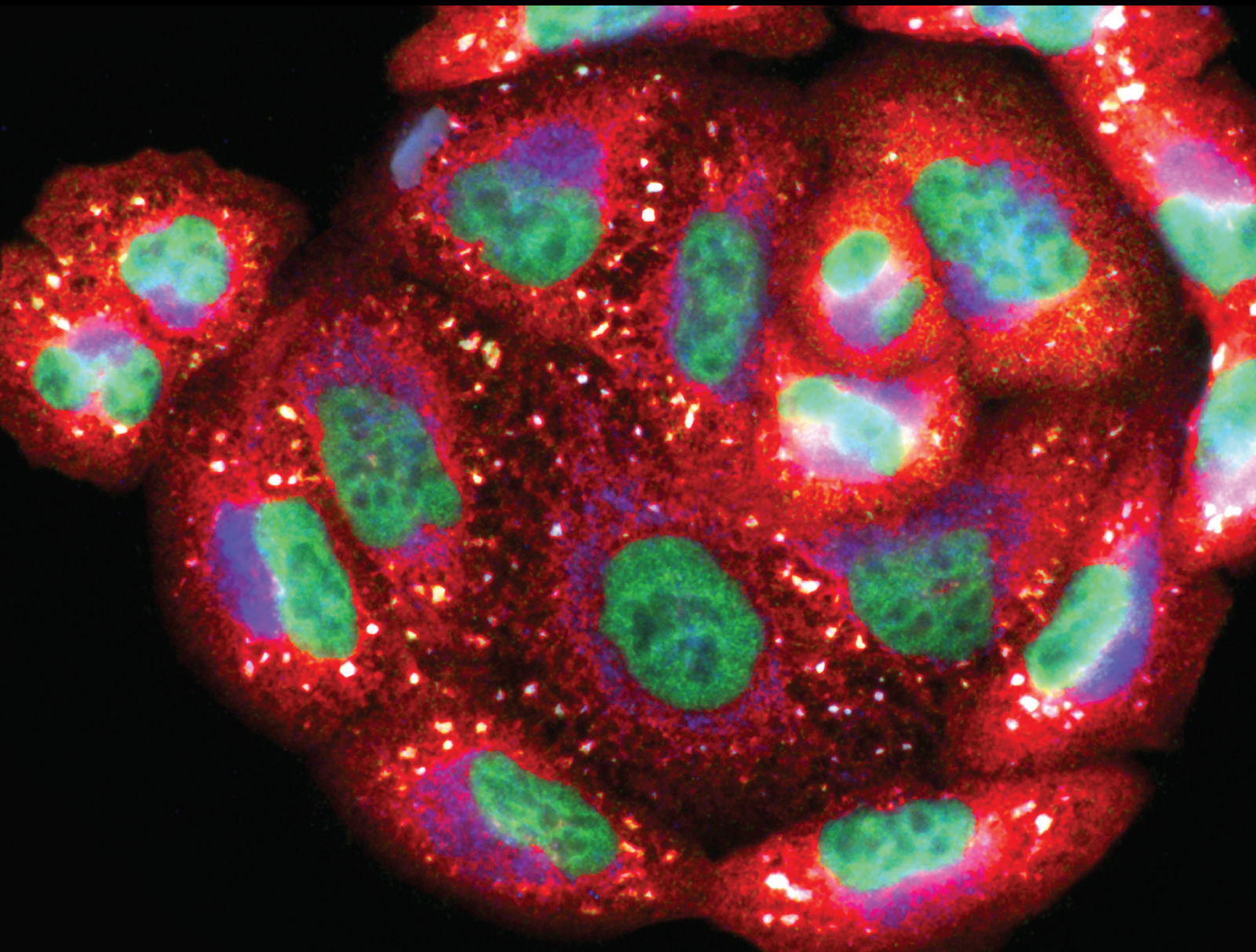


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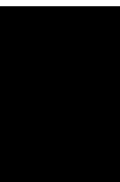
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Oxidative Medicine and Cellular Longevity

