59 ± 4.67*
IL-6 (pg/ml)	98.45 ± 6.38	42.36 ± 3.58*
IL-17 (pg/ml)	216.39 ± 11.23	117.96 ± 9.67*
IL-18 (pg/ml)	182.64 ± 13.62	98.63 ± 8.45*
TNF-α (pg/ml)	136.25 ± 7.12	86.23 ± 8.31*

* indicates a significant difference between the control and COS groups ($P < 0.05$).

and IL-6 [18]. Raised TLR-4 expression in intestinal epithelial cells is associated with an enhanced mucosal inflammatory response and subsequent dysfunction of the intestinal epithelial barrier [18]. Our study revealed suppressed expression of TLR-4 and consequently the mRNA and protein levels of IL-1β, IL-6, and TNF-α were correspondingly lower. The anti-inflammatory effects of COS on mice are proposed to arise from the COS inhibition of intestinal ETEC infection. It is interesting in our previous research, the mRNA levels of IL-1β, and IL-6 in the ETEC-challenged piglets consuming 300 mg/kg COS diet were significantly higher than those in the control group [6]. This discrepancy may be due to two reasons: firstly, it may take more times for complex network of cytokines in piglets to respond to the COS diet; thus the IL-1β and IL-6 mRNA levels in jejunal mucosa were still high during the sampling; secondly, the samples used for mRNA analysis are different, the jejunal mucosa was used for analysis in piglets model and the jejunal tissues were used in the mice model.

The findings here also indicated that the jejunal expression of IL-17 and IL-18 was reduced by the chitosan supplement. IL-17, manufactured in response to inducible (iTh17) and natural (nTh17) T helper cells, is a proinflammatory cytokine that recruits monocytes and neutrophils to areas of inflammation; it also stimulates local chemokine synthesis. It has been implicated in a number of autoimmune diseases [24]. On the other hand, IL-18, another proinflammatory cytokine, is strongly implicated in antitumour responses and host defence. Gene therapy studies that increase of IL-18 has been shown to provide test animals with protection against infection, tumour growth, and metastases. However, overexpression of IL-18 has been shown to result in emphysematous lesions in mice [25]. Chitosan lowered the bacterial load in jejunum, and the lower ETEC load may decrease intestinal expression of cytokines.

To summarise, chitosan supplements decrease the body weight of mice and are an effective prophylactic against in vivo infection of enterotoxigenic *E. coli*. The results presented here indicate that COS decreased the bacterial load, TLR-4, and cytokine biosynthesis. Further development of COS may provide an effective method to promote intestinal health and protection against enterotoxigenic *E. coli* infection.

Competing Interests

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

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Research Article

Effect of Previous High Glutamine Infusion on Inflammatory Mediators and Mortality in an Acute Pancreatitis Model

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Parenteral glutamine supplementation in acute inflammatory conditions is controversial. We evaluated the inflammatory and survival responses after parenteral glutamine infusion in sodium taurocholate-induced acute pancreatitis (AP) model. Lewis rats received 1 g/kg parenteral glutamine ($n = 42$), saline ($n = 44$), or no treatment ($n = 45$) for 48 h before AP induction. Blood, lung, and liver samples were collected 2, 12, and 24 h after AP to measure serum cytokines levels and tissue heat shock protein (HSP) expression. From each group, 20 animals were not sacrificed after AP for a 7-day mortality study. Serum cytokine levels did not differ among groups at any time point, but the intragroup analysis over time showed higher interferon-γ only in the nontreatment and saline groups at 2 h (versus 12 and 24 h; both $p \leq 0.05$). The glutamine group exhibited greater lung and liver HSP90 expression than did the nontreatment group at 2 and 12 h, respectively; greater liver HSP90 and HSP70 expression than did the saline group at 12 h; and smaller lung HSP70 and liver HSP90 expression than did the nontreatment group at 24 h (all $p \leq 0.019$). The 7-day mortality rate did not differ among groups. In experimental AP, pretreatment with parenteral glutamine was safe and improved early inflammatory mediator profiles without affecting mortality.

1. Introduction

Glutamine can become essential during hypercatabolic stress and under critical conditions, such as severe trauma, sepsis, inflammatory diseases, and burns [1]. Glutamine is a fuel source for lymphocytes and enterocytes, a substrate for glutathione and heat shock protein (HSP) synthesis, and a potential inhibitory agent for inflammatory cytokine release [2, 3]. These biological properties could contribute to improving gut barrier and lymphocyte function and to attenuate inflammatory responses [4].

In critically ill patients, glutamine supplementation has been suggested to properly support increased cell proliferation rates, gut barrier protection, and inflammatory dysfunction attenuation [5, 6]. The intravenous administration of glutamine can result in its earlier availability for cell use and could be advantageous for the achievement of rapid inflammatory modulation and protection of cells against damage in clinical critical care conditions. However, unexpected harmful effects of parenteral glutamine supply, mainly in patients with multiple organ failure, have been reported recently [7–9].

These observations have challenged the development of new guidelines for safe glutamine supplementation and have made apparent the need for new experimental studies to better understand this nutrient's mechanisms of action in critical illness. Experimental acute pancreatitis (AP) is an effective model for the study of systemic responses that can be applied to test immunomodulatory therapies [10]. The present study aimed to evaluate the impact of previous parenteral glutamine infusion on inflammatory mediator levels and mortality in acute critically ill conditions, using experimental AP as a systemic inflammation-reproducing model.

2. Methods

2.1. Animals. Adult male isogenic Lewis rats (n = 131, 300–350 g) were purchased from the Animal Laboratory of the Multidisciplinary Center for Research in Biological Science (Campinas, São Paulo, Brazil). Prior to the experiment, the animals were adapted for 5 days in metabolic cages at a controlled room temperature (22 ± 25°C) with a 12 h light/dark cycle and free access to water and standard rodent chow (Quimtia®; Nutrilav, Jundiaí, Brazil). All experimental procedures were approved by the Research Ethics Committee of the School of Medicine, University of São Paulo, São Paulo, Brazil.

2.2. Intravenous Access. Animals were anesthetized with an intraperitoneal injection of ketamine (Ketamin-S(+)®, 100 mg/kg body weight; Cristália, Itapira, Brazil) and xylazine (Rompum®, 8 mg/kg body weight; Bayer, São Paulo, Brazil). Intravenous access was achieved by jugular central venous catheterization (CVC), according to a standard technique, followed by connection to a swivel apparatus that allowed the animals to have free mobility [11, 12]. After CVC, all animals received 0.9% saline solution infusion for 24 h. After this period, the animals were randomized to receive 48 h intravenous infusion of 6 mL/day 0.9% saline solution (saline group, n = 44) or 1 g/kg body weight dipeptide alanyl-glutamine (Dipeptiven® 20%, Fresenius-Kabi, Bad Homburg, Germany; glutamine group, n = 42), or no infusion (nontreatment group, n = 45). All animals had access to a standard oral diet (AIN-93M) and water ad libitum during this period.

2.3. Experimental Acute Pancreatitis. After 72 h intravenous access, all animals were anesthetized with an intraperitoneal injection of 100 mg/kg body weight ketamine (Ketamin-S(+)®, Cristália) and 8 mg/kg body weight xylazine (Rompum®, Bayer). The pancreas was exteriorized through an abdominal incision and the pancreatic duct was catheterized using a 24-gauge angicatheter. AP was then induced by retrograde injection of 0.5 mL 3% sodium taurocholate solution (Sigma Chemical, St Louis, MO, USA), according to a standard technique [13–15]. Following AP induction, 71 animals were sacrificed after proper anesthetization at 2 h (saline group, n = 8; glutamine group, n = 9; nontreatment group, n = 10), 12 h (saline group, n = 9; glutamine group, n = 6; nontreatment group, n = 9), and 24 h (saline group, n = 7; glutamine group, n = 7; nontreatment group, n = 6) by cardiac puncture for blood and tissue (lung and liver) collection, and 60 animals (n = 20/group) were kept alive for mortality analysis.

2.4. Serum Cytokine Measurement. Blood samples were centrifuged at 1,000 ×g at 4°C for 10 min to obtain serum. Concentrations of cytokines (interleukin- [IL-] 1, IL-2, IL-4, IL-6, IL-10, interferon- [IFN-] γ, and tumor necrosis factor- [TNF-] α) were assessed in 500 μL serum by multiplex microsphere immunoassays, using a commercial kit for rats (RECYTMAG® 07-65K; Genesis Ltd., MO, USA). Plates were read in a Luminex analyzer (MiraiBio, Alameda, CA, USA), according to the manufacturer's instructions [16].

2.5. Heat Shock Protein Measurement. Approximately 100 mg lung tissue and 50 mg liver tissue were pulverized in liquid nitrogen. The material was homogenized in RIPA lysis buffer (100 mM Tris-HCl [pH 7.5], 1% sodium deoxycholate, 1% NP40, 150 mM NaCl, 0.1% sodium dodecyl sulfate [SDS]) plus protease inhibitors (1 mg/mL pepstatin A, 100 mM phenylmethylsulfonyl fluoride). The samples were then centrifuged at 14,000 ×g for 10 min at 4°C. The supernatants were collected and protein concentrations were quantified using the Bradford method (Bio-Rad Laboratories, Hercules, CA, USA).

Protein samples were added to sample buffer (2% SDS, 60 mM Tris [pH 6.8], 5% mercaptoethanol, 0.01% bromophenol blue) and subjected to electrophoresis in a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) system (1.5 M Tris-HCl, 10% SDS, 30% bis-acrylamide, 10% ammonia persulfate, and 3,3,5,5-tetramethylethylenediamine). Proteins were then transferred to nitrocellulose membranes using a semidry transfer apparatus (both from Bio-Rad Laboratories). The membranes were incubated in a blocking solution of 5% skim milk in TBST buffer (50 mM Tris buffer [pH 8.0], 100 mM NaCl, and 1% Tween 20) for 1 h at room temperature. Then, they were washed in TBST and incubated with the primary antibody against the protein of interest (HSP polyclonal goat anti-rat®; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. Subsequently, the membranes were incubated in a solution containing the peroxidase-conjugated secondary antibody (1 : 1000; Santa Cruz Biotechnology), and Super Signal detection (Pierce, Rockford, IL, USA) was performed. Protein expression was compared by gel densitometry using the ImageJ public domain software created by Wayne Rasband at the US National Institutes of Mental Health, which has been used previously for the determination of HSP70 and HSP90 [13].

2.6. Mortality Observation. After AP induction, 20 rats in each group remained under observation for a maximum of 7 days, with access to standard oral diet (AIN-93M) and water ad libitum. The animals were observed individually every 8 h for death registration. Animals that survived until 7 days after AP were sacrificed with an intraperitoneal injection of 80 mg/kg ketamine hydrochloride (Ketamin-S(+)®; Cristália) and 8.0 mg/kg xylazine hydrochloride (Rompum® 2%; Bayer).

2.7. Statistical Analysis. All inflammatory variables were compared using the Kruskal–Wallis and Behrens–Fisher tests, as Kolmogorov–Smirnov tests showed that they were not distributed normally. These comparisons were performed between groups at each time point and within groups over time. Mortality and survival data were evaluated by the Fisher test and Kaplan–Meier analysis, respectively. All analyses were based on a 5% level of significance and were performed

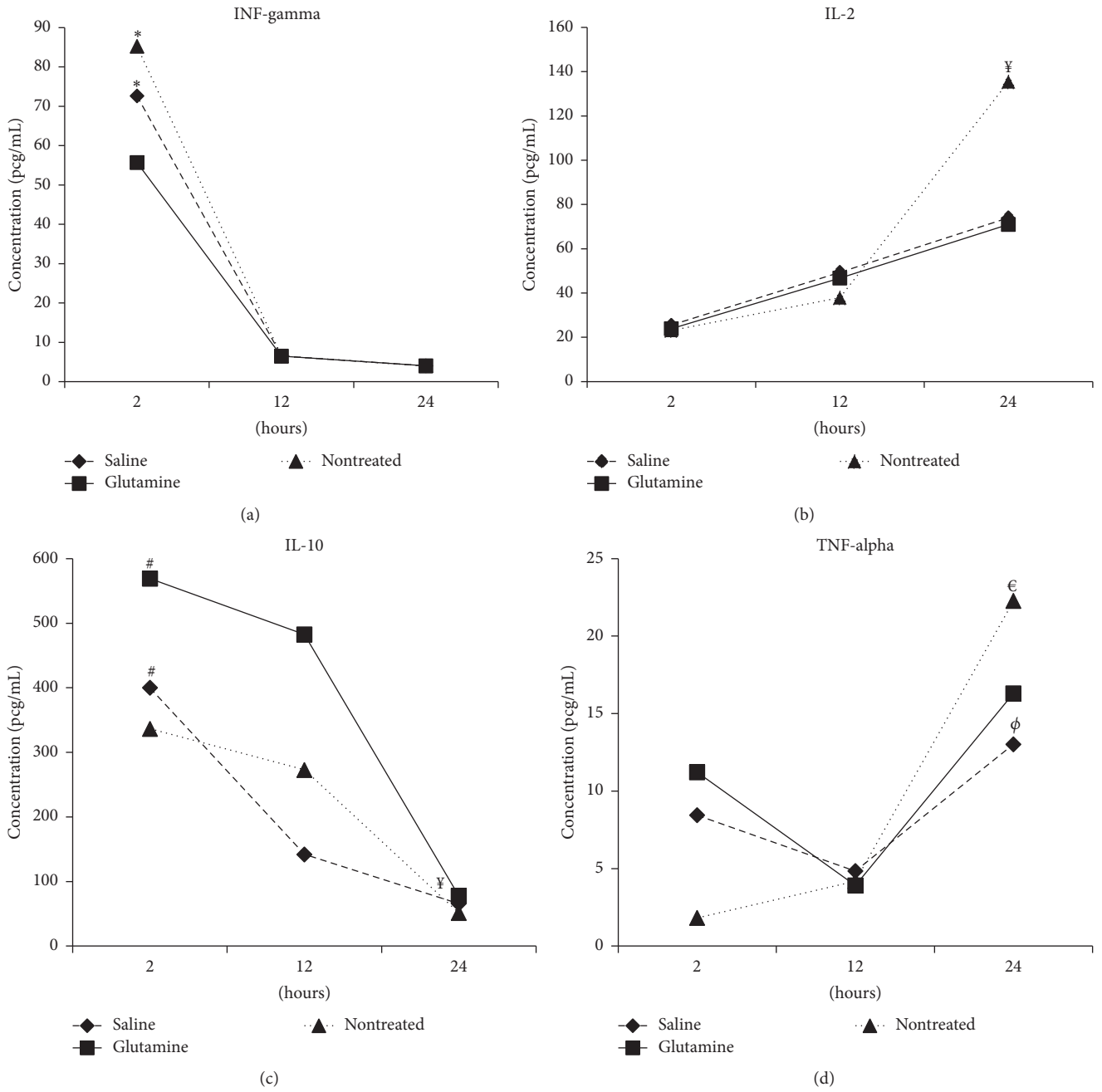

Figure 1: Median serum concentrations of interferon- (IFN-) gamma (a), interleukin- (IL-) 2 (b), IL-10 (c), and tumor necrosis factor- (TNF-) alpha (d) at 2, 12, and 24 h after acute pancreatitis induction with sodium taurocholate in Lewis rats previously treated or not treated with 48 h infusion of parenteral saline or glutamine. $^{*}p \leq 0.050$ versus 12 and 24 h; $^{¥}p \leq 0.050$ versus 2 and 12 h; $^{\#}p \leq 0.050$ versus 24 h; $^{€}p = 0.051$ versus 2 and 12 h; $^{\phi}p = 0.060$ versus 12 h.

using SPSS software (ver. 18.0 for Windows; SPSS, Chicago, IL, USA).

3. Results

3.1. Serum Cytokine Concentrations. Serum cytokine levels did not differ among groups at any time point (Table 1). Serum IFN-γ levels in the nontreatment and saline groups were significantly higher at 2 h after AP than at 12 h ($p = 0.026$ and 0.001, resp.) and 24 h ($p = 0.002$ and 0.050, resp.) after AP (Figure 1(a)). In addition, animals in the nontreatment group exhibited higher serum IL-2 levels (Figure 1(b)) and lower serum IL-10 levels (Figure 1(c)) at 24 h after AP than at 2 h (both $p < 0.001$) and 12 h ($p = 0.005$ and $p < 0.001$, resp.) after AP. Animals in the saline and glutamine groups exhibited only lower IL-10 levels at 24 h after AP relative to

Table 1: Serum cytokine levels in rats with acute pancreatitis, according to treatment and time point.

Cytokine	Treatment	Time point											
		2 h				12 h				24 h			
		Median	1° quartile	3° quartile	p value	Median	1° quartile	3° quartile	p value	Median	1° quartile	3° quartile	p value
IL-1a	Saline	14.41	4.67	47.89	0.552	15.60	11.40	31.86	0.574	1.84	1.84	46.08	0.583
	Glutamine	4.67	4.67	12.27		13.57	9.48	33.94		12.49	1.84	25.98	
	Nontreatment	8.47	4.67	47.89		8.57	6.13	17.86		72.61	1.84	128.00	
IL-4	Saline	3.98	3.98	8.90	0.970	5.91	4.88	9.23	0.464	1.30	1.30	13.10	0.398
	Glutamine	3.98	3.98	3.98		5.91	3.91	15.38		1.30	1.30	4.88	
	Nontreatment	3.98	3.98	3.98		2.17	1.35	6.98		19.72	1.30	42.82	
IL-2	Saline	25.50	22.32	31.21	0.833	49.40	10.97	72.34	0.814	74.03	27.12	117.21	0.249
	Glutamine	23.89	22.31	30.38		46.75	18.16	90.19		70.94	17.49	167.75	
	Non-treatment	23.10	17.69	33.71		37.89	24.44	48.50		135.50	83.31	166.00	
IL-6	Saline	200.00	76.71	289.50	0.644	220.00	50.13	12704.00	0.971	369.00	184.21	766.50	0.458
	Glutamine	178.00	33.11	452.00		331.25	29.70	1619.00		903.00	486.50	1089.00	
	Nontreatment	336.00	33.11	3135.00		1789.00	113.00	5319.00		464.50	293.00	599.00	
IL-10	Saline	400.00	294.50	560.00	0.288	142.00	99.08	691.00	0.964	65.83	34.62	183.18	0.553
	Glutamine	539.00	298.00	893.00		482.50	65.28	1521.00		77.82	54.16	125.41	
	Nontreatment	336.50	168.00	482.00		273.00	96.09	617.00		51.42	39.50	57.66	
IFN-γ	Saline	72.62	30.44	89.41	0.142	6.50	6.50	6.50	0.908	4.01	4.01	4.01	0.776
	Glutamine	55.67	55.67	64.15		6.50	6.50	6.50		4.01	4.01	4.01	
	Nontreatment	85.25	64.15	93.61		6.50	6.50	6.50		4.01	4.01	63.08	
TNF-α	Saline	8.44	1.82	15.97	0.390	4.84	2.17	5.86	0.862	13.02	9.19	23.27	0.579
	Glutamine	11.22	1.82	11.22		3.91	2.17	8.05		16.31	3.94	18.50	
	Nontreatment	1.82	1.82	8.53		4.19	2.43	9.21		22.27	14.12	38.39	

IL, interleukin; INF, interferon; TNF, tumor necrosis factor.

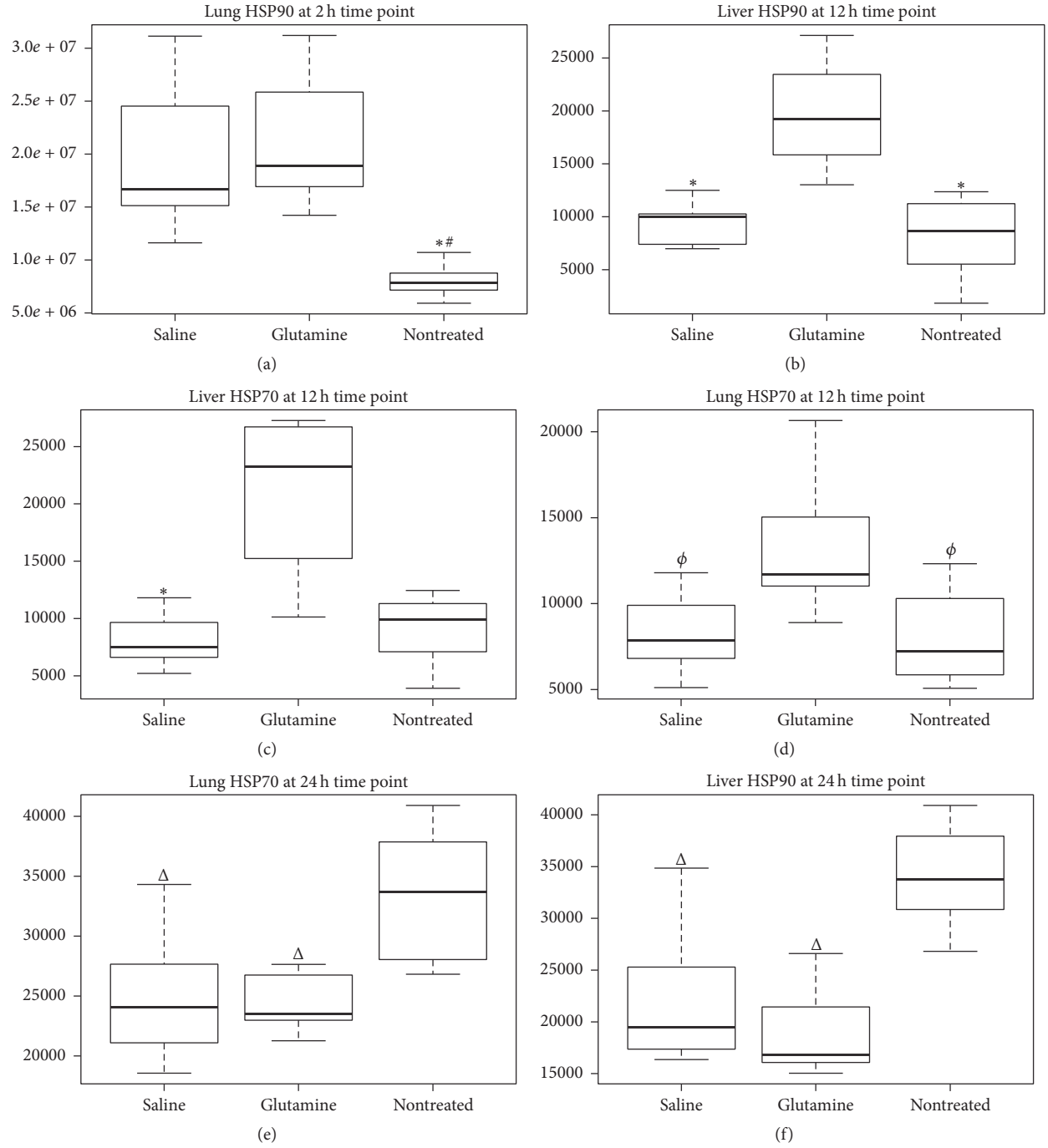

Figure 2: Lung and liver expression of heat shock proteins (HSPs) at different time points after acute pancreatitis induction with sodium taurocholate in Lewis rats previously treated or not treated with 48 h infusion of parenteral saline or glutamine. (a) Expression of HSP90 in lung tissue at 2 h. (b) Expression of HSP90 in liver tissue at 12 h. (c) Expression of HSP70 in liver tissue at 12 h. (d) Expression of HSP70 in lung tissue at 12 h. (e) Expression of HSP70 in lung tissue at 24 h. (f) Expression of HSP90 in liver at 24 h. Data are expressed as medians. $^{*}p \leq 0.050$ versus glutamine group; $^{\#}p \leq 0.050$ versus saline group; $^{\phi}p = 0.066$ versus glutamine group; $^{\Delta}p \leq 0.050$ versus nontreatment group.

2 h after AP (both $p < 0.001$; Figure 1(c)). No significant change in the serum IL-1, IL-4, IL-6, or TNF-α level occurred over time, although marginally nonsignificant higher TNF-α levels were observed at 24 h after AP in the nontreatment ($p = 0.051$ versus 2 and 12 h after AP) and saline ($p = 0.060$ versus 12 h after AP) groups (Figure 1(d)).

3.2. Heat Shock Protein Expression. Data on HSP expression are presented in Table 2. Animals in the glutamine group exhibited greater lung and liver HSP90 expression than did those in the nontreatment group at 2 h ($p = 0.007$; Figure 2(a)) and 12 h ($p = 0.001$; Figure 2(b)) after AP, respectively, and greater liver HSP90 and HSP70 expression

Table 2: Heat shock protein expression in rats with acute pancreatitis, according to treatment and time point.

Variable	Treatment	Time point											
		2 h				12 h				24 h			
		Median	1° quartile	3° quartile	p value	Median	1° quartile	3° quartile	p value	Median	1° quartile	3° quartile	p value
HSP70 lung	Saline	15615447	14285740	20267619		7848	6817	9901		24064	21086	27654	
	Glutamine	13645497	8114841	15673669	0.372	11684	11021	15036	0.066	23499	22976	26745	**0.0194**
	Nontreatment	12956058	10096912	16820669		7219	5854	10299		33718	28039	37863	
HSP90 lung	Saline	16654811	15125912	24525217		8079	6392	9511		14031	11409	28005	
	Glutamine	18875083	16923497	25849518	**0.009**	11742	9796	13052	0.065	8108	6184	9750	0.1067
	Nontreatment	8037912	7573083	12943462		8745	5670	10700		9094	7174	12214	
HSP70 liver	Saline	27154796	22116953	32188418		7497	6614	9658		24495	20696	24938	
	Glutamine	28513246	26185806	35924403	0.175	23252	15236	26715	**0.027**	15817	13442	20652	0.1495
	Nontreatment	34420660	30578261	41040332		9875	7099	11308		20686	14079	21387	
HSP90 liver	Saline	NS	NS	NS		10014	7396	10263		19507	17368	25291	
	Glutamine	NS	NS	NS	NS	19218	15843	23452	**0.008**	16841	16074	21436	**0.0045**
	Nontreatment	NS	NS	NS		8671	5527	11237		337601	30857	37946	

NS, nonsignificant expression to be detected; HSP, heat shock protein.

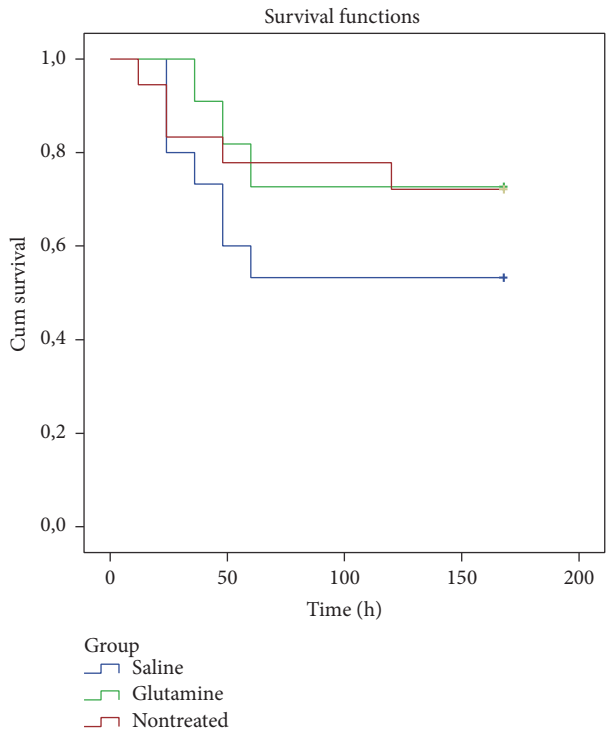

Figure 3: Seven-day mortality rates of Lewis rats treated or not treated with 48 h parenteral infusion of saline or glutamine before acute pancreatitis induction with sodium taurocholate.

than did those in the saline group at 12 h after AP ($p < 0.001$ and $p = 0.006$, respectively; Figures 2(b) and 2(c)). Marginally nonsignificant greater lung HSP70 expression was observed in animals in the glutamine group compared with the other groups at 12 h after AP ($p = 0.066$; Figure 2(d)). Lung HSP90 expression was greater in the saline group than in the nontreatment group at 2 h after AP ($p = 0.017$; Figure 2(a)). The nontreatment group presented increased lung HSP70 and liver HSP90 expression relative to the other groups at 24 h after AP ($p = 0.019$ and 0.004, respectively; Figures 2(e) and 2(f)).

3.3. Mortality. No significant difference in 7-day mortality was observed among groups (nontreatment, 28%; saline, 47%; glutamine, 27%) (Figure 3). In addition, the median interval of mortality occurrence did not differ among groups (nontreatment, 24–48 h; saline, 30–36 h; glutamine, 48 h).

4. Discussion

Our study aimed to contribute to the understanding of potential inflammatory mechanisms that may impact the risk-benefit balance of parenteral glutamine infusion in critical care. AP was chosen as a critical condition model due to the central roles of inflammatory mediators in its physiopathology and in multiple organ dysfunction syndrome, which is usually its primary cause of death [10]. In addition, the systemic effects of AP are similar to those observed in other critical conditions, such as septicemia, severe burns, and trauma [17]. Specifically, sodium taurocholate-induced AP has been reported to be a representative model of the disease, with severe and measurable systemic inflammatory response and multiple organ failure, as evidenced by lung, liver, and intestinal impairment in rats [18–21].

In human and experimental models, marked release of the proinflammatory mediators IL-1, IL-6, and TNF-α is the main detrimental finding associated with AP [15, 22]. This release is usually followed by increased release of anti-inflammatory mediators (e.g., IL-10), which may induce immunosuppression in the late stage of the disease [15]. In our study, high doses of parenteral glutamine infused for 48 h before the induction of experimental AP did not change serum levels of IL-1, IL-6, and TNF-α at any post-AP time point. Marginally nonsignificant increases in TNF-α levels were observed at 24 h after AP in the nontreatment and saline groups, but not in the glutamine group.

The glutamine group also did not show the significant decreases in IFN-γ level observed over time in the nontreatment and saline groups. In addition, the IL-2 levels were maintained overtime in the glutamine and saline groups and the decrease in IL-10 level, observed in all groups at 24 h after AP, occurred more slowly in the glutamine and saline groups than in the nontreatment group. Possible harmful effects and benefits associated with these cytokines must be interpreted in light of the timing of their release over the inflammatory stages of AP progression. For instance, IFN-γ and IL-2 can activate inflammation, but these cytokines also have benefits related to pathogen clearance that can be relevant in efforts to avoid infection in the later stages of critical aggression [23, 24].

Accordingly, immunotherapy with IFN-γ seems be detrimental in the early stage of AP (when inflammation has harmful effects) and beneficial in the later stage of the disease (when infectious complications and immunoparalysis are dominant causes of mortality) [25–28]. Similarly, decreased IL-2 release and 90% nonspecific mortality were observed after the intraperitoneal administration of lipopolysaccharide in mice with AP, and therapy with recombinant IL-2 reduced lipopolysaccharide-induced mortality in the later stages of the disease [24]. In addition, due to its potent contraregulatory effects, IL-10 has been found to be beneficial in a sodium taurocholate-induced AP model [29]. However, substantial release of this cytokine may hyperintensify its anti-inflammatory effect and favor immunoparalysis [30]. Therefore, the dynamics of IFN-γ, IL-2, and IL-10 release over time observed in animals in the glutamine group in this study seem to be protective, enabling the maintenance of immunocompetence for pathogen clearance in the later stages of AP progression.

In our study, marked effects on HSP expression were also observed in the glutamine group in relation to the other groups. This effect included increased liver HSP70 expression and a tendency for increased lung HSP70 expression 12 h after AP, as well as an early significant increase in lung and liver HSP90 expression. Xue et al. [31] reported improvement in the expression of heat shock transcription factor-1 (a master regulator of HSP expression) after parenteral infusion of glutamine in rats. HSP expression may be vital to cellular and tissue protection in the context of stress or injury, as HSPs act as molecular chaperones that stabilize and refold damaged intercellular proteins and prevent intracellular protein aggregation [32]. Indeed, the main metabolic and stress-signaling effects of glutamine in illness and injury seem to occur due its ability to induce HSP expression [33].

Increases in HSP70 expression induced by glutamine are associated with improvements in survival, tissue injury, and inflammatory response [32]. HSP90 also has cytoprotective properties, but most of its target proteins are kinases and transcription factors that can act as cellular regulators of gene expression, including the transcription of proinflammatory molecules via nuclear factor kappa B [34, 35]. However, we found no systemic detrimental increase in proinflammatory cytokines in parallel with increased HSP90 in the glutamine group. Moreover, compared with the nontreatment group, the increased liver HSP90 expression in the glutamine group occurred early after AP induction and was reduced significantly after 24 h. Because HSP expression increases in response to detrimental stimuli, this observation is highly suggestive of early liver homeostasis in response AP injury in the glutamine group [36].

Parenteral glutamine supplementation was recently associated with high mortality rates in critically ill patients with multiple organ failure [7, 8]. With consideration of systemic disturbances that could culminate in multiple organ failure, our AP model did not confirm this harmful effect, despite our parenteral infusion of high glutamine doses. The most recent multicentric trial showed that parenteral glutamine infusion did not change the mortality rate of patients in the surgical intensive care unit but also did not improve clinical outcomes [37]. In our study, the mortality rate was lower and death occurred later in animals in the glutamine group compared with the other groups, although these differences were not significant.

Our study has some limitations in addition to its experimental nature, which may limit the applicability of the findings to humans. First, glutamine was infused alone and before critical stress. As the release of inflammatory mediators is transient, this strategy was adopted to provide glutamine to cells and tissue in time to observe its modulatory effects on these mediators in our model. However, we do not know whether the same effect would be observed if glutamine were infused with other nutrients and in the presence of stress factors. In addition, parenteral supply of glutamine before critical stress cannot be applied fully in clinical practice. Second, saline was used as a parenteral control for glutamine. Saline hydration can attenuate AP by mitigating changes in pancreatic microcirculation and circulatory disorders of the intestinal wall, which facilitate bacterial translocation and perpetuate the inflammation mechanism [38]. These effects may explain the greater benefits of glutamine on systemic proinflammatory mediator profiles and tissue HSP expression in comparison with the nontreatment group than in comparison with the saline group.

Within these limitations, our data suggest that a high dose of parenteral glutamine protects against stress-induced organ damage by improving cytokine profiles and increasing HSP70 and HSP90 expression in our AP model. These protective effects are of particular interest for the treatment of critically ill patients. Further studies must seek to design a protocol for parenteral administration of glutamine that allows us to take clinical advantage of its potential benefits.

Competing Interests

Dan L. Waitzberg has received speaking honoraria from Fresenius-Kabi, the manufacturer of parenteral glutamine solution.

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Review Article

Role of Uric Acid Metabolism-Related Inflammation in the Pathogenesis of Metabolic Syndrome Components Such as Atherosclerosis and Nonalcoholic Steatohepatitis

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Uric acid (UA) is the end product of purine metabolism and can reportedly act as an antioxidant. However, recently, numerous clinical and basic research approaches have revealed close associations of hyperuricemia with several disorders, particularly those comprising the metabolic syndrome. In this review, we first outline the two molecular mechanisms underlying inflammation occurrence in relation to UA metabolism; one is inflammasome activation by UA crystallization and the other involves superoxide free radicals generated by xanthine oxidase (XO). Importantly, recent studies have demonstrated the therapeutic or preventive effects of XO inhibitors against atherosclerosis and nonalcoholic steatohepatitis, which were not previously considered to be related, at least not directly, to hyperuricemia. Such beneficial effects of XO inhibitors have been reported for other organs including the kidneys and the heart. Thus, a major portion of this review focuses on the relationships between UA metabolism and the development of atherosclerosis, nonalcoholic steatohepatitis, and related disorders. Although further studies are necessary, XO inhibitors are a potentially novel strategy for reducing the risk of many forms of organ failure characteristic of the metabolic syndrome.

1. Introduction

Uric acid (UA) is the end product of the metabolic pathway for purines, the main constituents of nucleotides. The pathway of UA generation is shown in Figure 1. Briefly, inosine monophosphate (IMP) is derived from de novo purine synthesis and from purine salvage. Hypoxanthine from IMP is catalyzed to xanthine and then to uric acid by xanthine oxidase (XO). De novo nucleotide synthesis generates IMP via ribose-5-phosphate, catalyzed to 5-phosphoribosyl-1-pyrophosphate (PRPP). In the salvage pathway, hypoxanthine-guanine phosphoribosyl transferase (HGPRT) plays an important role in generating IMP, thereby inhibiting UA generation.

Since humans are unable to catabolize UA to the more soluble compound allantoin due to lack of urate oxidase or uricase [1], the serum UA concentration is higher in humans than almost all other mammals. However, this high UA level in humans has been regarded as being beneficial in the presence of elevated oxidative stress [2]. UA is oxidized to allantoin and other metabolites via nonenzymatic oxidation

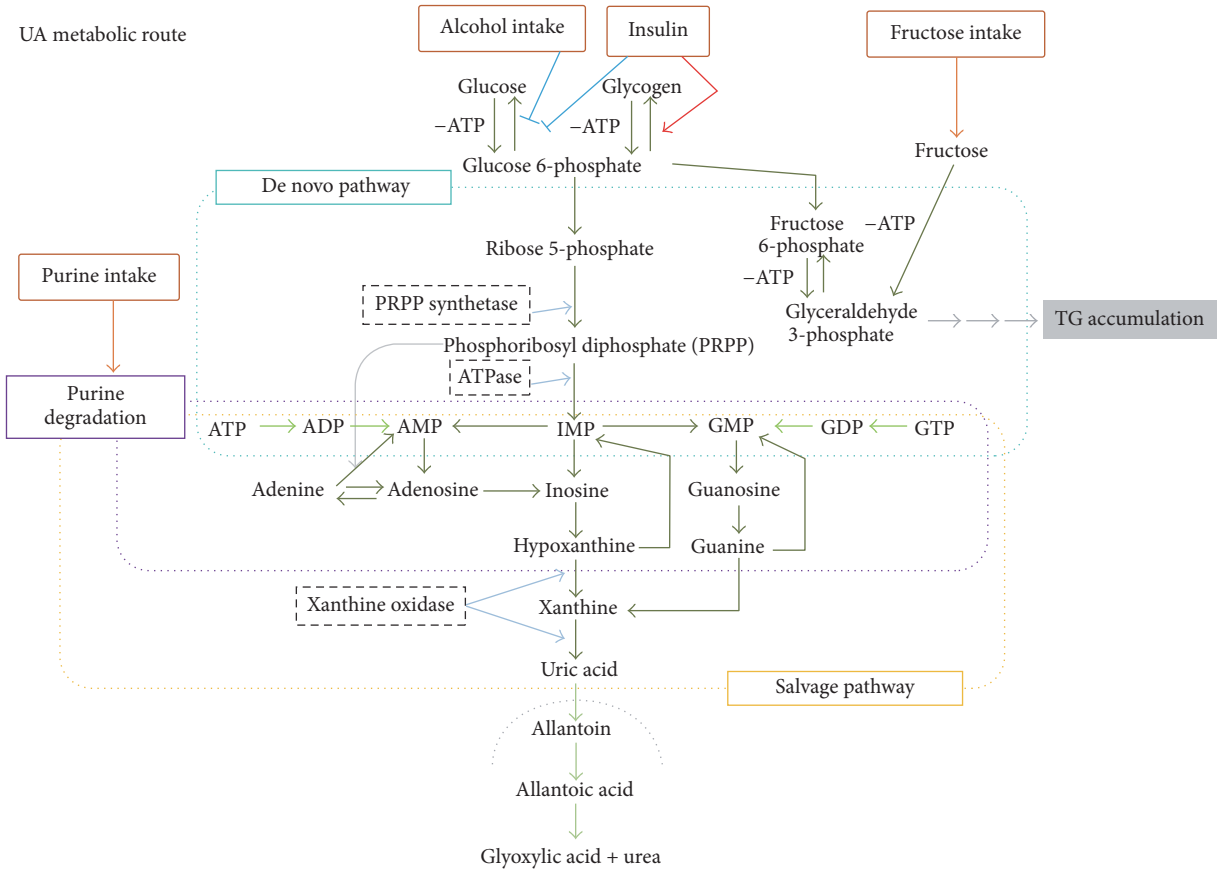

Figure 1: Metabolic pathways involving UA.

[3] and, thus, UA can function to neutralize prooxidant molecules, such as hydroxyl radicals, hydrogen peroxide, and peroxynitrite. UA shows the highest scavenging rate constant against $O_2^{-\bullet}$, with constants being low against $CH3^{\bullet}$ and t-$BuOO^{\bullet}$ [4]. UA directly (nonenzymatically) and preferentially deletes nitric oxide (NO) and forms 6-aminouracil in physiological environments or in association with antioxidants [5]. In vitro, UA has both an antioxidant effect on native LDL and a prooxidant effect on mildly oxidized LDL [6]. Allantoin does not have these effects. The mechanisms of these reactions vary among combinations of prooxidant molecules and solution polarities [7].

It has been suggested that this antioxidant effect of the high UA concentrations in humans contributes to neuroprotection in several neurodegenerative and neuroinflammatory diseases [8–14].

However, despite the potential antioxidant effect of UA itself, numerous studies have revealed close associations of serum UA concentrations and various disorders, most of which are included in the metabolic syndrome category. Thus, UA metabolism may be a so-called double-edged sword as regards the inflammatory and/or oxidative responses in many organs, though on the whole, its harmful effects appear to outweigh the benefits of UA in most cases.

In this review, we first explain the two putative molecular mechanisms underlying inflammation occurrence in relation to UA metabolism; one is inflammasome activation via UA crystallization and the other involves superoxide free radicals generated by XO. While the UA crystallization mechanism would be dependent on a high serum UA concentration, the latter may not necessarily reflect the serum UA concentration though XO activity does lead to the production of reactive oxygen species (ROS).

Subsequently, lines of research showing relationships between UA metabolism and the development of various disorders are introduced and discussed. Importantly, recent studies have demonstrated beneficial effects of XO inhibitors against the occurrence and/or progression of several disorders, particularly atherosclerosis and nonalcoholic steatohepatitis (NASH), both of which are associated with insulin resistance, hyperlipidemia, and/or obesity. In this review, atherosclerosis and NASH are discussed extensively, while studies of gout and chronic kidney diseases (CKD) are mentioned briefly. In conclusion, we propose that such XO inhibitors may be more useful for preventing a variety of disorders, such as atherosclerosis and NASH, than previously believed, probably via an anti-inflammatory effect.

2. Inflammation Occurrence Related to UA Metabolism

Among the disorders related to hyperuricemia, gout is the most representative and well known. Features of gout include painful arthritis affecting the limbs, caused by reduced UA

crystals in the joints. While symptoms of a gout attack are typical of an acute inflammatory response, as indicated by the presence of swelling, heat, rubescence, and pain, there are many disorders with mild but chronic inflammation which are very likely to be related to UA metabolism. In the latter case, superoxide free radicals generated by XO are key players leading to chronic inflammatory processes eventually resulting in impaired organ functions. Thus, we introduce two independent mechanisms underlying UA metabolism-induced inflammation.

2.1. Inflammasome Activation by Crystallized UA Particles. In 2002, the inflammasome concept was proposed to involve multiple proteins and to control the cleavage of prointerleukin 1 (IL-1) [15]. Initially, inflammasomes were considered to play a role in immune responses and serve as defense systems against pathogens [16, 17]. However, a line of subsequent studies has elucidated that inflammasomes are key players in the onsets of a wide range of diseases as well as host defense. Excessive metabolites, such as ATP or monosodium urate crystals (MUC), were also confirmed to be involved in the activation of inflammasomes, and inflammatory responses occurring via inflammasomes have been demonstrated to be linked to the onset and progression of human diseases, including gout, atherosclerosis and NASH, as described below in detail [18–24].

Inflammasomes are known to be divided into discernible patterns, depending on component proteins [16]. Among them, the NLRP3 inflammasome, comprised of three major components, Nod-like receptor 3 (NLRP3), apoptosis-associated speck-like protein containing a CARD (ASC) and caspase-1, has been well investigated. Maturations of both IL-1 and IL-18 by inflammasomes require a two-step mechanism. First, the Toll-like receptor ligands, such as lipopolysaccharide (LPS), activate the NF-κB pathway and upregulate the expression levels of interleukins, including pro-IL-1β and pro-IL-18. Subsequently, the inflammasome complex activated by pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) cleaves pro-IL-1β or pro-IL-18, resulting in the production of mature interleukins [15–17].

MUC also reportedly serve as a danger signal and trigger the activation of inflammasomes [18]. Although the mechanism of inflammasome activation by MUC has not been fully elucidated, the following mechanism was proposed. MUC stimulate the Toll-like receptor 2/4-Myd88 pathway and raise transcriptional levels of pro-IL-1β through the NF-κB pathway [25]. It is theorized that MUC-induced inflammasome activation is driven by two key factors. One is a decrease in the intracellular potassium concentration. Indeed, the addition of high potassium abrogated IL-1β release by MUC. The other is the generation of ROS, because an antioxidant, N-acetyl-cysteine, abolished IL-1β secretion by MUC [26]. Other studies have indicated the application of MUC to raise intracellular ROS levels. However, the relationship between intracellular K^+ level changes and ROS generation remains unknown, and future studies are expected to resolve this issue [27, 28]. Elevation of intracellular ROS mediates the detachment of thioredoxin-interacting protein (TXNIP) from thioredoxin and enables TXNIP to associate with NLRP3, leading to NLRP3 inflammasome activation [29, 30]. Thus, MUC accumulation promotes inflammatory responses through inflammasomes (Figure 2) and thereby promotes the onset of diseases, such as gout.

2.2. Superoxide Free Radicals Generated by XO. When mammalian xanthine dehydrogenase (XDH) is converted to XO under stressed conditions such as tissue damage and ischemia [31], superoxide anion and hydrogen peroxide are produced during molybdenum hydroxylase-catalyzed reactions in a molar ratio of about 1 : 3 [32]. The proteolytic activation from XDH to XO is required for superoxide generation [33]. In essence, XO oxidizes a variety of purines and pterins, classified as molybdenum iron-sulfur flavin hydroxylases. When XO reacts with xanthine, electrons are transferred from Mo, Fe-S, and FAD. XO produces FADH2, while XDH produces FADH. Only FADH2 reacts with O2 [34]. In the UA metabolic pathway, XO oxidizes hypoxanthine from nucleic acid metabolites into xanthine and xanthine into UA (Figure 1). XO, as well as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and the mitochondrial electron-transport chain, generates ROS [35].

ROS from XO might play physiological roles, especially in development. Treatment during pregnancy with allopurinol alters maternal vascular function involving β1-adrenergic stimulation and impairs the fetal α1-adrenergic vasoreflex response involving NO [36]. Fetal XO is activated in vivo during hypoxia and XO-derived ROS contributes to fetal peripheral vasoconstriction, leading to fetal defense against hypoxia [37]. XO depletion induces renal interstitial fibrosis, and renal epithelial cells from XOR (−/−) mice are more readily transformed into myofibroblasts [38]. Indeed, how ROS from XO directly and physiologically acts in vivo is unknown.

The tissue and cellular distributions of XO in mammals are highest in the liver and intestines due to XO-rich parenchymal cells [39]. Xanthine oxidoreductase (XOR) is present in hepatocytes, while XO is present in bile duct epithelial cells, concentrated toward the luminal surface. Moreover, in human liver disease, proliferating bile ducts are also strongly positive for XO [40]. Molybdenum supplementation significantly increased XO activities in the liver and small intestinal mucosa [41]. XO activity is low in human serum, the brain, heart, and skeletal muscle, while being rich in microvascular endothelial cells [42] and is also present in macrophages [43]. Circulating XO can adhere to endothelial cells by associating with endothelial glycosaminoglycans [44]. The study using electron spin resonance measurements revealed the contribution of increased XO activity to endothelial dysfunction in patients with coronary artery diseases [45].

XO activation is induced by LPS, angiotensin II, NADPH oxidase, hypoxia, hypoxia-inducible factor 1, and inflammatory cytokines such as IL-1β [46–49]. The release of XO is increased in hypercholesterolemia, chronic hyperammonemia, thermal trauma, beta-thalassemia, brain ischemia, and pulmonary artery hypertension [50–54]. Aging is another factor associated with elevated XO activity. Indeed, XO was

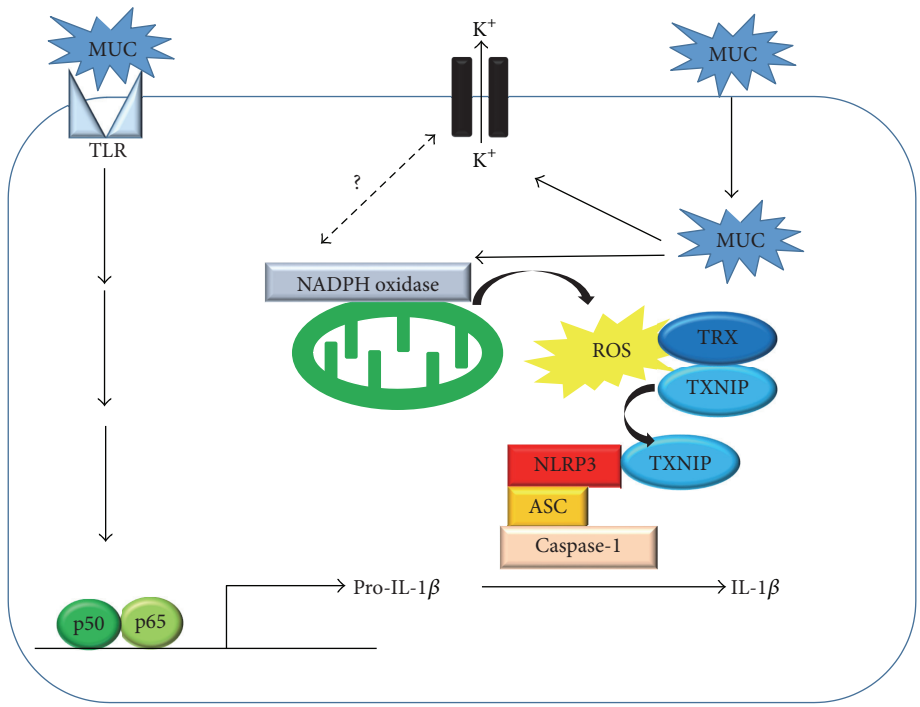

Figure 2: MUC induces inflammasome activation. MUC activates the NF-κB pathway through TLR2/4, thereby increasing the expressions of pro-IL-1β or pro-IL-18. At the same time, MUC induces ROS release from mitochondria. The generated ROS detaches TXNIP from thioredoxin and enables TXNIP to interact with the NLRP3 complex. The binding of TXNIP to NLRP3 activates inflammasomes, leading to the production of mature IL-1β or IL-18. MUC: monosodium urate crystals, TLR: Toll-like receptor, TXNIP: thioredoxin-interacting protein, TXR: thioredoxin, and ROS: reactive oxygen species.

significantly higher in the aortic walls and skeletal muscles of old rats than in those of their young counterparts. The correlation between plasma XO activity and age is observed in both humans and rats [55]. It appears that hyperglycemia itself has no impact on liver XO activity, though cardiac, renal, and brain XO activities were shown to be increased in rats with advanced diabetes [56, 57]. XO activity rises remarkably in ischemic congestive heart failure and XO localizes within CD68 positive macrophages [43]. The association between XO and ischemic reperfusion injury has been well investigated. XO is one of the major superoxide sources in ischemia/reperfusion injuries of the heart [58], forebrain [59], skin [60], liver [61, 62], and gastric mucosa [63], as well as multiple system organ failure after hind limb reperfusion [64]. XO activity, along with lipid peroxidation, myeloperoxidase activity and NO levels, is increased in the liver in response to renal ischemia/reperfusion in diabetic rats [65]. Ischemia/reperfusion injury is attributable to elevated XO activity and ATP depletion related to increasing hypoxanthine and xanthine levels during ischemia, and reperfusion provides O_2 for oxidation of these compounds [1].

Superoxide production by XO may also be enhanced by increasing the amount of its substrate, purine bodies. Excess fructose metabolism results in ATP depletion which is associated with degradation of AMP to hypoxanthine, followed by conversion to UA by XO [66]. Indeed, the serum UA level is upregulated in response to a fructose burden [67]. Inversely, UA stimulates fructokinase and fructose metabolism during fatty liver development [68]. ATP depletion, such as that characteristic of glycogen storage disease type 1 [69], hypoglycemia [70], exercise [71], and starvation [72], also increases UA production. Conditions associated with DNA turnover, such as tumor progression [73] and tumor lysis [74], are also mediated by XO.

Superoxide produced by XO is an important messenger inducing inflammation and signal transduction, leading to tissue damage. We found inflammatory cytokines to be induced via XO when foam cells form with lipid accumulation [75]. XO regulates cyclooxygenase-2 [76] in the inflammatory system, and XO appears to be critical for innate immune function [77]. XO increased Egr-1 mRNA and protein, as well as the phosphorylation of ERK1/2, while pretreatment with an ERK1/2 inhibitor prevented induction of Egr-1 by XO [78]. In addition, XO reportedly reduced SUMOylation of PPARγ in inflammatory cells [79]. ROS from XO augment TRB3 expression in podocytes [80].

As noted above, superoxide from XO has been suggested to play roles in various forms of inflammatory or ischemic pathophysiology (Figure 3), not necessarily involving hyperuricemia.

3. UA Metabolism and Chronic Renal Disease, Atherosclerosis, Heart Failure, and NASH

While gout is a disorder well known to be caused by the precipitation of UA crystals, the involvement of hyperuricemia in CKD is also widely recognized. The major causes of CKD have been regarded as diabetes mellitus and hypertension,

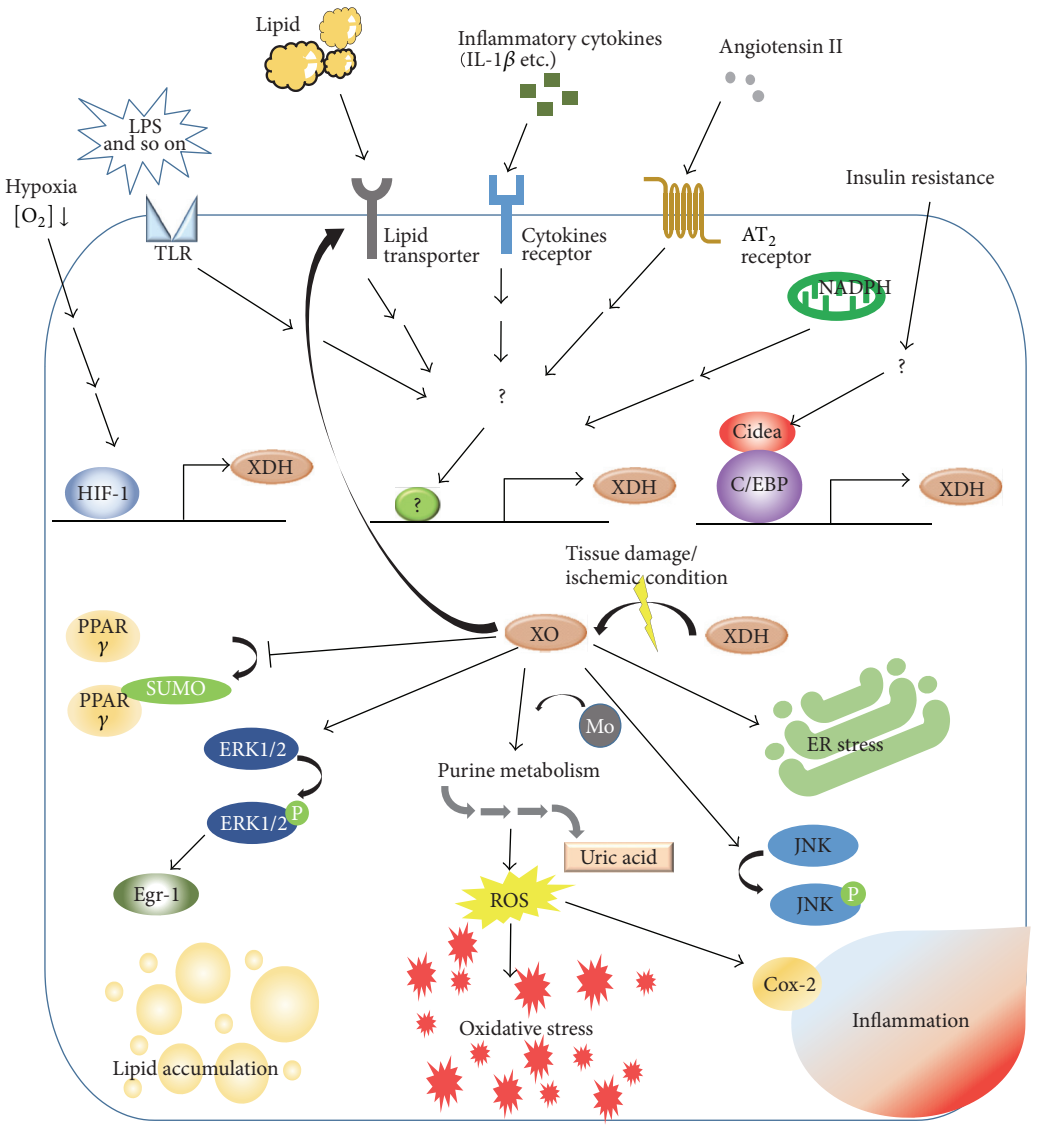

Figure 3: Involvement of XO in molecular pathologies related to inflammation; "causes and results."

and thus, hyperuricemia was long viewed as a consequence of CKD. In fact, loss of kidney function reduces the excretion of UA into urine, resulting in hyperuricemia. In contrast, recent studies demonstrated a significant association between serum UA and the development of CKD. While each metabolic syndrome component, including hyperglycemia, hyperlipidemia, and hypertension, was associated with an increased CKD risk, hyperuricemia was apparently an independent risk factor not influenced by the others. Therefore, hyperuricemia is both a cause and a consequence of CKD and is frequently associated with other metabolic syndrome features.

In terms of CKD pathogenesis, serum UA is likely to activate the renin-angiotensin system resulting in vascular smooth muscle cell proliferation [81] and to induce an epithelial-to-mesenchymal transition of renal tubular cells [82]. XO inhibitor treatment reportedly reduced intercellular adhesion molecule-1 (ICAM-1) expression in tubular epithelial cells [83] of mice. We speculate that UA itself and superoxide free radical generation both play roles in the molecular mechanisms underlying hyperuricemia-related CKD development, but further research is required to elucidate the complex mechanistic interactions between serum UA and CKD.

As mentioned in Section 2, both UA and superoxide free radicals are simultaneously produced by XO and might be the pathophysiological cause of these diseases. As shown in Figure 3, chronic inflammation is also involved in pathophysiological processes, generally exhibiting a close relationship with oxidative stress. ROS from XO induces LPS-induced JNK activation via inactivation of MAPK phosphatase-(MKP-) 1 [84] and XO regulates cyclooxygenase-2, one of the master regulators of inflammation [76]. Therefore, damage from UA, ROS, and UA-induced and/or ROS-induced inflammation might together contribute to the progression of certain diseases, and distinguishing which mechanism acts first is often difficult in lifestyle-related diseases.

3.1. Atherosclerosis, Vascular Dysfunction, and Heart Failure. Although the relationships between serum UA levels and atherosclerotic diseases, including hypertension [85, 86], have been documented, whether or not serum UA itself is an independent cardiovascular risk factor remains controversial as most hyperuricemic patients with cardiovascular diseases (CVD) have other complications such as hypertension, dyslipidemia, diabetes, and CKD as well, which are generally regarded as more established risk factors for CVD than hyperuricemia. Recently, however, a growing body of evidence from both clinical and basic research supports the hypothesis that hyperuricemia, partly via elevated XO activity, is an independent risk factor for hypertension and CVD.

Despite the association between hyperuricemia and hypertension having been recognized since the 19th century [85], it was not until recently that hyperuricemia was demonstrated to be an independent risk factor for hypertension development [87–93]. A recently published meta-analysis showed that the adjusted relative risk of developing hypertension was 1.48 for hyperuricemic patients [94], and this association was apparently much stronger in younger, early-onset hypertensive patients [86, 95]. Several clinical trials have demonstrated the beneficial effects of UA lowering therapy for hypertension [96–99]. In a trial targeting prehypertensive obese adolescents, administration of either allopurinol (XO inhibitor) or probenecid (uricosuric agent) lowered blood pressure [98]. Consistently, both allopurinol and benziodarone (uricosuric agent) reduced blood pressure in rats with hypertension induced by hyperuricemia [100, 101], suggesting that not only XO activity but also UA itself plays an important role in the pathogenesis of hypertension.

Besides the association with hypertension, hyperuricemia or gout has been confirmed to be related to the morbidity and the mortality of CVD [102–106]. According to a recently published meta-analysis [107], the relative risks of morbidity and mortality for coronary heart diseases were 1.13 and 1.27, respectively, in hyperuricemic patients as compared to controls. Several clinical studies have indicated the benefits of XO inhibitors for reducing the incidence of myocardial infarction [108], improving exercise tolerance in patients with stable angina [109], and enhancing endothelial function [110, 111]. However, interestingly, unlike the case of treating hypertension, uricosuric agents have failed to show any benefits in patients with hyperuricemia or gout [110, 112].

What are the mechanisms underlying the aforementioned association between hyperuricemia and atherosclerotic diseases? First, the role of XO in the pathogenesis of atherosclerosis merits attention. As described above, XO produces ROS when converting hypoxanthine into xanthine and then UA. XO is also expressed in endothelial cells [113] and was shown to be increased in the aortic endothelial cells of $ApoE^{-/-}$ mice [114], an established model of atherosclerosis. Since oxidative stress inactivates NO and leads to endothelial dysfunction [115], endothelial XO, especially given its enhanced expression during the development of atherosclerosis, contributes to vascular damage via ROS production.

Recently, we established that XO activity in macrophages also plays a key role in the development of atherosclerosis [75]. During atherosclerosis development, monocytes migrate beneath the endothelium and transform into macrophages, which then turn into foam cells by incorporating modified low density lipoproteins (LDL) (such as oxidized LDL and acetyl LDL) or very low density lipoproteins (VLDL). Foam cells contribute to the formation of unstable plaques by secreting inflammatory mediators and matrix-degrading proteases (such as matrix metalloproteinases (MMPs)) and by generating a prothrombotic necrotic core by eventually undergoing necrotic or apoptotic death [116]. We demonstrated that allopurinol treatment ameliorated aortic lipid accumulation and calcification of the vessels of ApoE-KO mice and that allopurinol markedly suppressed the transformation of J774.1 murine macrophages or primary cultured human macrophages into foam cells in response to stimulation with acetyl LDL or VLDL. The expressions of scavenger receptors (SR-A1, SR-B1, and SR-B2) and VLDL receptors in J774.1 cells were upregulated by XOR overexpression and downregulated by siRNA-mediated XOR suppression, raising the possibility that XO activity in macrophages positively regulates foam cell formation by increasing the uptake of modified LDL or VLDL. Conversely, expressions of ABCA1 and ABCG1, which regulate cellular cholesterol efflux, were decreased by XOR overexpression and increased by XOR knockdown. Furthermore, allopurinol suppressed the expressions of inflammatory cytokines such as IL-1β, IL-6, IL-12, and TNFα, and the expressions of VCAM1, MCP-1, and MMP2, which were upregulated in J774.1 cells transformed into foam cells by atherosclerogenic serum. Subsequently, febuxostat, another XO inhibitor, was also demonstrated to attenuate the development of atherosclerotic lesions in $ApoE^{-/-}$ mice [114]. That study showed XO expression to be increased in macrophages infiltrating atherosclerotic plaques and that febuxostat diminished the ROS level in the aortic walls of $ApoE^{-/-}$ mice. The authors demonstrated that cholesterol crystals (CCs) increased endogenous XO activity and ROS production in macrophages and that CCs enhanced not only IL-1β release via NLRP3 inflammasome activation but also secretions of other inflammatory cytokines such as IL-1α, IL-6, and MCP-1 from macrophages, processes which in turn were suppressed by febuxostat or ROS inhibitors. The significance of NLRP3 inflammasome activation in macrophages by CCs was verified by the observation that atherosclerosis in high-cholesterol diet fed LDL receptor-(LDLR-) deficient mice was alleviated by transplanting bone marrow from NLRP3-deficient, ASC-deficient, or IL-1α/β-deficient mice [117]. Taking these observations together, we can reasonably speculate that XO in macrophages enhances foam cell formation, ROS production, and NLRP3 inflammasome activation, all three of which exacerbate inflammation and plaque formation, thereby contributing to the development of atherosclerotic diseases [75, 114–116].

Independently of XO, UA itself is widely recognized to exert direct effects on vascular functions. Vascular endothelial cells express several UA transporters [118] and incorporated UA impairs NO production and leads to endothelial dysfunction [118, 119]. In vascular smooth muscle cells, UA stimulates proliferation and ROS production and inhibits NO production via increased angiotensin II expression [81, 120]. As noted above, not only XO inhibitors but also uricosuric

agents markedly lowered blood pressure, especially in studies targeting early-stage hypertensive patients [98] and those using animal models [100, 101]. The results obtained suggest that UA presumably contributes to early-stage hypertension by promoting renal vasoconstriction via reduced NO production and activation of the renin-angiotensin system [86, 98].

3.2. Nonalcoholic Steatohepatitis. The number of nonalcoholic fatty liver disease (NAFLD) patients including those with NASH has been increasing worldwide and a portion of NASH patients will progress to hepatocarcinoma onset [121–123]. Therefore, numerous investigations have been performed in efforts to elucidate the causes of NASH.

NASH is characterized by fat deposition, inflammation and fibrosis in the liver, and a two-hit mechanism of onset has been proposed [124–126]. This hypothesis is that fatty liver formation and subsequent injuries, including inflammation and oxidative stress, cause NASH pathology [127]. Interestingly, recent studies have raised the possibility that UA is among the risk factors for NASH pathology. We discuss the relationship between UA and NASH below.

3.2.1. Serum UA Is a Predictor of NAFLD/NASH Onset and Progression. Many clinical studies have been carried out to investigate the relationship between serum UA levels and NAFLD/NASH progression. For example, a cohort study in Korea found the serum UA level to be a useful marker for predicting NAFLD development because the serum UA concentration correlated positively with the 5-year incidence rate of NAFLD [128]. Their conclusion is supported by another study showing that serum UA levels of NAFLD patients are higher than those of control groups [129]. In addition, there are also studies demonstrating that serum UA is a risk factor for the development and/or progression of NAFLD including NASH [130–132].

Consistent with these observations, hepatic XO activities and serum UA levels are reportedly increased in murine NAFLD/NASH models [133, 134]. Moreover, a fraction of NAFLD/NASH patients also have obesity, and hypertrophic adipocytes were also reported to secrete UA [135]. Taken together, these results indicate serum UA to be a good parameter for predicting the development of NAFLD/NASH, and that XO inhibitors or uricosuric agents might have potential as treatments for ameliorating the features of NAFLD.

3.2.2. The Mechanism of UA-Induced NAFLD/NASH Progression. As described above, increasing serum UA or XO activity apparently plays important roles in NAFLD/NASH onset and progression. Interestingly, UA was reported to induce fat depositions by enhancing lipogenesis in hepatocytes. Fructose treatment of HepG2 cells reportedly increased both the intracellular UA concentration and triglyceride (TG) accumulation, while allopurinol, an XO inhibitor, suppressed this fructose-mediated TG deposition. Moreover, the application of UA alone was demonstrated to increase intracellular TG contents as well as ROS generation in mitochondria [136]. As a mechanism of UA-induced TG accumulation, the authors asserted that the elevation of intracellular ROS by UA raised both the citrate concentration and ATP citrate lyase activity via enhanced phosphorylation at S455, resulting in the induction of lipogenesis. These observations are supported by those of another study in which pretreatment with antioxidants inhibited the elevation of triglyceride contents by UA [137]. The authors asserted that ROS generation by UA evoked endoplasmic reticulum stress, leading to upregulation of lipogenic genes, such as acetyl CoA carboxylase1 and FASN [137].

ROS generation by UA is considered to depend on NADPH oxidase activation [136, 138, 139]. For example, UA reportedly promotes translocation of the NADPH oxidase subunit NOX4 into mitochondria [136]. It was also reported that UA treatment raises NADPH oxidase activity and alters its localization, leading to lipid oxidation [139]. In addition, XO may also function as a source of ROS generation because XO activity is upregulated in the livers of murine NASH models.

Collectively, these observations indicate that UA enhances fatty acid synthesis by regulating lipogenesis and induces ROS generation by regulating NADPH oxidase activity and upregulating fatty acid synthesis, thereby contributing to NASH development.

3.2.3. Inflammasome Participation in NASH Progression. As described elsewhere, UA is involved in inflammasome activation. Recent investigations have provided convincing evidence that inflammasomes are key players in NASH development. An initial study revealed that inflammasome impairment exacerbated the NASH progression induced by feeding a methionine-choline deficient diet for 4 weeks to ASC or IL-1 KO mice [140]. Subsequent studies, however, found that inflammasomes themselves exacerbate NASH symptoms. For example, it was reported that NLRP3 deficiency prevents liver fibrosis in response to a choline diet deficient in amino acids [141]. In addition, caspase-1 deficient mice were also resistant to developing steatosis or fibrosis while being fed a high-fat diet [142]. Moreover, other groups have demonstrated that diets which lead to NASH also increase the expressions of inflammasome components [143–145].

Taking these lines of evidence together, in the initial stage of NASH, inflammasomes appear to exert a protective effect, but continuous inflammasome activation appears to cause excessive productions of inflammatory cytokines, ultimately resulting in liver injury. Although, to date, numerous factors playing important roles in NASH progression have been identified, UA also appears to be a key participant in the onset of NAFLD/NASH.

3.3. Insulin Resistance, Diabetes, and Hyperlipidemia. Hyperuricemia was reportedly found to be related to insulin resistance in several clinical analyses [146–152]. In addition, several meta-analyses have suggested the UA level to be positively associated with the development of type 2 diabetes mellitus (DM) [153–156], although Mendelian randomization studies did not support circulating UA as being among the causes of DM development [157, 158]. In metabolic syndrome patients, an oxidative stress marker, the myeloperoxidase level, was decreased by allopurinol and endothelial function improved [159]. On the other hand, rapid UA reduction

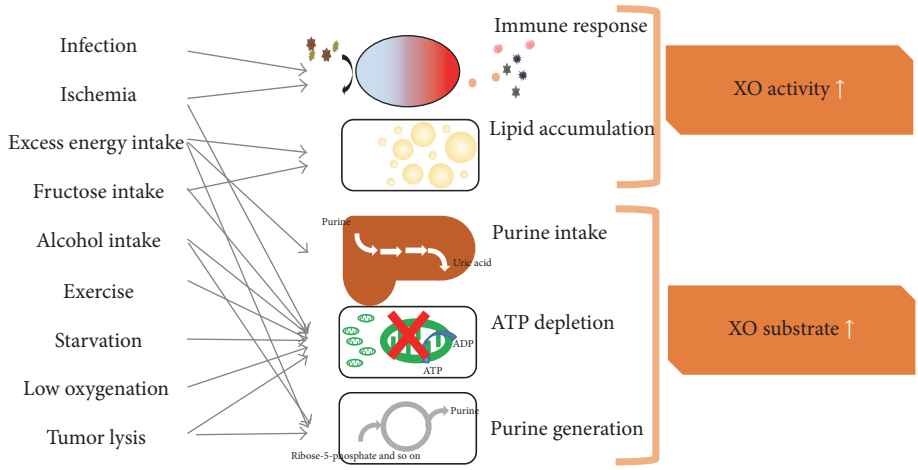

Figure 4: Increased catalyst activity of XO, originating from pathological and physiological events. Involvement of XO in pathophysiological processes suggests applications of XO inhibitors to the treatment of various disorders.

achieved by rasburicase, a urate oxidase, in obese subjects with high UA resulted in increasing the markers of systemic and skeletal muscle oxidative stress while having no effect on insulin sensitivity [160].

Furthermore, excess fructose intake is one of the major causes of the development of obesity with hyperuricemia, fatty liver, and metabolic syndrome. Fructose is metabolized by fructokinase to fructose-1-phosphate and results in a drop in both intracellular phosphate and ATP levels [161]. The intracellular phosphate decrease stimulates AMP deaminase (AMPD), the enzyme catalyzing the degradation of AMP to inosine monophosphate and eventually UA. Activated AMPD increases the expressions of gluconeogenesis genes, that is, PEPCK and G6Pase, via inhibition of AMP-activated protein kinase (AMPK) [162]. AMPD also increases lipogenesis through AMPK inhibition. AMPK phosphorylation was decreased in HepG2 cells treated with UA. The UA increased fructose-induced TG accumulation and decreased β-hydroxybutyrate levels, dose-dependently, while allopurinol, a XO inhibitor, blocked it. Because UA is the downstream product of AMPD and allopurinol abolished fructose-induced lipid accumulation, AMPD effects on AMPK appeared to depend on UA [163]. UA activates the transcription factor ChREBP, which triggers a vicious cycle of fructokinase transcription and accelerated fructose metabolism [68]. Via these mechanisms, activated AMPD and increased UA production tend to promote fat accumulation and glucose production.

UA is considered to be an antioxidant in human blood, though UA induces oxidative stress in cells [164]. UA raised NADPH oxidase activity and ROS production in mature adipocytes. The stimulation of NADPH oxidase-dependent ROS by UA resulted in the activation of MAP kinase p38 and ERK1/2, a decrease in NO bioavailability, and increases in both protein nitrosylation and lipid oxidation [138]. Increased UA production, in turn, generates mitochondrial oxidants. Mitochondrial oxidative stress inhibits aconitase in the Krebs cycle, resulting in citrate accumulation and the stimulation of ATP citrate lyase and fatty acid synthase, ultimately leading to de novo lipogenesis [136]. In hepatocytes treated with high UA, oxidative stress is increased, which activates serine (rat Ser307 and human Ser312) phosphorylation of IRS-1. This activity impairs Akt phosphorylation, thereby resulting in acute hepatic insulin resistance after exposure to high UA levels [165]. Therefore, UA-induced lipid accumulation and oxidative stress are responsible for the development of insulin resistance and diabetes.

4. Beneficial Effects of XO Inhibitors

Involvement of increased XO catalyst activity in pathophysiological processes (Figure 4) suggests applications of XO inhibitors to the treatment of various disorders. At present, XO inhibitors, including allopurinol, oxypurinol, febuxostat, and topiroxostat, are widely used for treating gout and hyperuricemia. Furthermore, XO inhibitors have been experimentally or clinically shown to exert beneficial effects by lowering serum UA and oxidative stress.

Febuxostat preserved renal function in 5/6 nephrectomized rats with and without coexisting hyperuricemia and prevented diabetic renal injury in streptozotocin-treated rats [166, 167]. Febuxostat also ameliorated tubular damage, diminished macrophage interstitial infiltration, and suppressed both proinflammatory cytokine activities and oxidative stress [168]. Febuxostat also reduced the induction of endoplasmic reticulum stress, as assessed by GRP-78 (glucose-regulated protein-78), ATF4 (activating transcription factor-4), and CHOP (C/EBP homologous protein-10) [169]. The clinical significance of measuring the serum UA level and XO inhibition for renal protection has largely been established by the results of recent studies [170–173].

On the other hand, beneficial effects of XO inhibitors on atherosclerosis and NASH constitute an evolving concept that has yet to be proven. In rats with fructose-induced metabolic syndrome, febuxostat treatment reversed hyperuricemia, hypertension, dyslipidemia, and insulin resistance [174]. The beneficial effects of XO inhibitors on NASH are

rarely reported, except by our research group [134], because animal models of NASH with obesity, inflammation, and fibrosis have been difficult to establish. NASH in response to the MCD diet, as used in our studies, caused primarily inflammation and also made the mice lean, such that no benefit of XO inhibition was obtained [134]. Thus, we next used a high-fat diet containing trans-fatty acids and a high-fructose diet to induce NASH development in our animal models. Another report showed that inhibition of XO activity also significantly prevents hepatic steatosis induced by a high-fat diet in mice. XO has also been indicated to regulate activation of the NLRP3 inflammasome [175].

Atherosclerosis has been far more extensively investigated than NASH, both clinically and experimentally. Tungsten, acting as an XO inhibitor, has an inhibitory effect on both atherosclerosis and oxidative stress [176]. We reported for the first time that more specific XO inhibition, using allopurinol rather than tungsten on macrophages, resulted in the inhibition of foam cell formation and reduced atherosclerotic lesions in ApoE-KO mice, independently of the serum lipid profile [75]. We also identified phenotypic changes of macrophages in response to allopurinol, such as alterations of gene expressions involved in lipid accumulation. Moreover, both XO overexpression and knockdown of XO expression revealed VLDL receptors to be dramatically upregulated by XO. Febuxostat was also proven to have similar effects in terms of reducing the atherosclerotic lesions in ApoE-KO mice, and oxidative stress was reduced in macrophages from atherosclerotic lesions [113]. Febuxostat also suppressed LPS-induced MCP-1 production via MAPK phosphatase-1-mediated inactivation of JNK [84]. As a strategy for suppressing atherosclerosis, XO inhibition is expected to act on either macrophages or inflammatory cells.

XO inhibitors also improve endothelial function and prevent vascular remodeling. Oxypurinol reduces O_2^- radical dot production and improves endothelial function in blood vessels from hyperlipidemic experimental animals [69]. XO inhibition can also provide protection from radiation-induced endothelial dysfunction and cardiovascular complications [177]. Allopurinol treatment prevents hypoxia-induced vascular remodeling in the lung [178]. However, controversy persists as to whether the effect of XO on endothelial function is clinically relevant as an interventional target [49]. Pretreatment with XO inhibitors has beneficial effects on ischemia/reperfusion injuries of the intestine [179], in the impaired liver [61, 62], the edematous brain [180], kidneys with contrast induced nephropathy [181], and coronary ischemia [182]. XO inhibitors prevent postischemic O_2^- generation [183].

5. Conclusion

Inflammation related to UA metabolism is induced via either inflammasome activation by UA crystal precipitation or free radical production in response to XO activity. In addition to gout, many disorders are known to be related to UA metabolism and XO inhibitor treatments have been shown to be effective for preventing the onset and/or the progression of such disorders. In particular, atherosclerosis and NASH are diseases for which relationships with UA metabolism were not immediately recognized, but rodent model studies revealed the importance of UA metabolism maintenance for managing these disorders. We believe the impact of UA metabolism on many diseases accompanying chronic inflammation to have been underestimated. Future studies are anticipated to reveal the pathological contribution of serum UA and/or XO activity to the specific processes underlying various disorders. Further study of the detailed molecular mechanisms is clearly warranted.

Abbreviations

UA: Uric acid
MUC: Monosodium urate crystal
NASH: Nonalcoholic steatohepatitis
XO: Xanthine oxidase
LPS: Lipopolysaccharide
TIMP: Tissue inhibitor of metalloproteinases
MCP-1: Monocyte chemoattractant protein 1
NADPH: Nicotinamide adenine dinucleotide phosphate
CKD: Chronic kidney disease
ICAM-1: Intercellular adhesion molecule-1.

Competing Interests

The authors have no competing interests regarding the publication of this report to declare.

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Review Article

Therapeutic Effects of Quercetin on Inflammation, Obesity, and Type 2 Diabetes

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In previous studies, abdominal obesity has been related to total low-grade inflammation and in some cases has resulted in insulin resistance and other metabolism related disorders such as diabetes. Quercetin is a polyphenol, which is a derivative of plants, and has been shown *in vitro* as well as in a few animal models to have several potential anti-inflammatory as well as anticarcinogenic applications. The substance has also been shown to aid in the attenuation of lipid peroxidation, platelet aggregation, and capillary permeability. However, further research is called for to gain a better understanding of how quercetin is able to provide these beneficial effects. This manuscript reviewed quercetin's anti-inflammatory properties in relation to obesity and type 2 diabetes.

1. Introduction

The world has seen a rapid increase in the incidence of diabetes generally, and type 2 diabetes has become the most common metabolic disease globally. This has been in part due to a growth in obesity, especially excess visceral adiposity, in addition to the associated "metabolic syndrome" which encompasses insulin resistance, hyperglycaemia, dyslipidaemia, and hypertension [1].

Inflammation occurs in response to various pathological stimuli and tissue injury, and chronic inflammation and the activation of the immune system may be mainly responsible for the process of obesity-related metabolic diseases such as type 2 diabetes [2, 3]. Key characteristics of type 2 diabetes include insulin secretion defects and insulin resistance in liver, adipose tissue, and skeletal muscles. Diabetes and associated complications are a result of the inflammatory processes [4]. Interventional studies have confirmed the aspects of inflammation occurring within type 2 diabetes pathogenesis. Metformin and peroxisome proliferator-activated receptor- (PPAR-) γ agonist has been shown to use anti-inflammatory mechanisms to effectively lower the occurrence of type 2 diabetes and its related complications [5]. These studies further indicate that drugs aimed at hypoglycaemia may also have anti-inflammatory and therefore antidiabetic characteristics.

Unique biological elements of the flavonoid quercetin (found in fruits and vegetables) contain potential mental and physical health benefits. Among these are disease resistance; enhanced mental and physical performance; the ability to inhibit lipid peroxidation; stimulation of mitochondrial biogenesis; and other anti-inflammatory, antiviral, and antioxidant properties [6, 7].

This study will review the effects of quercetin as a dietary supplement. Quercetin's impact on inflammation, disease resistance, and overall health will be reviewed with the goal of summarising its main potential therapeutic applications. The data within the peer-reviewed literature are being considered within the study, which will be based on the cellular, molecular, and fundamental functions of possible therapy through the use of Quercetin.

2. Inflammatory Markers of Obesity

The development of insulin resistance, diabetes, and a higher possibility of contracting cardiovascular disease has been linked to obesity [8]. Examinations of overweight and obese adults have revealed altered circulating levels of inflammatory cytokines such as interleukin-1β (IL-1β), interleukin-6 (IL-6), tumour necrosis factor-alpha (TNF-α), or C-reactive protein (CRP) [9]. Although body fat generally has been correlated with serum levels of inflammatory proteins, abdominal obesity has shown a stronger correlation than either Body Mass Index (BMI) or total body fat [9], for all individuals, including those in good health.

Hermsdorff et al. associated abdominal fat accumulation with concentrations of IL-6, CRP, and complement factor C3 [10]. Some researches have demonstrated a correlation between the fats in abdomens and CRP concentration within individuals that are not obese. The evidence points to an increased risk associated with visceral adipose tissue vis-a-vis subcutaneous adipose tissue [11].

3. Inflammatory Markers of Type 2 Diabetes

Type 2 diabetes patients undertaking a more active lifestyle to reduce weight have been shown to exhibit improvement in a variety of factors. These factors include the count of white blood cell, CRP, serum amyloid A (SAA), and proinflammatory cytokines such as TNF-α, IL-6, and IL-1β [10, 12]. Additionally, irrespective of the level of insulin resistance and obesity at the outset, an independent risk factor for the development of type 2 diabetes seems to be subclinical chronic inflammation.

Among type 2 diabetes indicators, CRP measurement is a relatively inexpensive, standardised, and readily available measure. Other indicators described in prospective studies include the level of white blood cells, proinflammatory cytokines, chemokines, and numerous inflammatory biomarkers including fibrinogen and sialic acid [13–15]. Several prospective studies show high sensitivity-CRP (hs-CRP) levels are a good predictor of the future advancement of type 2 diabetes in nondiabetic individuals regardless of fat distribution and insulin resistance. A measurement of CRP that is incredibly sensitive has been created to accurately detect CRP at lower levels. There has been current meta-analysis and appraisal of CRP-related studies further demonstrated that higher levels of CRP are correlated with the increased risk for type 2 diabetes [16].

4. Dietary Sources of Quercetin

Flavonols, primarily in the form of glycosides, exist in part that is edible within numerous plants including leafy vegetables, many fruits, bulbs and tubers, herbs, spices, tea, and also wine [17]. Among these, quercetin has the highest amount of flavonol molecules. Furthermore, the majority of quercetin-type flavonols consumed consist of quercetin glycoside conjugates in which quercetin is related to 1-2 glucose residues. As a result, within the average diet there are relatively fewer quantities of quercetin aglycones [18].

Quercetin levels in food have been found to be impacted by growing conditions. For example, in the case of tomatoes, a higher quercetin aglycone level has been found in those organically grown as compared to those grown using traditional growing techniques.

Dietary consumption of quercetin differs across countries. Flavonoid daily intake (in which about 75% is quercetin) ranges from a low of 5 milligrams per day to a high of 80 milligrams. Key among the variables influencing the level is the amount of fruits and vegetables and tea consumed. On average, men as well as older individuals consume relatively lower levels of quercetin; and seasonal consumption (i.e., summer versus winter levels) showed no significant difference. Flavonol intake levels in the United States are about 13 milligrams per day for adults, with quercetin accounting for about 75% [19]. In northern China, quercetin intake is found to be only about 4.37 milligrams per day, with the chief flavonol crop being apples with 7.4%, followed by potatoes at 3.9%, and lettuce and oranges at 3.8% each [20]. Averages quercetin consumption in the Chinese city of Harbin followed a similar but more diverse blueprint: 4.43 milligrams per day, made up of apples (3.7%), potatoes (2.5%), celery (2.2%), eggplant (2.2%), and *Actinidia* (1.6%) [21]. Japanese average and median intake are much higher at 16.2 and 15.5 milligrams per day, respectively.

5. Anti-Inflammatory Effect of Quercetin

Extracts from *Mexican oregano* have demonstrated anti-inflammatory properties through decreasing the production of reactive oxygen species (ROS) and nitric oxide (NO) [22]. Similarly, many phenolic compounds are shown to inhibit secretion and production of proinflammatory cytokines. Although the oregano extracts contain several distinct flavonoids including quercetin, luteolin, and scutellarein glycosides, it is unclear which flavonoid triggers the bioactivity.