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Research Article

In Vitro Modulation of Endogenous Antioxidant Enzyme Activities and Oxidative Stress in Autism Lymphoblastoid Cell Line (ALCL) by Stingless Bee Honey Treatment

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Autism has been associated with a low antioxidant defense mechanism, while honey has been known for decades for its antioxidant and healing properties. Determination of stingless bee honey (KH) effects on antioxidant enzyme activities and oxidative damage in Autism Lymphoblastoid Cell Line (ALCL) was performed. ALCL and its normal sibling pair (NALCL) were cultured in RPMI-1640 medium at 37°C and 5% CO₂. ALCL was treated with 400 µg/mL KH (24 h), and oxidative stress marker, malondialdehyde (MDA), and antioxidant enzyme activities (catalase (CAT), glutathione peroxidase (GPx), and superoxide dismutase (SOD)) were measured via enzyme-linked immunosorbent assay (ELISA), while deoxyribonucleic acid (DNA) damage was determined via comet assay. Low SOD activity ($p < 0.05$) and high MDA level ($p < 0.05$) were observed in ALCL compared to NALCL. Higher grade (Grades 2 and 3) of DNA damage was highly observed ($p < 0.05$) in ALCL compared to NALCL, whereas lower grade (Grades 0 and 1) DNA damage was highly detected ($p < 0.05$) in NALCL compared to ALCL. KH treatment caused a significant increase in SOD and GPx activities ($p < 0.05$) in ALCL compared to untreated ALCL. Correspondingly, KH treatment reduced the Grade 2 DNA damage ($p < 0.05$) in ALCL compared to untreated ALCL. CAT activity showed no significant difference between all three groups, while the MDA level showed no significant difference between treated and untreated ALCL. In conclusion, KH treatment significantly reduced the oxidative stress in ALCL by increasing the SOD and GPx antioxidant enzyme activities, while reducing the DNA damage.

1. Introduction

Autism spectrum disorder (ASD), also recognized as autism, is an extremely heritable disorder. It is also known as a heterogeneous neurodevelopmental disorder with impaired social, verbal, or nonverbal interaction; sensory anomalies; stereotyped and repetitive behaviors or interests; and varying levels of intellectual disability [1]. Medical comorbidities such as epilepsy, sleep disturbances, gastrointestinal dysfunction, immune dysfunction, and mental retardation may accompany the core behavioral abnormalities mentioned [2].

The prevalence of ASD was estimated to be 1 in 160 children worldwide [3]. Up to the present, various aspects from environmental factors to epigenetic perspective and immunological relativity had been linked to autism. However, no valid determinant had been approved as the underlying cause of ASD [4]. Excessive oxidative stress and antioxidant defense mechanism impairment had been correlated with autism [4]. Oxidative damages triggered by the imbalance of the excessive production of reactive oxygen species and weaken antioxidant defense may contribute to the pathogenesis of the disorder and clinical signs and symptoms [4].

Regulation of redox balance is the main focus of current reports on autism and antioxidant therapy as the main player. Clinical trials had been carried out by using various kinds of antioxidants such as vitamin C, glutathione, fish oil, melatonin, carnosine and zinc, and vitamin B6-magnesium combination [4]. It was reported that the treatment with high doses of vitamin C improved the behavioral symptoms of autism [5]. Other than antioxidants, nutraceutical supplementation on ASD individuals such as dietary fatty acids, herbal preparation, and probiotics also showed convincing outcomes on reducing the progress of ASD [6].

The correlation of redox imbalance and potential redox therapy with ASD pathogenesis inspired the interest in studying *kelulut* honey (KH), a natural product, on autism cell lines. Widely considered as having antioxidant properties, the total antioxidant activity of honey is contributed by its complex chemical components such as phenols, flavonoids, phenolic acids, proteins, amino acids, and vitamins [7]. Honey is also reported to exhibit other therapeutic activities such as antimicrobial, anti-inflammatory, antineoplastic, wound healing, and protective effects on the liver and cardiovascular system [8]. In this study, the modulatory effect of KH antioxidant properties on oxidative stress levels in Autism Lymphoblastoid Cell Line (ALCL) was determined by measuring the endogenous antioxidant enzyme activities and DNA damages in cells.