Recently, quercetin has been shown to inhibit *in vitro* production of cyclooxygenase (COX) and lipoxygenase (LOX) which are typically induced by inflammation [23]. The anti-inflammatory effect has been supported by *in vivo* experiments as well. Examples of quercetin's inhibitory qualities include the significant blocking of proinflammatory cytokines in cultured fibroblasts [24]. 10 μM quercetin downregulated the production of COX-2, the Nuclear Factor-kappa B (NF-κB), and NO [25]. 10–25 μM quercetin inhibited the level of NO and TNF-α [26]. Other properties of 50 and 100 μM quercetin include reducing the secretion of IL-6 and TNF-α in LPS-stimulated RAW 264.7 microphages [27]; while at 25 and 50 μM it proved to be the most efficient blocker of TNF-α secretion in macrophages. Finally, at low concentrations, quercetin (less than 50 μM) also stimulated anti-inflammatory cytokine IL-10 [28]. Similarly, 25 μM quercetin blocked IL-1β, IL-6, IFN-γ, and TNF-α secretion in human whole blood induced by LPS [29]. Meanwhile, by inhibiting NF-κB activation, quercetin at less than 10 μM inhibited the production of NO, IL-6, monocyte chemoattractant protein-1 (MCP-1), TNF-α, iNOS, and COX-2 in RAW 264.7 cells [25]. Quercetin can also

inhibit proinflammatory cytokines. A six-week regiment of 150 milligrams of quercetin taken daily by human subjects significantly lowered cytokine TNF-α serum concentrations [30]. Quercetin has also been shown to reduce pancreatic histopathological damage and lower the mRNA and protein level of NF-κB, IL-1β, IL-6, and TNF-α in rats [31].

Despite the extensive evidence for quercetin's anti-inflammatory effect, the mechanism for its success is not well understood. Potential influences could be the inhibition the molecular level of COX-2 and iNOS, NF-κB, AP-1, or mitogen-activated protein kinase (MAPK) [32]. Interrupting these enzymes would have anti-inflammatory impact. NO, a proinflammatory mediator, is synthesised by iNOS due to the reactions by proinflammatory compounds like LPS. Furthermore, other studies have demonstrated that the pretreatment with quercetin inhibits iNOS and NO production induced by LPS and counteracts the oxidative stress created by the unregulated NO production [33]. Meanwhile, NF-κB and AP-1, both inhibited by quercetin, are significant transcriptional features in modulating proinflammatory cytokines [34–36]. In rat aortic endothelial cells, quercetin significantly reduces the production of NF-κB and AP-1 activity [37]. Quercetin has also proven to be an effective pretreatment to combat apoptosis cell death. In addition, quercetin would inhibit phosphorylation of stress-activated protein kinases (JNK/SAPK) and the p38 MAPK, which are responsible for the inhibitory effect of cell growth. The wide array of evidence points to quercetin as potentially a powerful weapon in the fight against the inflammatory disease. It could also potentially prove useful to cells involved in allergic inflammation [38].

6. Antiobesity Effect of Quercetin

Research suggested that quercetin downregulated adipogenesis and apoptosis by decreasing the action of adipogenesis-related enzymes; meanwhile, levels of MAPK as well as its substrate acetyl-CoA carboxylase (ACC) were upregulated [39]. Simultaneously apoptosis was induced and a drop in the levels of ERK and JNK phosphorylation was seen. The implication is that quercetin works to block adipogenesis actions through stimulating the MAPK signal pathway. At the same time quercetin induced the apoptosis of mature adipocytes by controlling the important ERK and JNK pathways.

Other authors have shown a role for quercetin in the regulation of hepatic gene expression and lipid metabolism [40]. Studies showed that quercetin prevents high-fat diet (HFD) induced obesity in C57B1/6 mice, perhaps by regulating lipogenesis. Mice fed a quercetin supplement saw a significant lowering of HFD induced obesity compared to those fed the HFD without the supplement. Specifically, the supplement-fed mice experienced a reduction in body weight, liver weight, and amount of total white adipose tissue. Quercetin appears to have modified the gene profiles of genes related to lipid metabolism including Fnta, Pon1, Pparg, A1dh1b1, Apoa4, Abcg5, Gpam, Acaca, Cd36, Fdft1, and Fasn.

7. Antidiabetic Effect of Quercetin

The antidiabetic qualities of quercetin involve the stimulation of glucose uptake through an MAPK insulin-dependent mechanism. Stimulation of the mechanism in skeletal muscles has resulted in the translocation of glucose transporter 4 (GLUT4). This role for MAPK is distinct from its role in the liver where it reduces the production of sugar mostly through the downregulation of the key gluconeogenesis enzymes [41].

Studies have been conducted on effects of quercetin in animals with type 2 diabetes [42]. Those that received quercetin showed lower glucose plasma levels relative to the control group and do not experience increase or decrease in insulin measured by the homeostasis model. Animals that received a 0.08% portion of quercetin showed a variety of other improvements including an increase in plasma adiponectin and HDL-cholesterol, decreases in plasma total cholesterol and plasma triacylglycerols, and increases in specific liver enzymes activities important in the detoxification processes. Quercetin has further been shown to play a role in improved renal functioning in diabetic nephropathic rats by blocking the overexpression of connective tissue growth factor (CTGF) and transforming growth factor-β1 (TGF-β1). End-stage renal disease is closely associated with diabetic nephropathy. Studies show that TGF-β1 and CTGF have an essential impact on the DN pathophysiological systems involved. Studies examined the impact of quercetin on TGF-β1 and CTGF renal functions in streptozotocin- (STZ-) induced diabetic Sprague-Dawley rats [43]. Results showed that rats treated with quercetin saw a reduction in their weight ratio of kidney and body. The expressions of CTGF and TGF-β1 are higher in the renal tissues. For those that received quercetin, the overexpression was reduced.

Finally, quercetin has been shown to produce an effective *in vitro* block against lens aldose reductase and additionally prevents polyol accumulation [44]. For humans, quercetin has shown to help decrease the seriousness of numbness, jolting pain, and irritation for patients with type 2 diabetes neuropathy. It has further been shown that active treatment with quercetin can improve various quality-of-life matrices [45].

8. Conclusions

Evidence in various studies seems to connect abdominal obesity, type 2 diabetes, and chronic low-grade inflammation. Researchers have begun to view type 2 diabetes more in terms of inflammation as more confirming evidence has been found. Adipose tissue appears to create changes in cellular composition and the production of proinflammatory cytokines and chemokines. Through an increase of antioxidative activities, NF-κB regulation, the reduction of proinflammatory enzymes activity, and the reduction of cytokine levels, quercetin has shown itself to be a strong anti-inflammation weapon. These positive results have been found in both animal and human studies and support the use of quercetin in fighting inflammatory disease. Nevertheless, continued evaluations are needed to uncover the exact mechanisms through which quercetin functions in order to satisfactorily

address safety concerns. It is hoped this study will reignite interest in the anti-inflammatory properties of quercetin and encourage the public to explore vegetarian diets and natural medicines.

Competing Interests

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

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Research Article

N-Acetyl-L-cysteine Protects the Enterocyte against Oxidative Damage by Modulation of Mitochondrial Function

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The neonatal small intestine is susceptible to damage caused by oxidative stress. This study aimed to evaluate the protective role of antioxidant N-acetylcysteine (NAC) in intestinal epithelial cells against oxidative damage induced by H_2O_2. IPEC-J2 cells were cultured in DMEM-H with NAC and H_2O_2. After 2-day incubation, IPEC-J2 cells were collected for analysis of DNA synthesis, antioxidation capacity, mitochondrial respiration, and cell apoptosis. The results showed that H_2O_2 significantly decreased ($P < 0.05$) proliferation rate, mitochondrial respiration, and antioxidation capacity and increased cell apoptosis and the abundance of associated proteins, including cytochrome C, Bcl-XL, cleaved caspase-3, and total caspase-3. NAC supplementation remarkably increased ($P < 0.05$) proliferation rate, antioxidation capacity, and mitochondrial bioenergetics but decreased cell apoptosis. These findings indicate that NAC might rescue the intestinal injury induced by H_2O_2.

1. Introduction

The neonatal small intestine is particularly vulnerable to damage induced by endotoxin, and this damage may be involved in plasma and intracellular production of reactive oxygen species (ROS), resulting in cell apoptosis, reducing antioxidative capacity and mitochondrial dysfunction [1–3]. The intestinal epithelium, the border between the body and the environment, is the main place to transport the nutrient. And the enterocyte is the main target of harmful factors and stress, for example, toxin and ROS [4]. Moreover, a large of evidence suggests that oxidant derivatives and ROS are produced in excess by the inflamed mucosa and may be pathogenic factors in some intestinal diseases [5, 6]. Oxidative stress generated by an imbalance between ROS and antioxidants contributes to the pathogenesis of arthritis, cancer, cardiovascular, liver, and respiratory diseases [7]. ROS is generic and includes a wide variety of molecules, free radicals, or ions derived from molecular oxygen, for instance, singlet oxygen (O_2), superoxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^{\bullet}$) [8]. ROS elicits a wide spectrum of responses [9]. Low doses of ROS are mitogenic and promote cell proliferation, while intermediate doses of ROS induce temporary or permanent growth arrest, and high doses of ROS cause cell death [9]. H_2O_2 is an abundant and stable form of ROS, responding

to inflammation, cellular dysfunction, and apoptosis, which ultimately lead to tissue and organ damage. Mitochondrion is the main target of intracellular oxidative stress and is regarded as the main source for endogenous ROS. Previous studies showed that an acute, noncytotoxic dose of H_2O_2 caused a delay fragmentation of the mitochondrial reticulum and depressed the mitochondrial membrane potential and maximal respiratory rate [10]. Therefore, H_2O_2-induced damage is a reproducible and simple model to cause oxidative stress.

N-Acetylcysteine (NAC), the precursor of L-cysteine, is known as an antioxidant that acts as a source of thiols and functions in glutathione synthesis, glutathione peroxidase (GPx) activity, and detoxification and acts directly on reactive oxidant radicals as a superoxide scavenger which interacts with ROS such as $HO^{\bullet}$ and H_2O_2 [7]. The previous study showed that weaning increased the concentrations of NO and H_2O_2 in the serum in postweaning piglets, and feeding antioxidant-containing diets could prevent the ROS-induced damage and suppress oxidative stress [11]. There is growing evidence that NAC might be a promising agent to improve intestinal health in piglets [12]. NAC supplementation could alleviate the mucosal damage and improve the absorptive function of the small intestine in lipopolysaccharide-(LPS-) challenged piglets [13]. NAC regulates antioxidative responses, cell apoptosis, and epidermal growth factor gene expression under acetic acid challenges [6]. However, the mechanisms by which NAC exerts protective effects in intestinal damage are incompletely understood.

We hypothesize that NAC enhances cell growth and mitochondrial bioenergetics and decreases cell apoptosis on H_2O_2-induced oxidative damage in intestinal cells. The present study was designed to test this hypothesis using a model of H_2O_2-induced damage of intestinal porcine epithelial cells (IPEC-J2).

2. Materials and Methods

2.1. Cell Culture. The reagents and cell culture refer to our previous study [14]. High-glucose (25 mM) Dulbecco's modified Eagle's (DMEM-H), fetal bovine serum (FBS), and antibiotics were procured from Invitrogen (Grand Island, NY, USA). Plastic culture plates were manufactured by Corning Inc. (Corning, NY, USA). Unless indicated, all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

IPEC-J2 cells were seeded and cultured with DMEM-H medium containing 10% FBS, 5 mM l-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a 5% CO_2 incubator. After an overnight incubation, the cells were changed to culture in basal medium containing 0 or 800 μM NAC. The following day, 0 or 100 μM H_2O_2 was added for 4 hours and then the mediums were changed as before. The cells were collected for further research after 2-day incubation.

2.2. Cell Viability Assay. About 1×10^4 cells per well of IPEC-J2 cells were seeded in 96-well plates and grown as usual. After incubation in 0, 500, 650, 800, or 1000 μM NAC medium for 24 h, then 100 μM H_2O_2 were added for 4 h. The wells were washed and fresh basal medium was replaced. Cell Counting Kit-8 (CCK-8) was added to each well, incubated for 2 h, and read on the spectrophotometer at 450 nm; the measured absorbance is proportional to the number of viable cells.

2.3. DNA Synthesis Measurement. IPEC-J2 cells (1×10^4) were seeded in 96-well plates and cultured for a 2-day period. DNA synthesis during cell proliferation in all treatment groups was quantified using 5-ethynyl-2′-deoxyuridine (EdU; Invitrogen) incorporation using Cell-Light EdU Kit (Rui Bo Biotechnology Limited Company, Guangzhou, China), as described in our previous studies [1]. Briefly, IPEC-J2 cells were cultured in DMEM-H mediums containing 50 μM EdU for 1 h. An Olympus BX51 microscope (Olympus, Japan) was used to observe EdU-positive cells. Images of the Apoll® 567 Hoechst 33342 were captured. The percentage of EdU-positive cells was expressed as the ration of red nuclei cells to blue nuclei cells in at least five different microscopic fields randomly selected for counting at 200-fold magnification.

2.4. Detection of Antioxidation Capacity. IPEC-J2 cells (50×10^4) were seeded in 10 cm dishes for determination of total antioxidant capacity (T-AOC) and lactate dehydrogenase (LDH) using their corresponding assay kits (Nanjing Jiancheng, Nanjing, China) according to the manufacturer's instructions [2]. All samples were measured by UV/visible spectrophotometer-UV-2450 (SHIMADZU, Kyoto, Japan) to get the results.

2.5. Flow Cytometry Analysis. IPEC-J2 cells (10×10^4) were seeded in 6-well cell culture plates for flow cytometry analysis. After a 2-day period of culture in DMEM-H medium containing 0 or 800 μM NAC and 0 or 100 μM H_2O_2, medium and cells were collected separately. About 1×10^6 cells were pelleted at 16 000 ×g for 5 min. The supernatant was removed and 1 mL of 70% cold ethanol was slowly added during vigorous mixing. Samples were stored at 4°C. Cells were washed once with ice-cold PBS and resuspended in 1 mL of staining reagent containing 50 mg/mL PI and 100 mg/mL RNase for 30 min in the dark. To assess apoptosis, harvested cells were stained with PI/Annexin-V-FITC (KeyGEN, Nanjing, China) according to the manufacturer's instructions. Cell cycle arrest and apoptosis were analyzed by flow cytometry (BD FACSCalibur, USA). Fluorescence of PI and Annexin-V-FITC was monitored at 630 nm and 525 nm, respectively.

2.6. Metabolic Assays. The XF-24 Extracellular Flux Analyzer and Cell Mito Stress Test Kit from Seahorse Biosciences were used to examine the effects of NAC treatment on mitochondrial respiration in H_2O_2-induced cells as described by Tan et al. [15]. After a 2-day period of culture, the basal medium was changed prior to the bioenergetic measurements to serum-free unbuffered (without sodium bicarbonate) DMEM medium base supplemented with 2 mM L-glutamine, 25 mM D-glucose, and 1 mM sodium pyruvate, at pH 7.4 ± 0.1 at 37°C. To measure indices

of mitochondrial function, oligomycin, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), and rotenone and antimycin A were injected sequentially at the final concentrations of 0.5, 1, and 1 μM, respectively. This allowed for an estimation of the contribution of non-ATP–linked oxygen consumption (proton leak) and ATP-linked mitochondrial oxygen consumption (ATP production). The maximal respiration capacity was determined using the FCCP-stimulated rate. The spare respiratory capacity was represented by the maximal respiratory capacity subtracted from the baseline oxygen consumption rate (OCR). The residual oxygen consumption that occurred after addition of rotenone and antimycin A was ascribed to nonmitochondrial respiration and was subtracted from all measured values in the analysis [1]. Owing to the effects of NAC on IPEC-J2 proliferation, total cellular protein was determined and used to normalize mitochondrial respiration rates.

2.7. Detection of TCA Cycle Intermediates by GC-MS. IPEC-J2 cells (50×10^4) were seeded in 10 cm dishes for GC-MS analysis as described by Morita et al. [16]. Briefly, cells were washed with PBS and treated by 0.25% trypsin. And then cells were collected and pelleted at 1000 ×g for 5 min. After being quenched using 500 μL of prechilled 50% (v/v) methanol, cells were centrifuged at 1000 ×g for 5 min and then removed and added 500 μL of prechilled 100% (v/v) methanol. Cells were measured by an Agilent 7890B-5977A GC-MS equipped with HP-5ms (30 m × 250 μm × 0.25 μm) capillary column (Agilent J&W, Santa Clara, CA, USA). All metabolites were previously validated using authentic standards (Sigma).

2.8. Western Blotting Analysis. Cells were rinsed twice using PBS, harvested, pelleted by centrifugation, and lysed in RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl at PH 7.4), plus a protease inhibitor cocktail and phosphatase inhibitors. Protein concentrations of cell homogenates were measured using the BCA method and bovine serum albumin as standard, as described by our previous studies [1]. All samples were adjusted to an equal concentration. Soluble proteins were subjected to SDS-PAGE and transferred to PVDF membranes, blocked with 5% nonfat milk in TBS-with 0.05% Tween-20 for 1 h, and incubated overnight with the following primary antibodies overnight at 4°C with gentle rocking: cytochrome C (1 : 1,000; Cell Signaling Technology), Bax (1 : 1,000; Cell Signaling Technology), caspase-3 (1 : 1,000; Cell Signaling Technology), Bcl-XL (1 : 400; Santa Cruz Biotechnology, Dallas, TX), cleaved caspase-3 (1 : 400; Santa Cruz Biotechnology, Dallas, TX), or β-actin (1 : 400; Santa Cruz Biotechnology, Dallas, TX), followed by horseradish peroxidase-linked secondary antibodies. The protein bands were visualized using a chemiluminescent reagent. The density of the protein bands was determined using the Alpha Imager 2200 software (Alpha Innotech Corporation) and normalized the data with inner control.

2.9. Statistical Analysis. Results are expressed as mean ± SEM. The statistical analysis was performed by one-way ANOVA using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). Probability values < 0.05 were considered statistically significant.

3. Results

3.1. Effects of H_2O_2 and NAC on the Cell Viability of IPEC-J2 Cells. Viability assay of IPEC-J2 cells was performed by firstly treating the cells with different concentrations of NAC (0, 500, 650, 800, and 1000 μM, resp.) for one day and then with 100 μM H_2O_2 for 4 h. The results indicated that 100 μM H_2O_2 decreased IPEC-J2 cell viability, while addition of NAC enhanced cell viability of H_2O_2-treated IPEC-J2 cells in a dose-dependent manner, and 800 and 1000 μM NAC addition showed the best promotion effects compared with the 0 and 500 μM NAC treatment in H_2O_2-treated cells ($P < 0.05$) (Figure 1). The results of EdU incorporation illustrated in Figure 2 have showed that the percentages of EdU-positive cells were significantly decreased in response to H_2O_2 treatment ($P < 0.05$), while addition of NAC to cells showed a tendency to increase the percentages of EdU-positive cells compared with NC group.

3.2. Mitochondrial Bioenergetics. The results of mitochondrial respiration in IPEC-J2 cells are shown in Figure 3. Addition of 100 μM H_2O_2 gradually decreased ($P < 0.05$) individual parameters for basal respiration, proton leak, maximal respiration, nonmitochondrial respiration, and ATP production in cells while addition of NAC elevated the rate of mitochondrial respiration in 100 μM H_2O_2-treated cells ($P < 0.05$) but not in normal cells.

3.3. TCA Cycle Intermediates. The relative content of pyruvic acid, lactic acid, and TCA cycle intermediates (citric acid, alpha-ketoglutarate, succinic acid, fumaric acid, and malic acid) of IPEC-J2 cells are illustrated in Figure 4. Addition of 100 μM H_2O_2 significantly decreased lactic acid, and TCA cycle intermediates compared with the NC treatment ($P < 0.05$). Compared to NC treatment, addition of 800 μM NAC significantly decreased the content of pyruvic acid and lactic acid ($P < 0.05$), while there were no differences in the contents of pyruvic acid, lactic acid, succinic acid, fumaric acid, malic acid and α-ketoglutaric acid between NAC and NAC + H_2O_2 treatments ($P > 0.05$).

3.4. Antioxidative Capacity. The concentrations of T-AOC and LDH are presented in Figure 5. Compared with the NC group, 100 μM H_2O_2 significantly decreased the concentrations of T-AOC but increased the concentrations of LDH ($P < 0.05$). However, in H_2O_2-treated cells, addition of NAC markedly increased the concentrations of T-AOC and decreased LDH leakage into the culture medium ($P < 0.05$).

3.5. Cell Apoptosis. Cell apoptosis was analyzed by Annexin-V-FITC/PI staining, the results showed that compared with the NC group, 100 μM H_2O_2 significantly increased the percentage of both early and late apoptosis of cells, and 800 μM NAC addition also increased the apoptosis rate ($P < 0.05$). However, in H_2O_2-treated cells, addition of 800 μM

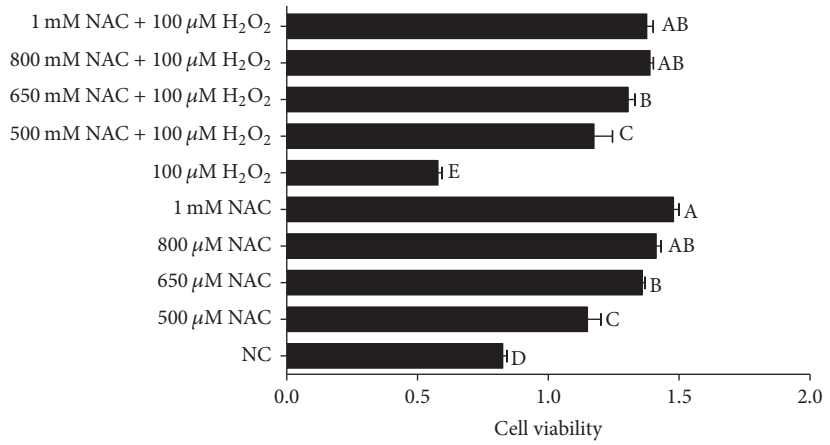


Figure 1: Cell proliferation in IPEC-J2 cells. Cells were treated with 0 (NC) to 1000 μM NAC and 0 or 100 μM H_2O_2, respectively, for a 2-day period. Cell viability was quantified by CCK-8 assay. Data are expressed as means ± SEM of at least three independent experiments. $^{a-e}$Values with different letters are significantly different ($P < 0.05$).

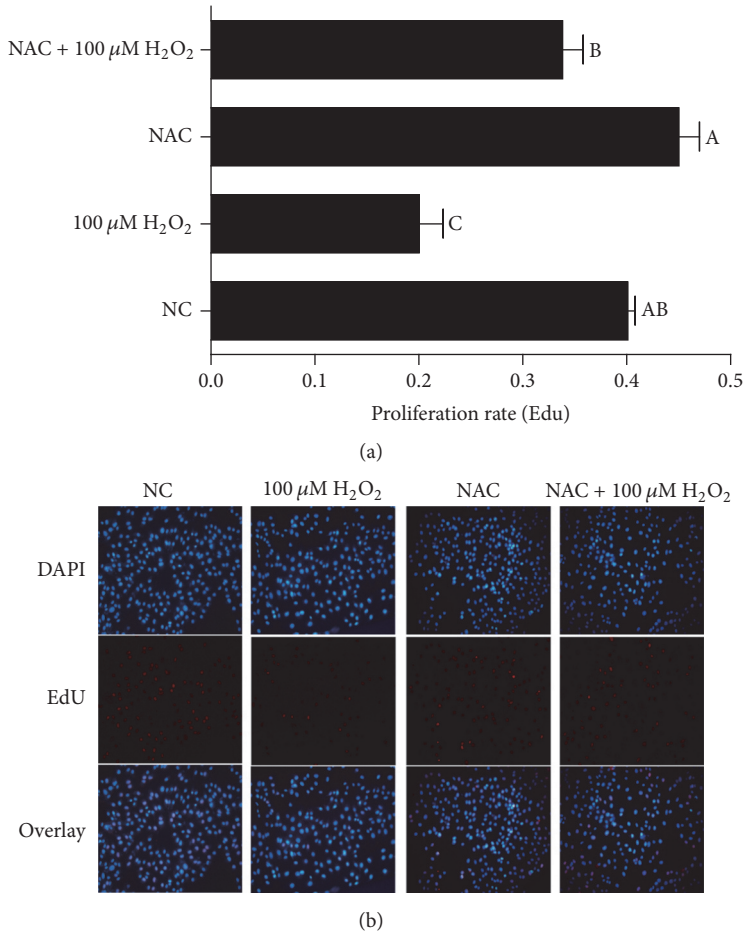


Figure 2: DNA synthesis in IPEC-J2 cells. DNA synthesis during the proliferation of IPEC-J2 cells was quantified by EdU incorporation (red color) using Cell-Light™ EdU Kit (Rui Bo Biotechnology Limited Company, Guangzhou, China). Nuclei are shown in blue color. Cells were treated with 0 (NC) or 800 μM NAC and 0 or 100 μM H_2O_2, respectively. (a) The percentage of EdU-positive cells (the number of red nuclei versus the number of blue nuclei in at least five different microscopic fields of vision). (b) Representative images of EdU staining (magnification ×200) of cells. Data are expressed as means ± SEM of at least three independent experiments. $^{a-c}$Values with different letters are significantly different ($P < 0.05$).

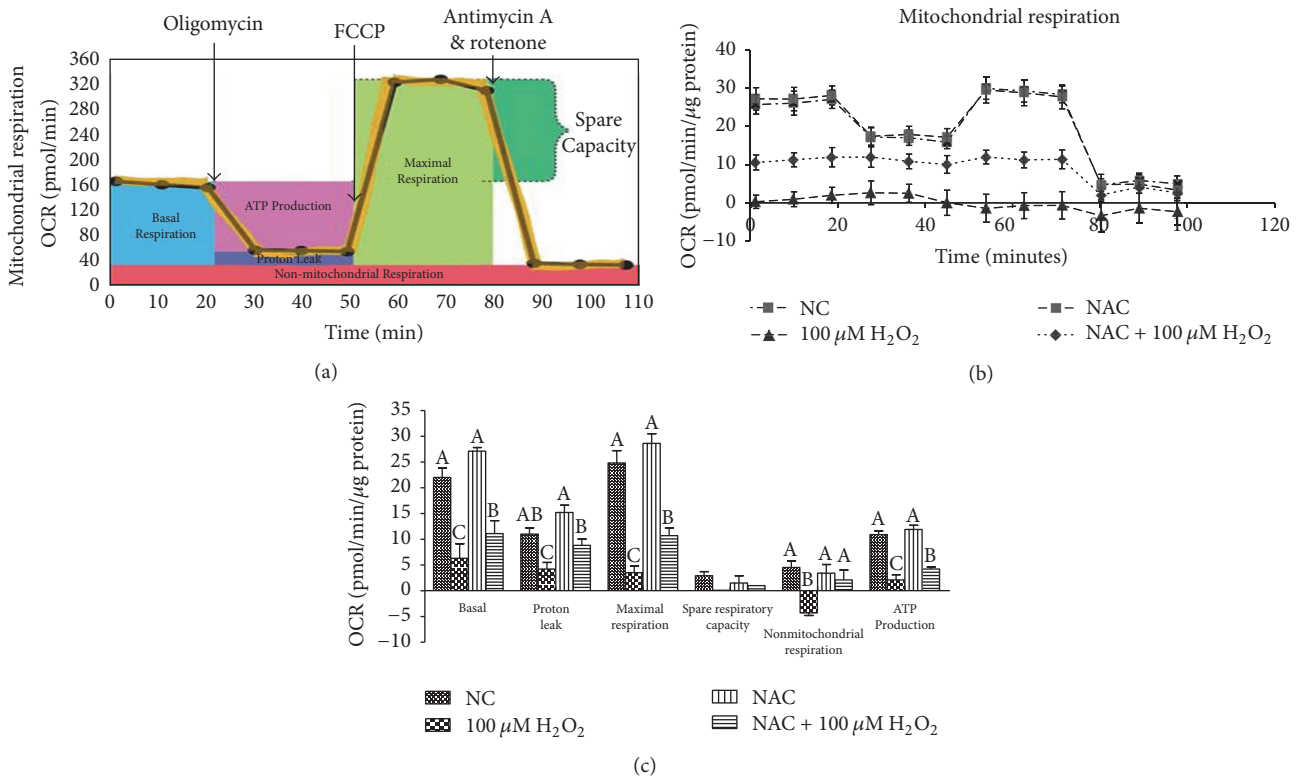


Figure 3: Mitochondrial respiration of IPEC-J2 cells measured by the XF-24 Extracellular Flux Analyzer and Cell Mito Stress Test Kit from Seahorse Biosciences (North Billerica, MA, USA). (a) Schematic and (b) oxygen consumption rate (OCR) assessed by extracellular flux analysis. OCR was measured under basal conditions followed by the sequential addition of oligomycin (0.5 μM), FCCP (1 μM), rotenone (1 μM), or antimycin A (1 μM). Each data point represents an OCR measurement. (c) Individual parameters for basal respiration, proton leak, maximal respiration, spare respiratory capacity, nonmitochondrial respiration, and ATP production were determined. Cells were treated with 0 (NC) or 800 μM NAC and 0 or 100 μM H_2O_2, respectively. Data were expressed as means ± SEM of at least three independent experiments. $^{a–c}$Values with different letters are significantly different ($P < 0.05$).

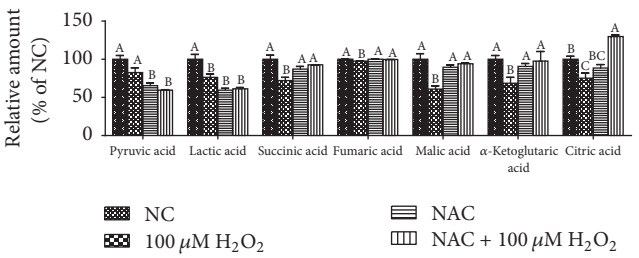


Figure 4: The TCA cycle intermediates, pyruvic acid, and lactic acid of IPEC-J2 cells measured by an Agilent 7890B-5977A GC-MS equipped with HP-5ms (30 m × 250 μm × 0.25 μm) capillary column (Agilent J&W, Santa Clara, CA, USA). Cells were treated with 0 (NC) or 800 μM NAC and 0 or 100 μM H_2O_2, respectively. Data were expressed as means ± SEM of at least three independent experiments. $^{a–c}$Values with different letters are significantly different ($P < 0.05$).

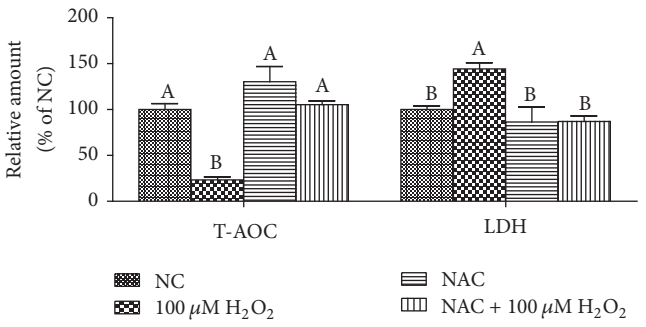
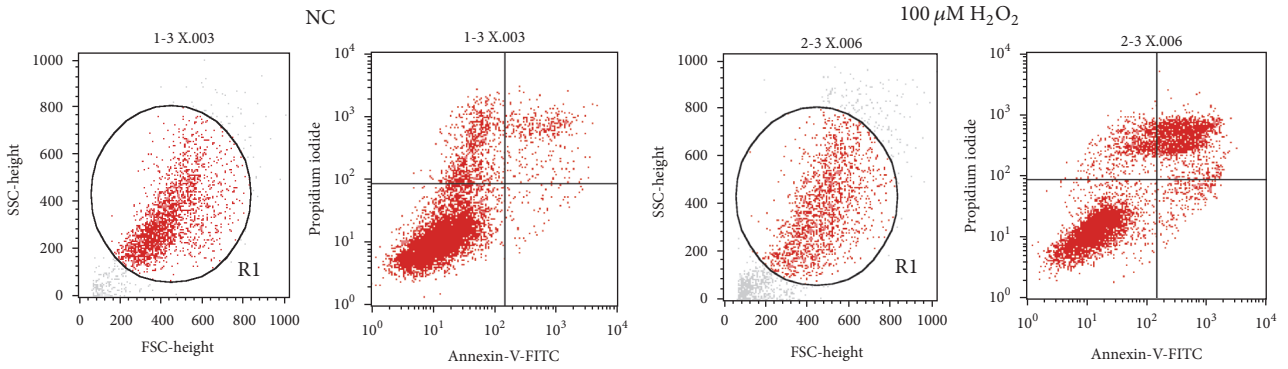


Figure 5: The concentrations of T-AOC and LDH in the IPEC-J2 cells. Cells were treated with 0 (NC) or 800 μM NAC and 0 or 100 μM H_2O_2, respectively. Data were expressed as means ± SEM of at least three independent experiments. $^{a–b}$Values with different letters are significantly different ($P < 0.05$).

NAC significantly decreased the percentage of early and late apoptosis ($P < 0.05$) (Figure 6).

3.6. The Relative Protein Expression Levels of Cell Apoptosis. The relative expression levels of cytochrome C, Bax, B-cell lymphoma/leukaemia-XL (Bcl-XL), cleaved caspase-3, and total caspase-3 proteins are shown in Figure 7. Addition of 100 μM H_2O_2 significantly increased protein levels for cytochrome C, Bcl-XL, cleaved caspase-3, and total caspase-3 proteins ($P < 0.05$), while addition of 800 μM NAC



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Y parameter: propidium iodide (log)

Quad	Events	% Gated
UL	780	8.78
UR	338	3.80
LL	7704	86.69
LR	65	0.73

File: 2-3 X.006
Y parameter: propidium iodide (log)

Quad	Events	% Gated
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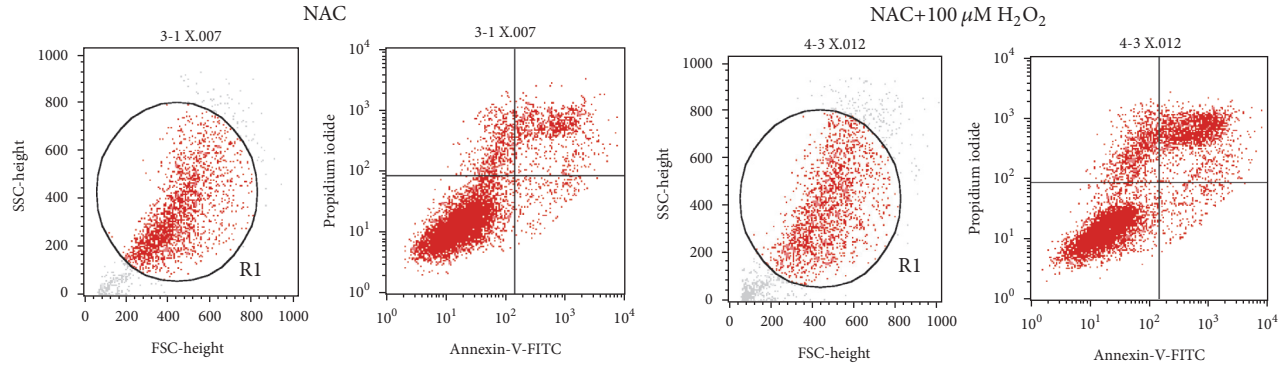
LR	229	3.28



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UR	819	9.32
LL	7247	82.47
LR	196	2.23

File: 4-3 X.012
Y parameter: propidium iodide (log)

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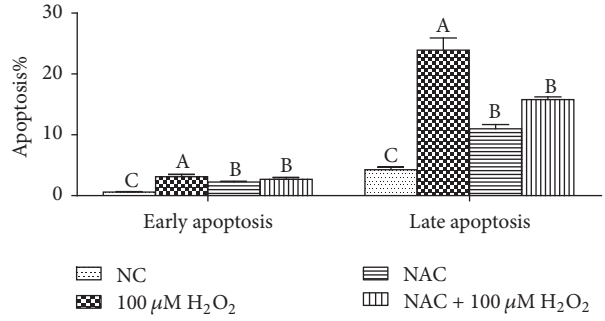
LR	219	2.90

(a)



(b)

FIGURE 6: Cell apoptosis in the IPEC-J2 cells. (a) Representative flow cytometry diagrams and (b) apoptosis rate. Cells were treated with 0 (NC) or 800 μM NAC and 0 or 100 μM H_2O_2, respectively. Data were expressed as means ± SEM of at least three independent experiments. $^{a-c}$Values with different letters are significantly different ($P < 0.05$).

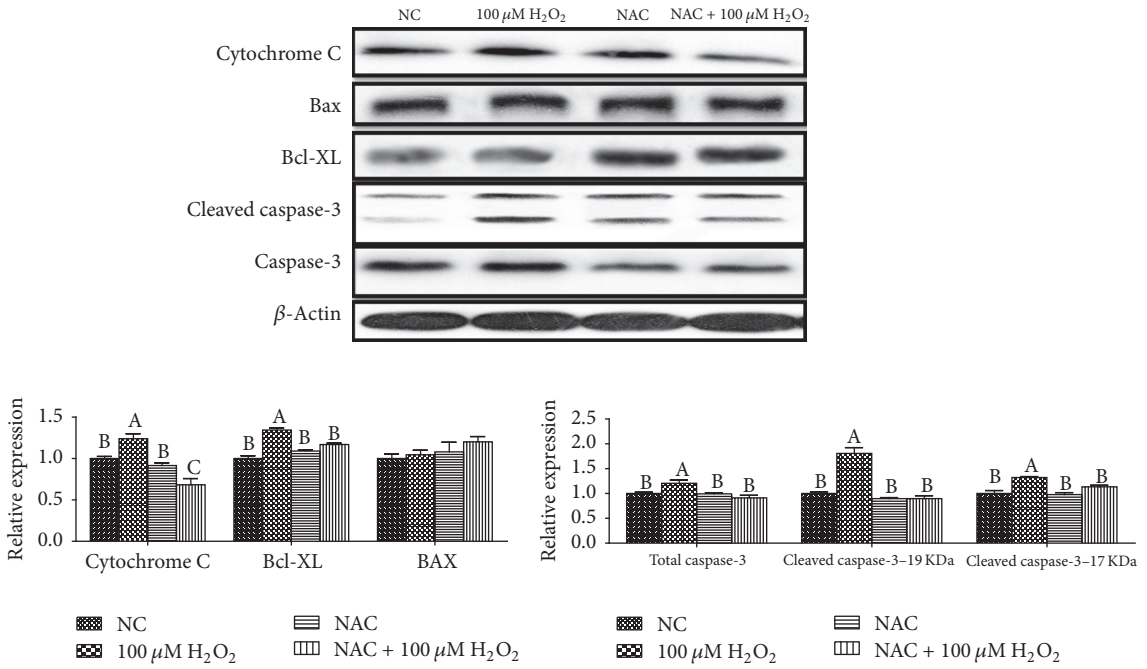


Figure 7: Abundances of proteins (cytochrome C, Bax, Bcl-XL, cleaved caspase-3, and caspase-3) in IPEC-J2 cells determined by western blot analysis. Cells were treated with 0 (NC) or 800 μM NAC and 0 or 100 μM H_2O_2, respectively. Data were expressed as means ± SEM of at least three independent experiments. $^{a-c}$Values with different letters are significantly different ($P < 0.05$).

significantly decreased the above parameters in 100 μM H_2O_2-treated cells ($P < 0.05$).

4. Discussion

NAC has promising effects in different diseases, including cancer, liver toxicity, cardiovascular diseases, and metal toxicity [17], due to its role in attenuating pathophysiological processes including oxidative stress, apoptosis, and mitochondrial dysfunction [18]. In addition, NAC could attenuate inflammation in the liver of LPS-treated mice [19]. Similarly, supplement of NAC has been reported to improve growth performance and energy status, reduce inflammation, and ameliorate tissue damage [12]. Recently, Yi et al. have found that NAC could stimulate protein synthesis and inhibit proteolysis in IPEC-1 cells [20]. In the present study, we found that NAC could not only ameliorate H_2O_2-induced cell growth inhibition, but also attenuate mitochondrial dysfunction in H_2O_2-treated cells. Furthermore, NAC downregulated the mitochondria-depended apoptosis in H_2O_2-treated cells. Therefore, our data suggest that NAC might repair intestinal damage through improving the mitochondrial function.

Intragastric or intraperitoneal administration of H_2O_2 could decrease growth performance and caused oxidative stress [21–23]. Furthermore, previous studies showed that addition of H_2O_2 (300 μM) to chicken intestinal epithelial cells for 24 h significantly decreased cell survival and SOD activity [24]. Similarly, the results indicated that H_2O_2 at 100 μM for 4 h decreased the growth of IPEC-J2. Additionally, the percentages of EdU-positive cells were decreased in response to 100 μM H_2O_2 treatment. In addition, the reports show that excess intracellular ROS level could cause oxidative damage to lipids, DNA, and proteins via apoptosis [25]. In the present study, mitochondrial function was destroyed in H_2O_2 treatment, which is in accordance with results from Fan et al. [10]. These data indicate that H_2O_2 induces mitochondrial ROS production and then leads to DNA damage in IPEC-J2 cells. The report by Yi et al. indicated that NAC increased the growth of IPEC-1 cells and suggested that NAC at low concentrations (<1 mM) could stimulate cell growth [20]. In line with these results, this experiment showed that addition of NAC at 500–1000 μM to 100 μM H_2O_2 treatment increased the cell viability. And adding NAC at 800 μM to 100 μM H_2O_2 increased the cell proliferation, indicating that NAC might improve the H_2O_2-induced cell growth damage.

There is increasing evidence supporting that NAC improve redox status and directly react with oxidative metabolites [12, 26, 27]. NAC protects cells against oxidative stress through reducing glutathione (GSH) and interacting with ROS [12]. In this study, H_2O_2 exposure induced oxidative stress evidenced by decreased cell viability, inhibited T-AOC, and increased leakage of LDH, while NAC treatment markedly improved antioxidant system. Mitochondria are the powerhouses of the cell, producing a considerable share of cellular ATP and playing a central role in cellular function and metabolism [28]. Our previous report showed that mitochondrial dysfunction was observed with decrease in the basal respiration, maximal respiration, and nonmitochondrial respiration after LPS treatment [1]. The present data also demonstrated that mitochondrial function damage induced by H_2O_2 was observed, showing decrease in basal respiration, proton leak, maximal respiration, spare respiratory capacity,

nonmitochondrial respiration, and ATP production. In vivo, NAC has been determined to improve mitochondrial uncoupling and respiration in inflamed intestines [26]. Our results showed that NAC could improve mitochondrial bioenergetics in H_2O_2-treated cells. The previous studies have reported that NAC improved oxygen delivery [29] and systemic oxygen consumption [30] and regulated mitochondrial TCA cycle metabolism by stimulation of carbon flux through pyruvate dehydrogenase, a key enzyme for hepatocellular mitochondrial energy metabolism by acetyl-CoA supply [31, 32]. Our results were in agreement with these previous ideas. The results showed that NAC influenced metabolism of cellular pyruvic acid, lactic acid, succinic acid, malic acid, and citric acid, which contribute to mitochondrial redox balancing and are transported into mitochondria to affect ATP production by oxidative phosphorylation [33, 34].

Reports showed that ROS and mitochondrial dysfunction could mediate apoptosis, indicating that ROS are important in cellular apoptosis [35]. Based on studies from various cell types, it is increasingly clear that NAC could inhibit the cell apoptosis [12, 36, 37]. Flow cytometry analysis showed that 100 μM H_2O_2 significantly induced cell apoptosis, while NAC could attenuate this effect of H_2O_2 by inhibiting cell apoptosis at both early and late stages, which is consistent with Mayer and Noble and Shen et al.'s studies [38, 39]. Unbelievably, this inhibition of NAC on cell apoptosis is only observed in H_2O_2 treated cells and NAC induced cell apoptosis in normal cells, which require further research. Cytochrome c is released from mitochondria due to formation of a channel, the mitochondrial apoptosis-induced channel, in the outer mitochondrial membrane, and serves a regulatory function as it precedes morphological change associated with apoptosis. The report showed that apoptosis was mediated via the intrinsic pathway. Loss of mitochondrial membrane potential increased release of cytochrome C in cytosol and activated some proapoptotic molecules (Bax, cleaved caspase-9, caspase-3, and so on) and caused downregulation of Bcl-2 which happened in a dose-dependent manner [40]. The previous study has found that supplementation with NAC attenuated caspase-3 protein expression in the small intestine of LPS-challenged pigs [13]. In the current study, we noted that the relative proteins expressions of cell apoptosis were elevated in IPEC-J2 cells after H_2O_2 treatment but were reduced when NAC was added into IPEC-J2 cells pretreated with H_2O_2. Thus, the beneficial effects of NAC may be associated with attenuating cell apoptosis.

In summary, H_2O_2 induced mitochondrial dysfunction and cell apoptosis, while NAC promoted DNA synthesis, mitochondrial bioenergetics, and mitochondria-depended apoptosis in intestinal epithelial cells. Possible mechanisms for the cytoprotective effect of NAC on H_2O_2-induced damage in IPEC-J2 cells scavenged the H_2O_2 and then improved cell proliferation, TCA cycle, and mitochondria function and reduced cell apoptosis and death. Results from these studies have important implications for the use of NAC in the clinical management of oxidative damage in the neonatal pigs.

Disclosure

Hao Xiao and Miaomiao Wu are joint first authors.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

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Clinical Study

The Effect of Immunonutrition on the Postoperative Complications in Thymoma with Myasthenia Gravis

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Object. To test whether preoperative immunonutrition is efficacious in reducing postoperative complications in patients of thymoma with myasthenia gravis (MG). *Material and Methods*. A total of 244 patients operated on for thymoma with myasthenia gravis were prospectively assigned to two groups, each receiving seven-day preoperative and seven-day postoperative nutrition. The patients in immunonutrition group were given oral immunonutrition (IN). The patients in control group received oral standard nutrition. Immunonutritional and inflammatory biomarkers (IgA, IgG, IgM, CD3t, CD4t, CD8t, CD4t/CD8t ratio, NK-cell, prealbumin, albumin, white blood cells counts, and C-reactive protein) and clinical variables (age, gender, BMI, performance status, type of thymoma, type of MG, operative time, pathology, operative approach, postoperative complications, quantity of drainage, hospital stays) were examined. *Results*. A significant reduction in the length of hospital stay, quantity of drainage, and postoperative complications was observed in the IN group ($p < 0.05$). An increase in the level of IgA, IgG, IgM, CD3+T, CD4+T, CD4+T/CD8+T, WBC, CRP, and NK-cell in the IN group was observed after thymectomy, while a decrease was seen with regard to prealbumin and albumin ($p < 0.05$). *Conclusion*. Preoperative immunonutrition support is effective in reducing postoperative complications in patients of thymoma with MG. It helps to lower the risk of postoperative infectious complications and hospital stays.

1. Introduction

Thymoma is a rare disease but accounts for about 50% of tumors in the anterior mediastinum [1, 2]. Thymectomy is the golden standard for the treatment of thymoma due to its malignant potential [3–5]. Considering its immunological characteristics, a lot of scholars have recommended performing en bloc resection of thymic cells, because this is an important prognostic factor in the treatment of thymoma [6, 7]. It is self-evident that complications after operation are unavoidable, which mainly include pneumonia, infection of incision, and respiratory insufficiency [8]. Besides, thymoma usually is associated with many autoimmune diseases, including systemic lupus erythematosus (SLE), polymyositis, Good's syndrome, pure red cell aplasia (PRCA), and myasthenia gravis (MG) which is the most common symptom complicated with the thymoma [9]. Although approximately 50% of the patients have a chance of drug- and symptom-free life after the operation [10, 11], the morbidity and mortality could be increased during postoperation [12], and many of them are the independent factors affecting the prognosis, especially the MG. If postthymectomy myasthenic crisis occurred, it can make the pneumonia, which is a common complication, life-threatening [13]. But it is difficult to predict when such complications will come up after the operation, and by now, there has been no effective approach to preventing these complications or reducing the incidence.

Immunonutrition (IN), which is the combination of standard nutritional formulas and immunonutrients, has been widely exploited recently. The immunonutrients are glutamine, arginine, and polyunsaturated fatty acids (omega-3), among others, which can improve the immunity and nutrition effectively. They can modulate inflammatory responses and enhance protein synthesis and then increase immune responses. It has been proved that the perioperative application of immunonutrition is an effective and more prevailing

Table 1: Composition of immunonutrition.

Composition (per 100 ml)	Amount (g)
Proteins, g	7.6
L-arginine, g	1.0
Ribonucleic Acid, g	0.2
Fats (total), g	3.9
Saturated fatty acids	1.8
Medium chain triglycerides	1.12
Monounsaturated	0.73
Polyunsaturated	1.3
Linoleic acid	0.6
U-3 fatty acids	0.77
Carbohydrates, g	13.3
Sucrose, g	10.5
Lactose, g	<0.02
Soluble fibers, g	1.4
Energy, kcal	141
Iron, mg	1.7
Zinc, mg	2.2
Copper, μg	169
Vitamin A, μg	200
Vitamin D, μg	3.3
Vitamin E, mg	4.5
Vitamin B1, mg	0.33
Vitamin B2, μg	0.33
Vitamin B6, μg	0.33
Vitamin B12, μg	1
Vitamin C, mg	33
Water, g	77

therapeutic strategy [14, 15]. As they can reduce infectious and other postoperative complications, they have been extensively applied in many kinds of disease and status, especially in digestive tumor and malnourished patients [16–19]. Strong evidence, including some randomized, double-blind trials, demonstrates that perioperative immunonutrition support significantly reduced the incidence of pneumonia and hospital stay [20]. Meanwhile, some studies reported that the treatment of immunoglobulin is effective for the MG crisis and severe MG. In some cases, intravenous immunoglobulin could be an alternative approach when therapeutic plasma exchange is unsuitable because of its high possibility of developing cardiopulmonary failure [21]. Some other studies reported that improving the nutrition and immunity rapidly is beneficial for the inflammation [22]. But it is still unknown whether or not immunonutrition is effective in reducing the morbidity and mortality in the perioperative period of thymectomy.

As such, the object of this study was to examine whether preoperative immunonutrition is efficacious in reducing postoperative complications in patients of thymoma with MG.

2. Materials and Methods

2.1. Patients. A prospective, nonrandomized, interventional, single-blind cohort study was designed. 244 well-nourished patients of thymoma with MG from January 2012 to December 2015 were enrolled. No patient received preoperative chemoradiotherapy. Informed consent was obtained from all the patients.

2.2. Feeding Regimens. The oral immunonutrition (IN) supplement group receiving immune-enriched diet was compared with the oral standard nutrition (SN) supplement group receiving the standard nutrition diet, which started 7 days prior to thymectomy and lasted 7 days postoperatively with the parenteral alimentation on the day of surgery. The ingredients of formula are listed in Table 1.

We performed blood test on postoperative 1st, 3rd, 5th, and 7th days and preoperative first day, respectively. Outcome measures included clinical variables (age, gender, BMI, performance status, type of thymoma, type of MG, operative time, pathology, operative approach, postoperative complications, quantity of drainage, and hospital stays) and immunonutritional and inflammatory biomarkers (IgA, IgG, IgM, CD3t, CD4t, CD8t, CD4t/CD8t ratio, NK-cell, prealbumin, albumin, white blood cells counts, and C-reactive protein).

2.3. Statistical Analyses. We performed statistical comparison using Chi squared test, Student's t-test, and Fisher's exact test for the comparison. It was considered statistically significance at $p < 0.05$. Statistics analysis was performed using SPSS v 18.00.

3. Results

102 (41.8%) subjects under investigation received the immunonutrition support, and 142 (58.2%) patients did not. Concerning baseline characteristics between the two groups, no significant difference was observed with respect to age, gender, BMI, performance status, type of thymoma, type of MG, operative time, pathology, or operative approach. Comparatively a significant decrease in the duration of hospital stay, quantity of drainage, and postoperative complications was observed in the IN group ($p < 0.05$) (Table 2).

As shown in Table 3, 56 (23.0%) patients experienced postoperative complications for the entirety, and 21 (20.6%) of the 102 patients did in IN group ($p < 0.05$). Pneumonia and myasthenic crisis remain the most common postoperative complications; with regard to pneumonia, the incidence of pneumonia in the IN group was significantly lower than in the control group ($p < 0.05$). The IN group was inclined to reduce the total infectious complications rate ($p < 0.05$). The significant relativity between fast reconversion of WBC, CRP, prealbumin, albumin, and immunonutrition support was observed through the analysis of subgroups with infectious complications (Figures 1(a), 1(b), 1(c), and 1(d)).

On the other hand, decreased hospital stays, length of extubation, intensive care unit stay, and pneumonia with the IN group were observed significantly through the analysis of subgroups with myasthenic crisis (Table 4).

Table 2: Patient characteristics between two groups.

	Immunonutrition ($n = 102$)	Standard nutrition ($n = 142$)	p
Age	52.3 ± 12.4	53.2 ± 13.1	>0.05
Gender			>0.05
Male	48 (47.1%)	67 (47.2%)	
Female	54 (52.9)	75 (52.8%)	
BMI	22.8 ± 4.1	23.0 ± 4.0	>0.05
Performance status			>0.05
1	20 (19.6%)	30 (21.1%)	
2	66 (64.7%)	92 (64.8%)	
3	16 (15.7%)	20 (14.1%)	
Type of thymoma			>0.05
I	22 (21.6%)	31 (21.8%)	
II	78 (76.5%)	108 (76.1%)	
III	2 (1.9%)	3 (2.1)	
Type of MG			>0.05
I	22 (21.6%)	33 (23.2%)	
II	63 (61.9)	85 (59.9%)	
III	12 (11.8%)	16 (11.3%)	
IV	5 (4.9%)	8 (5.6%)	
V	0	0	
Operative time (min)	155.6 ± 62.8	159.8 ± 68.2	>0.05
Pathology			>0.05
A	6 (5.9%)	8 (5.6%)	
AB	42 (41.2%)	60 (42.3%)	
B1	14 (13.7%)	19 (13.4%)	
B2	33 (32.4%)	44 (31.0)	
B3	4 (3.9%)	7 (4.9%)	
C	3 (2.9)	4 (2.8%)	
Operative approach			>0.05
Right chest (VATS)	74 (72.5%)	104 (73.2%)	
Sternal (OPEN)	16 (15.7%)	22 (15.5%)	
Subxiphoid (VATS)	12 (11.8%)	16 (11.3%)	
Postoperative complications	21 (20.6%)	35 (24.6%)	<0.05
Quantity of drainage	452.2 ± 112.5	485.3 ± 123.5	<0.05
Hospital stays	16.5 ± 8.4	20.4 ± 9.4	<0.05

The preoperative inflammatory and immune markers including IgA, IgG, IgM, CD3+T, CD4+T, CD8+T, CD4+T/CD8+T, NK-cell, prealbumin, albumin, WBC, and CRP between the two groups were not statistically different. On the first postoperative day, no significant difference was observed in the mentioned markers between the two groups. However, on the third postoperative day, the levels of WBC, CRP, and NK-cell dramatically decreased in the IN group ($p < 0.05$). On the fifth day, in contrast to the significant decrease in the IN group concerning the level of WBC, CRP, and NK-cell, an increase was seen in the level of prealbumin and albumin ($p < 0.05$). Although CD8+T showed no significant differences, an increase in the level of IgA, IgG, IgM, CD3+T, CD4+T, and CD4+T/CD8+T in the IN group was observed on the seventh day, the same as the value of prealbumin and albumin. The level of WBC, CRP, and NK-cell was significantly lower than that in SN group (Table 5).

4. Discussion

Thymectomy is the most common tumor of anterior mediastinum, and 30–50% of the thymoma patients have myasthenia gravis [1]. About 15–20% of MG patients will develop myasthenic crisis, which is a life-threatening emergency always caused by infections [23–25]. Thymectomy may reduce the morbidity of this status [26], while thymectomy

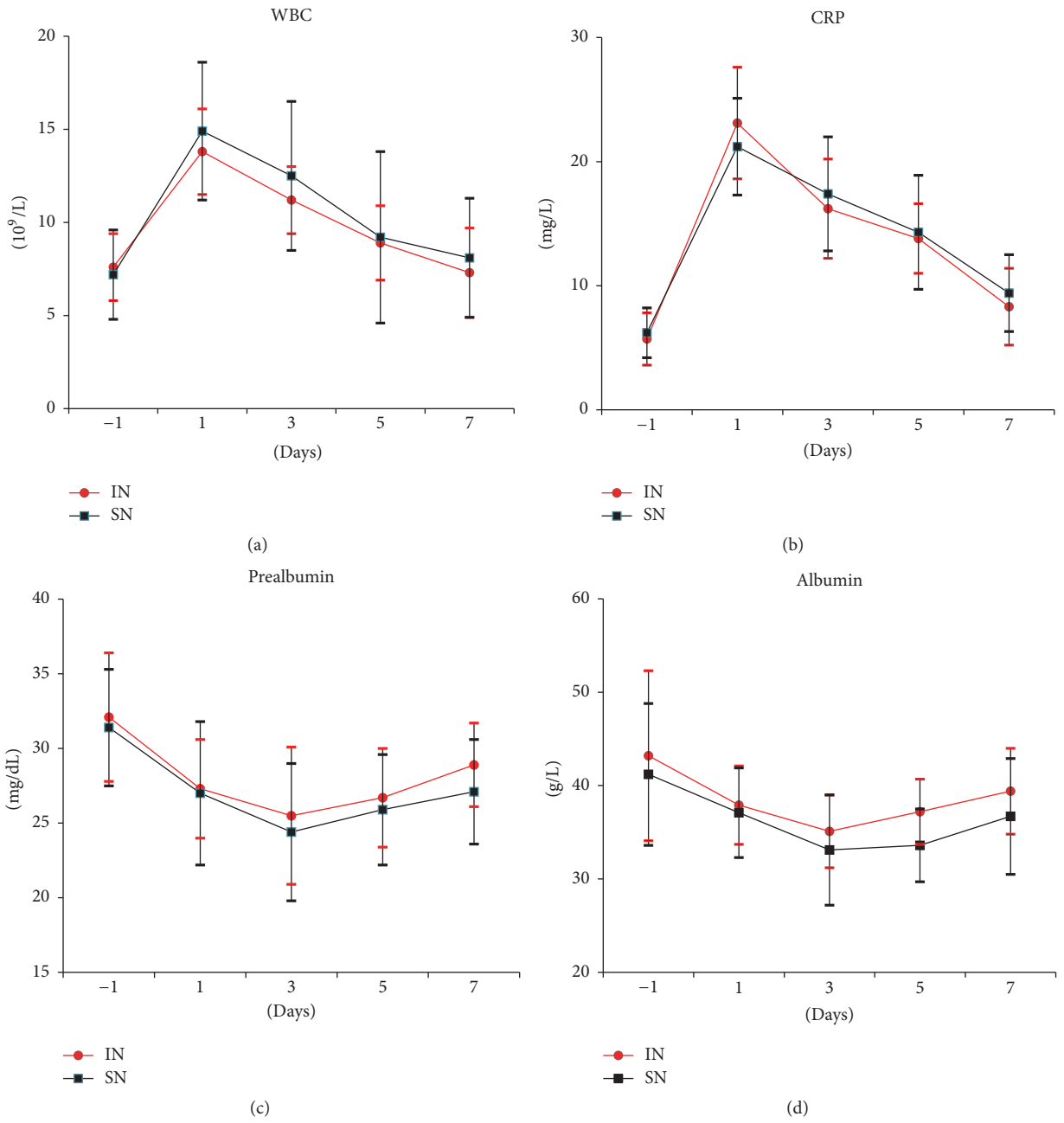

Figure 1

was always accompanied with high postoperative complications, especially infectious complications including pneumonia, which limited its efficacy. Given that immunosuppression caused by surgical stress is one of the most important factors in developing complications [27], the effects of immunonutrition on outcome after major operation for cancer or after severe injury have been frequently reported; however, the effects for thymoma have never been mentioned. Therefore we designed this study to examine the effect of immunonutrition on the postoperative complications in thymoma with myasthenia gravis.

Immunonutrition can not only modulate inflammatory responses and enhance protein synthesis and then increase immune responses, but also promote the control of the immune obstacle during the early postoperative phase and improve intestinal blood supply and oxygen metabolism [28–31]. Lots of studies have demonstrated that perioperative immunonutrition support significantly reduced the incidence of infectious complications. In their research for patients undergoing major abdominal cancer surgery, Giger et al. concluded that perioperative immunonutrition support significantly reduces inflammation and postoperative complications compared with postoperative diet administration alone [18]. In addition, Rowan and his collaborators published their results that perioperative immunonutrition may lead to significant reductions of postoperative complications

Table 3: Details of postoperative complications between two groups.

	IN ($n = 21$)	SN ($n = 35$)	p
Pneumonia	7 (33.3%)	14 (40%)	<0.05
Mediastinal infection	1 (4.8%)	1 (2.9%)	>0.05
Infection of incision wound	1 (4.8%)	1 (2.9%)	>0.05
Pleural effusions	1 (4.8%)	1 (2.9%)	>0.05
Myasthenic crisis	8 (38.1)	14 (40%)	<0.05
Atrial fibrillation	1 (4.8%)	1 (2.9%)	>0.05
Vocal cord palsy	0	1 (2.9%)	>0.05
Phrenic nerve injury	1 (4.8%)	1 (2.9%)	>0.05
Sternum separation	1 (4.8%)	1 (2.9%)	>0.05

Table 4: Comparison of subgroups of postoperative myasthenic crisis between two groups.

	IN ($n = 8$)	SN ($n = 14$)	p
Hospital stays	20.5 ± 4.5	22.6 ± 6.7	<0.05
Length of extubation	6.4 ± 4.5	8.5 ± 6.4	<0.05
ICU stay	7.5 ± 4.5	9.8 ± 6.3	<0.05
Quantity of drainage	455.5 ± 112.4	474.4 ± 128.3	>0.05
Pneumonia	4 (50%)	10 (71.4%)	<0.05
Operative time	160.4 ± 68.6	168.5 ± 75.4	>0.05

in high-risk head and neck cancer patients [32]. Moreover, Silvestri et al. compared the clinical characteristics of patients after pancreaticoduodenectomy between immunonutrition group and control group and then obtained the result that immunonutrition helps to lower the risk of postoperative infectious complications and of hospital stays [33–35]. In our cohort, we obtained similar results with patients of thymoma with MG; the morbidity of postoperative complications, especially infectious complications, was significantly reduced in IN group.

Although some studies showed no significant difference with hospital stay in immunonutrition group [33, 36], different outcome was observed in our study. We attribute the result to the choice of the subjects. Moya and his team studied the effect of immunonutrition on the patients under colorectal resection in an enhanced recovery after surgery (ERAS) protocol and drew a conclusion that the average postoperative hospital stay was not significantly different between the two groups. Their median postoperative hospital stay, which was only 5 days, maybe interfered by the applications of ERAS [33]. Song and his collaborators reviewed 9 studies involving 785 patients undergoing gastric resection concerning enteral immunonutrition and obtained a result that length of hospitalization was not improved, but they analysed that their outcome with heterogeneity caused by different compositions, different timing of administration of immunonutrient, insufficient quantity of sample size, and number of eligible researches in most of sensitive analyses with subgroup analysis may impair their conclusions [36]. We surmised that it is the heterogeneity that contributes to the different results.

In the analysis of the subgroup of myasthenic crisis, the hospital stay, length of extubation, and ICU stay were significantly lower in IN group than those in SN group. The patients with myasthenic crisis were inclined to be accompanied by high morbidity of pneumonia, especially ventilator associated pneumonia. Rabinstein and Mueller-Kronast reported that pneumonia was significantly associated with extubation failure, which considerably prolonged intensive care unit stay and hospital stay [37]. In our research, there were more patients with myasthenic crisis in the SN group, which may contribute to the longer hospital stay, extubation, and ICU stay. It is assumed that the different morbidity of pneumonia may contribute to the hyperdispersion of the subjects investigated. Immunonutrition provides a new option for improving the prognosis of our patients.

Song and his collaborators conducted a meta-analysis on the patients from when they underwent surgery for gastric cancer to enteral immunonutrition. They came to the conclusion that the immune markers increased in immunonutrition group [36]. Shimogawa et al. reported that the inflammatory markers including CRP and WBC count were decreased in the IN group of patients with severe intracranial hemorrhage during the acute stage [38]. In our study, the increased immune markers were observed five days postoperatively and the decreased inflammatory markers were observed to begin on the third postoperative day. Although there was still significant difference between the two groups on the seventh postoperative day, there has been no clinical significance because the value of CRP and WBC count has restored to normal in both groups. With the analysis of the subgroup of infectious complications, there was significant difference with the reconversion of WBC count, CRP, prealbumin, and albumin between the two groups. The morbidity of myasthenic crisis was lower in IN group.

Calder reported that ω-3 polyunsaturated fatty acids (ω-3 PUFAs), as a main component of the immune nutrients, ensured the maintenance of membrane fluidity and the sufficient function of membrane proteins. Its dysbolism maybe leads to many human diseases including immune disorders or cancers [39]. Furthermore, it demonstrates strong anti-inflammatory and immunomodulatory effects via influencing the synthesis of eicosanoid mediators prostaglandins (PGs), thromboxanes (TXs), and leukotrienes (LTs). In addition to this, its production stimulated peripheral monocytes [40]. Tossou et al. reported that tryptophan played the role of regulating physiological functions and intestinal permeability in the alimentary tract [41]. Hyeyoung Kim reviewed the glutamine, another essential immune nutrient. He pointed out that glutamine always played an essential role in nitrogen transport in vivo and served as a substrate for renal ammoniagenesis. Moreover, it influenced the expression of heat shock proteins and stimulates nucleotide synthesis and activates signaling mediators [42–44]. Arginine, as the important component part of immune nutriment, stimulates anabolic hormone release, improves nitrogen balance, and has immunostimulatory and thymotrophic functions. It also plays roles in nitrogen metabolism, creatine, and polyamine

Table 5: Inflammatory and immunobiomarkers between two groups.

	IN					SN				
	D−1	D1	D3	D5	D7	D−1	D1	D3	D5	D7
IgA (mg/ml)	3.5 ± 1.4	2.1 ± 1.4	2.6 ± 1.2	2.9 ± 1.3	3.4 ± 1.2*	3.4 ± 1.3	2.0 ± 1.4	2.5 ± 1.3	2.7 ± 1.4	3.2 ± 1.1
IgG (mg/ml)	16.5 ± 6.3	10.2 ± 4.5	11.3 ± 4.6	14.5 ± 4.3	16.3 ± 5.1*	16.3 ± 5.7	10.2 ± 4.4	11.0 ± 4.4	13.5 ± 4.8	15.4 ± 4.5
IgM (mg/ml)	1.5 ± 0.4	1.1 ± 0.4	1.2 ± 0.5	1.3 ± 0.4	1.6 ± 0.3*	1.5 ± 0.3	1.0 ± 0.3	1.1 ± 0.3	1.2 ± 0.4	1.4 ± 0.4
CD3+T (10^5/ml)	18.5 ± 8.4	10.6 ± 3.5	12.4 ± 3.5	15.4 ± 3.8	19.2 ± 4.5*	17.8 ± 8.3	11.1 ± 3.8	12.3 ± 3.6	15.2 ± 3.6	17.8 ± 4.0
CD4+T (10^5/ml)	9.5 ± 5.4	5.8 ± 2.8	6.2 ± 3.2	7.5 ± 3.5	9.4 ± 4.8*	9.6 ± 4.8	6.0 ± 2.8	6.3 ± 3.6	7.2 ± 3.4	9.4 ± 4.9
CD8+T (10^5/ml)	8.4 ± 3.5	4.5 ± 3.2	5.3 ± 3.3	6.5 ± 3.2	7.8 ± 3.2	8.6 ± 3.5	4.5 ± 3.3	5.5 ± 3.3	6.4 ± 3.1	7.7 ± 3.1
CD4+T/CD8+T	1.1 ± 0.1	1.6 ± 0.3	1.2 ± 0.2	1.5 ± 0.4	1.4 ± 0.3*	1.2 ± 0.2	1.6 ± 0.2	1.5 ± 0.3	1.4 ± 0.3	1.5 ± 0.3
Pre-ALB (mg/dL)	32.1 ± 4.3	27.3 ± 3.3	25.5 ± 4.6	26.7 ± 3.3*	28.9 ± 2.8*	31.4 ± 3.9	27.0 ± 4.8	24.4 ± 4.6	25.9 ± 3.7	27.1 ± 3.5
ALB (g/L)	43.2 ± 9.1	37.9 ± 4.2	35.1 ± 3.9	37.2 ± 3.5*	39.4 ± 4.6*	41.2 ± 7.6	37.1 ± 4.8	33.1 ± 5.9	33.6 ± 3.9	36.7 ± 6.2
NK cell (%)	60.5 ± 12.1	80.4 ± 15.4	75.5 ± 13.3*	67.6 ± 13.5*	62.5 ± 12.1*	61.4 ± 11.7	79.3 ± 16.4	75.4 ± 13.2	68.5 ± 13.1	65.4 ± 12.5
WBC counts	7.6 ± 1.8	13.8 ± 2.3	11.2 ± 1.8*	8.9 ± 2.0*	7.3 ± 2.4*	7.2 ± 2.4	14.9 ± 3.7	12.5 ± 4.0	9.2 ± 4.6	8.1 ± 3.2
CRP (mg/l)	5.7 ± 2.1	23.1 ± 4.5	16.2 ± 4.0*	13.8 ± 2.8*	8.3 ± 3.1*	6.2 ± 2.0	21.2 ± 3.9	17.4 ± 4.6	14.3 ± 4.6	9.4 ± 3.1

IN, immunonutrition; D, day; WBC, white blood cell count; CRP, C-reactive protein; Alb, albumin; Pre-Alb, prealbumin; IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M; *significantly different compared with SN.

synthesis [45]. On the whole, immunonutrition, including ω-3 PUFAs, glutamine, and arginine, could modulate inflammatory responses, enhance nitrogen balance and protein synthesis, increase host immune responses, and then shorten the course of disease, reduce postoperative complications, and improve the prognosis of patients.

Interestingly, there is also significant difference between quantity of drainage and IN group. We speculate that this result may contribute to the fast reconversion of the albumin and prealbumin because it was proven that preoperative immunonutrition could provide a significant increase in prealbumin levels [46]. Xu et al. compared the effects on nutritional status between immunonutrition pharmaceutics group and standard nutrition group and reached a conclusion that higher IgG, CD4/CD8 ratio, and prealbumin were significantly relevant with immunonutrition support [47].

Though we exclude the malnutrition subjects so as to reduce the bias as much as possible, some limitation still exists in our study. For one thing, distribution of characteristics of subjects restricted by the single centre may be different from general regularity. For another thing, the sample size for the subgroups is not big enough.

5. Conclusion

Preoperative immunonutrition support is effective in reducing postoperative complications in patients of thymoma with MG. It helps to lower the risk of postoperative infectious complications and hospital stays.

Abbreviation

MG: Myasthenia gravis
IN: Immunonutrition
BMI: Body mass index
WBC: White blood cell count
CRP: C-reactive protein
Alb: Albumin
Pre-Alb: Prealbumin
IgA: Immunoglobulin A
IgG: Immunoglobulin G
IgM: Immunoglobulin M
NK-cell: Natural killer cell
SLE: Systemic lupus erythematosus
PRCA: Pure red cell aplasia
SN: Standard nutrition
ERAS: Enhanced recovery after surgery
ICU: Intensive care unit
ω-3 PUFAs: ω-3 polyunsaturated fatty acids
PGs: Prostaglandins
TXs: Thromboxanes
LTs: Leukotrienes.

Competing Interests

The authors declare that there has no conflict of interests.

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Research Article

Veronicastrum axillare Alleviates Lipopolysaccharide-Induced Acute Lung Injury via Suppression of Proinflammatory Mediators and Downregulation of the NF-κB Signaling Pathway

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Veronicastrum axillare is a traditional medical plant in China which is widely used in folk medicine due to its versatile biological activities, especially for its anti-inflammatory effects. However, the detailed mechanism underlying this action is not clear. Here, we studied the protective effects of *V. axillare* against acute lung injury (ALI), and we further explored the pharmacological mechanisms of this action. We found that pretreatment with *V. axillare* suppressed the release of proinflammatory cytokines in the serum of ALI mice. Histological analysis of lung tissue demonstrated that *V. axillare* inhibited LPS-induced lung injury, improved lung morphology, and reduced the activation of nuclear factor-κB (NF-κB) in the lungs. Furthermore, the anti-inflammatory actions of *V. axillare* were investigated *in vitro*. We observed that *V. axillare* suppressed the mRNA expression of interleukin-1β (IL-1β), IL-6, monocyte chemotactic protein-1 (MCP-1), cyclooxygenase-2 (COX-2), and tumor necrosis factor-α (TNF-α) in RAW264.7 cells challenged with LPS. Furthermore, pretreatment of *V. axillare in vitro* reduced the phosphorylation of p65 and IκB-α which is activated by LPS. In conclusion, our data firstly demonstrated that the anti-inflammatory effects of *V. axillare* against ALI were achieved through downregulation of the NF-κB signaling pathway, thereby reducing the production of inflammatory mediators.

1. Introduction

Acute lung injury (ALI) is a prototypical inflammatory disease. ALI's most significant pathological feature is the acute lung inflammation, including inflammatory cell recruitment and the release of proinflammatory mediators [1, 2]. Lipopolysaccharide (LPS) is the major component of the cell wall in Gram-negative bacteria. LPS can be used to produce a classical animal model of ALI associated with the activation of monocytes, overflow of pulmonary neutrophils, increased levels of alveolar-capillary permeability, and diffuse alveolar damage [3]. During the initiation of the host defense process, LPS is recognized by Toll-like receptor 4 (TLR4), which then activates several intracellular signaling pathways, among which nuclear factor-κB (NF-κB) pathway is the predominant one. The activation of NF-κB eventually leads to the upregulation of several inflammatory factors [4]. Therefore, interfering with NF-κB signaling pathway is a promising therapeutic regimen for the treatment of acute inflammatory disease, like ALI.

Veronicastrum axillare is a folk medicine which is widely used in many regions in East Asia to treat ascites, burns, and snakebites, among other ailments [5]. Our previous work indicated that *V. axillare* significantly inhibits ethanol-induced gastric ulcers [6]. Recently, it was shown that the ethyl acetate (EtOAc) extracts of *V. axillare* displayed potent anti-inflammatory activity *in vivo* using xylene-induced mouse ear edema model [7]. Nevertheless, effects of *V. axillare*

against ALI and potential anti-inflammatory mechanisms are poorly understood. Here, we assessed the anti-inflammatory effects of *V. axillare in vivo* employing LPS-induced ALI mice. We also explored the underlying mechanisms of this action, including the possible inhibition on the activation of macrophages.

2. Materials and Methods

2.1. Animals and Chemical Reagents. Male ICR mice (20 ± 2 g) were purchased from the Shanghai Laboratory Animal Research Center and housed in the Animal Experimental Center, Zhejiang Chinese Medical University. Four mice in one cage were housed in a room with controlled lighting (12 h light/dark cycle) and temperature (20 ± 2°C), and the air was filtered by a ventilation system with relative humidity of 50%. Mice were fed with a standard laboratory diet (AIN-93 formulation for experimental animals, Xietong Biotechnology, Nanjing, China) and water *ad libitum* and received careful care to fulfill the requirement of animal welfare during experimental periods. The animal experimental protocols described in this study were in accordance with the regulations from the Animal Experimental Center, Zhejiang Chinese Medical University, and were approved by Zhejiang Chinese Medicine University Animal Care Committee.

LPS (*Escherichia coli* O111:B4) and alkaline phosphatase-conjugated secondary antibody (anti-rabbit IgG) were purchased from Sigma (St. Louis, MO, USA). Primary rabbit monoclonal antibodies against phospho-IκB-α (pS36), IκB-α, and β-tubulin were purchased from Epitomics (Burlingame, CA, USA). Primary rabbit monoclonal antibodies phospho-NF-κB p65 (Ser536) were purchased from Cell Signaling (Beverly, MA, USA). All the other reagents were obtained from Sangon Biotechnology (Shanghai, China) unless indicated specifically in each section.

2.2. Preparation of V. axillare Aqueous Extract. Whole *V. axillare* plants were gathered in Lishui, Zhejiang Province, China, and identified by Professor Zhensheng Yao (School of Pharmacy, Zhejiang Chinese Medical University, China). The aqueous extract of *V. axillare* was prepared from air-dried and powdered whole plants. In brief, the powdered plants were soaked for 0.5 h in 20°C with 1 L water. The samples were then boiled for 1.5 h with gentle heat three times in 100°C. The decoction was then concentrated to 140 g/L for storage at 4°C in the fridge in the dark. The extract was diluted to the corresponding concentration with pure water before use [6].

2.3. Experimental Protocols for the Animal Studies. After 4 d of adaptation, the mice were randomly divided into five groups ($n = 8$) as follows: (1) control group (normal saline, 10 mL/kg orally); (2) LPS-induced model group (LPS, 1 mg/kg via tail vein injection); (3) *V. axillare* low-dose group (*V. axillare*, 300 mg/kg orally and LPS injection); (4) *V. axillare* high-dose group (*V. axillare*, 1200 mg/kg orally and LPS injection); and (5) dexamethasone-treated group (DEX and LPS injection). The mice were orally pretreated with *V. axillare* (300 or 1200 mg/kg) for 3 consecutive days, while mice from other groups (control, LPS, and DEX group) received the normal saline solutions. The mice in the DEX-treated group were given DEX orally (2 mg/kg) 1 h before LPS challenge. One hour after the final *V. axillare* treatment, all animals except for the control group were challenged with LPS via tail vein to induce ALI. The mice were sacrificed 6 h after the LPS tail injection [8]. Blood samples were collected and centrifuged at 3000 rpm for 10 min at 4°C to obtain serum, which was then stored at −80°C for further experiments. Lung tissues were harvested simultaneously from mice and soaked in 10% formalin.

2.4. Histological Evaluation of the Lungs. Mice lung tissues were immediately dehydrated and embedded in paraffin. Paraffin sections (4 μm, 6–10 per lung) were sliced and stained with hematoxylin and eosin (H&E) using a standard conventional method. Pathological sections were observed under a light microscope at 400x amplification. Photos were taken by the attached camera (Nikon).

Immunostaining was performed according to Lin's experimental method [9]. The sections were blocked with BSA (Sangon, Shanghai) and then incubated with NF-κB primary antibody (Santa Cruz) overnight at 4°C. Horseradish peroxidase- (HRP-) conjugated secondary antibodies were used for 1 h incubation. Finally, the sections were treated with *Dolichos biflorus* agglutinin (DBA) as a color reagent and counterstained with hematoxylin. The sections were viewed with the light microscope, and the pathological pictures were analyzed by ImageJ software.

2.5. Cell Culture and Cell Viability Assay. The murine macrophage RAW264.7 cell line was donated by Professor Fuliang Hu (College of Animal Sciences, Zhejiang University, China). The cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 g/mL streptomycin at 37°C in 5% CO_2 atmosphere. The viability of RAW264.7 cells incubated with *V. axillare* was determined by using cell counting kit-8 (CCK-8, Dojindo, Japan). RAW264.7 cells were seeded in 96-well plates at a density of 10^4 cells/well. After incubating for 24 h, the cells were treated with various concentrations of *V. axillare* extract for an additional 24 h. CCK-8 reagent (10 μL) was added to the cells and then further incubated for 2 h. The OD values of each well were measured using a microplate reader at 450 nm (Bio-Rad Model 550, CA).

2.6. Measurement of Inflammatory Cytokines. Levels of various inflammatory cytokines, including interleukin-6 (IL-6), IL-10, monocyte chemotactic protein-1 (MCP-1), interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), and IL-12p70, were assessed using a cytometric bead array (CBA) mouse inflammation kit (BD Biosciences). Serum samples were diluted 25-fold as instructed by the manufacturer.

2.7. RNA Extraction and Quantitative Real-Time PCR (qPCR). Total cellular RNA was extracted using an RNA extraction kit (Aidlab Biotechnologies, Beijing, China) following the

Table 1: Primers used for qRT-PCR experiments.

Gene	Primer sequence	GenBank accession number
IL-1β	5′-CCAACAAGTGATATTCTCCATGAG-3′ 5′-ACTCTGCAGACTCAAACTCCA-3′	NM_008361.3
IL-6	5′-CTCTGCAAGAGACTTCCATCC-3′ 5′-GAATTGCCATTGCACAACTC-3′	NM_031168.1
IL-10	5′-CTATGCTGCCTGCTCTTACTG-3′ 5′-CAACCCAAGTAACCCTTAAAGTC-3′	NM_010548.2
TNF-α	5′-CCACGCTCTTCTGTCTACTG-3′ 5′-ACTTGGTGGTTTGCTACGAC-3′	NM_013693.2
MCP-1	5′-AAGAAGCTGTAGTTTTTGTCACCA-3′ 5′-TGAAGACCTTAGGGCAGATGC-3′	NM_011333.3
COX-2	5′-GAAATATCAGGTCATTGGTGGAG-3′ 5′-GTTTGGAATAGTTGCTCATCAC-3′	NM_011198.3
GAPDH	5′-GAGAAACCTGCCAAGTATGATGAC-3′ 5′-TAGCCGTATTCATTGTCATACCAG-3′	NM_008084.2

Table 2: Effect of *V. axillare* on serum inflammatory cytokines in LPS-challenged mice[a].

Group	Dose (mg/kg)	MCP-1 (ng/mL)	IFN-γ (ng/mL)	TNF-α (pg/mL)	IL-6 (ng/mL)	IL-10 (pg/mL)	IL-12p70 (pg/mL)
Control	—	ND	ND	ND	ND	ND	ND
Model	—	98.27 ± 10.69	5.22 ± 0.31	1897.57 ± 93.31	96.00 ± 10.66	237.15 ± 2.04	100.60 ± 8.08
V. axillare	300	81.31 ± 6.52*	6.44 ± 0.62	1290.10 ± 250.03*	83.17 ± 8.17	225.95 ± 9.78	63.72 ± 2.57*
V. axillare	1200	60.45 ± 2.42**	3.02 ± 0.09**	711.36 ± 32.64**	33.82 ± 4.47**	118.84 ± 7.28**	25.15 ± 2.04*
Dexamethasone	2	67.93 ± 1.18**	2.69 ± 0.64**	850.81 ± 58.44**	52.05 ± 7.37**	180.21 ± 11.53**	26.96 ± 0.52**

[a]Values are the means ± SD ($n = 8$ for each group). $^{*}p < 0.05$ and $^{**}p < 0.01$ indicate a significant difference compared to the model group. ND: not detected.

directions of the manufacturer. For each sample, cDNA was synthesized using the Prime Script RT reagent kit (TaKaRa, Dalian, China). The primer sequences are shown in Table 1 and were synthesized by Sangon Biotechnology Co., Ltd. qPCR was executed in an automated thermal cycler (Eppendorf, Hamburg, Germany) in a final volume of 25 μL (2 μL cDNA, 12.5 μL SYBR® Premix Ex Taq, 1 μL of each primer, 10 μmol/L, and 8.5 μL ddH_2O). The cycling reaction was performed using a standard two-step PCR reaction.

2.8. Western Blotting Analysis. For western blotting analysis, cells were washed twice with chilled PBS and then lysed in lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% NP-40, 10% glycerol, 2 mM DTT, 1 mM leupeptin, and 1 mM PMSF) on ice for 30 min. Obtained cell lysates were cleared by centrifugation at 12,000 ×g for 15 min at 4°C. Cellular proteins (30 μg) were then mixed with sampling buffer and further boiled for 5 min at 95°C. Then, proteins (10 μg) were separated by SDS-PAGE gels and then transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% nonfat milk at room temperature and then probed with primary polyclonal antibodies overnight at 4°C. After that, the membranes were washed with TBST and then incubated with secondary antibody for 1 h at room temperature. Then, the membranes were washed again three times in TBST. Finally, the immune reactive protein bands on the membrane were visualized using 10 mL alkaline phosphatase in a color development buffer.

2.9. Statistical Analysis. All values were presented as the mean ± SD for at least three independently performed experiments. The data's statistical comparison was performed by one-way analysis of variance (ANOVA), followed by the t-test. Statistical significance was set at $p < 0.05$. All statistical tests were performed using SPSS 19.0.

3. Results

3.1. V. axillare Reduced LPS-Induced Augmentation of Serum Cytokines and Attenuated the Pathological Injuries in Mouse Lungs. In order to assess the protective effect of *V. axillare* in mice with ALI, serum inflammatory cytokines and pulmonary histopathology were evaluated. *V. axillare* (300 or 1200 mg/mL) was administered intragastrically 3 days before LPS injection. As demonstrated in Table 2, LPS stimulation increased inflammatory cytokine production, including IL-6, MCP-1, TNF-α, and IL-12p70 in mouse serum. However, the following pretreatment with *V. axillare* (1200 mg/kg) and DEX (2 mg/kg) dramatically inhibited the release of inflammatory cytokines in ALI mice.