Trigona spp. is the bee species involved in the production of KH. Derived from multiflora sources, KH was reported to be ten times higher in antioxidant properties [9, 10]. Reports from the previous studies showed that the total phenolic content (TPC) of the KH was higher compared to New Zealand manuka honey [9, 10] and the total flavonoid content (TFC) was higher from Portuguese heather honey and Spanish rosemary honey [10, 11]. Additionally, ferric-reducing antioxidant potential (FRAP) assay showed that the antioxidant activity of KH was higher than that was found in New Zealand manuka honey [12].

2. Materials and Methods

2.1. KH Sample. The KH sample was purchased from Persatuan Usahawan Lebah Kelulut Darul Naim (DRONESS), Kelantan, Malaysia. KH was produced by *Trigona* spp. (a stingless bee species) and produced from a multiflora source. The honey was obtained from Kelantan, Malaysia, where the beekeepers are the community of the DRONESS. The optimum dose of KH at 400 $\mu\text{g}/\text{mL}$ was obtained from the previous viability assay (unpublished data) and used as the treatment on ALCL. The honey sample was prepared in the culture medium on the day of the assay.

2.2. Cell Culture. Three variants of Autism Lymphoblastoid Cell Line (ALCL) were used as the autism model and its non-affected siblings (NALCL) for the normal control. Cell lines were provided by the "Autism Genetic Resource Exchange" (AGRE; Los Angeles, CA, USA). The cell lines were cultured in the "Roswell Park Memorial Institute" (RPMI) 1640 media (Sigma-Aldrich, German) supplemented with 15% (v/v) fetal bovine serum (FBS; Sigma-Aldrich, German), 2 mM L-

glutamine (Gibco, USA), 100 U mL⁻¹ penicillin, and 100 U mL⁻¹ streptomycin (Gibco, USA) at 37°C and 5% CO₂. The cell lines used were from the 8th to the 10th passage. The cells were divided into three groups: ALCL, KH-treated ALCL, and NALCL. Oxidative stress marker such as malondialdehyde (MDA) and antioxidant enzyme activities such as catalase (CAT), glutathione peroxidase (GPx), and superoxide dismutase (SOD) were measured using specific "enzyme-linked immunosorbent assay (ELISA) kits" (Cayman Chemical, USA) while deoxyribonucleic acid (DNA) damage was measured using "comet assay" (Sigma-Aldrich, USA). All methods mentioned below were based on the manufacturer's protocols.

2.3. Superoxide Dismutase (SOD) Assay. Cells were collected by 10 min centrifugation (1,000–2,000 $\times g$) (4°C). The cell pellet was homogenized and sonicated in cold 20 mM HEPES buffer (pH 7.2) (70 mM sucrose, 210 mM mannitol, and 1 mM EGTA). It was then centrifuged at 1,500 $\times g$ (5 min) (4°C). The supernatant was used for the analysis.

The reactions were started by adding 20 μL xanthine oxidase (diluted) to the samples. The procedure was done according to the manufacturer's protocols. The readings were recorded at 440–460 nm.

2.4. Glutathione Peroxidase (GPx) Assay. Cells were collected by centrifugation (1,000–2,000 $\times g$) (10 min) (4°C). Pellet was homogenized in a cold buffer and centrifuged at 10,000 $\times g$ (15 min) (4°C). The supernatant was used for the analysis.

The samples were concentrated using an Amicon centrifuge concentrator to achieve the optimum enzymatic activity. The reactions were started by adding 20 μL of cumene hydroperoxide to the samples. The procedure was done according to the manufacturer's protocols. The readings were recorded at 340 nm (five time points).

2.5. Catalase (CAT) Assay. Cells were collected by centrifugation (1,000–2,000 $\times g$) (10 min) (4°C), homogenized and sonicated on ice in 1–2 mL of cold buffer. It was then centrifuged (10,000 $\times g$) (15 min) (4°C). The supernatant was used for analysis.

The reactions were started by adding H₂O₂ to the samples. The procedure was done according to the manufacturer's protocols. The readings were recorded at 540 nm.

2.6. Malondialdehyde (MDA) Assay. 2 $\times 10^7$ cells were collected in 1 mL of cell culture media. The cells were homogenized and sonicated on ice, and the whole homogenate was used in the assay. Cell lysates do not need to be diluted before assaying.

100 μL of sample and 100 μL SDS solution were added to 5 mL vial. 4 mL of the color reagent was added to the sides of vials and placed on a floating foam tube holder. The vials were added to boiling water (1 h) followed by an ice bath (10 min). The procedure was done according to the manufacturer's protocols. The readings were recorded at 530–540 nm.

2.7. Comet Assay. Comet assay was done to determine DNA damage using "single-cell gel electrophoresis" (SCGE). Slides were covered with 0.6% of "Normal Melting Agarose"

(NMA), a mixture of cell suspension, 0.6% of “Low Melting Agarose” (LMA), and 0.6% of LMA (without cell). The solidification process was done at 4°C. Slides were then immersed in the “lysing buffer” (2.6 M NaCl, 100 mM Na₂EDTA, 10 mM Tris, and 1% Triton-X, pH 10) (4°C) (1 h).

Before electrophoresis, the slides were left in the electrophoresis solution (pH 13) (20 min). Electrophoresis was done at 4°C (20 min) (25 V and 0.3 A). Finally, the slides were neutralized, stained with ethidium bromide, and analyzed using a fluorescent microscope. The degree of DNA damage is graded into five groups based on the amount of the DNA in the tail (Table 1).

2.8. Statistical Analysis. All statistical analyses were performed using SPSS version 23.0 with statistical significance set at $p < 0.05$. Differences between the variables between ALCL, KH-treated ALCL, and NALCL were compared using one-way ANOVA. Three variants of ALCL were used, and all assays were performed in triplicate, with 24 h incubation time of KH treatment.

3. Results

3.1. Endogenous Antioxidant Enzyme Activities in ALCL and KH Treatment. CAT activity showed no significant difference between the normal group of cell lines (NALCL) and the autism group (ALCL; $p > 0.05$; Figure 1). KH treatment on ALCL for 24 h incubation time showed no significant difference with untreated ALCL ($p > 0.05$). All three groups of cell lines showed no significant difference in CAT activity ($p > 0.05$).

SOD activity was found to be significantly lower in the autism cell lines (ALCL) compared to NALCL ($p < 0.05$; Figure 2). Interestingly, the SOD activity was successfully restored and significantly increased with 24 h KH treatment relative to the untreated ALCL and NALCL groups ($p < 0.05$).

GPx activity in NALCL did not significantly differ from the ALCL group ($p > 0.05$; Figure 3). On the contrary, the KH-treated ALCL group showed a significant increase in GPx activity relative to untreated ALCL with 24 h treatment ($p < 0.05$).

3.2. Oxidative Damages in ALCL and KH Treatment. MDA concentrations were found to be significantly higher in the autism group (ALCL) than the normal group, NALCL ($p < 0.05$; Figure 4). However, MDA concentration in treated ALCL did not significantly decrease with KH treatment compared to the ALCL and NALCL groups ($p > 0.05$).

Figure 5 represents the outcomes of DNA damage and demonstrates that Grade 0 (no DNA damage) and Grade 1 (low-level DNA damage) are significantly higher in the normal cell lines (NALCL) compared to the autism cell lines, ALCL ($p < 0.05$). However, Grade 2 (medium-level DNA damage) and Grade 3 (high-level DNA damage) are significantly higher in ALCL ($p < 0.05$). Interestingly, the 24 h KH treatment on ALCL significantly reduced the Grade 2 DNA damage compared to the untreated group ($p < 0.05$).

TABLE 1: Grades of DNA damage.