After staining with H&E, histopathological changes in the lungs of each group were observed. A lot of apparent structural damage was found in the pulmonary tissue from the mice of LPS-treated model group, including increased alveolar wall thickness, inflammatory cell aggregation, and pulmonary hemorrhage (Figure 1). These injuries were not apparent in the control group. Pretreatment with 1200 mg/kg

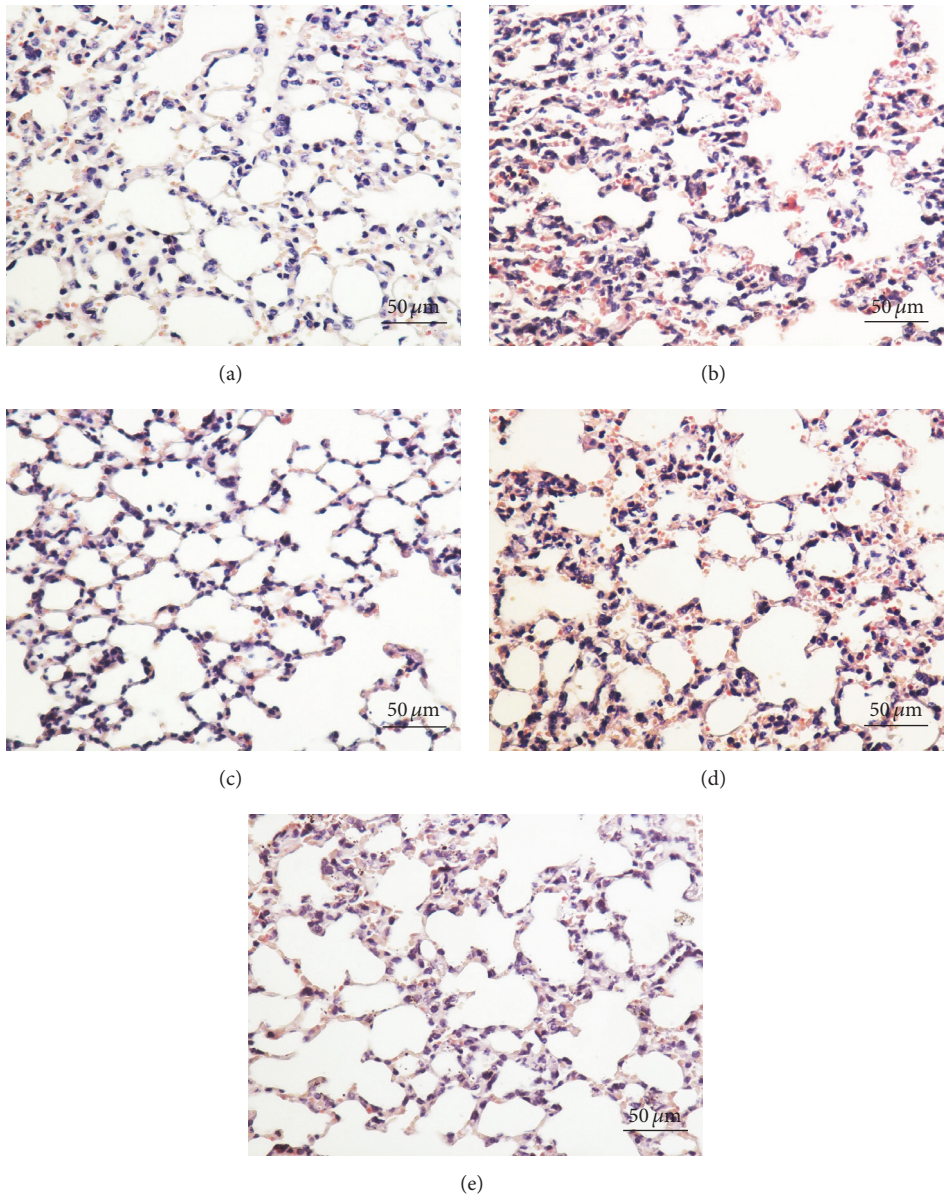

Figure 1: Effects of *V. axillare* on histopathological changes in lung tissues in LPS-induced ALI mice. Representative lung sections from (a) control group; (b) LPS-induced ALI model group; (c) mice injected with LPS and treated with DEX (2 mg/kg); (d and e) mice injected with LPS and given *V. axillare* (400 or 1200 mg/kg) are shown. Histological changes were evaluated by H&E staining and microscopy (original magnification: 200x, scale bar: 50 μm).

V. axillare and 2 mg/kg DEX significantly inhibited LPS-induced histological changes. These results indicated that *V. axillare* protected against LPS-induced lung tissue damage.

We further evaluated NF-κB activation in the lung sections using immunohistochemistry. The NF-κB p65 subunit was stained brown and was primarily localized in the nuclei of inflammatory cells (Figure 2). The expression of NF-κB in the LPS-treated model group was higher than in the control group. Both *V. axillare* and DEX treatment reduced the positive percentage and density of the NF-κB p65 subunit significantly ($p < 0.05$), indicating that both *V. axillare* and DEX may have inhibited the activation of NF-κB in the lungs.

3.2. Effects of V. axillare on RAW264.7 Cell Viability. In order to observe whether *V. axillare* had any toxic effects on cells and to find a suitable concentration for application in the subsequent *in vitro* experiments, we examined the effects of *V. axillare* on cell viability in RAW264.7 cells using a CCK-8 assay. Figure 3 shows that there were no cytotoxic effects in RAW264.7 cells treated with *V. axillare* within proper concentrations (up to 900 μg/mL). Based on these results, we

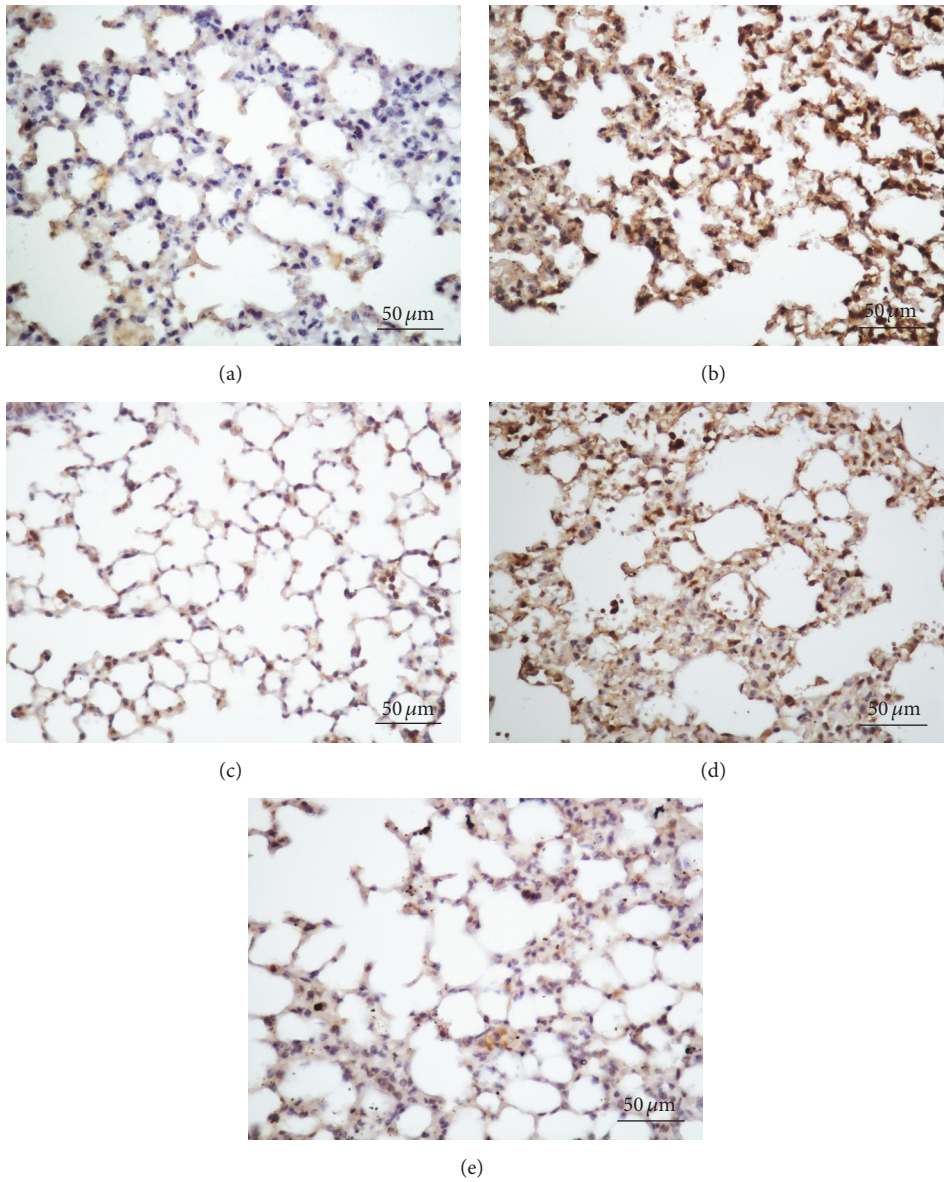

Figure 2: Representative immunohistochemical analysis of NF-κB from (a) the control group, (b) the LPS-treated model group, (c) mice treated with DEX (2 mg/kg), and (d and e) mice treated with *V. axillare* (400 or 1200 mg/kg). Magnification: 200x, scale bar: 50 μm.

selected suitable concentration ranges during the following *in vitro* experiments.

3.3. V. axillare Modulated Several Key Inflammatory-Related Gene Expressions in LPS-Stimulated RAW264.7 Macrophages. To assess the effect of *V. axillare* on inflammatory mRNA expression in LPS-stimulated RAW264.7 cells, we analyzed the levels of several inflammatory genes (IL-1β, IL-6, TNF-α, MCP-1, and COX-2) through real-time PCR. The RAW264.7 cells were stimulated with LPS alone or LPS and *V. axillare* for 6 h. We selected this time point because the mRNA expression of the proinflammatory genes reached a peak at this point according to our previous studies [10]. As shown in Figure 4, after stimulation with LPS, the expression of IL-1β, IL-6, TNF-α, MCP-1, and COX-2 was upregulated. Importantly, this upregulation was not observed in RAW264.7 cells cultured with *V. axillare* alone. Pretreatment with *V. axillare* (250, 500, and 750 μg/mL) significantly reduced the mRNA expression of these genes in a dose-dependent manner.

3.4. V. axillare Inhibits LPS-Induced Phosphorylation of p65 and Prevented IκB-α Degradation in RAW264.7 Macrophages. The genes repressed by *V. axillare* are normally controlled by the transcription factor NF-κB. Phosphorylation and degradation of IκB-α lead to the phosphorylation of NF-κB p65 subunit. This phenomenon enables the release and translocation of the free NF-κB into the nucleus, where it binds specifically to the κB binding sites on DNA [11]. Therefore,

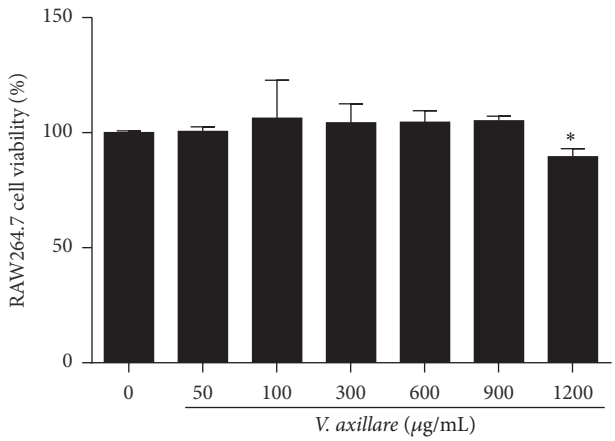

Figure 3: Effects of *V. axillare* on cell viability in RAW264.7 cells. Various concentrations of *V. axillare* were treated to the cells for 24 h, and the cell viability was measured by CCK-8 assay, as described in the Materials and Methods. The values represent the mean ± SD from three independent experiments ($^*p < 0.05$ indicates a significant difference versus untreated control cells by Student's *t*-test).

we examined whether *V. axillare* altered these processes. As shown in Figure 5, LPS stimulation decreased the IκB-α protein level in untreated cells, which was consistent with the increased level of phosphorylated p65 and IκB-α. In contrast, pretreatment with *V. axillare* at 250, 500, and 750 μg/mL significantly reduced the phosphorylation of p65 and IκB-α induced by LPS, indicating that *V. axillare* does exert an anti-inflammatory effect through the inhibition of LPS-induced NF-κB signaling pathway.

4. Discussion

In this study, we illustrated that, after pretreatment with *V. axillare*, the expression of proinflammatory cytokines (MCP-1, IFN-γ, TNF-α, IL-6, and IL-12p70) in the serum of mice with LPS-induced ALI was notably reduced. We also found that *V. axillare* improved pulmonary histology in these mice. These findings collectively suggest that *V. axillare* may be effective for ALI prevention induced by LPS. In order to research the mechanisms of *V. axillare* action, we used RAW264.7 cells to evaluate changes in inflammatory mediators, cytokines, and signaling pathways.

During the progression of inflammation, proinflammatory cytokines such as TNF-α and IL-6 are released by macrophages to protect the body from tissue injury or infection [12]. Furthermore, COX-2 is known as the rate-limiting enzyme that not only participates in the synthesis of prostaglandin E2, but also contributes to the inflammatory reaction [13]. The activation of macrophages leads to high expression level of MCP-1. The release of MCP-1 will cause the migration of tissue macrophages and blood monocytes to the site of inflammation, thereby promoting chronic inflammatory dysfunctions. Therefore, the mRNA expression levels of these inflammatory mediators are significant indicators during inflammation. In our study, the stimulation of LPS to the RAW264.7 cells led to considerable changes of several proinflammatory genes, including IL-1β, IL-6, COX-2, MCP-1, and TNF-α, which correlate well with previous studies [14]. *V. axillare* also decreased the mRNA expression of these genes. These results provide direct evidence that *V. axillare* inhibited the development of inflammation through decreasing proinflammatory gene expressions in LPS-stimulated macrophages.

LPS is a TLR4 agonist in macrophages [15]. The association between TLR4 and LPS results in the fast activation of NF-κB [16]. The expression of various cytokines, chemokines, cell-adhesion molecules, and growth factors in the lungs is closely regulated by the activation of NF-κB [17]. Thus, the suppression of the proinflammatory mediators by the modulation of intracellular signaling in macrophages could be used for treating LPS-induced pulmonary diseases [18]. Our *in vitro* data suggest that NF-κB was activated after LPS stimulation, evidenced by the degradation of IκB-α and the phosphorylation of NF-κB p65, which correlate well with previous findings [6, 14]. These responses were prevented by *V. axillare* pretreatment. Similar results were acquired by immunohistochemical staining of less activated NF-κB *in vivo*. These results suggest that the anti-inflammatory effect of *V. axillare* might be mediated via the inhibition of the NF-κB signaling pathways.

Macrophages play an important role during the innate immunity of the host response against many types of infections by producing various inflammatory mediators and adhesion molecules, which activate the immune system [19]. A large number of infection-associated disorders have been linked to the aberrant activation of macrophages, including ALI, sepsis, and septic shock. Therefore, agents which can inhibit the production/release of those inflammatory mediators can be developed as potential anti-inflammatory agents [20]. To further understand the underlying mechanisms of the anti-inflammatory effects of *V. axillare*, we evaluated the effects of *V. axillare* on the LPS-induced TLR4 signaling pathway in RAW264.7 cells. Previous mechanistic studies indicate that LPS-stimulated TLR4 activation was the most prominent mechanism of macrophage activation and can activate two different signaling pathways: the MyD88-dependent and the TRIF-dependent pathways [21, 22]. The MyD88-dependent pathway plays a critical role in the regulation of macrophage activation, thereby promoting the activation of MAPKs and NF-κB. It is known that LPS-induced activation of MAPKs and NF-κB leads to the expression of several proinflammatory mediators in macrophages [23, 24]. We demonstrated that *V. axillare* extracts significantly suppressed LPS-induced phosphorylation of p65 and IκB-α in RAW264.7 macrophages, which was consistent with our *in vivo* data.

In terms of its chemical composition, the previous studies have found that *V. axillare* contains flavonoids, phenols, phenylpropanoids, terpenoids, tannins, and so forth [5]. Recently, three new phenylpropanoid glycosides isolated from *V. axillare* exerted strong anti-inflammatory effects, including procumboside A. It is the major anti-inflammatory constituent of the EtOAc fraction of *V. axillare*. Interestingly, the anti-inflammatory effect of procumboside A seems to

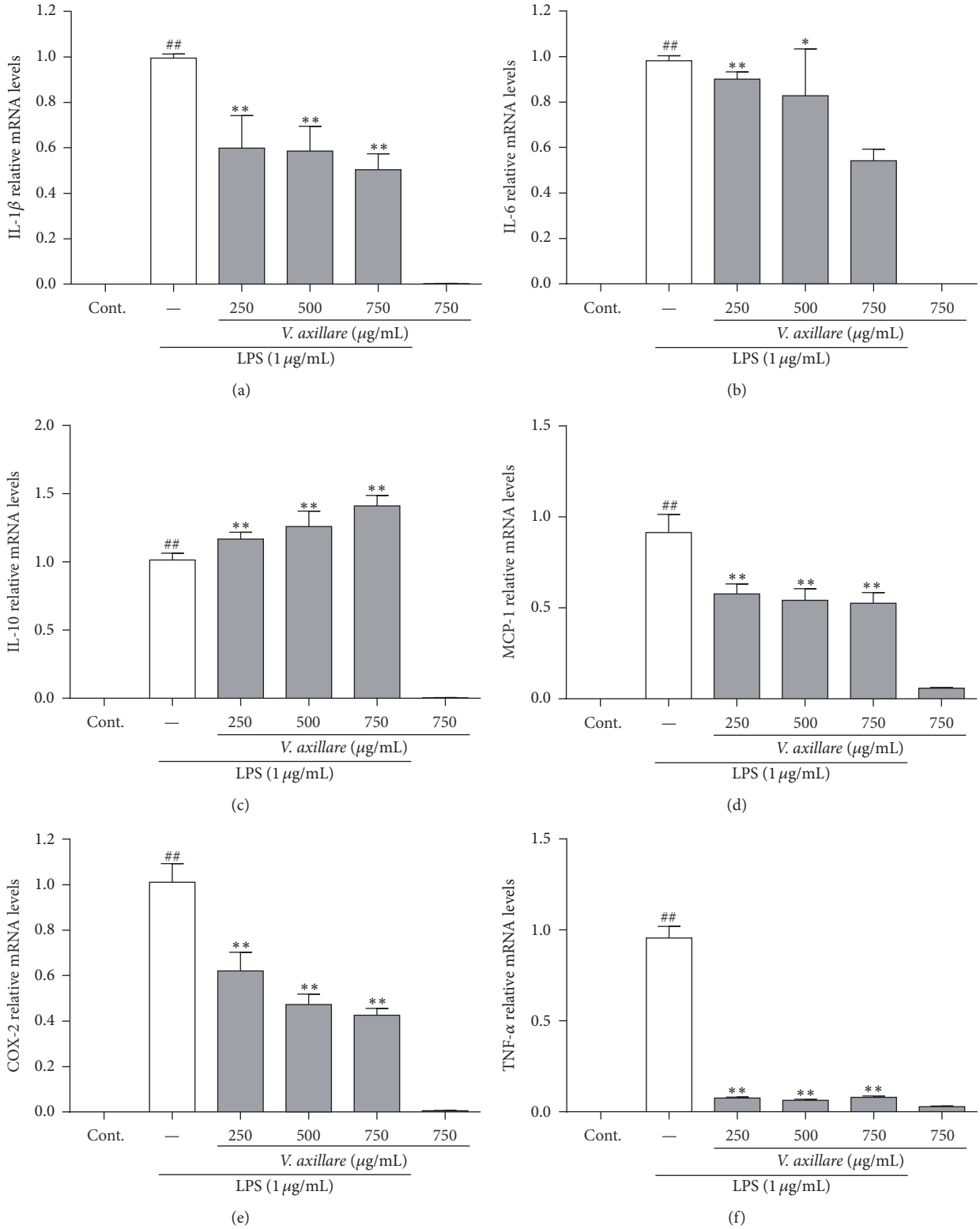

Figure 4: Effects of *V. axillare* on the gene expression of key proinflammatory genes in LPS-stimulated RAW264.7 cells. Various concentrations of *V. axillare* (250, 500, and 750 μg/mL) were treated to the cells for 1 h before incubation with LPS (1 μg/mL) for 6 h. Additionally, one group was treated with *V. axillare* (750 μg/mL) alone to observe the effects of *V. axillare* on the RAW264.7 without LPS stimulation. The mRNA expression of (a) IL-1β, (b) IL-6, (c) IL-10, (d) MCP-1, (e) COX-2, and (f) TNF-α was analyzed by qRT-PCR. Data are presented as the mean ± SD from three independent experiments. Student's t-test was performed to compare individual groups ($^{*}p < 0.05$ and $^{**}p < 0.01$ indicate a significant difference compared with the LPS group; $^{\#\#}p < 0.01$ indicates a significant difference compared with the untreated group).

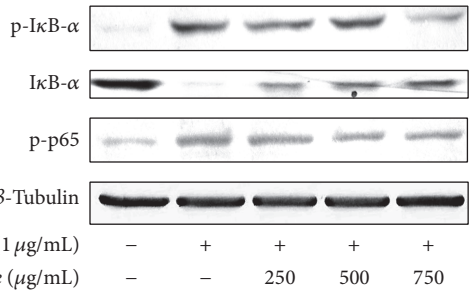

Figure 5: *V. axillare* inhibits LPS-induced phosphorylation of p65 and prevented IκB-α degradation in RAW264.7 macrophages. Cells were pretreated with *V. axillare* for 1 h before incubation with LPS for 30 min. Levels of phosphorylated p65 and total and phosphorylated IκB-α were determined by western blot analysis. β-Tubulin was used as a loading control.

be stronger than that of dexamethasone which is also used as a positive drug in a previous study [7]. Nevertheless, the interactions among active compounds in the natural products are complex with potential synergistic/antagonistic effects, which attracts great interest in TCM research. Hence, it is valuable to study the interaction among the aforementioned compounds in *V. axillare* in the future.

Taken together, our study confirmed that the aqueous extract of *V. axillare* exerted strong anti-inflammatory effects by attenuating LPS-induced lung injury. *V. axillare* prevented the overproduction of inflammatory cytokines in ALI mice. Histological analysis indicated that *V. axillare* significantly improved lung injury and decreased pulmonic NF-κB activation. We also noticed that *V. axillare* could downregulate the mRNA expressions of IL-1β, IL-6, IL-10, MCP-1, COX-2, and TNF-α in LPS-activated RAW264.7 cells. These effects seem to be mediated, at least in part, by the inhibition of NF-κB activation, which provides first-hand evidence for the development of anti-inflammatory drugs in the future. Further studies about the active components in *V. axillare* are required for better understanding the protective effects against ALI.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

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Research Article

In Vitro Anti-Inflammatory Effects of Three Fatty Acids from Royal Jelly

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Trans-10-hydroxy-2-decenoic acid (10-H2DA), 10-hydroxydecanoic acid (10-HDAA), and sebacic acid (SEA) are the three major fatty acids in royal jelly (RJ). Previous studies have revealed several pharmacological activities of 10-H2DA and 10-HDAA, although the anti-inflammatory effects and underlying mechanisms by which SEA acts are poorly understood. In the present study, we evaluated and compared the *in vitro* anti-inflammatory effects of these RJ fatty acids in lipopolysaccharide-stimulated RAW 264.7 macrophages. The results showed that 10-H2DA, 10-HDAA, and SEA had potent, dose-dependent inhibitory effects on the release of the major inflammatory-mediators, nitric oxide, and interleukin-10, and only SEA decreased TNF-α production. Several key inflammatory genes have also been modulated by these RJ fatty acids, with 10-H2DA showing distinct modulating effects as compared to the other two FAs. Furthermore, we found that these three FAs regulated several proteins involved in MAPK and NF-κB signaling pathways. Taken together, these findings provide additional references for using RJ against inflammatory diseases.

1. Introduction

Royal jelly (RJ) is a viscous secretion from the mandibular and hypopharyngeal glands of worker bees *(Apis mellifera)* and is known as an essential food for the queens [1]. RJ is also an important functional substance that has been widely used in commercial products, dietary supplements, and cosmetics [2]. RJ has been shown to possess versatile bioactive properties such as antibacterial [3], immunomodulatory [4], antiviral [5], wound-healing [6], growth promoting [7], antioxidant [8], nephroprotective [9], and anti-inflammatory [10] activities. Fresh RJ consists of water (50–60%), lipids (3–8%), proteins (18%), carbohydrates (7–18%), and other trace elements [11]. The lipid composition of RJ comprises 80–85% fatty acids, together with proteins that contribute to its biological activities [12].

Fatty acids (FAs) can be classified as long-chain (more than 12 C), medium-chain (6–12 C), and short-chain (less than 6 C) fatty acids, of which medium-chain fatty acids (MCFAs) exist mostly in the free form [13]. The major MCFAs found in RJ are *trans*-10-hydroxy-2-decenoic acid (10-H2DA), 10-hydroxydecanoic acid (10-HDAA), and sebacic acid (SEA) (Figure 1) [14]. *Trans*-10-hydroxy-2-decenoic acid (10-H2DA), an unsaturated hydroxyl fatty acid, is predominant and is one of the most extensively studied MCFAs in RJ, constituting more than 50% of the free FAs. A saturated hydroxyl fatty acid, 10-HDAA, comprises 60–80% of the total FAs, together with 10-H2DA. SEA (1, 10-decanedioic acid), a dicarboxylic fatty acid, accounts for 3.3% of the FA family found in the RJ [15, 16]. Chemical characteristics of FAs in RJ have been determined by gas chromatography-mass spectrometry (GC-MS), high performance liquid chromatography (HPLC), and ultraperformance liquid chromatography (UPLC) methods from lyophilized royal jelly [17–19]. Previous studies have investigated the ability of SEA to inhibit histone deacetylase [20] and modulate the estrogen receptor [21]. Both 10-H2DA and 10-HDAA have been shown to possess diverse pharmacological activities such as immunomodulatory [22], estrogenic [21, 23], and anti-inflammatory effects [24] *in vitro*. *In vivo* models demonstrated that 10-H2DA effectively protected against the depression and anxiety in mice when

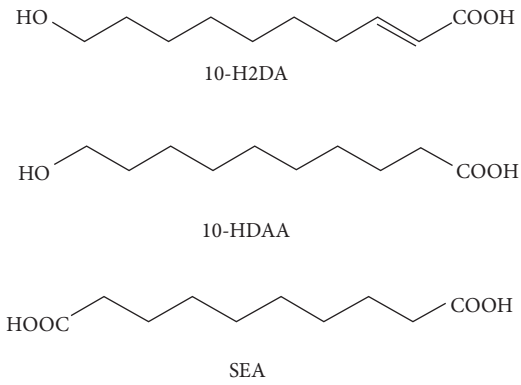


Figure 1: Chemical structures of 10-H2DA, 10-HDAA, and SEA.

intraperitoneally administered [25]. However, the pharmacological activities of SEA have remained elusive. Owing to similarities in the chemical structures of the three above-mentioned MCFAs, we hypothesized that SEA may also exhibit similar pharmacological activities.

Inflammation is an important host response of tissues to injury or infection, which may be triggered by chemical toxins, mechanical injuries, and many other reactions. Cell cytokines, like interleukin-6 (IL-6), IL-10, and tumor necrosis factor-α (TNF-α), play important roles in mediating inflammation [26]. Mitogen-activated protein kinases (MAPKs) comprise protein kinases that participate in the regulation of key cellular processes like inflammatory responses. Extracellular signal-regulated kinases (ERKs), p38, and c-Jun N-terminal kinase (JNK) are the major classes of MAPKs that play important roles in pathogenesis [27, 28]. Additionally, extensive studies have focused on determining the function and regulation of the nuclear factor-κB (NF-κB) signaling pathway [29].

Previous studies have reported that 10-H2DA inhibits lipopolysaccharide- (LPS-) induced IL-6 production in a dose-dependent manner [30] and 10-H2DA inhibits LPS and IFN-β induced NO production via inhibition of NF-κB [31]. Additionally, 10-HDAA could also inhibit LPS-induced NO production by inhibiting the translation of IRF-1 [32]. However, studies concerning the biological activities of other FAs in the RJ, like 10-HDAA, and SEA are still limited. In the present study, we investigated and compared the anti-inflammatory activities of 10-H2DA, 10-HDAA, and SEA in LPS-stimulated RAW 264.7 cells. Our results revealed for the first time that all the three FAs exerted strong biological activities via multiple mechanisms and provided evidence for further functional usage of RJ.

2. Materials and Methods

2.1. Chemicals and Reagents. 10-H2DA, 10-HDAA, SEA, and LPS (*Escherichia coli* 0111:B4) and alkaline phosphatase-conjugated secondary antibody (anti-rabbit IgG) were purchased from Sigma (St. Louis, USA). Primary antibodies against β-tubulin, phospho-ERK1/2, phospho-JNK1/2, phospho-c-Jun (pS63), phospho-IκBα (pS36), and phospho-p65 were purchased from Abcam (Cambridge, Massachusetts, USA). Primary antibody against phospho-p38 (Thr180/Tyr182) was purchased from Cell Signaling Technology (Danvers, MA, USA). Griess reagent, $NaNO_2$, and other chemicals of analytical grade were purchased from Sangon Biotechnology, Co. Ltd. (Shanghai, China).

2.2. Cell Culture and Cell Viability Assay. Murine macrophage RAW 264.7 cells were a generous gift from Professor Zongping Xia (Life Sciences Institute, Zhejiang University, China). Cells were cultured in high glucose DMEM containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 U/mL streptomycin at 37°C in a 5% CO_2 atmosphere. 10-H2DA, 10-HDAA, and SEA were dissolved in dimethyl sulphoxide (DMSO) before adding them to the culture media at the indicated concentrations. The cell viability was measured using the CCK-8 (cell counting kit-8) (Dojindo, Japan) according to the manufacturer's instructions. Cells were seeded into 96-well cell culture plates and were cultured in the presence of various concentrations of 10-H2DA, 10-HDAA, and SEA after 24 h incubation. After 24 h, the cells were incubated with 10 μL of CCK-8 at 37°C for 2 h. The optical density (OD) was measured at 450 nm using a microplate reader (Bio-Rad, Model 550, CA).

2.3. Determination of NO and Inflammatory Cytokine Production. Murine RAW 264.7 cells (1×10^5) were seeded into 24-well plates and cultured for 24 h. Thereafter, the cells were pretreated with specified concentrations of 10-H2DA, 10-HDAA, and SEA for 1 h, following which they were stimulated with 1 μg/mL LPS. After the 24 h incubation, the cell supernatants were collected, dispensed, and stored at −80°C until further testing. The amounts of the inflammatory-related cytokines (IL-6, IL-10, and TNF-α) in the cell culture supernatants were measured using enzyme-linked immunosorbent assay (ELISA) kits (Boster Company, Wuhan, China). The generation of NO was measured with Griess reagent [33]. The optical density was measured at 450 nm for IL-6, IL-10, and TNF-α and 550 nm for NO.

2.4. RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR). Total RNA from RAW 264.7 cells was extracted using commercial RNA extraction kits (Aidlab Biotechnologies Co. Ltd., Beijing, China) according to the manufacturer's protocols. The concentration of RNA in the samples was measured by NanoDrop spectrophotometer (ND-2000, NanoDrop Technologies, USA) and stored at −80°C until further use. For cDNA synthesis, one microgram of the RNA sample was used with the PrimeScript RT Master Mix (TaKaRa, Dalian, China). The primers presented in Table 1 were synthesized by Sangon Biotechnology (Shanghai, China). Quantitative real-time PCR was performed using StepOne Plus (Applied Biosystems, Carlsbad, CA, USA) with a SYBR Premix Ex Taq (TaKaRa, Dalian, China) via

Table 1: Primer sequences used for qRT-PCR experiments.

Gene	Primer sequence	Product size (bp)	GenBank accession no.
IL-6	5′-CTCTGCAAGAGACTTCCATCC-3′ 5′-GAATTGCCATTGCACAACTC-3′	210	NM_031168.1
IL-10	5′-CTATGCTGCCTGCTCTTACTG-3′ 5′-CAACCCAAGTAACCCTTAAAGTC-3′	221	NM_010548.2
iNOS	5′-TTTCCAGAAGCAGAATGTGACC-3′ 5′-AACACCACTTTCACCAAGACTC-3′	294	NM_010927.3
COX-2	5′-GAAATATCAGGTCATTGGTGGAG-3′ 5′-GTTTGGAATAGTTGCTCATCAC-3′	237	NM_011198.3
TNF-α	5′-CCACGCTCTTCTGTCTACTG-3′ 5′-ACTTGGTGGTTTGCTACGAC-3′	169	NM_013693.2
HO-1	5′-ACATTGAGCTGTTTGAGGAG-3′ 5′-TACATGGCATAAATTCCCACTG-3′	241	NM_010442.2
GAPDH	5′-GAGAAACCTGCCAAGTATGATGAC-3′ 5′-TAGCCGTATTCATTGTCATACCAG-3′	212	NM_008084.2

a standard two-step PCR. The reaction volume was 10 μL per well in a 96-well plate format. Specificity was confirmed by carrying out dissociation curve analysis. The housekeeping gene *GAPDH* was used to normalize the expression of the other target genes, and the results were expressed as $2^{-\Delta\Delta Ct}$ [34].

2.5. Cellular Protein Extraction and Immunoblot Analysis. RAW 264.7 cells were pretreated with the assigned concentrations of 10-H2DA, 10-HDAA, and SEA for 1 h and then stimulated with 1 μg/mL LPS for 30 min. Further, the cells were put on ice and washed immediately with precold PBS twice. The cytoplasmic proteins were lysed with NP40 mixed with protease inhibitors and phosphatase inhibitors (Roche, Basel, Switzerland), and the cell lysate was collected using cell scrapers (Corning, New York), following which it was vortexed and put on ice for 10 min to remove the cell debris. Equal amounts of cellular protein (30 μg) were then mixed with Laemmli's sample buffer and boiled at 95°C for 10 min. The concentration of the protein was measured by the BCA protein assay kit (Weiao Biotechnology, Shanghai, China). The proteins were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride membrane (Millipore, Billerica, MA). Skim milk (5%) dissolved in Tris-buffered saline Tween 20 was used to block the nonspecific binding sites for 30 min at room temperature. The membranes were blotted using specific antibodies in combination with AP-conjugated anti-rabbit secondary antibodies with a 1 : 5000 dilution. The protein bands on the membranes were developed by the NBT/BCIP method [35] and the results were evaluated using Quantity One software.

2.6. Statistical Analysis. Data are expressed as the means ± SEM for the indicated number of independently performed experiments. Statistical comparison of the data was performed by Student's *t*-test or one-way ANOVA following Student–Newman–Keuls method. P values < 0.05 were considered statistically significant. Statistical tests were carried out using SPSS software version 22.0 and GraphPad Prism 6.0 software.

3. Results

3.1. Effects of 10-H2DA, 10-HDAA, and SEA on RAW 264.7 Cell Viability. To ensure that 10-H2DA, 10-HDAA, or SEA do not have any toxic effects on cell metabolism and to determine the optimal concentrations for further experiments, the effects of these fatty acids on cell viability were assessed in RAW 264.7 cells using the CCK-8 assay. Figure 2 shows the results of cell viability after 24 h of incubation using different concentrations of 10-H2DA (Figure 2(a)), 10-HDAA (Figure 2(b)), and SEA (Figure 2(c)). Treatments with the three fatty acids (0, 1, 2.5, and 5 mM) for 24 h did not cause any significant changes in the viability compared to the control group. The acids also did not show any toxicity in RAW 264.7 cells at concentrations up to 5 mM; however, higher concentrations (8 mM) were found to be toxic. Statistically significant decreases in cell survival were detected at concentrations up to 8 mM. Based on these results, we chose FA concentrations up to 5 mM for the subsequent experiments.

3.2. Effects of 10-H2DA, 10-HDAA, and SEA on the Production of NO and Inflammatory-Related Cytokines in LPS-Stimulated RAW 264.7 Cells. NO production was estimated using Greiss' reaction and is shown in Figure 3. The inflammatory cytokines in the cell medium were analyzed using ELISA assays as shown in Figures 4(a)–4(c). After a 24 h incubation period,

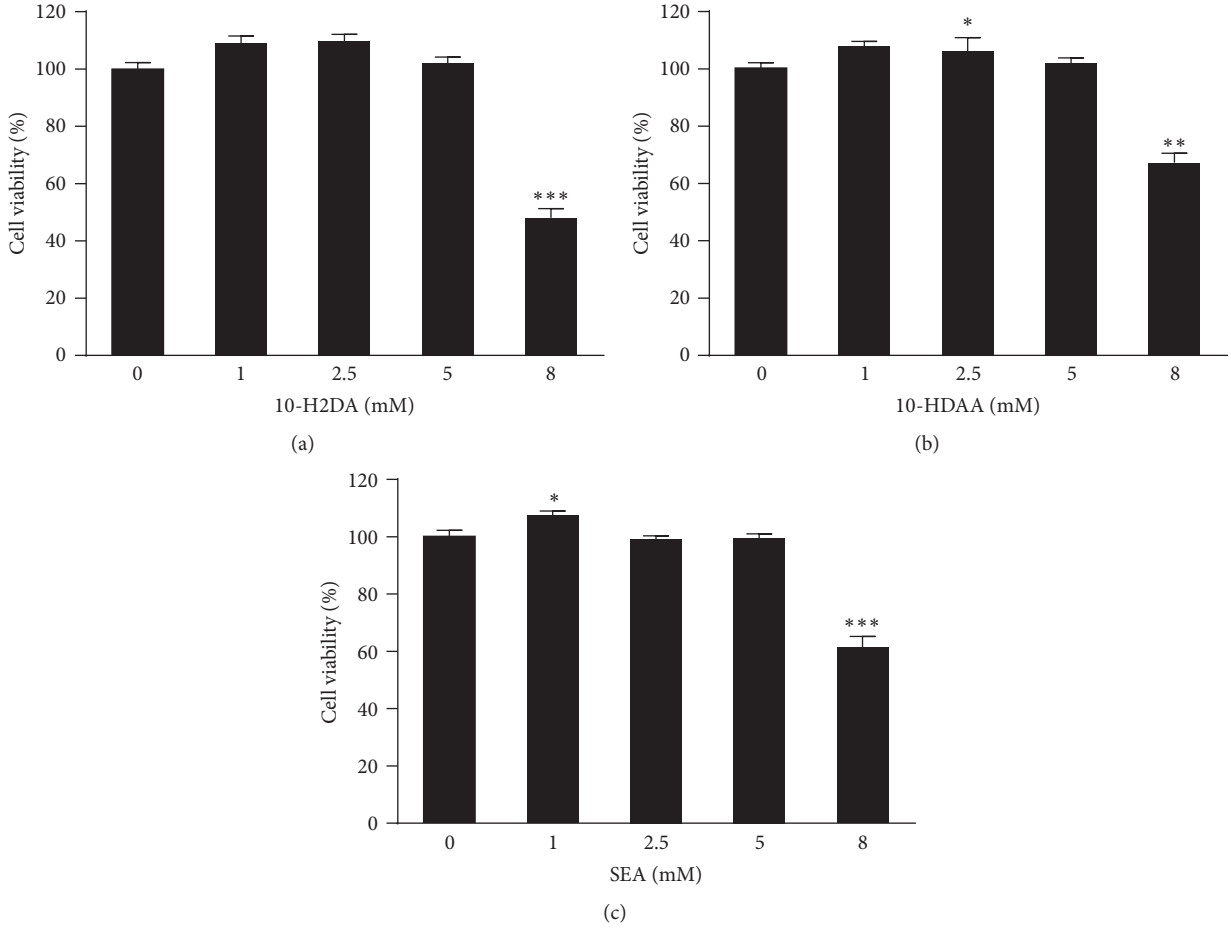


Figure 2: Effects of 10-H2DA, 10-HDAA, and SEA on the viability of RAW 264.7 cells. RAW 264.7 cells were treated with the indicated concentrations of 10-H2DA (a), 10-HDAA (b), and SEA (c) (0–8 mM) for 24 h, and the results are expressed as percentages of surviving cells over control cells, by CCK-8 assays. The data are the means ± SEMs for three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus control cells by Student's t-test.

very low amounts of NO and the three cytokines, IL-6, IL-10, and TNF-α, were detected in the absence of LPS. When the cells were stimulated with LPS (1 μg/mL), the production of NO and the cytokines was found to increase markedly. However, pretreatment with the three FAs for 1 h inhibited NO and IL-10 production in a dose-dependent manner. 10-HDAA and higher concentrations of 10-H2DA decreased IL-6 production. On the other hand, SEA exerted strong inhibitory effects on the production of TNF-α, which was different compared to those seen with the other two FAs.

3.3. Effects of 10-H2DA, 10-HDAA, and SEA on the mRNA Expression of Key Inflammatory-Mediators and Cytokine Genes in LPS-Stimulated RAW 264.7 Cells. To evaluate the effects of 10-H2DA, 10-HDAA, and SEA on mRNA expressions in LPS-stimulated RAW 264.7 cells, we chose six key genes involved in the inflammatory response and measured their gene expression using quantitative real-time PCR. The RAW 264.7 cells were stimulated with LPS alone or LPS and 10-H2DA, 10-HDAA, and SEA for 6 h. LPS caused significant increase in the transcription of the inflammatory-related genes (Figures 5(a)–5(f)). Pretreatment with the three FAs decreased IL-10, iNOS, and COX-2 mRNA expressions (Figures 5(b)–5(d)). Additionally, low doses of 10-H2DA and SEA (1 mM) slightly increased the mRNA expression of IL-6; however, 10-HDAA, 10-H2DA, and SEA, at a concentration of 5 mM, each, inhibited the mRNA expression of IL-6. Compared to that in the LPS-stimulated group, 10-H2DA and 10-HDAA (2.5, 5 mM) increased the mRNA expression of TNF-α; however, SEA caused strong inhibition of TNF-α mRNA transcription. Similar effects of SEA appeared to occur with HO-1 mRNA expression, and 10-H2DA exhibited a stronger enhancement of HO-1 production as compared to 10-HDAA.

3.4. Effects of 10-H2DA, 10-HDAA, and SEA on MAPK and NF-κB Signaling Pathways in LPS-Stimulated RAW 264.7 Cells. To further clarify the molecular mechanisms underlying 10-H2DA-, 10-HDAA-, and SEA-mediated effects

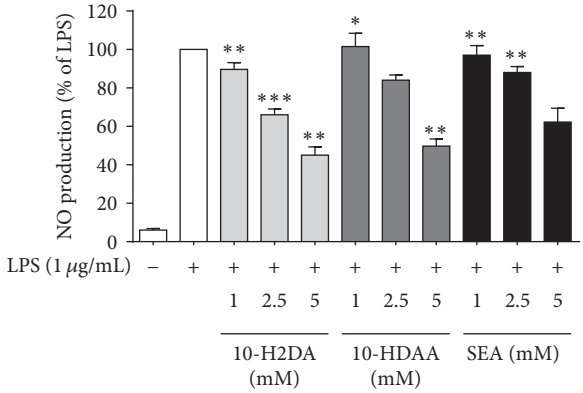


Figure 3: Effects of 10-H2DA, 10-HDAA, and SEA on LPS-induced NO production in RAW 264.7 cells. Cells were pretreated with/without indicated concentrations of 10-H2DA, 10-HDAA, and SEA for 1 h and then stimulated with LPS (1 μg/mL) for 24 h. Control values were obtained in the absence of LPS and the three fatty acids. The values are presented as percentages of NO in comparison with LPS-treated cells. The data are the means ± SEMs for three independent experiments. Individual groups were compared by Student's t-test ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with the LPS group).

on inflammation-related genes, we next used Western blot analysis to characterize the effects on LPS-induced phosphorylation using specific antibodies. Cells were investigated by examining the changes in the expression levels of IκBα and phosphorylated c-Jun, ERK1/2, JNK1/2, p38, and p65. As shown in Figure 6, the expression levels of each of these proteins were rapidly activated after treatment with 1 μg/mL LPS. Dose-course experiments showed that all the three FAs attenuated the phosphorylation of c-Jun. Despite the similar modulating effects against the production of inflammatory cytokines, these FAs showed distinct regulatory effects on MAPK and NF-κB proteins. It can be observed that these RJ FAs increased the phosphorylation levels of ERK1/2 when compared to that in the LPS group. The phosphorylation of c-Jun was inhibited by all the RJ FAs. Nevertheless, only SEA blocked the activation of p38 and JNK1/2. With regard to the NF-κB proteins, 10-HDAA and SEA showed inhibitory effects against NF-κB activation, since they blocked the activation of p-p65 and SEA (2.5 mM) upregulated the protein abundance of IκBα.

4. Discussions

The market for functional foods has been expanding annually over the past decades [36]. Natural bee products like honey, propolis, and royal jelly have attracted increasing attention, with the anti-inflammatory activity of propolis being well known and widely studied [37, 38]. Investigations of honey and RJ in inflammation are relatively rare and generally focus on its major proteins. Previous studies reported that the major RJ and honey glycoprotein Apalbumin1 (Apa1) showed significant stimulatory effects on TNF-α production by murine peritoneal macrophages [39]. Similar results were also observed in major honeybee royal jelly protein 1 (MRJP1) [40]. Nevertheless, our studies provide first-hand evidence that the FAs in the RJ showed distinctive anti-inflammatory effects.