Grade of genotoxicity	DNA damage
Grade 0 (no damage)	<5%
Grade 1 (low-level damage)	5%–20%
Grade 2 (medium-level damage)	21%–40%
Grade 3 (high-level damage)	41%–95%
Grade 4 (total damage)	>95%

4. Discussion

4.1. Modulation of Endogenous Antioxidant Enzyme Activities in ALCL by KH. SOD, CAT, and GPx play a vital role in protecting cells from oxidative stress [4]. They are the three antioxidant enzymes measured in this study. During the oxidative chain, superoxide is the primary ROS produced and will be further converted to hydrogen peroxide (H₂O₂) by SOD and to water by CAT and/or GPx activities [4]. The vital functions of endogenous antioxidant defense are to halt the ROS production and deactivate the propagated ROS [4]. Oxidative stress is the consequence of the imbalance between the uncontrolled production of ROS and the compromised defense mechanism which eventually caused oxidative damages [4].

Previous studies have reported that the CAT activity is lower in erythrocytes of autistic patients. Other studies showed the reduction of the total GSH levels and altered GPx, SOD, and CAT activities in patients with autism compared to the controls [4]. In this study, the three vital antioxidant enzymes CAT, SOD, and GPx were measured to determine the differences of activities in three groups of cell lines. The findings that showed no significant difference in CAT activities between the ALCL and NALCL groups were in line with the other findings from a study conducted on Saudi autistic children [13]. Nonetheless, KH treatment failed to enhance the activity of CAT in ALCL.

A study showed that a higher level of H₂O₂ (a type of ROS) was generated in ASD children, as indicated by the decrease of superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities in erythrocytes [14]. Our data revealed that the activity of SOD was significantly lower in ALCL compared to NALCL. Interestingly, the SOD activity could be restored and significantly increased with KH treatment. The ability of KH to regenerate the SOD activity in the autism cell lines could be contributed by the antioxidant capacity of the honey. Reports from other studies using an antioxidant source of treatments such as boiled camel milk [15] and vitamin C and E [16] similarly showed a significant increase in SOD activity in ASD and sickle cell patients, respectively. Likewise, hesperetin and nanohesperetin (antioxidant) treatments also showed enhanced SOD expression and ameliorated social behavior deficits in autistic Wistar-Albino rats [17].

Based on the previous studies, autistic patients showed a significantly lower plasma GPx than controls [18, 19]. On the contrary, Thomas et al. reported that the GPx activity in sickle cell patients was significantly increased after they received vitamin C and E antioxidant treatments [16]. Camel

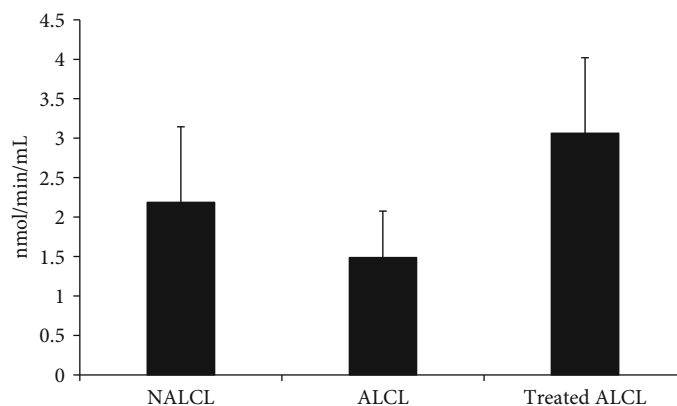


FIGURE 1: CAT activities in different groups of cells, normal (NALCL), autism (ALCL), and KH-treated ALCL. Results are expressed as mean \pm SD.

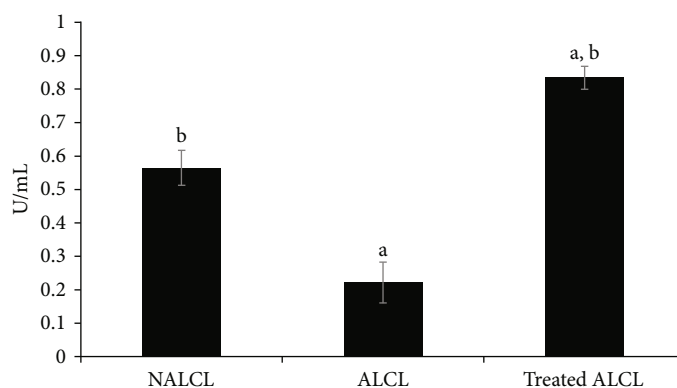


FIGURE 2: SOD activities in different groups of cells, normal (NALCL), autism (ALCL), and KH-treated ALCL. Results are expressed as mean \pm SD. ^aDenotes $p < 0.05$ compared to NALCL. ^bDenotes $p < 0.05$ compared to ALCL.