Macrophages are innate immune cells which could be activated and release various inflammatory cytokines and chemokines when induced by LPS [41]. During the inflammatory process, NO is generated in the macrophages by inducible NO synthase (iNOS) in response to LPS. Similar to a previous study [32], we found that 10-H2DA and 10-HDAA significantly inhibited LPS-induced NO production and iNOS mRNA expression. Moreover, we observed that SEA also attenuated the production of NO and decreased iNOS gene expression, but the effect was weaker compared to the other two FAs. Kohno et al. showed that MRJP3 and some lower molecular compounds in RJ efficiently inhibited the production of proinflammatory cytokines in LPS and IFN-γ costimulated mouse peritoneal macrophages [42]. Our ELISA results showed that the three FAs decreased LPS-induced production of IL-6 at higher concentrations and SEA had strong inhibitory activity against TNF-α production, thus indicating that 10-H2DA, 10-HDAA, and SEA are part of those active lower molecular compounds. A previous study has shown that LPS-induced mRNA expression of IL-6 and TNF-α was not decreased by 10-HDAA at the time points of 3, 6, 12, and 24 h [32]. In our study, we also detected that 10-HDAA showed a slight inhibition of IL-6 production and had no significant effect on TNF-α production at various concentrations. These findings are in agreement with the results of previous studies, indicating that 10-H2DA, 10-HDAA, and SEA could reduce inflammatory responses by inhibiting mRNA expression of target genes.

Cyclooxygenase-2 (COX-2) and IL-10 are two important cytokines that are closely related to the inflammatory process [43, 44]. Cyclooxygenase (COX) enzymes catalyse the committed step in prostanoid synthesis, converting free arachidonic acid into the prostaglandin (PG) precursors. COX-2 is induced by proinflammatory stimuli; drugs that block COX-2 activity could have anti-inflammation actions [45]. IL-10 is known as a key anti-inflammatory cytokine which is activated during the resolution stage of inflammation [37]. LPS-induced mRNA levels of COX-2 and IL-10 were markedly reduced by pretreatment with the three FAs in a dose-dependent manner suggesting that they showed similar regulation of those transcriptional genes. Additionally, we observed a remarkable enhancement in the effect of 10-H2DA (5 mM) on HO-1 mRNA expression. HO-1 mediates an important pathway with anti-inflammatory effects in different experimental models and could be a potential therapeutic target in human inflammatory diseases [46]. Nevertheless, SEA reduced the expression of HO-1 dose dependently, and 10-HDAA showed a slight increase at higher concentrations, suggesting that these FAs possess different modulating mechanisms during the inflammation process.

Previous study indicated that 10-H2DA suppression was likely to be mediated via blocking the p38 kinase and JNK-AP-1 signaling pathways and that 10-H2DA had no effect

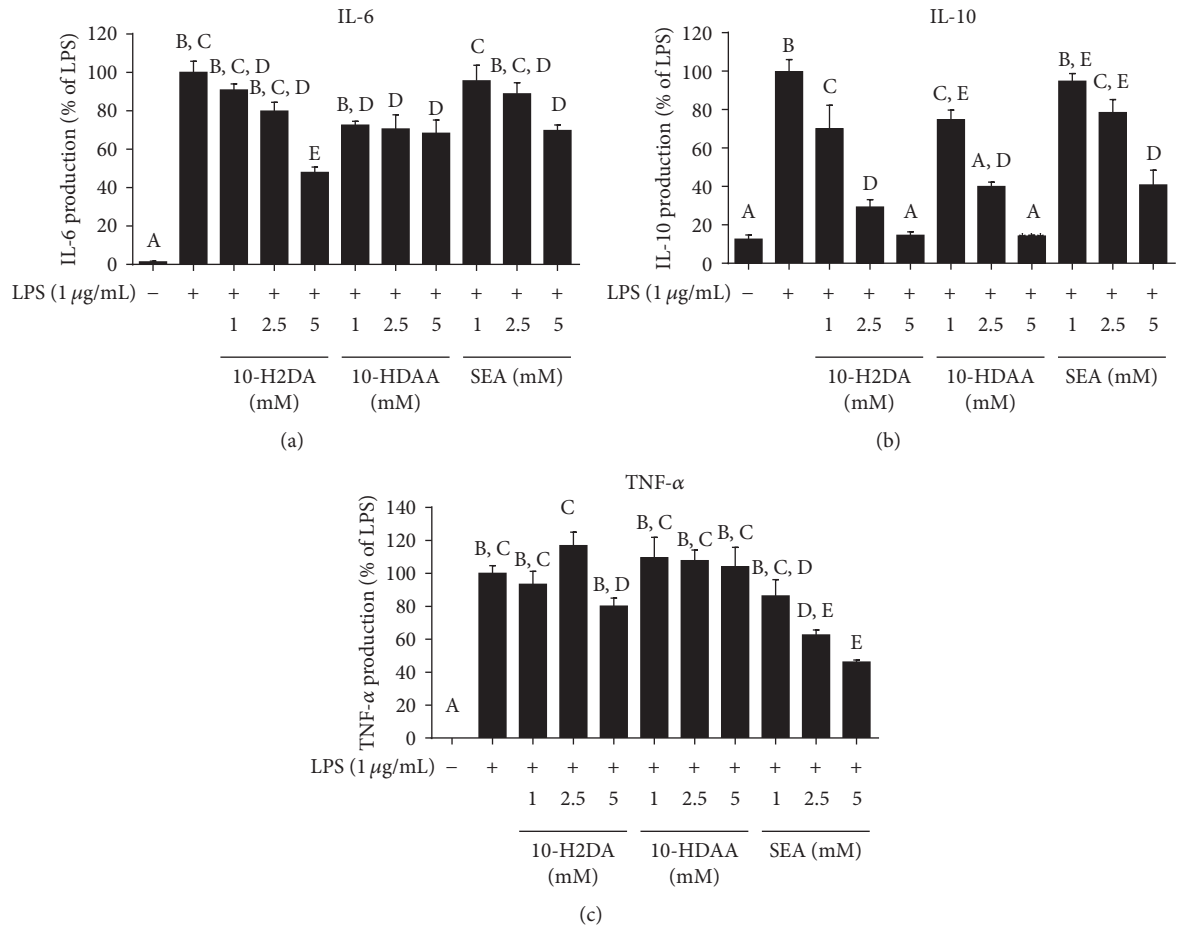


Figure 4: Effects of 10-H2DA, 10-HDAA, and SEA on LPS-induced IL-6, IL-10, and TNF-α production in RAW 264.7 cells. Cells were pretreated with/without indicated concentrations of 10-H2DA, 10-HDAA, or SEA for 1 h and then stimulated with LPS (1 μg/mL) for 24 h. Control values were obtained in the absence of LPS or the treatment. The values are presented as percentages of IL-6 (a), IL-10 (b), and TNF-α (c) in comparison with LPS-treated cells, respectively. The data are the means ± SEMs for three independent experiments. One-way ANOVA with the Student–Newman–Keuls method was performed to compare all groups; means with different letters are significantly different ($P < 0.05$).

on ERK1/2, NF-κB DNA-binding activity, and IκBα degradation in TNF-α induced rheumatoid arthritis synovial fibroblasts [24]. In our study, the expression levels of all MAPK pathway proteins were rapidly phosphorylated after treatment with 1 μg/mL LPS. 10-H2DA blocked the phosphorylation of ERK1/2 at 2.5 mM and slightly elevated the phosphorylation at 5 mM. 10-HDAA inhibited JNK1/2 phosphorylation at 2.5 mM. SEA (5 mM) regulated the phosphorylation of ERK1/2 and p38 and markedly reduced JNK1/2 phosphorylation. JNK pathway could be strongly activated by proinflammatory agents and was an important event in the cellular response to stress [47]; SEA showed great potential in mediating JNK signaling pathways. In addition, all the three FAs reduced the expression level of p-c-Jun, the AP-1 heterodimer involved in MAPK pathways. NF-κB plays a crucial role in regulating inflammatory process and thus becomes target for developing novel anti-inflammation treatments [41]. NF-κB is combined with IκB, an inhibitory protein which keeps NF-κB in an inactive state in the cytoplasm. Induced by LPS, phosphorylation of IκB in the NF-κB/IκB protein complex can release NF-κB to translocate from the cytoplasm into the nucleus and then phosphorylated p65 could activate the correlated target genes [48]. Previous research has demonstrated that 10-H2DA could inhibit NF-κB activation via suppressing NO production and IκB-ζ mRNA expression and transcription stimulated by LPS and IFN-β. Also, immunoblotting revealed that 10-H2DA did not inhibit LPS-induced IKK-α phosphorylation and IκBα degradation [30]. In our study, 10-H2DA showed modest attenuating effects on IκBα and enhanced the phosphorylation of p65, suggesting that 10-H2DA regulated LPS stimulation through more than one single pathway. Nevertheless, 10-HDAA showed no significant effects on those NF-κB pathway proteins. Notably, SEA exerted a strong suppressive effect on p65 phosphorylation, indicating that SEA inhibited transcription of target genes including iNOS, IL-10, TNF-α, and COX-2 mRNA by suppressing the activity of NF-κB.

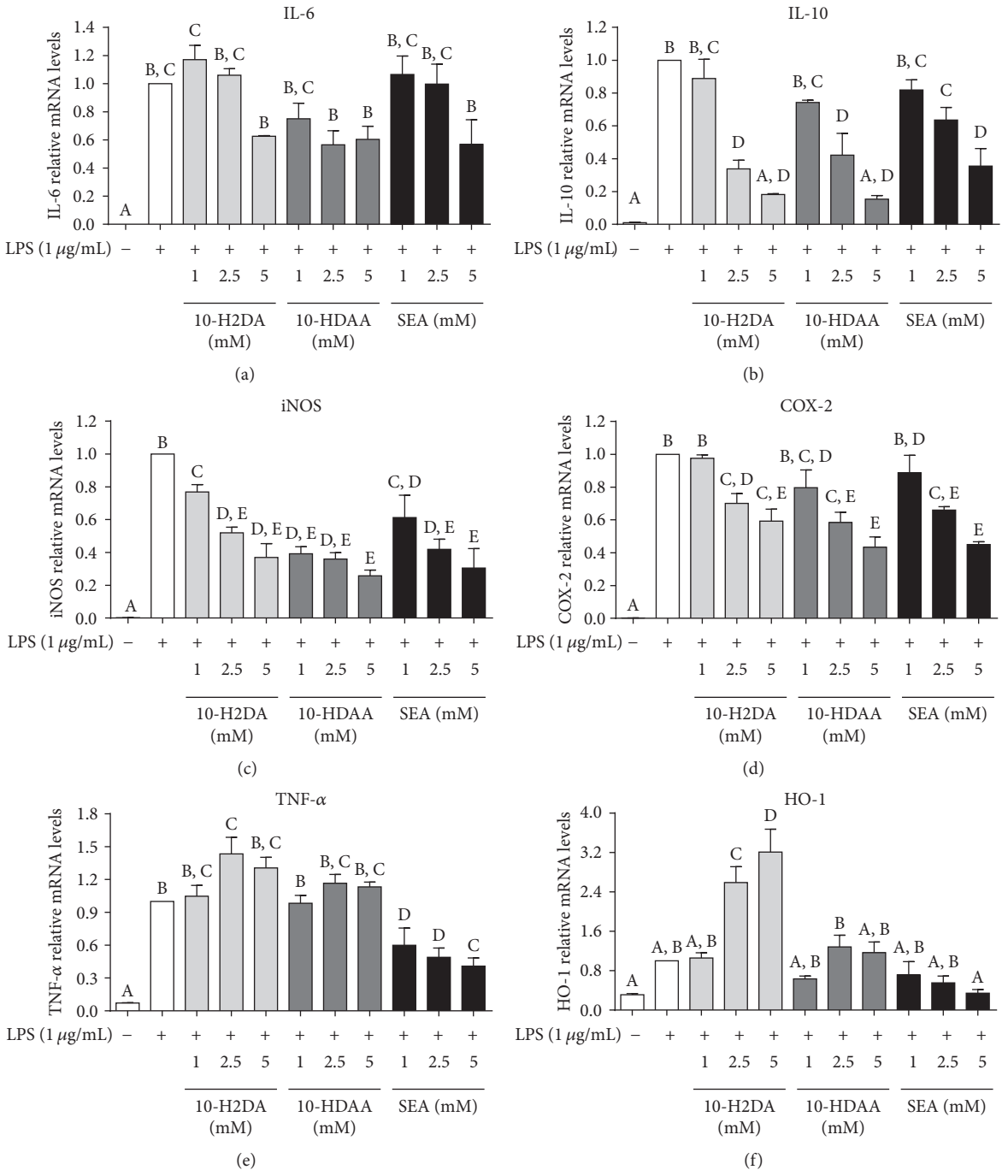
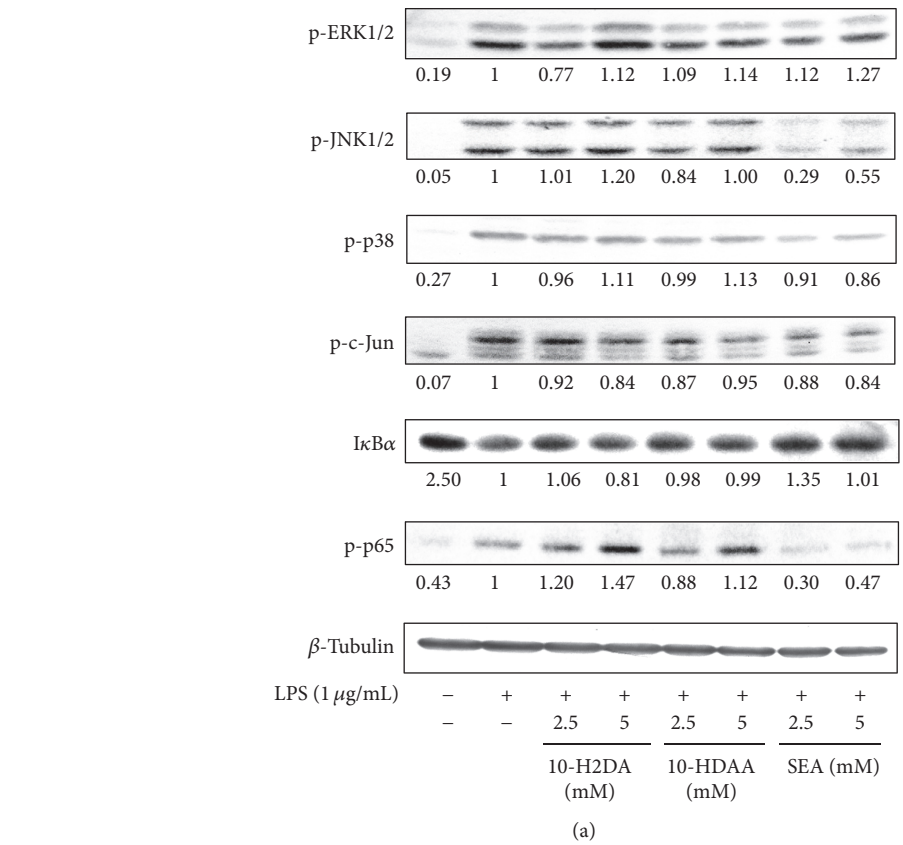


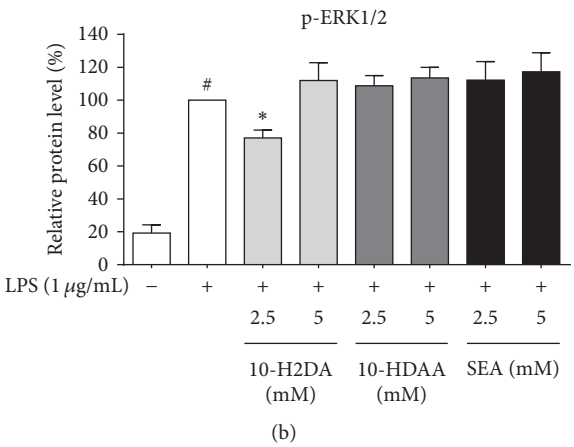
Figure 5: Effects of pretreatment with 10-H2DA, 10-HDAA, and SEA on the mRNA expression of key inflammatory-mediators and cytokine genes in LPS-stimulated RAW 264.7 cells. Effects of 10-H2DA, 10-HDAA, and SEA on the mRNA expression of key inflammatory-mediators and cytokine genes in LPS-stimulated RAW 264.7 cells. Cells were pretreated with 10-H2DA, 10-HDAA, and SEA in designed concentrations for 1 h and then stimulated with LPS (1 μg/mL) for 30 min. The mRNA levels of IL-6 (a), IL-10 (b), iNOS (c), COX-2 (d), TNF-α (e), and HO-1 (f) were quantified using qRT-PCR and normalized to GAPDH and the levels of gene expression in the LPS group were set to 1. Data shown represent means ± SEM values from three independent experiments. One-way ANOVA with the Student–Newman–Keuls method was performed to compare all groups; means with different letters are significantly different ($P < 0.05$).

5. Conclusion

The present study explored the *in vitro* anti-inflammatory effects of three major FAs from RJ. The results clearly showed that all three FAs present are responsible for the previously reported anti-inflammatory property of RJ, and the role of SEA should be noted. Our findings also indicate that 10-H2DA has the strongest *in vitro* anti-inflammatory effect



(a)



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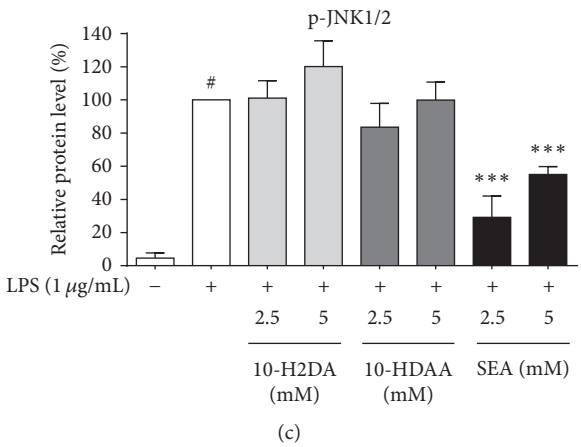

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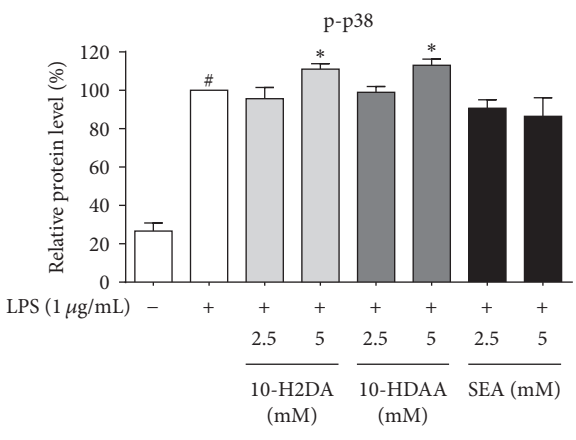

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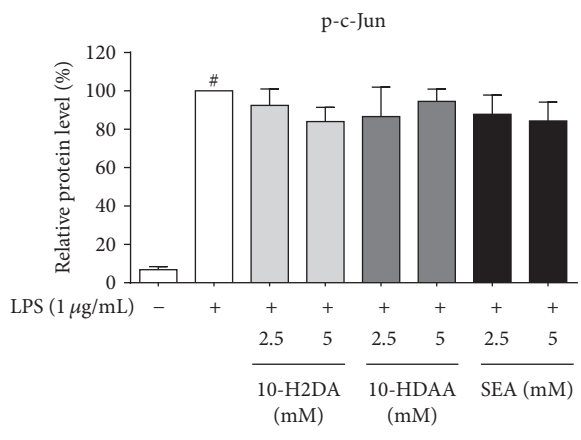

(e)

Figure 6: Continued.

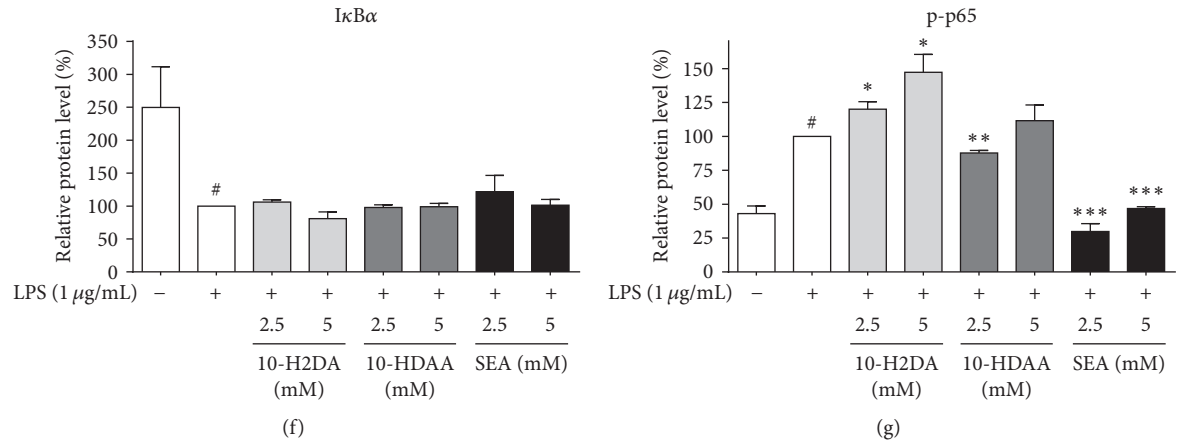

Figure 6: Effects of 10-H2DA, 10-HDAA, and SEA on the phosphorylation of MAPK and NF-κB pathways in LPS-stimulated RAW 264.7 cells. (a) RAW 264.7 cells either were pretreated with 10-H2DA, 10-HDAA, and SEA at the indicated concentrations for 1 h or received no such pretreatment. They were then stimulated with LPS (1 μg/mL) for 30 min. Whole cell lysates were analyzed by Western blotting analysis using specific antibodies. The relative expression of proteins was quantified using Quantity One software, comparing with β-tubulin. Data shown are the representative of three independent experiments with similar results. (b–g) The intensity of corresponding bands was measured by densitometry and normalized to β-tubulin. The values are the means ± SEMs. Individual groups were compared by Student's t-test ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with the LPS group; $^{\#}P < 0.05$ compared with untreated group).

among the three FAs tested, suggesting a possible therapeutic potential against inflammatory diseases.

Abbreviations

10-H2DA:	Trans-10-hydroxy-2-decenoic acid
10-HDAA:	10-hydroxydecanoic acid
SEA:	Sebacic acid
LPS:	Lipopolysaccharide
NO:	Nitric oxide
TNF-α:	Tumor necrosis factor-α
IL:	Interleukin
iNOS:	Induced nitric oxide synthase
COX-2:	Cyclooxygenase-2
HO-1:	Heme oxygenase-1
NF-κB:	Nuclear factor-κB
AP-1:	Activator protein-1
MAPK:	Mitogen-activated protein kinase.

Competing Interests

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

Acknowledgments

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Research Article

Regulation of Autophagy-Related Protein and Cell Differentiation by High Mobility Group Box 1 Protein in Adipocytes

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High mobility group box 1 protein (HMGB1) is a molecule related to the development of inflammation. Autophagy is vital to maintain cellular homeostasis and protect against inflammation of adipocyte injury. Our recent work focused on the relationship of HMGB1 and autophagy in 3T3-L1 cells. *In vivo* experimental results showed that, compared with the normal-diet group, the high-fat diet mice displayed an increase in adipocyte size in the epididymal adipose tissues. The expression levels of HMGB1 and LC3II also increased in epididymal adipose tissues in high-fat diet group compared to the normal-diet mice. The *in vitro* results indicated that HMGB1 protein treatment increased LC3II formation in 3T3-L1 preadipocytes in contrast to that in the control group. Furthermore, LC3II formation was inhibited through HMGB1 knockdown by siRNA. Treatment with the HMGB1 protein enhanced LC3II expression after 2 and 4 days but decreased the expression after 8 and 10 days among various differentiation stages of adipocytes. By contrast, FABP4 expression decreased on the fourth day and increased on the eighth day. Hence, the HMGB1 protein modulated autophagy-related proteins and lipid-metabolism-related genes in adipocytes and could be a new target for treatment of obesity and related metabolic diseases.

1. Introduction

In the past 30 years, the global prevalence of obesity has increased among all age groups. Obesity leads not only to an increase in adipose tissue mass but also to the infiltration of proinflammatory cells and secretion of inflammatory cytokines [1, 2]. Therefore, obesity is characterized by low-grade inflammation in local and systemic sites as demonstrated by robust secretion of proinflammatory cytokines, including IL-6, as well as active recruitment of leukocytes [3]. Substantial evidence supports the hypothesis indicating that inflammation may contribute to insulin resistance, which further induces a series of diseases such as diabetes, hypertension, fatty liver disease, and coronary heart disease, thereby threatening human health [4, 5]. However, the mechanism underlying inflammation remains unclear.

Autophagy includes three basic forms, namely, macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA) [6]. Macroautophagy (henceforth termed autophagy) is a lysosomal degradation pathway, which can degrade the organelles, longevity protein, and lipid drops and thus provide energy for the body [7, 8]. When the body faces various pressures caused by acute stress, autophagy plays a key role in maintaining the stability of the internal environment, particularly in regulating apoptosis and resisting the invasion of pathogenic microorganisms [9]. Self-renew, repair, and differentiation of cells are important for metabolism and maintenance of energy balance. Studies have shown that autophagic dysfunction is closely related to metabolic disorders, such as insulin resistance, diabetes, obesity, and osteoporosis [10].

High mobility group box 1 protein (HMGB1) is a nonhistone nuclear factor and a highly conserved protein. HMGB1 can bind to chromosomal DNA to adjust the refactoring of chromatin [11, 12]. HMGB1 is abundant in the vast majority of mammalian cells [13] and plays a key role as a signal molecule extracellularly [14]. HMGB1 can be passively released from necrotic cells or actively secreted from inflammatory cells [15, 16]. Aseptic injury to cells increases the level of HMGB1 in serum and tissues [17]. As such, HMGB1 is associated with low-grade inflammation diseases, such as obesity and type 2 diabetes [18]. Some research found that HMGB1 interacted with autophagy through its different receptors, outside the cells by receptor of advanced glycation end products (RAGE), within the nucleus through heat shock protein beta-1 (HSPB1), and within the cytoplasm through BECN1 [19]. These findings suggested that HMGB1 was involved in the process of autophagy. However, little is known about how HMGB1, autophagy, and adipocytes interact to regulate adipocyte development and differentiation. The present research mainly focused on the effects of HMGB1 on autophagy and cell differentiation in adipocytes.

2. Materials and Methods

2.1. Reagents. Antibodies were obtained from the following sources: HMGB1 and GAPDH from Abcam, LC3 from Cell Signaling, and p62 from Proteintech Group. Secondary antibodies against rabbit or mouse were bought from Beyotime. The following reagents were purchased from Sigma: 1-methyl-3-isobutylxanthine, dexamethasone, insulin, Oil-Red-O dye, and hematoxylin and eosin. The recombinant HMGB1 protein was obtained from Sino Biological. The negative control siRNA and siRNA HMGB1 were purchased from Invitrogen. TRIzol reagent and SuperScript III Reverse Transcriptase were also purchased from Invitrogen. SYBR® Select Master Mix was obtained from ABI.

2.2. Animals and Diet. C57BL/6 mice were purchased from Vital River Laboratory Animal Technology Co., Ltd., in Beijing. Rearing environment indoor temperature was controlled at 20°C to 25°C, relative humidity within 40% to 60%, lights 12 h every day, along with free drinking water in the cage. Six-week-old male mice were randomly divided into two groups, namely, the normal-diet (ND) group and high-fat diet (HFD) group, with 10 mice in each group. Mice were fed correspondingly with standard chow (10% kcal in fat) or HFD (45% kcal in fat) for 16 weeks. Mice's epididymal adipose tissue was extracted for the experiments.

2.3. Hematoxylin and Eosin Staining. Mouse epididymal adipose tissue samples were fixed in 4% paraformaldehyde and paraffin-embedded, then cut into 4 μm thick sections, and deparaffinized in xylene and rehydrated in a descending ethanol series. The sections were stained with hematoxylin and eosin using standard pathologic procedures. Finally, photomicrographs of all sections were taken by Leica DM500 microscope.

2.4. Adipocyte Differentiation and Treatment of Recombinant HMGB1 Protein. 3T3-L1 preadipocytes were maintained and cultured in Dulbecco's Modified Eagle's Medium (DMEM) with existing 10% heat inactivated fetal bovine serum and 1% penicillin/streptomycin at 37°C in 5% CO_2. 3T3-L1 preadipocytes were first cultured in a medium supplemented with 10 μg/mL insulin, 1 μM dexamethasone, and 0.5 mM 1-methyl-3-isobutylxanthine for 2 days. The medium was then replaced by a medium containing only insulin. After 2 days, this maintenance medium was discarded. Differentiated adipocytes were cultured in DMEM containing 10% fetal bovine serum [1, 20].

To invest the effects of HMGB1 protein on autophagy in various differentiation periods of 3T3-L1 adipocytes, the adipocytes were treated with HMGB1 protein 24 h before differentiation. In detail, the 3T3-L1 preadipocytes were induced at 0, 2, 4, 6, 8, and 10 days, and the 0.2 μg/mL recombinant HMGB1 proteins were added at days −1, 1, 3, 5, 7, and 9 and removed at days 0, 2, 4, 6, 8, and 10.

2.5. Cell Transfection. 3T3-L1 preadipocytes were grown to approximately 50% to 70% confluence in a six-well plate and then transfected with 75 pmol HMGB1 siRNA and control siRNA by using Lipofectamine 2000 in accordance with the manufacturer's instructions. After transfection for 6 h, the medium was replaced with a normal medium.

2.6. Oil-Red-O Staining. Differentiated adipocytes were stained by Oil-Red-O, as follows: 0.5 g Oil-Red-O powder was dissolved in 100 mL of isopropanol overnight. The solution was filtered and stored in a brown bottle at 4°C. After the medium was discarded, cells were fixed with 4% formaldehyde for 2 h at room temperature. Cells were then washed with PBS and stained with the Oil-Red-O dye solution for 1 h. Subsequently, cells were washed with PBS and treated with 60% isopropanol for 5 min. Finally, cells were washed and photographed under a microscope.

2.7. Western Blot Analysis. 3T3-L1 cells were collected and lysed in ice-cold lysis buffer (5.0 mM Tris buffer, pH 7.4, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% deoxycholic acid, 1 mM EDTA, 1 mM PMSF, 2 μg/μL aprotinin, 2 μg/μL leupeptin, and 1 mM NaF) for 40 min and then subsequently boiled and quantified. After the samples were resolved by SDS-PAGE for 2 h, proteins were transferred to nitrocellulose membranes at 200 V for 90 min. Membranes were blocked in 5% nonfat milk for 2 h and then incubated with specific primary antibodies for 2 h or overnight at 4°C. After being washed with Tris-buffered saline with Tween (TBST) thrice, membranes were incubated with peroxidase-conjugated secondary antibodies for 1 h. Finally, membranes were washed with TBST and developed by enhanced chemiluminescence. Blots were quantified using Amersham Imager 600 and analyzed with Image J.

2.8. Quantitative PCR. Total RNA was purified using TRIzol reagent and converted into cDNA by SuperScript III Reverse Transcriptase. The sequences of primers for PCR

amplification were as follows: GAPDH: forward, 5′-AGGTCGGTGTGAACGGATTTG-3′; reverse, 5′-TGTAGACCATGTAGTTGAGGTCA-3′ [21]; PPAR-γ: forward, 5′-TTTTCAAGGGTGCCAGTTTC-3′; reverse, 5′-TCTGTGACGATCTGCCTGAG-3′; FABP4: forward, 5′-TCCAGTGAAAACTTTGATGATTAT-3′; reverse, 5′-ACGCATTCCACCACCAGTTTATCA-3′ [22]. Quantitative PCR analysis was performed using SYBR Select Master Mix in 7500 Fast Real-Time PCR System. The thermal profile for real-time PCR was 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min, and then 95°C for 30 s and 60°C for 15 s. Relative expression was calculated using the $2^{-\Delta\Delta CT}$ method. All samples were normalized to GAPDH.

2.9. Enzyme-Linked Immunosorbent Assay (ELISA). The level of peripheral blood HMGB1 was detected in mice at 16 weeks in accordance with the ELISA kit's protocol. The spectrophotometry (OD) value of the microplate reader was set at 450 nm wave length. The HMGB1 concentration in the testing samples can be calculated using the standard curve.

2.10. Statistics. Data are presented as means ± SEM. Significance was assessed by Student's t-test between individual comparisons. One-way analysis of variance with Bonferroni's corrections was used for multiple comparisons. Calculations were performed using SPSS version 19.0 statistic software. Differences were considered statistically significant at $^{*}P < 0.05$.

3. Results

3.1. Effects of HFD on Body Weight and Epididymal Adipose Tissue. To determine the changes in HMGB1 and autophagy protein LC3 in epididymal adipose tissues in obese mice, male C57BL/6 mice were fed with HFD for 16 weeks. The mean body weight for each group is shown in Figure 1(a). With the extension of time, the body weight significantly increased in the HFD group. After 16 weeks, the mean body weight of the HFD group was about 1.5 times that of the ND group. Moreover, the epididymal adipose tissue of the HFD group increased notably (Figures 1(b) and 1(c)). We also found that the adipocyte sizes in the HFD group were significantly larger than those in the ND group (Figure 1(d)).

3.2. HMGB1 and LC3 Were Upregulated in High-Fat Diet Mice. HMGB1 was assessed in epididymal adipose tissue and serum by Western blot and ELISA, respectively. Compared with the ND group, the HFD group showed that HMGB1 expression increased in both epididymal adipose tissues and peripheral blood of obese mice (Figures 1(e), 1(f), and 1(h)). Western blot was also used to detect the expression of autophagic protein LC3 in epididymal adipose tissues. The LC3II protein markedly increased, reaching 3.29 times that of the normal-diet groups (Figures 1(f) and 1(g)). These data suggested that HFD promoted HMGB1 production and activated autophagy in the epididymal adipose tissues in mice.

3.3. Exogenous HMGB1 Protein Promoted LC3II Formation and P62 Degradation in 3T3-L1 Preadipocytes. To determine the functional role of exogenous HMGB1 in autophagy, 3T3-L1 preadipocytes were treated with 0.2 μg/mL recombinant HMGB1 protein for 24 h, which did not affect the normal cell proliferation [7] and HMGB1 protein expression in cells (Figures 2(a) and 2(d)). However, compared with the control group, the treatment group showed that the expression of autophagic protein LC3II increased (Figures 2(a) and 2(b)), whereas the P62 protein expression decreased significantly (Figures 2(a) and 2(c)).

3.4. Knockdown of HMGB1 Weakened LC3II Formation and P62 Degradation. We further studied the changes in autophagy when HMGB1 was downregulated by specific siRNA. Western blot analysis revealed that the knockdown of HMGB1 significantly reduced HMGB1 protein expression (Figures 3(a) and 3(b)). Furthermore, the LC3II protein decreased obviously, whereas the P62 protein increased markedly compared with the control group (Figures 3(c) and 3(d)).

3.5. Effects of HMGB1 Protein on Various Differentiation Periods of 3T3-L1 Adipocytes. To further explore the effects of HMGB1 on autophagy in various differentiation periods of 3T3-L1 adipocytes, we added 0.2 μg/mL HMGB1 protein in the differentiation stages of adipocytes for 24 h. Oil-Red-O staining was performed to detect intracellular lipid droplets. As shown in Figures 4(a) and 4(b), 3T3-L1 preadipocytes were successfully induced into the adipocytes in the control group. No significant difference was observed in the HMGB1 treatment group. Interestingly, with the differentiation of adipocytes, the LC3II protein expression increased gradually. However, after adding exogenous HMGB1 for 24 h, the LC3II expression increased in the differentiation on the second and fourth days but decreased in the differentiation on the eighth and tenth days compared with the control group (Figures 4(c) and 4(e)). On the contrary, the P62 protein expression decreased in the differentiation on the second and fourth days (Figures 4(c) and 4(f)). Finally, the expression of adipocyte differentiation related genes peroxisome proliferator-activated receptor- (PPAR) γ and FABP (fatty acid-binding protein) 4 was measured. No significant difference in PPAR-γ was found between the two groups (Figure 4(g)), but the FABP4 expression significantly decreased in 4 days and increased in 8 days (Figure 4(h)). These data indicated that the HMGB1 protein exerted different effects on autophagy and lipid metabolism in the early and late stages of adipocyte differentiation.

4. Discussion

The adipose tissue plays an important role in whole-body energy homeostasis. The two main types of adipose tissues are white and brown. White adipose tissue is primarily responsible for energy storage of the body [23], whereas brown adipose tissue is mainly responsible for energy degradation through uncoupling protein 1 (UCP1) [24]. Obesity is characterized by an excess of white adipose tissue [25] and leads to adipocyte dysfunction, thereby increasing the risk for insulin

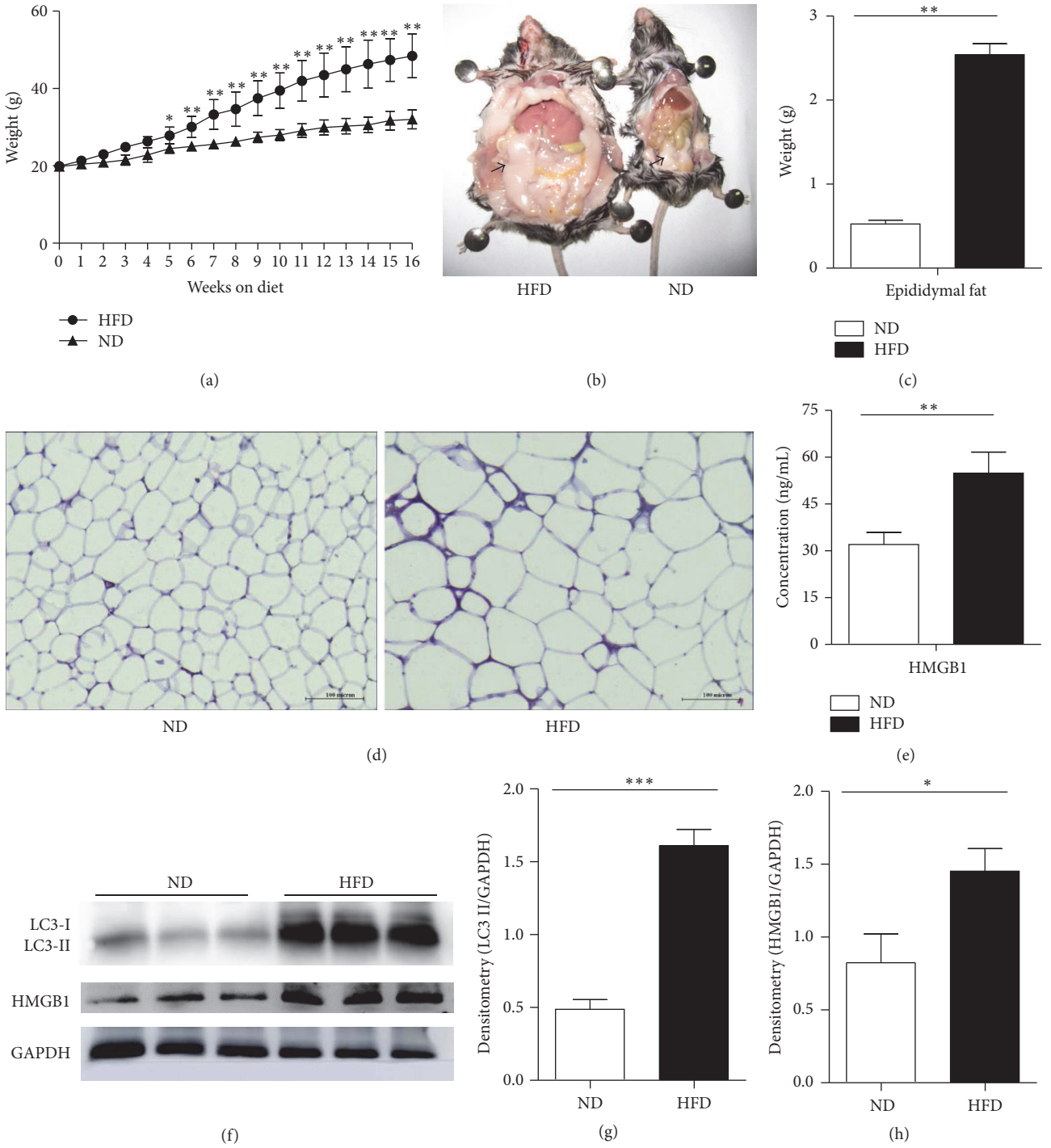

Figure 1: Effects of HFD on HMGB1 and LC3. After male mice were fed with HFD and ND for 16 weeks, serum was separated, and the epididymal adipose tissue was extracted. (a) The change of the body weight. (b) The epididymal adipose tissue, HFD (left) and ND (right). The black arrow pointed to epididymal adipose tissue. (c) The weight of epididymal adipose tissue. (d) HE staining of epididymal adipose tissue. (e) Levels of HMGB1 in peripheral blood were analyzed by ELISA. (f) The expression of autophagy protein LC3II and HMGB1 was analyzed by Western blot. (g-h) The protein levels were quantified through normalization with GAPDH, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ for comparisons between ND and HFD mouse epididymal adipose tissues.

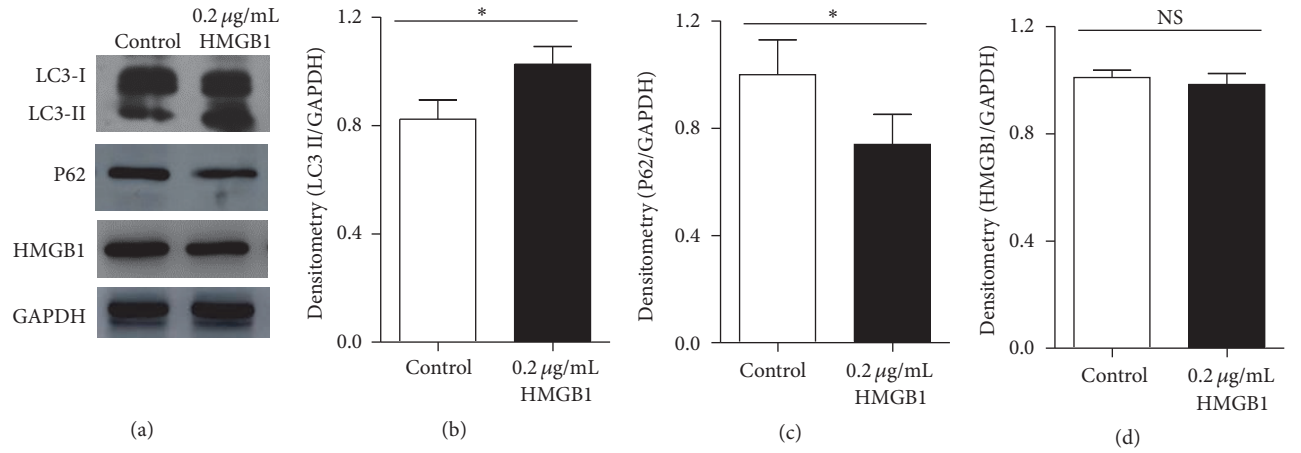

Figure 2: Exogenous HMGB1 activated autophagy. 3T3-L1 preadipocytes were treated with HMGB1 protein (0.2 μg/mL) or PBS for 24 h accordingly. (a) Total protein was extracted, and LC3II, P62, and HMGB1 proteins were analyzed by western blot. (b–d) LC3II, P62, and HMGB1 protein were quantified and normalized to GAPDH, $^{*}P < 0.05$ versus the control. NS: not significant.

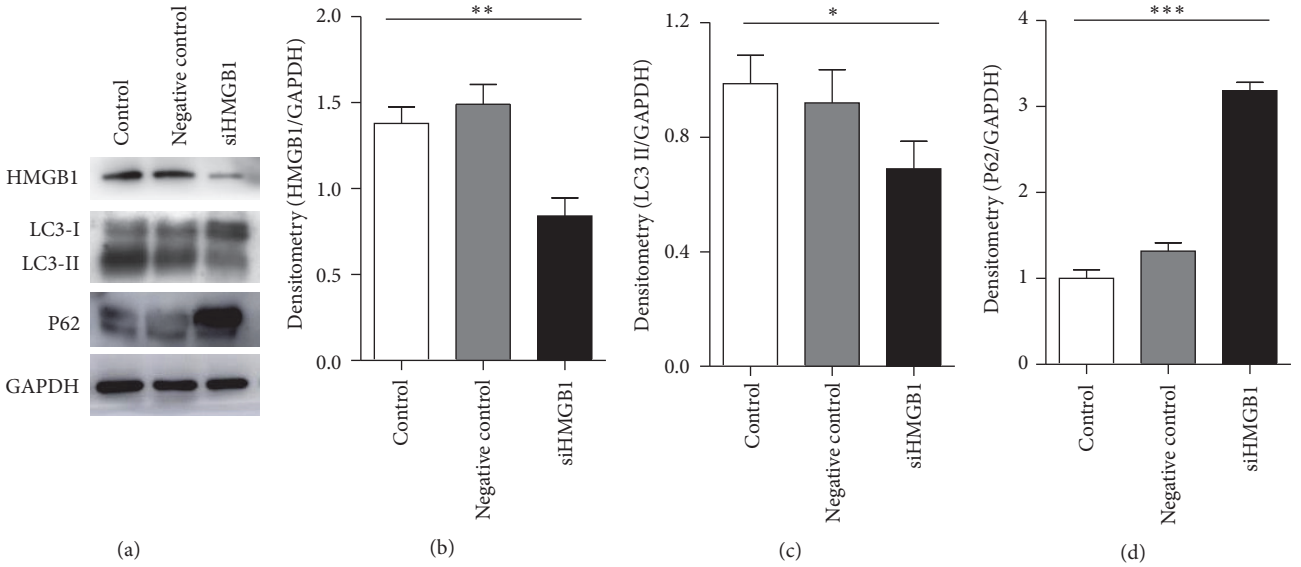

Figure 3: Autophagy was weakened by siRNA knockdown of HMGB1. HMGB1 siRNA or control siRNA was transfected into 3T3-L1 preadipocytes by lipo2000. (a) The expression levels of HMGB1, LC3, and P62 were examined by Western blot. (b–d) Quantification of protein levels normalized by GAPDH. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, versus the control.

resistance, type 2 diabetes mellitus, and cardiovascular diseases [26, 27].

HMGB1 is produced as a damage-associated molecular pattern molecule (DAMP) by necrotic cells or activated immune cells [14]. Studies showed that HMGB1 protein expression increased in the adipose tissues of obese mice [28]. Our data also confirmed the increased expression of HMGB1 in the adipose tissues and peripheral blood of mice induced by HFD.

Autophagy is a process which passes intracytoplasmic ingredients to lysosomes for degradation and provides energy to the body [29]. Autophagy not only is very important in the maintenance of cellular homeostasis but also participates in the metabolism of lipid droplets and lipogenesis. Research showed that, in both *in vivo* and *in vitro* experiments, the inhibition of autophagy increases lipid storage. This finding may link to the reduction of the degradation of triacylglycerol. In addition, knockdown of autophagy genes Atg5 and Atg7 in adipocytes can improve insulin sensitivity and relieve obesity [30]. Therefore, autophagy played an important role in regulating obesity-related metabolic disorders [31]. The suppression of autophagy also reduced the expression of adipogenesis-related genes, such as PPAR-γ and C/EBPs. Previous studies proved that the knockdown of Atg7 decreased TG accumulation in 3T3-L1 preadipocytes, generated obese mice with decreased weight and white adipose mass, and then enhanced insulin sensitivity. These findings suggest that autophagy regulated lipid metabolism.

Some research found that HMGB1 activated an autophagic response to oxidative stress [32], and the loss of

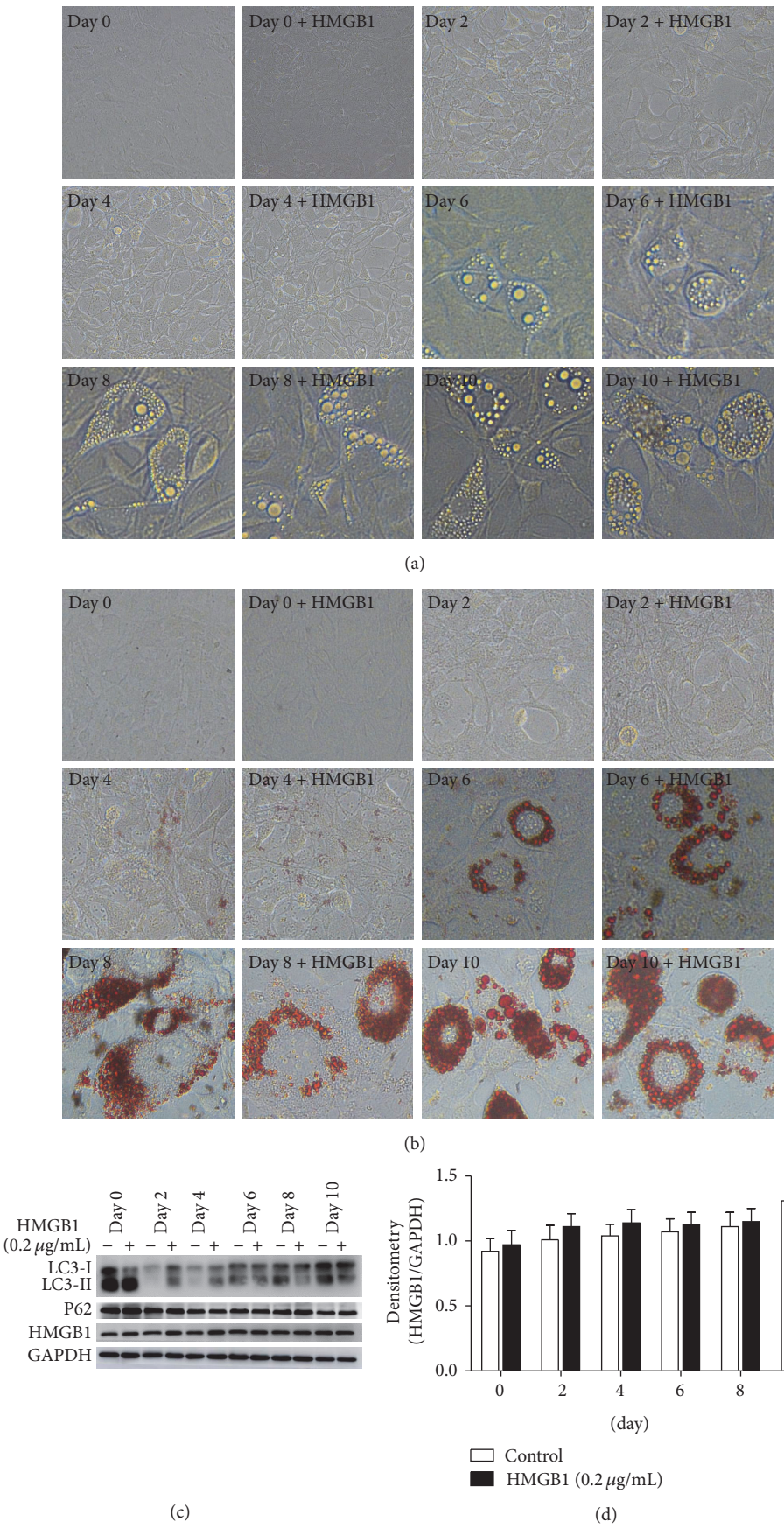

Figure 4: Continued.

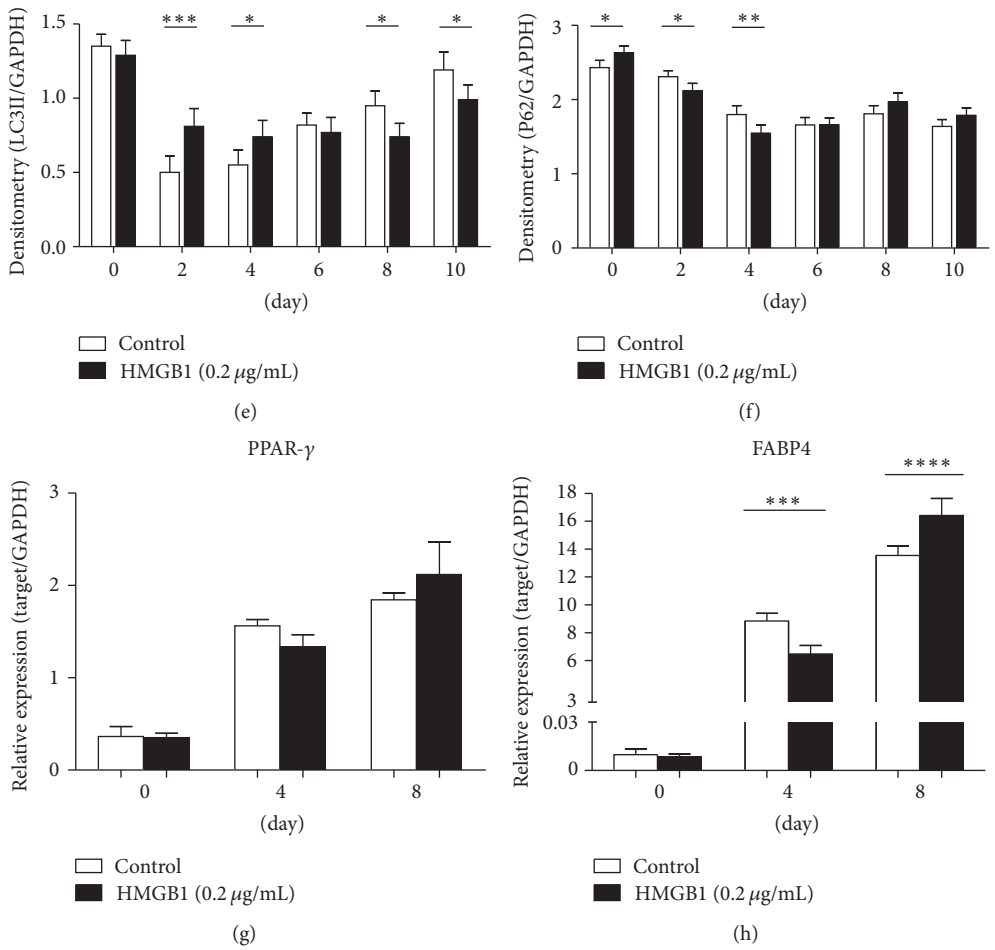

Figure 4: Effects of recombinant HMGB1 protein on autophagy in various differentiation periods of 3T3-L1 adipocytes. Differentiation of 3T3-L1 preadipocytes was induced at 0, 2, 4, 6, 8, and 10 days. The adipocytes were added with 0.2 μg/mL recombinant HMGB1 protein 24 h before differentiation. (a) Pictures were taken with a bright-field microscopic images of differentiating cells. (b) Oil-Red-O staining (200 magnification). (c) The expression of proteins HMGB1, LC3, and P62 was detected by Western blot. (d–f) HMGB1, LC3, and P62 protein were quantified and normalized to GAPDH. (g-h) Levels of PPAR-γ and FABP4 were determined by quantitative PCR in differentiation at 0, 4, and 8 days, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$ versus the day itself (control).

HMGB1 inhibited autophagy. When HMGB1 decreased, the autophagy weakened. We observed the change of autophagy in 3T3-L1 preadipocytes when HMGB1 was disturbed by specific small interfering RNA. Our results showed that autophagy was weakened when the expression of HMGB1 was suppressed in 3T3-L1 preadipocytes. HMGB1 also played a vital role in regulating autophagy in response to metabolic stress and oxidative damage [33]. However, little is known about the connections between the HMGB1 protein and the change of autophagy in adipose tissues of mice. Our results displayed that HMGB1 increased along with autophagy activated in adipose tissue of obese mice induced by HFD. This finding implied that activated autophagy may be a protective mechanism under cell stress state induced by HFD.

To gain insights into the effects of HMGB1 on autophagy in adipocytes, *in vitro* studies were performed in adipocytes. 3T3-L1 preadipocytes were first stimulated with the recombinant HMGB1 protein to observe the expression of autophagy-related protein LC3 (microtubule-associated protein 1A/1B-light chain 3) and P62. A cytosolic form of LC3 (LC3I) is conjugated to phosphatidylethanolamine to form LC3-phosphatidylethanolamine conjugate (LC3II), which is recruited to autophagosomal membranes [34, 35]. Thus, the formation of LC3II appears to be correlated with the induction of autophagy [36]. P62 serves as a link between LC3 and ubiquitinated substrates, and the inhibition of autophagy correlates with the increased levels of p62. Our results showed that LC3II increased and P62 decreased when cells were treated with recombinant HMGB1 protein. This finding suggests that exogenous HMGB1 induced autophagy in 3T3-L1 preadipocytes. In addition, as small interfering RNA knocked down the expression of HMGB1, autophagy appeared to be weakened. These data indicated that HMGB1 played an important role in regulating the autophagy in 3T3-L1 preadipocytes.

We further examined whether HMGB1 proteins were related to adipocyte differentiation and autophagy. 3T3-L1 adipocytes were cotreated with HMGB1 protein and adipocyte differentiation-inducing agent. We observed HMGB1 activated autophagy in the early differentiation within 2 and

4 days but weakened autophagy in differentiation after 8 and 10 days. Presumably, the activation of autophagy resisted external stress in the early differentiation stage but was unable to resist this constant pressure in the late stage of differentiation. These results demonstrated that HMGB1 played a different role in the differentiation of 3T3-L1 adipocytes. Several transcription factors, such as PPAR-γ, are involved in the differentiation of preadipocytes into mature adipocytes [37]. FABP4, a lipid chaperone, is expressed in adipocytes and plays an important role in the regulation of insulin sensitivity [38]. Our results showed that FABP4 increased within 4 days but inversed within 8 days. This finding was consistent with the expression of LC3, which suggested that HMGB1 regulated autophagy and further altered lipid metabolism in adipocytes.

5. Conclusion

This study demonstrated that HFD induced HMGB1 protein and LC3II production in adipose tissues. HMGB1 can regulate autophagy and alter lipid metabolism in adipocytes. These results not only provided a new theoretical basis for HMGB1 and autophagy in adipocytes but also presented a new target for the treatment of obesity and its related metabolic diseases.

Competing Interests

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

Acknowledgments

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Research Article

Effects of Glutamate and Aspartate on Serum Antioxidative Enzyme, Sex Hormones, and Genital Inflammation in Boars Challenged with Hydrogen Peroxide

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Background. Oxidative stress is associated with infertility. This study was conducted to determine the effects of glutamate and aspartate on serum antioxidative enzymes, sex hormones, and genital inflammation in boars suffering from oxidative stress. *Methods*. Boars were randomly divided into 4 groups: the nonchallenged control (CON) and H_2O_2-challenged control (BD) groups were fed a basal diet supplemented with 2% alanine; the other two groups were fed the basal diet supplemented with 2% glutamate (GLU) or 2% aspartate (ASP). The BD, GLU, and ASP groups were injected with hydrogen peroxide (H_2O_2) on day 15. The CON group was injected with 0.9% sodium chloride solution on the same day. *Results*. Dietary aspartate decreased the malondialdehyde (MDA) level in serum ($P < 0.05$) compared with the BD group. Additionally, aspartate maintained serum luteinizing hormone (LH) at a relatively stable level. Moreover, glutamate and aspartate increased transforming growth factor-β1 (TGF-β1) and interleukin-10 (IL-10) levels in the epididymis and testis ($P < 0.05$) compared with the BD group. *Conclusion*. Both glutamate and aspartate promoted genital mRNA expressions of anti-inflammatory factors after oxidative stress. Aspartate more effectively decreased serum MDA and prevented fluctuations in serum sex hormones after H_2O_2 challenge than did glutamate.

1. Introduction

Artificial insemination has been shown to be a successful reproductive management approach to improve livestock production efficiency. Artificial insemination facilitates the use of high-genetic-merit boars for inseminating a group of sows [1]. Therefore, boars with a high reproductive capacity can improve efficiency with significant genetic effects [2].

However, many risk factors can cause reproductive dysfunction in boars, such as harsh environments and mental stress and disease [3–6]. Cumulative evidence indicates that whole body oxidative stress (OS) is related to all these risk factors. The imbalance between oxidation and antioxidation and the elevation of reactive oxygen species (ROS) are believed to cause defective spermatogenesis and sperm dysfunction in sexually mature boars [7]. Approximately 25% of infertile men showed high levels of semen ROS, while fertile men did not [8]. In mammals, spermatozoal membranes have many polyunsaturated fatty acids (PUFAs) and are sensitive to ROS attack, which can damage membrane and morphological integrity, impair cellular functions, and promote sperm apoptosis and impaired sperm motility [7]. Additionally,

most infertile men have been shown to suffer from acute or chronic inflammation of the genitourinary tract [9]. Cytokines and ROS may interact in mediating the toxic effects of inflammation [10]. Previous reports found a positive correlation between seminal ROS generation and seminal plasma proinflammatory cytokines, such as interleukin-8 (IL-8), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) [11]. A sustained inflammatory/oxidative environment can damage healthy cells, which may lead to infertility and cause economic losses. In light of these factors, a strategy to minimize oxidative stress and genital inflammation in boars is required.

In recent years, many antioxidant therapies have been used to enhance reproductive ability [12–14]. These compounds can directly and indirectly influence the concentration of ROS metabolism-regulating processes [15–17]. As nutritional supplements for animals, glutamate and aspartate were found to possess antioxidative activity. Dietary supplementation with 2% glutamate enhanced the antioxidant system and improved body weight in piglets after diquat challenge [18], suggesting it can ameliorate the damage caused by acute oxidative stress. Moreover, glutamate modulated the body weight [19], regulated the release of hormones [20] and lipid metabolism [21], and improved gastrointestinal absorption [22]. Dietary supplementation with aspartate at a dose of 0.5–1% restored the intestinal barrier, improved liver metabolism, and enhanced energy status in piglets challenged with lipopolysaccharide [23, 24]. These effects may be due to its contributions to the tricarboxylic acid cycle and the production of ATP [25]. Because glutamate and aspartate have beneficial health and antioxidative effects, they may protect boars suffering from acute oxidative stress. Thus, we evaluated the effects of glutamate and aspartate supplementation on the antioxidative enzymes and reproductive system of boars under conditions of acute oxidative stress. Hydrogen peroxide (H_2O_2) was introduced to establish the oxidative stress model [26].

2. Materials and Methods

2.1. Experimental Design. Sixteen large white boars (6 months old, 85 ± 3.2 kg BW) were housed in individual metabolic cages equipped with a nipple drinker and a feeder in the room, and the temperature was maintained at 25°C. All boars were fed a basal diet (Table 1), which was formulated to meet the nutritional needs of 6-month-old boars according to NRC (1998), for 5 days. Then, they were divided into 4 groups to undergo different treatments: (1) nonchallenged control (CON, basal diet + 2% alanine, saline-challenged); (2) H_2O_2-challenged control (BD, basal diet + 2% alanine, H_2O_2-challenged); (3) H_2O_2 + 2% glutamate treatment (GLU, basal diet + 2% glutamate, H_2O_2-challenged); and (4) H_2O_2 + 2% aspartate treatment (ASP, basal diet + 2% aspartate, H_2O_2-challenged). Three diets were prepared to be isonitrogenous by introducing alanine into the basal diet. All groups were treated with a single intraperitoneal injection (i.p.) of 10% H_2O_2 (1 mL/kg body weight) on day 15, except for the CON group, which was injected with the same volume of 0.9% sodium chloride solution. The dosage of H_2O_2 used in this study was based on a previous study [26]. The amino acids (purity > 99%) used in this study were purchased from Beijing Chemclin Biotech Co., Ltd. (Beijing, China). Other chemicals were purchased from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China).

Table 1: The composition of basal diet.

Items	Basal diet
Ingredient (%)	
Corn	64
Soybean meal	22
Wheat bran	6
Fish meal	4
Premix*	4
Composition	
Crude protein (%)	13.80
Metabolism energy (MJ/kg)	13.25
Calcium (%)	0.85
Phosphorus (%)	0.72

*Composition: vitamin A, 400000 U; vitamin D, 380000 U; vitamin E, 1200 U; vitamin K, 360 mg/kg; vitamin B_1, 145 mg/kg; vitamin B_2, 135 mg/kg; vitamin B_6, 85 mg/kg; vitamin B_{12}, 0.58 mg/kg; niacin, 600 mg/kg; calcium pantothenate, 350 mg/kg; folate, 90 mg/kg; biotin, 12 mg/kg; choline chloride, 15 g; copper, 0.4 g; iron, 3.3 g; manganese, 0.5 g; cobalt, 10 mg; iodine, 10 mg; and selenium, 8 mg.

2.2. Sample Collection. All boars were anesthetized using sodium pentobarbital and then killed by jugular puncture on day 22. The blood, testis, and epididymis samples were collected. This study was performed in accordance with the Declaration of Helsinki and ratified by the Laboratory Animal Care Advisory Committee at the Institute of Subtropical Agriculture, the Chinese Academy of Sciences [31]. Blood was collected from the jugular vein of boars. Serum samples were prepared by centrifugation of blood samples at 2000 rpm (or 500 ×g) for 10 min at 4°C and then stored at −80°C until use. The testis and epididymis were weighed after slaughter, and the organ coefficient was calculated as follows [32]: organ coefficient (%) = organ weight/body weight of boar × 100%. A small portion of the testis and epididymis was immediately frozen in liquid nitrogen and maintained at −80°C for subsequent analyses of gene expression.

2.3. Measurements of Specific Enzymes and Hormones in Serum. The serum concentrations of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and malondialdehyde (MDA) were analyzed to determine the serum antioxidant capacity. They were measured using kits from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China) [33]. Kits from Beijing North Institute of Biological Technology (Beijing, China) were used to determine the serum concentrations of reproductive hormones, such as follicle-stimulating hormone (FSH), luteinizing hormone (LH), and testosterone (T2) [34].

2.4. RNA Extraction and cDNA Synthesis. Total mRNA from liquid nitrogen-pulverized testis and epididymis was

Table 2: Primers used in this study.

Gene[1]	Accession number	Primer squence (5′-3′)	Size (bp)
IL-6	NM_001252429.1	F: CCTCTCCGGACAAAACTGAA R: TCTGCCAGTACCTCCTTGCT	118 [27]
IL-10	NM_214041.1	F: CTGCCTCCCACTTTCTCTTG R: TCAAAGGGGCTCCCTAGTTT	95 [28]
IL-1β	NM_214055.1	F: AGTGGAGAAGCCGATGAAGA R: CATTGCACGTTTCAAGGATG	113
TGF-β1	NM_214015.1	F: TTT CGC CTC AGT GCC CA R: GCCAGAATTGAACCCGTTAA	78 [27]
TNF-α	NM_214022.1	F: CCACGCTCTTCTGCCTACTGC R: GCTGTCCCTCGGCTTTGAC	168 [29]
GAPDH	NM_001206359.1	F: AAGGAGTAAGAGCCCCTGGA R: TCTGGGATGGAAACTGGAA	140 [30]

[1]IL-6: interleukin-6; IL-10: interleukin-10; IL-1β: interleukin-1β; TGF-β1: transforming growth factor-β1; TNF-α: tumor necrosis factor-α; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

extracted using TRIzol reagent (Invitrogen, USA) according to the manufacturer's recommendation [35]. RNA integrity was confirmed by agarose gel electrophoresis. The RNA concentrations were determined by measuring the absorbance at 260 nm in a spectrophotometer [36]. Reverse transcription was performed with a 2 mg RNA sample using PrimeScript™ RT reagent kit (TaKaRa) according to the manufacturer's instructions. The cDNA was synthesized with a PrimeScript 1st-Strand cDNA Synthesis Kit (TaKaRa, Japan) [37].

2.5. Quantification of mRNA by Real-Time PCR Analysis. Primers were designed with Primer 5.0 based on the cDNA sequence of boars to amplify target DNA (Table 2). GADPH was used as a reference gene to normalize target gene transcript levels. Real-time PCR was conducted with a total volume of 25 μL containing 12.5 μL SYBR® Premix Ex Taq (Tli RNase H *Plus*), 2 μL template (<100 ng), and 1 μL of each of the forward and reverse primers (10 μM). The PCR protocol was 10 min at 95°C, followed by 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. Amplification efficiency for each target gene was determined by plotting the threshold cycle (Ct) versus log (initial cDNA). The relative quantification of target gene expression was evaluated by $2^{-\Delta\Delta CT}$ method [36].

2.6. Statistical Analysis. All statistical analyses were carried out using SigmaPlot 12 software. First, tests for normal distribution (Shapiro-Wilk test) and equal variance were performed. If both tests were positive, one-way analysis of variance was used followed by post hoc Tukey's test. Values with different letter are significantly different ($P < 0.05$), while values with the same letter are not significantly different ($P > 0.05$).

3. Results

3.1. Organ Coefficient. The final body weights and testis coefficients are shown in Table 3. The H_2O_2 challenge did not affect the body weight of boars but increased the testis coefficient 7 days after the treatment compared with the CON group ($P < 0.05$). However, diets with and without glutamate and aspartate supplementation had no significant impact on the testis coefficient ($P > 0.05$).

3.2. Concentrations of MDA, SOD, and GSH-Px in Serum. Figure 1 shows that the H_2O_2 administration disturbed the balance between oxidation and antioxidation in boars. The MDA concentration in serum significantly increased after H_2O_2 challenge compared with the CON group ($P < 0.05$). Dietary aspartate significantly reduced the MDA level in serum compared with the BD group ($P < 0.05$), and the value was similar to that of the CON group ($P > 0.05$). Dietary glutamate slightly decreased the MDA level in serum, but the changes were not significant compared with the BD group ($P > 0.05$).

Intraperitoneal injection with H_2O_2 did not affect the SOD level in serum, and dietary supplementation with glutamate and aspartate also had little influence on SOD concentration ($P > 0.05$). The serum GSH-Px concentration in the BD group was significantly increased 7 days after H_2O_2 challenge compared with the CON group ($P < 0.05$). Dietary supplementation with glutamate and aspartate had little effect on the GSH-Px level compared with the BD group ($P > 0.05$).

3.3. Sex Hormones in Serum. The concentrations of FSH, LH, and T2 in boar serum were determined, and the results are shown in Figure 2. The H_2O_2 challenge had little impact on serum FSH, but supplementation with glutamate and aspartate significantly decreased the FSH level in serum compared with the CON group ($P < 0.05$). In contrast to FSH, the LH concentration was significantly higher, and the T2 level was significantly lower in the BD group than that in the CON group ($P < 0.05$). Dietary aspartate maintained serum LH at a stable level under oxidative stress, and its value showed no significant difference from that of the CON group ($P > 0.05$). However, dietary supplementation with glutamate and aspartate significantly decreased the serum T2 level compared with the BD group ($P < 0.05$).

Table 3: Final body weight, testis weight, and testis coefficient after H_2O_2 challenge.

	CON	BD	GLU	ASP
Final BW (kg)	82.67 ± 2.52	87.67 ± 1.53	87.67 ± 6.35	81.00 ± 2.00
TW (g)	137.07 ± 15.86[b]	343.23 ± 38.06[a]	375.40 ± 60.51[a]	321.13 ± 70.67[a]
T coefficient (%)	0.17 ± 0.02[b]	0.39 ± 0.05[a]	0.43 ± 0.04[a]	0.40 ± 0.09[a]

BW = body weight; TW = testis weight; and T coefficient = testis coefficients.
Values are means ($n = 4$), with their standard deviation represented by mean ± STD. [a,b]Mean values with different letters were significantly different ($P < 0.05$).

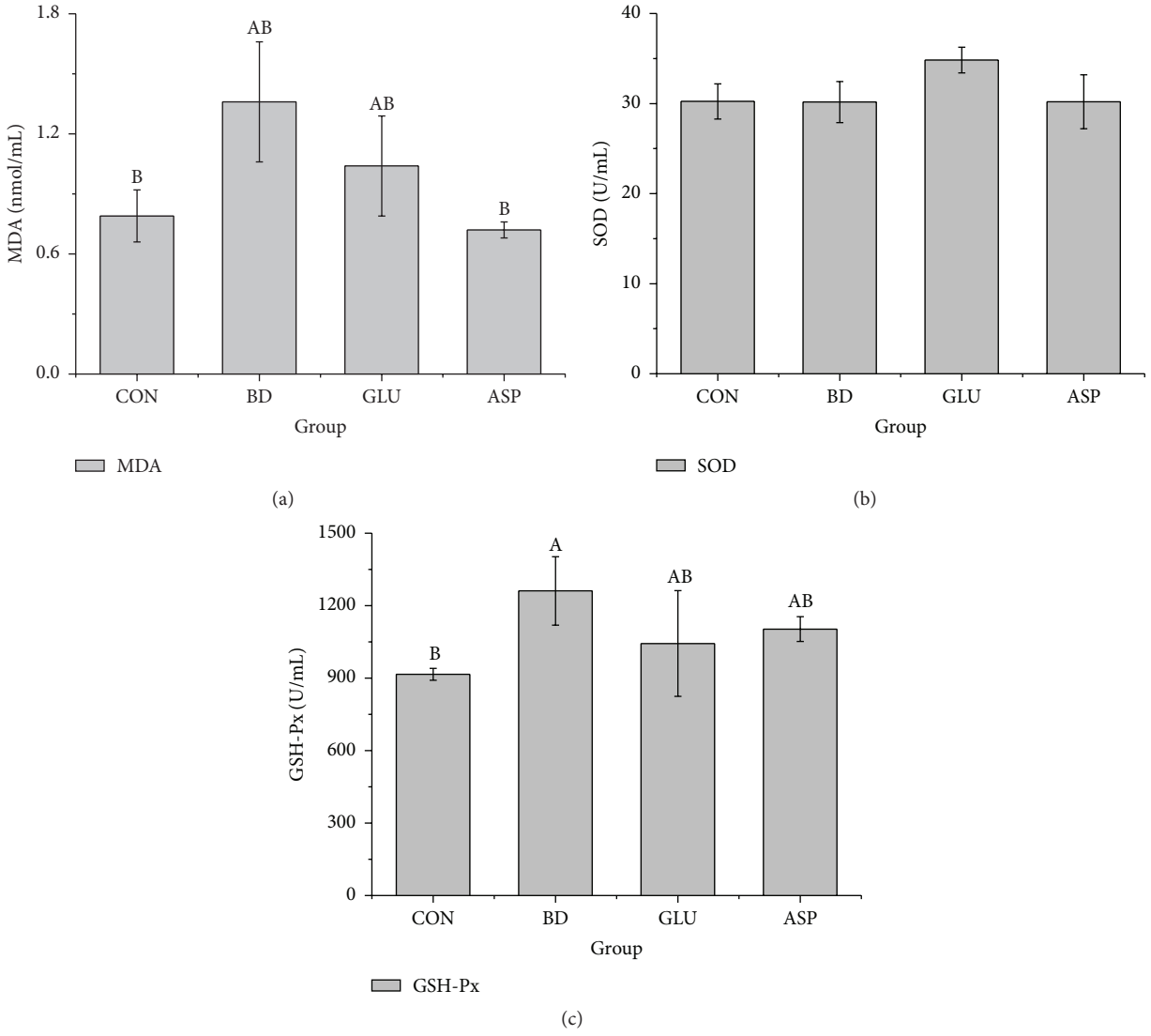

Figure 1: The effects of glutamate and aspartate on serums SOD, MDA, and GSH-Px after H_2O_2 challenge. SOD: superoxide dismutase; MDA: malondialdehyde; and GSH-Px: glutathione peroxidase. Values are means ($n = 4$), with their standard deviation represented by vertical bars. [A,B,C]Mean values with different letters were significantly different ($P < 0.05$).

3.4. Expression of Inflammatory Genes in Testis and Epididymis. The relative mRNA expressions of inflammatory factors (TGF-β1, IL-10, IL-6, IL-1β, and TNF-α) in boar testis and epididymis after H_2O_2 challenge were analyzed. The results (Figure 3) showed that inflammatory factors (IL-6, IL-1β, and TNF-α) were markedly upregulated in the testis after H_2O_2 challenge compared with the CON group. Dietary glutamate failed to decrease the IL-6 and TNF-α expressions in the testis and epididymis compared with the BD group, but it significantly upregulated TGF-β1 in the testis and both TGF-β1 and IL-10 in epididymis compared with the BD group ($P < 0.05$). Dietary aspartate upregulated TGF-β1 expression in the testis and IL-10 expression in the epididymis compared with the BD group ($P < 0.05$). Additionally, dietary aspartate

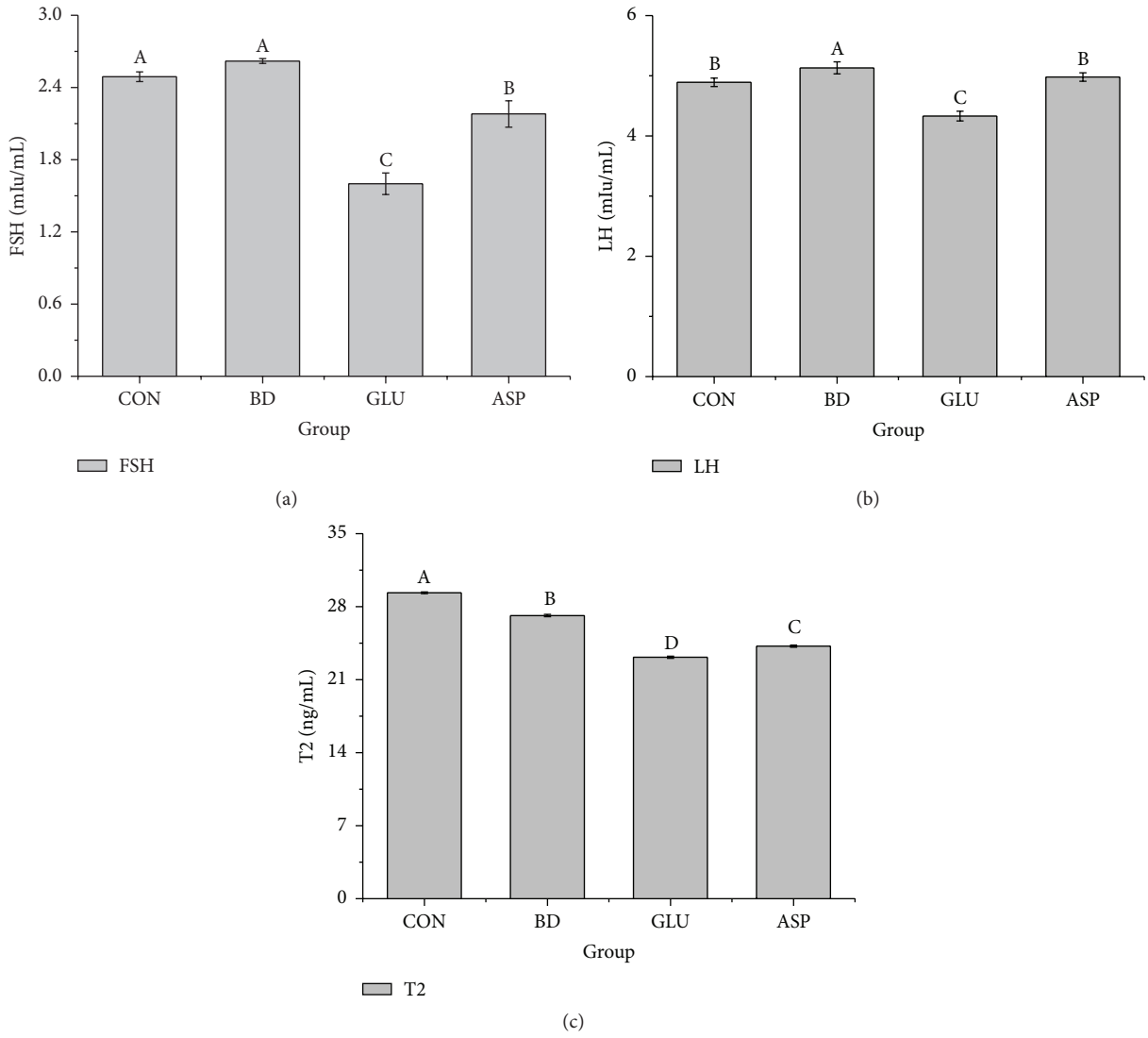

Figure 2: The effects of glutamate and aspartate on serum sex hormones (FSH, LH, and T2) after H_2O_2 challenge. FSH: follicle-stimulating hormone; T2: testosterone; and LH: luteinizing hormone. Values are means ($n = 4$), with their standard deviation represented by vertical bars. ^{A,B,C,D}Mean values with different letters were significantly different ($P < 0.05$).

decreased the IL-1β and TNF-α expressions in testis compared with the BD group ($P < 0.05$).

4. Discussion

ROS are products of normal cellular metabolism. However, once the balance between the generation of ROS and antioxidant scavenging activity is disturbed, oxidative stress occurs [38, 39]. Many studies have indicated that the most common ROS, such as H_2O_2, ROO^-, and OH^-, can lead to sperm damage and deformity and eventually male infertility [7]. Lipids are considered the most susceptible biomolecules and are abundant in the sperm plasma membrane and other cell membranes in the form of polyunsaturated fatty acids (PUFAs) [40]. ROS attack these PUFAs, leading to lipid peroxidation and elevated generation of MDA, which has been used to monitor the degree of peroxidative damage [40]. H_2O_2 is a highly reactive oxygen species. It can freely disperse into the mitochondria and lead to the generation of massive ROS levels. Peritoneal administration of 10% H_2O_2 (1 mL/kg body weight) resulted in oxidative stress [26]. Consistent with a previous study, the data from Figure 1 show that H_2O_2 injection (BD group) increased the serum MDA concentration compared with the CON group, suggesting that H_2O_2 had successfully induced systemic oxidative stress in boars. Notably, dietary glutamate and aspartate were reported to significantly alleviate the oxidative stress of piglets seven days after H_2O_2 or diquat challenge [18, 26] and would possibly have a better effect with prolonged use. Thus, to protect against oxidative stress, we fed the boars 2% glutamate or 2% aspartate prior to the H_2O_2 challenge. All boars were slaughtered and sampled seven days after H_2O_2 challenge to determine whether glutamate and aspartate rapidly alleviated the oxidative stress in boars as they did in piglets [26].

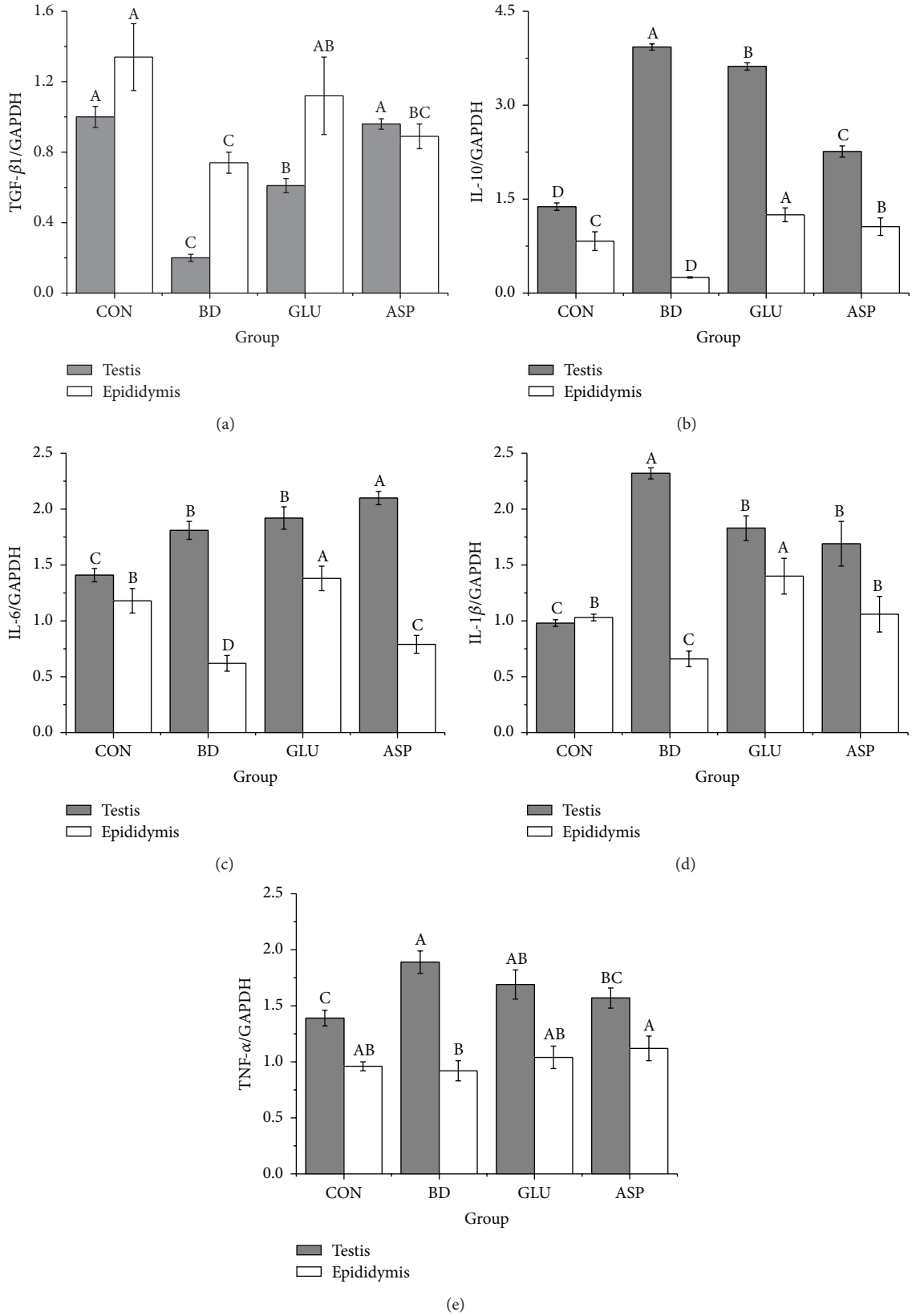

Figure 3: The relative mRNA expression of inflammatory-related factors (TGF-β1, IL-10, IL-6, IL-1β, and TNF-α) in boar's testis and epididymis after H_2O_2 challenge. TGF-β1: transforming growth factor-β1; TNF-α: tumor necrosis factor-α; and IL: interleukin. Values are means ($n = 4$), with their standard deviation represented by vertical bars. ^{A,B,C,D}Mean values with different letters were significantly different ($P < 0.05$).

To study the effects of glutamate and aspartate on boars under oxidative stress, we analyzed their impact on serum sex hormones, serum antioxidative enzymes, and genital inflammation. Hormones are signaling molecules. They can be transported to distant organs to regulate physiology and behavior via the circulatory system. The biosynthesis and secretion of hormones are regulated by other hormones, plasma concentrations of ions or nutrients, neurons and mental activity, and environmental changes [41]. Changes in hormone concentrations can also reflect the state of homeostasis [42]. Testosterone (T2) is a male sex hormone and is predominantly produced in the testis. It has an important role in sexual and reproductive development [43]. Fluctuations in T2 levels affect the sex drive, sperm production, and fat distribution [43, 44] and are associated with overall health in boars. A previous study revealed that testicular inflammation was related to a significant decrease in T2 production [45]. In this study, the H_2O_2 challenge decreased the serum T2 level in the BD group compared with that in the CON group, and administration of glutamate and aspartate failed to increase the T2 level. Combining the results of inflammatory gene analysis in testis, we found similar results indicating that H_2O_2 caused testicular inflammation along with downregulated T2 levels in serum. However, dietary glutamate and aspartate had little effect on testicular inflammation.

The course of normal spermatogenesis not only relies on the testicular secretion of T2 but also relies on the normal pituitary secretion of FSH and LH [46]. In males, FSH stimulates a number of downstream targets in Sertoli cells to affect spermatogenesis. Conversely, Sertoli cells are stimulated by FSH, which produces inhibin. This compound provides negative feedback to the anterior pituitary to decrease FSH secretion. LH stimulates Leydig cells to produce T2, which provides negative feedback to the anterior pituitary and hypothalamus [47]. In this study, a slight fluctuation in LH level was observed in the serum after H_2O_2 injection. However, it is difficult to determine the physiological significance of changes in these hormones because the mechanisms of FSH and LH regulation in boars under oxidative stress are still unclear. However, we analyzed the effect of glutamate or aspartate on preventing hormone disorders under oxidative stress by comparing the effects of different treatments on boar hormone concentrations. The results suggested that aspartate had positive effect on maintaining serum LH at a relatively stable level.

Glutamate administration can alleviate diquat-induced oxidative stress by enhancing SOD and T-AOC levels and inhibiting lipid oxidation and MDA generation [18]. Nevertheless, several studies suggested that glutamate accumulation in the brain increases oxidative stress [48]. Glutamate accumulation also has been reported to generate NO and stimulate cyclic guanosine monophosphate formation. Therefore, the relationship between glutamate and ROS appears to be complex [48]. Addition of aspartate was reported to prevent growth suppression of weaned pigs after LPS challenge [24]. However, our previous study found that administration of aspartate did not facilitate growth performance and showed little effect on relieving oxidative stress induced by diquat [18]. In this study, we found that aspartate was capable of reducing the MDA level in boar serum, while glutamate failed to alleviate H_2O_2-induced oxidative stress in boars. Boars in the BD group even had higher serum GSH-Px level than those in the GLU group. Whether these differences in their effects on oxidative stress are due to the distinct gender and age of the pigs requires further investigation.

Inflammatory factors are involved in oxidative stress [49]. Previous studies have shown that inflammation is a manifestation of increased oxidative stress [49]. Conversely, inflammatory cells also produce many mediators, such as metabolites of arachidonic acid, chemokines, and cytokines, which further recruit inflammatory cells to the site of injury and produce more ROS [49]. Under these circumstances, antioxidants in seminal plasma help prevent oxidative stress [50]. However, seminal plasma antioxidants cannot reach the testis, and the sperm must rely on epididymal/testicular antioxidants and their own intrinsic antioxidant capacity for protection during spermatogenesis and epididymal storage [51]. Glutamate and aspartate belong to the arginine family of amino acids, as well as proline, glutamine, asparagine, ornithine, citrulline, and arginine. They are interconvertible via complex interorgan metabolism in most mammals, including pigs. Both of these amino acids are predominantly absorbed in the small intestine [52]. However, the intestinal mucosa will preferentially use dietary glutamate rather than other amino acids [53]. Once glutamate and aspartate are absorbed by enterocytes, they are utilized as fuels or participate in the synthesis of other amino acids, such as alanine, arginine, and others, and then enter the systemic circulation [26]. Notably, arginine affects purine metabolism in testis tissues by activation of adenosine production, the salvage pathway, and ATP regeneration and shows protective effects on male metabolic and reproductive function [54]. Thus, dietary glutamate and aspartate are believed to be beneficial in enhancing testicular defense systems and reducing epididymal/testicular inflammation induced by oxidative stress.

To evaluate the effect of dietary glutamate and aspartate on genital inflammation in boars, we determined the mRNA expressions of TGF-β1, IL-10, IL-6, IL-1β, and TNF-α in boar testis and epididymis samples. TGF-β1 is a key regulator of male reproductive function [55]. The steroidogenesis of Leydig cells, the organization of peritubular myoid cells, and testis development and spermatogenesis are all modulated by testicular TGF-β1, and it is also involved in the tight balance between proliferative and apoptotic responses in the Leydig cells [55]. Another anti-inflammatory cytokine, IL-10, plays a role in upregulating monocyte production of soluble TNF-α and the IL-1β receptor antagonist [56]. It protects endothelial function after an acute inflammatory stimulus by limiting local increases in superoxide anion [57]. In this study, dietary supplementation with glutamate and aspartate caused varying degrees of upregulation of TGF-β1 and IL-10 mRNA expression in the testis and epididymis. These findings suggested that glutamate and aspartate protect boar testis and epididymis from inflammation by increasing TGF-β1 and IL-10 levels. However, the organ coefficient results also showed that there was a slight swelling in boar testis after H_2O_2 challenge, suggesting that the testes were suffering

from chronic inflammation. Even when boars were fed glutamate or aspartate, the swelling was not being relieved. The testicular inflammation indicated that the production and release of large amounts of ROS can trigger the immune responses and stimulate the secretion of numerous biological substances (such as leukocytes), which resulted in increased inflammation [9]. Because the antioxidant capacity of the testis and epididymis is very important in preventing oxidative stress-induced damage [7], the amount and activity of major antioxidant enzymes, especially SOD, catalase, and glutathione/glutathione peroxidase (GSH-Px), in the testis and epididymis are of great importance. Whether the testicular inflammation we found in this study was caused by the failure to elevate antioxidant enzyme activities in testis requires further research.

In conclusion, this study showed that dietary supplementation with glutamate and aspartate had little effect on increasing SOD and GSH-Px concentrations. They also failed to maintain FSH and T2 at a stable level in serum. Both glutamate and aspartate were unable to decrease the mRNA expressions of inflammatory factors (IL-1β, IL-10, and TNF-α) in testis and epididymis after H_2O_2 challenge. However, glutamate and aspartate promoted the genital mRNA expressions of anti-inflammatory factors (TGF-β1 and IL-10) after oxidative stress. Aspartate was more effective than glutamate in decreasing MDA levels and preventing the fluctuations of LH in boar serum.

Competing Interests

The authors declare that they do not have any commercial or associative interest that represents a conflict of interests in connection with the work submitted.

Authors' Contributions

Tiejun Li and Jiming Yao contributed equally to this work.

Acknowledgments

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Research Article

High-Methionine Diet Attenuates Severity of Arthritis and Modulates IGF-I Related Gene Expressions in an Adjuvant Arthritis Rats Model

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Rheumatoid arthritis, a synthesized form of adjuvant arthritis exhibited throughout many animal species, inhibits liver function and circulation of IGF-I and contributes to the degradation of skeletal muscle mass. One of the primary goals of the present study is determining whether a high-Methionine (high-Met) diet is capable of reducing the adverse effects of arthritis, namely, loss of body mass. Following adjuvant injection, forty arthritic rats were randomly assigned to either a control group with a basal diet or a high-Met group with the same basal diet + 0.5% Methionine. After 14 days all rats were terminated. The high-Met group exhibited an increase in body weight and food intake in comparison with the control group ($P < 0.05$). High-Met diet debilitated arthritis-induced surges in the gastrocnemius in both atrogin-1 and the MuRF1 expressions; however, it was observed to have little to no effect on atrogin-1 and MuRF1 gene expression in soleus. At the same time, high-Met diet rats experienced a rise in IGF-I, with lowering of IGFBP-3 gene expression in the gastrocnemius and the soleus. These data suggest that arthritis severity can be partly attenuated by high-Met diet.

1. Introduction

Adjuvant arthritis is a synthesized variant of rheumatoid arthritis which may be induced in rats via Freund's adjuvant intradermal injection. Following a ten-day period from the point of injection, chronic inflammation and polyarthritis become evident, along with a decrease in general body and muscle mass [1]. Inflammatory cachexia is considered to be a multifactorial process, resulting from a surge in inflammatory regulating entities and exhibiting endocrine modifications [2]. With respect to effects within the endocrine system, an increase in glucocorticoid secretion is observed [3] alongside inhibited release of anabolic hormones, namely, growth hormones (GH), testosterone, and insulin-like growth-factor-1 (IGF-I) [4]. As such, decreases in weight during chronic inflammation may occur partially due to insufficient IGF-I levels present within body.

Muscle atrophy in arthritic rats has been linked to up-regulation of atrogin-1 and MuRF-1 which are E3 ubiquitin-ligating enzyme genes and belong to the ubiquitin-proteasome proteolytic pathway [5]. Meanwhile, muscle wasting is also linked to the changes of the muscle IGF-IGFBPs system [1]. IGF-I has been analysed as the prevalent positive coordinator that designates levels of muscle mass [6]. The hormone facilitates a surge in generating muscle mass and aids in assessing satellite cells while additionally inhibiting the upregulation of atrogin-1/MuRF1 [7]. Furthermore, injecting IGF-I eliminated arthritis-induced proliferation in both muscle gene expression factors atrogin-1 and IGFBP-3 [8].

Studies have concluded that the signaling pathway of the insulin-like growth-factor-I (IGF-I) [9] facilitates muscle tissue generation. The mechanism is catalyzed by presence of amino acids, in particular, Methionine (Met). A lack of Met is correlated with a lesser muscle mass [10] and further research

has delineated supplementing additional Met into a diet to be highly effective in maintaining healthy muscle mass [11].

The assistive nature of high-Met diets in stimulating skeletal muscle development is well established; however, high-Met diet in dysfunctions related to skeletal muscle states is much less clear. It remains to be seen also, given the minimal research at present, whether high-Met diet mediation may assist in muscle degradation as a result of arthritis which modifies local expression of IGF-I interactions with IGFBPs.

The primary goal of this study therefore is to determine whether a high-Met diet could mitigate the adverse effects of adjuvant arthritis. Research has been undertaken into the resultant dietary effects between expressions of atrogenes, IGF-I, IGFBP-3, and IGFBP-5.

2. Material and Methods

2.1. Animals and Experimental Design. The present research complied with all Laboratory Animal Ethical Commission guidelines of the Tianjin Hospital of China. Forty male rats at five weeks of age were purchased from the Tianjin Laboratory Animal Public Service Center. Arthritic rats were afflicted as such via intradermal injection, administered in the right paw, and suspension rate 4 mg heat-treated (Freud's adjuvant) Mycobacterium butyricum with 0.1 mL paraffin oil, administered in isoflurane anesthesia conditions. 72 hours after this procedure, rats were placed into cages, 2 rats/cage, and cared for under ambient temperature and lighting: 20–22°C and lights switched on between the hours of 7:30 and 19:30.

Water was in free supply for the rats, which were split into two groups following adjuvant injection. The control group was fed a standard rodent diet [12], whereas the variable high-Met diet group received a surplus 0.5% quantity of Methionine. Food allocations per cage were calculated over a 24-hour period through measurement of the differences between the feeding and what remained at the end of the period. Intake was expressed in g per single rat, per 100 g of total weight. All rats were terminated following a 14-day period. The arthritic index of individual rats was delineated by grading each paw with a value between 0 and 4, with the following criteria: 0: no evidence of swelling, erythema, 1: minor erythema/swelling in at least one digit, 2: the entirety of the paw being evidently subject to moderate inflammation, 3: erythema being evident alongside inflammation spreading to the wrists, and 4: ankylosis and inability to maneuver ankle joints due to severe inflammation. Final scores were deduced through a collective total of the derived scores from each rat paw. Researchers expunged trunk blood into deheated tubes, allowed a clotting period and centrifuged with resultant serums kept at −20°C. Livers and gastrocnemius and soleus muscles (on the left side) were extrapolated, then dissected, and stored to freezing temperatures using liquid nitrogen, kept at −80°C.

2.2. RNA Extraction and RT-PCR. Gastrocnemius samples of 100 mg were assimilated. RNA was extrapolated through the TRIzol reagent (Invitrogen, USA). DNase I (Invitrogen, USA) was then added to treat the sample, in accordance with provided guidelines. RNA concentration and integrity were solidified through use of an agarose gel electrophoresis. In the case of RT-PCR analysis, 1 μg gastrocnemius RNA was utilised in cDNA creating with both oligo (dT) 20 and Superscript II reverse transcriptase (provided by Invitrogen, USA). In this case, primers were selected through analysis of existing research to delineate the optimal primer [13] with 18S rDNA as a reference gene. Thermal cycles can be delineated thus; a preincubation period of 95°C for a duration of 10 seconds, next, 40 cycles at 95°C, with denaturing spanning for 15 seconds, and a 60°C annealing stage subsequently occurring for a 30-second period, with 72°C extension procedures for 30 seconds, were undertaken. Final analyses were expressed in relation to 18S rDNA gene, whereby the prevalence of 18S rDNA was set to 1, utilizing a cycle limit of $2(\Delta\Delta CT)$ method.

2.3. Western Blot of IGFBP-3 and IGF-I Determination. IGF product concentration was measured through Western blot. A 2 mL product quantity was diluted within a sample buffer, to then be subjected to intense heating temperatures of 90°C for 2 minutes; samples were then placed onto 1% SDS-12.5% polyacrylamide gels and electrophoresed within a nonreducing context. Homogenous values of intestinal mucosa proteins were extrapolated using a polyacrylamide gel prior to relocation to a polyvinylidene difluoride (PVDF) membrane (sourced from Millipore, Bedford, MA, USA). Specimens then underwent incubation alongside primary IGFBP-3 antibodies (Abcam, US) for a duration of 12 hours at 4°C. PVDF membranes were subsequently kept in a warm environment alongside secondary antibodies goat anti-rabbit IgG-HRP (with products purchased from Santa Cruz Biotechnology, CA, USA) for 2 hours at a temperature of 25°C. Western blots were depicted via a superior chemiluminescence detection kit (Amersham, Arlington Heights, IL, USA). Images were procured with an Alpha Imager 2200 software application provided by the Alpha Innotech Corporation (CA, USA). Uniform distributions were observed among β-actin reference proteins across the groups. The protein expression value was ascertained to be the densitometry ratio of both IGFBP-3 and β-actin.

2.4. Statistical Analysis. Statistical analyses were undertaken via SPSS 25.0 (Chicago, IL, USA). Student's t-tests were utilised to ascertain the variation across the groups, with the statistical significance threshold at $P < 0.05$. Observations are delineated here as the means ± mean standard error (SEM).

3. Results

Arthritis score indices (ASI) developments through measurements of food consumption and weight gain are demonstrated in Figure 1. Throughout the initial stages of illness, prior to day 10 following the adjuvant injection, the arthritic rats developed minor inflammatory reaction within the injected paw. Scores for the arthritic symptoms in the paw ranged between 2 and 4 (Figure 1(a)); however, this was

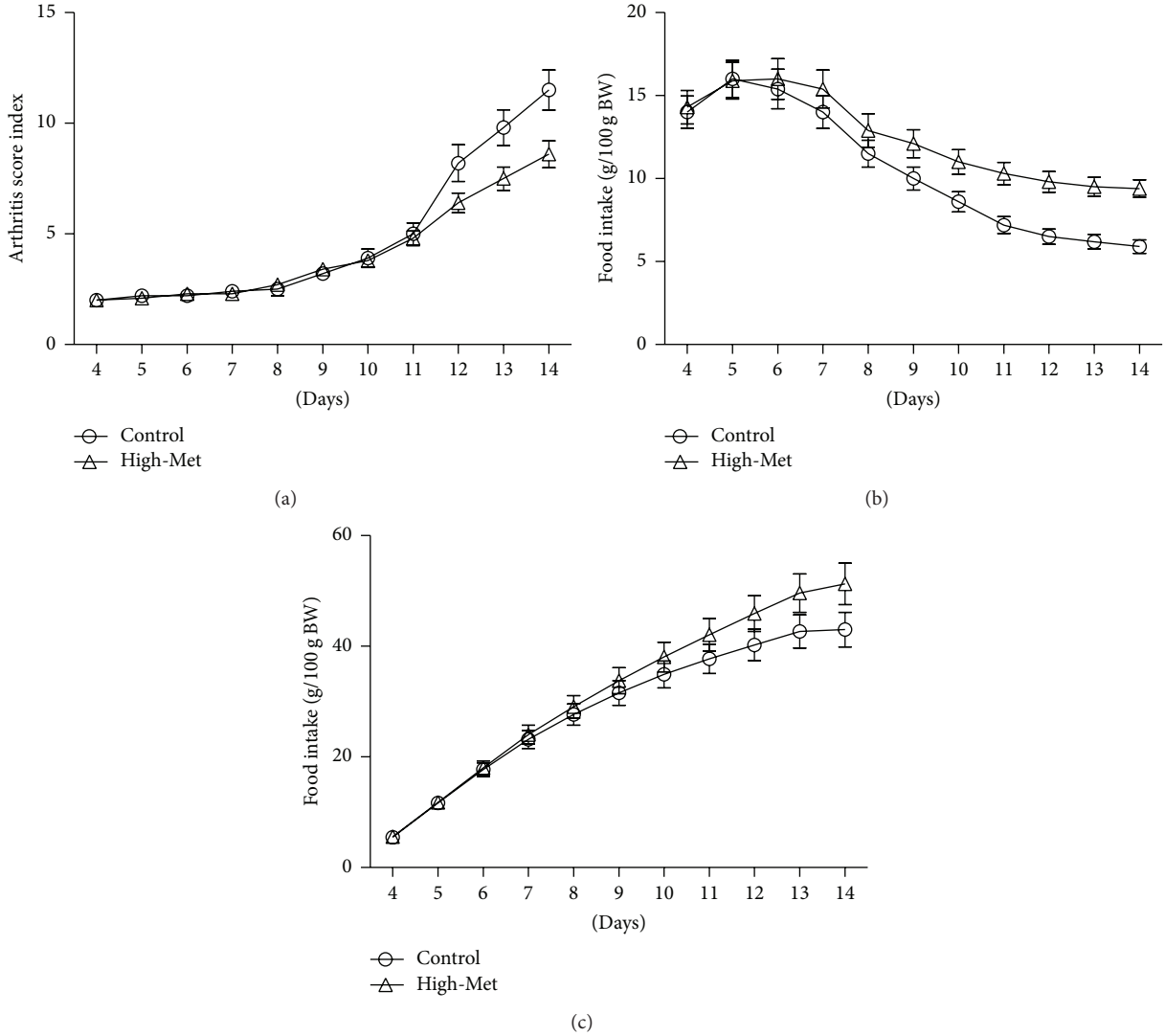

Figure 1: Arthritis index score (a) and consumption of food per 100 g/body weight (b), with the aggregate weight gain development (c) in days 4–14, following adjuvant injections within both the high-Met and control groups. Increased ASI corresponds to decreased food intake. Conversely, high-Met diets led to a rise in food consumption in comparison with the control group. Arthritic rats in the variable group collectively amassed a greater weight gain than the base group. Data are expressed as means ± SEM ($n = 10$).

not the case for paws which did not undergo injection. By the tenth day, the poly-articular inflammation began, and by day 14, for the control group, ASI reached the maximal value.

Arthritic symptoms were correlated with a reduction in food consumption ($P < 0.05$) for the 14-day duration (Figure 1(b)). Across both groups, food consumption was lowest between days 13 and 14, which correlated with a steep incline in the ASI. High-Met diet-fed arthritic rats increased their food consumption (Figure 1(b)). In accordance with the hypotheses, control group weight gain was significantly lower than the high-Met diet group, from the ninth day ($P < 0.05$, Figure 1(c)).

It appears that a high-Met diet leads to surges in the concentration of IGF-I found within the high-Met samples in comparison to the control group (Figure 2). As can be observed in Figure 3(a), the high-Met diet also led to decreased atrogin-1 and MuRF1 mRNA, observed in the gastrocnemius, with values below that of the control group ($P < 0.05$). At the same time, Figure 3(b) demonstrates that a high-Met diet did not affect atrogin-1 or MuRF1 mRNA levels within soleus contexts in comparison to the base group ($P < 0.05$). Figure 4(a) demonstrates that a high-Met diet had no effect in the gastrocnemius on IGFBP-5 mRNA compared to the control group. Conversely, IGF-I mRNA levels were elevated significantly ($P < 0.05$), and IGFBP-3 mRNA significantly declined ($P < 0.05$) for the gastrocnemius as compared to the control group. Identical trends were observed in the soleus, with high-Met diet raising IGF-I mRNA levels ($P < 0.05$) and dropping IGFBP-3 mRNA ($P < 0.05$), with no statistically significant effect on IGFBP-5 mRNA levels (Figure 4(b)).

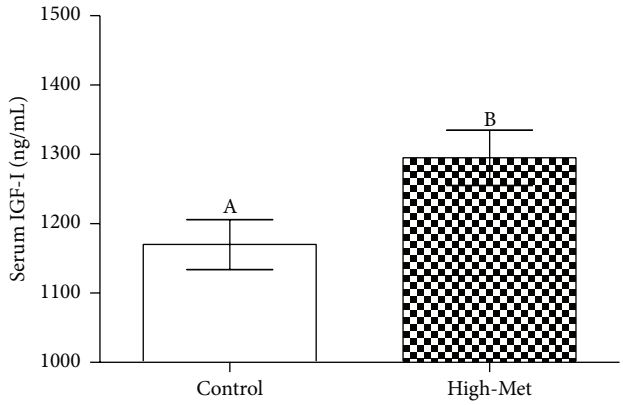

Figure 2: Serum levels of IGF-I when arthritic rats ate a high-Met diet. Data are shown here as means ± SEM ($n = 8$). [A,B]The varying letters indicate statistical significance in the divergences of the high-Met and control groups ($P < 0.05$).

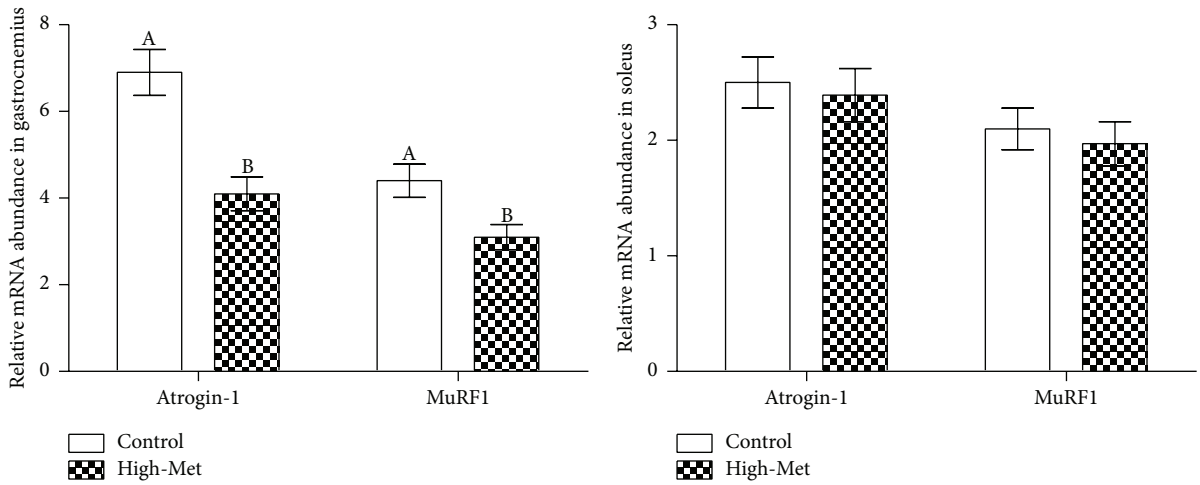

Figure 3: Results of high-Met diet upon the level of mRNA in gastrocnemius (a) and soleus (b). Data is presented as means ± SEM ($n = 8$). [A,B]The varying characters indicate the statistically significant differences between the high-Met and control groups ($P < 0.05$).

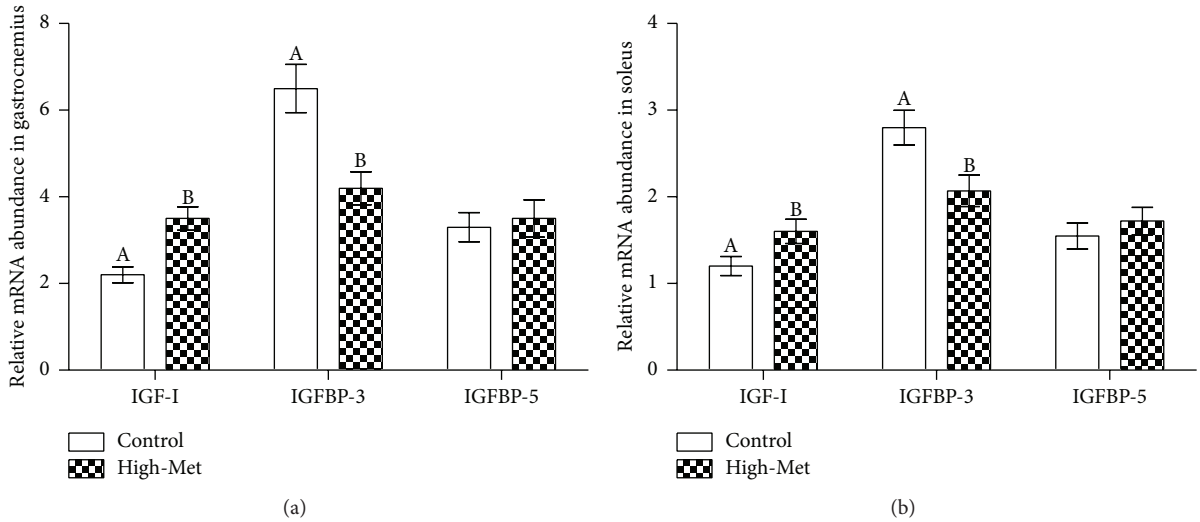

Figure 4: Results of high-Met diet upon the level of mRNA in gastrocnemius (a) and soleus (b). Data is presented as means ± SEM ($n = 8$). [A,B]The varying characters indicate the statistically significant differences between the high-Met and control groups ($P < 0.05$).

4. Discussion

The current research suggests that feeding arthritic rats a high-Met diet causes their food consumption to increase, as well as overall weight, in addition to serum IGF-I levels. Plasma IGF-I response levels to dietary Met have been noted in existing research [14]. However, as discussed by Nagao and colleagues [15], the regulatory implications of dietary Met were autonomous from plasma IGF-I concentration variations. High-Met diets resulted in raised serum level of IGF-I, which may be responsible for the arthritic intensity in rats. In the case of healthy humans with typically functioning IGF-I, IGF-I will lead to greater muscle protein synthesis [16]. Also, IGF-I has the capability to minimize the effects of negative nitrogen balancing throughout caloric reductions and an increased muscle mass within hypophysectomized animals [6]. Studies have also discussed the potential for IGF-I administration to minimize the debilitating effects of chronic arthritis [13]; such conclusions may accommodate to the present findings that a high-Met diet may moderately inhibit arthritic hindrances relative to body weight and overall reduce debilitating aspects of arthritis.

Arthritis behaves differently between the gastrocnemius and soleus, whereby muscle atrophy is observed to be much greater in the former than in the latter. Upregulation of arthritis-induced atrogenes atrogin-1 and MuRF1 showed values for the gastrocnemius to be higher than in samples extrapolated from the soleus. Furthermore, arthritic implications on IGF-I and IGFBP-3, in addition to factors of myogenic maintenance, are greater in the gastrocnemius. Preserving oxidative as a means to prevent muscle atrophy over several chronic diseases is evidenced by the oxidative muscle displaying efficacious responses to the antioxidant system relative to the glycolytic muscle [17]. Reactions from IGF-I administration follow the same pattern. In the arthritic rats' gastrocnemius, high-Met diets lowered atrogin-1 and MuRF1. Conversely, a high-Met diet had no effect on atrogin-1 and MuRF1 gene expression in the arthritic rats' soleus samples.

IGF-I regulation and relative proteins which bind to it behave in different ways in the liver and skeletal muscle. In the present research, it was found that high-Met diets facilitated the circulation of IGF-I; however, no effect was observed in skeletal muscle IGF-I mRNA. In the same vein, IGF-I generation in the liver varies as opposed to its mechanism in all other organs and tissues. Administration of clenbuterol, for instance, a $\beta 2$-adrenoceptor agonist, catalyzes a rise in IGF-I and IGFBP-5 gene expression as observed soleus contexts, but circulation of IGF-I is inhibited [18].

Some researches purported that locally procured IGF-I should be prioritized over IFG-I as a preserver of muscle strength and mass [6]. Along these lines, there is elevated IGF-I gene expression found within gastrocnemius contexts only, within arthritic rats. Yet muscle deterioration is more apparent in the former than the latter [13]. A fractional amendment to IGF-I circulation was found to have a great efficacy in catalyzing weight gain as well as gastrocnemius mass within the subject pool of arthritic rats.

The results of this study are indicative of a high-Met diet providing a crucial contribution to inhibiting muscle atrophy. Surges in the level of IGFBP-3 were significantly evident in soleus samples of the arthritic rats, however, not as steep as those noted from the gastrocnemius [1]. Increases in IGFBP-3 are also evident in existing literature; after 48-hour period following injury, this time is denoted as the preliminary recovery stage, during which inflammatory cells in the muscles are abundant, particularly in the vicinity of macrophages [19]. The current study ascertained that IGFBP-3 is primarily abundant in macrophages in the near vicinity of the afflicted tissue [19]. Overexpressing IGFBP-3 and inhibiting the binding of IGF-I receptors have autonomous IGF-I effects in reducing the overgeneration of cells [20]. In addition, IGFBPs prevention utilizing IGF-I aptamer gave rise to augmented tissue regeneration and benefitted from increases in recovery duration, from fast twitch as a result of myotoxic damage [5]. It may be deduced that reductions in IGFBP-3 in muscles following high-Met diets can indeed supplement the anabolic effect of IGF-I.

To conclude, data procured in this study strongly support the hypotheses and existing data that attenuation of arthritis may be obtained through an enhanced high-Met diet. In the case of the lab rats, high-Met diets catalyzed a surge in serum IGF-I levels and inhibited the level of a high-Met diet for atrogin-1, as well as MuRF1. Decreases in IGFBP-3, as well as the active engagement in myogenic regulatory elements, have been shown to have a positive effect in muscle retention.

Competing Interests

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

Acknowledgments

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Review Article

Diet-Intestinal Microbiota Axis in Osteoarthritis: A Possible Role

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Intestinal microbiota is highly involved in host physiology and pathology through activity of the microbiome and its metabolic products. Osteoarthritis (OA) is a common form of arthritis characterized by articular cartilage destruction and osteophyte formation. Although various person-level risk factors, such as age, sex, and obesity, have been proposed for the pathogenesis of OA, the underlying links between these person-level factors and OA are still enigmatic. Based on the current understanding in the crosstalk between intestinal microbiota and these risk factors, intestinal microbiota could be considered as a major hidden risk factor that provides a unifying mechanism to explain the involvement of these person-level risk factors in OA.

1. Intestinal Microbiota

All metazoan, from invertebrates to vertebrates, have intestinal microbiota. Intestinal microbiota is highly associated with various aspects of metazoan physiology, such as development, metabolism, and immunity, and is decidedly involved in the pathogenesis of various metazoan diseases, such as inflammatory bowel disease, obesity, and cancer [1, 2]. Even for some invertebrates that have a relative simple of intestinal microbiota, such as *Caenorhabditis elegans* (one bacterial species) and *Drosophila melanogaster* (5–20 species), the intestinal microbiota still has profound influence in host physiology, like host longevity [1]. The influence of gut microbiota on host physiological functions and pathogenesis of diseases may result from activity of the microbiome and its metabolic products [1]. Although these years have witnessed a compelling progression on the involvement of dysbiosis of intestinal microbiota in rheumatoid arthritis [3], the roles of intestinal microbiota or its metabolic products in the pathogenesis of osteoarthritis (OA) remain to be known.

OA is one of the most common joint disorders worldwide. The exact etiology of OA is still unknown, but various risk factors have been reported, including person-level factors, such as age, sex, obesity, and diet, and joint-level factors, including injury, malalignment, and abnormal loading of the joints [4–6]. Regarding person-level factors, a number of explanations have been proposed to explain the involvement of person-level factors in OA; however, most of these hypotheses have not been demonstrated experimentally, and even some have been challenged by later observational studies and clinical trials. In this paper, we try to explain the involvement of some person-level factors in OA from aspect of intestinal microbiota and try to highlight the importance of intestinal microbiota or its metabolic products in the pathogenesis of OA.

2. Age and OA

Age is regarded as the main predictor of OA [6]; however, the underlying mechanisms behind the increased prevalence and incidence of OA with age remain to be known. Although a number of explanations, including oxidative damage, thinning of cartilage, muscle weakening, and a reduction in proprioception, have been proposed to explain the involvement of age in OA, none of these hypotheses have been demonstrated empirically.

With cell culture-dependent and culture-independent studies, older people show significant difference in gut microbiota from that of younger adults, such as lower diversity of gut microbiota, greater proportion of *Bacteroides* spp., and distinct abundance pattern of *Clostridium* groups [7, 8]. The alteration of gut microbiota may regulate the age-related physiology, such as immune responses, cognitive function,

and organ disorders [7, 8]. For example, dysbiosis of intestinal microbiota highly precedes and predicts age-onset intestinal barrier dysfunction in *Drosophila*, while targeting of the aging-related dysbiosis of intestinal microbiota alleviates these age-related dysfunctions and increases life span in *Drosophila* [9, 10]. Thus, it is utterly possible that the aging-related dysbiosis of intestinal microbiota contributes to age as a risk factor for OA.

3. Gender and OA

It is an interesting observation that the prevalence and severity of OA in hip, knee, and hand are higher in women than in men, and they also increase around menopause [11]. For example, with one study with 4733 subjects in Alberta, Canada, the prevalence of knee OA is 6.3% for males and 8.9% for females, while the prevalence of hip OA is 4.4% for males and 7.6% for females [12]. This discovery suggests presence of sex difference in OA prevalence and incidence, with females generally at a higher risk. This compelling investigation results in a hypothesis that hormonal factors, like oestrogen, have role in the development of OA. However, later conclusions from observational studies and clinical trials challenge this hypothesis [13, 14], and other explanations have been proposed for the discrepancy between men and women in OA, such as differences in volume of cartilage, bone strength, and muscle strength.

Male mice have more bacteria overall in the feces compared to female mice; however, female mice show more significant diurnal oscillation in intestinal microbiota than that of male mice [15], indicating that gender has remarkable influence on intestinal microbiota. Indeed, gender-dependent influence in intestinal microbiota has been observed in various animal models [16], such as fish, mice, and human. For example, with 16S DNA sequencing of intestinal microbiota in fecal samples of 39 men and 36 postmenopausal women with similar dietary background and age, the abundance of *Bacteroides* genus and the abundance of *Bilophila* are lower in men than in women, while higher presence of *Veillonella* and *Methanobrevibacter* genera is observed in men compared to women [17]. Thus, the difference in intestinal microbiota may be responsible for the higher risk of OA in women. Similarly, female mice have higher incidence (1.3–4.4 times) of type 1 diabetes (T1D), compared to male mice [18, 19]. Similar to estrogen therapy which has little effect on OA [13, 14], the protection of males against T1D does not correlate with blood androgen concentration [18]. However, germ-free (GF) mice lack the gender bias, while colonization of GF mice with some lineages of overrepresented microbiota in male mice (i.e., *Enterobacteriaceae* family) restores the gender bias for T1D [18]. Interestingly, transplantation of gut microbiota from adult males to immature females alters the recipient's microbiota, leading to elevated testosterone, reduced islet inflammation and autoantibody production, and finally increased T1D protection [19]. These results show that intestinal microbiota regulates disease fate in individuals; thus it is possible that intestinal microbiota contributes to gender as a risk factor for OA.

4. Obesity and OA

One of the well-known risk factors for OA is obesity [20, 21]. C57BL/6J male mice fed a high-fat diet (HFD) for 12 weeks have greater body weight and also exhibit features consistent with knee OA, compared to the control mice [22]. With 1,764,061 observed subjects, for a median (interquartile range) of 4.45 (4.19 to 4.98) years, overweight or obesity increases the risk of OA at knee, hip, and hand, especially at the knee: overweight and (grades I and II) obesity increase knee OA risk 2-, 3.1-, and 4.7-fold, respectively [23]. With one study with 4733 subjects in Alberta, Canada, obesity (BMI $> 30\,kg/m^2$) is remarkably associated with the prevalence of knee and hip OA [12]. However, the mechanism by which obesity boosts OA is enigmatic. The link between obesity and OA was contributed to excessive joint loading as a result of increased body weight; however, a more complex aetiology for obesity-induced OA has been indicated, such as disturbed lipid metabolism, low-grade inflammation, and adipokines on joint tissues.

It is well known that intestinal microbiota is associated with the establishment and development of obesity. Obesity is associated with phylum-level changes in the microbiota (i.e., ratio of Firmicutes/Bacteroidetes), reduced bacterial diversity, and altered representation of bacterial genes and metabolic pathways [24, 25]. In genetically obese *ob/ob* mice and obese people, ratio of Firmicutes to Bacteroides is augmented, which promotes production of biologically active metabolites, such as short chain fatty acids (SCFAs), including acetate, propionate, and butyrate, from soluble dietary fibers (i.e., fructans) and resistant starch, leading to higher energy extraction from indigestible carbohydrates and to adipogenesis in the liver [24, 25]. As the dysbiosis of intestinal microbiota contributes to obesity and to other obesity-related conditions including insulin resistance and systemic inflammation, it is reasonable that intestinal microbiota is associated with pathogenesis of OA.

5. Diet and OA

Several dietary factors have been reported to be involved in pathogenesis of OA, such as vitamins [26], amino acids [5], and magnesium [27]. However, further studies are needed to better define the association between OA and these dietary factors and to better understand the underlying mechanism for these dietary factors to regulate OA. Intestinal microbiota is highly shaped by dietary nutrients [7, 28, 29]. For example, little amount of single amino acids supplementation (0.5% (w/w) L-arginine or 1.0% (w/w) L-glutamine) has shown significant influence on intestinal microbiota, such as the ratio of Firmicutes/Bacteroidetes [28, 29]. The possible reason for nutrient-induced change of intestinal microbiota is that nutrient alters the microenvironment for intestinal microbiota, such as composition and metabolism of intestinal microbiota, and immune responses of host. Glutamine supplementation promotes mouse intestinal secretory IgA (SIgA) production and IgA^+ plasma cell numbers through T cell-dependent (e.g., IL-5, IL-6, and IL-13) and T cell-independent pathways [e.g., transforming growth factor

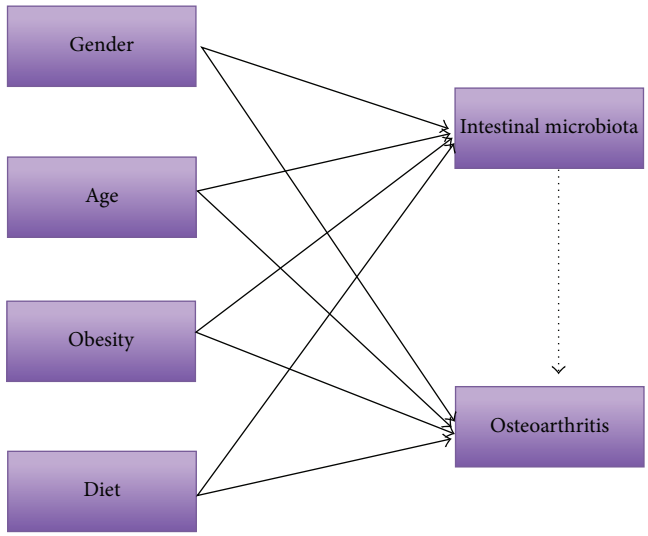

Figure 1: Intestinal microbiota in osteoarthritis (OA). Age, sex, obesity, and diet are risk factors in the etiology of OA (full line) and are associated with the alteration of intestinal microbiota (full line). Thus, it is possible that the intestinal microbiota is involved in the pathogenesis of OA (dotted line).

(TGF-β), a proliferation-inducing ligand (APRIL), and B cell-activating factor (BAFF)] of SIgA induction, which are largely dependent on glutamine's effect on intestinal microbiota [30]. Dietary chitosan supplementation significantly shapes the intestinal microbiota in mouse model [31]. Although chitosan has little effect on the richness indices of intestinal microbiota in mouse jejunum, ileum, and feces, it highly affects the microbiota diversity in the jejunum, ileum, and feces of mouse [31]. Chitosan also alters the component of intestinal microbiota, including lowering the ratio of Firmicutes : Bacteroidetes, decreasing the Bacteroidales in the feces, and increasing the Lactobacillalesin the feces [31]. Indeed, chitosan supplementation decreases mouse body weight through its effect on intestinal microbiota [31]. These interesting investigations indicate that nutrient affects host physiological functions largely dependent on intestinal microbiota. Thus, the nutrients-intestinal microbiota axis may be implicated in pathogenesis of OA. If so, the manipulation of nutrients-intestinal microbiota axis is auspicious to prevent and treat OA. Indeed, oral supplementation of resveratrol has significant anti-OA effects in HFD-induced OA model in mouse through recovery in joint structure and type II collagen expression in cartilage and inhibition in the degradation of type II collagen into C-telopeptide of type II collagen (CTX-II) and chondrocyte apoptosis [22].

6. Conclusion

The intestinal microbiota is profoundly associated with pathogenesis of various diseases, such as inflammatory bowel disease, obesity, and cancer [1, 2]. Here we describe the rationale for the hypothesis that intestinal microbiota is a major hidden risk factor for OA and an important explanation for person-level risk factors in OA (Figure 1). Although bacterial lipopolysaccharide has been suggested as hidden risk factor for OA [32], it is fruitful to explore the OA patient related changes in microbiota composition, bacterial diversity and bacterial genes, and metabolic pathway. The understanding on the association between intestinal microbiota and OA could facilitate the development of new approaches to diagnosing and treating OA. In particular, with the knowledge of intestinal microbiota in pathogenesis of OA, the manipulation of nutrient-intestinal microbiota- bacterial metabolite axis has potentials to prevent and treat OA.

Disclosure

Yusheng Li and Wei Luo are considered as co-first authors.

Competing Interests

The authors declare that there are no competing interests.

Authors' Contributions

Yusheng Li and Guanghua Lei conceived the study. Yusheng Li, Wei Luo, and Zhenhan Deng collected the data. Yusheng Li and Guanghua Lei wrote the paper.

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