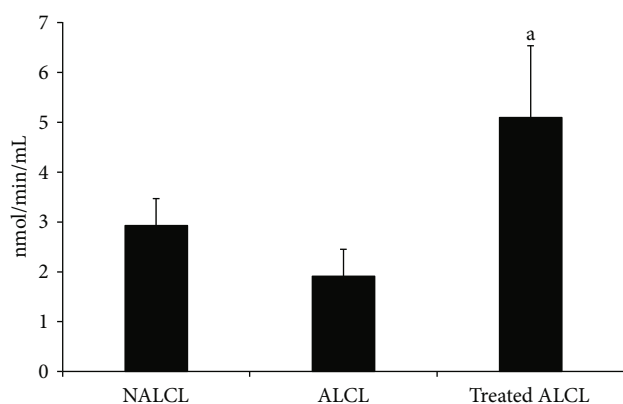


FIGURE 3: GPx activities in different groups of cells, normal (NALCL), autism (ALCL), and KH-treated ALCL. Results are expressed as mean \pm SD. ^aDenotes $p < 0.05$ compared to ALCL.

milk and hesperetin intervention also showed significant increment levels in GPx and enhanced GPx expression in autistic patients and autistic Wistar-Albino rats, besides improving the autistic behavior [15, 17]. Similar findings were determined in this study, where GPx activity was found to be significantly higher after 24h KH treatment in the autism cell lines, ALCL. The substantial increase in GPx

and SOD activities in the treated autism cell lines may indicate the capacity of KH in modulating and restoring the activity of the endogenous antioxidant enzymes in autism cell lines.

4.2. Oxidative Stress and the Reduction of DNA Damage following KH Treatment in ALCL. Lipids especially polyunsaturated fatty acids (PUFA) are oxidized by ROS which further initiating oxidative damage [4]. Malondialdehyde (MDA) is the product of lipid oxidation that has been established as an oxidative damage marker [4]. Numerous studies have indicated increased oxidative stress levels in individuals with ASD [20]. In addition to the elevation of serum lipid peroxides, the markers of lipid peroxidation, and urinary isoprostane levels [20], elevated levels of cytokines and xanthine oxidase have also been noted in the blood circulation of autistic patients, both of which generate ROS [20].

MDA concentration has been reported to be significantly higher in autism [19, 21]. Plasma thiobarbituric acid-reacting substances (TBARS), which include MDA, were significantly higher in autism. These findings support the hypothesis that excessive ROS production and oxidative damages correlated with ASD [4]. Similarly, in our study, the MDA concentration was significantly higher in ALCL than in NALCL. This data revealed that autistic individuals have significantly

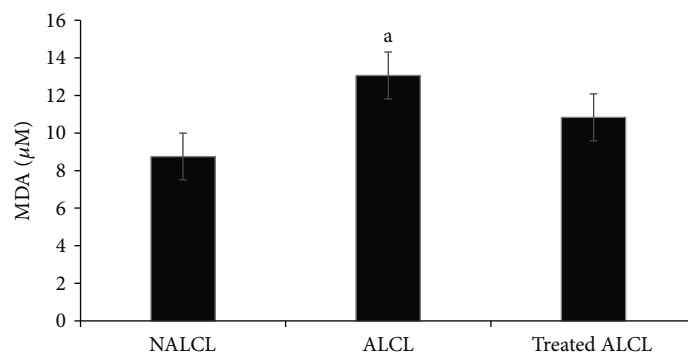


FIGURE 4: MDA concentrations in different groups of cells, normal (NALCL), autism (ALCL), and KH-treated ALCL. Results are expressed as mean \pm SD. ^aDenotes $p < 0.05$ compared to NALCL.

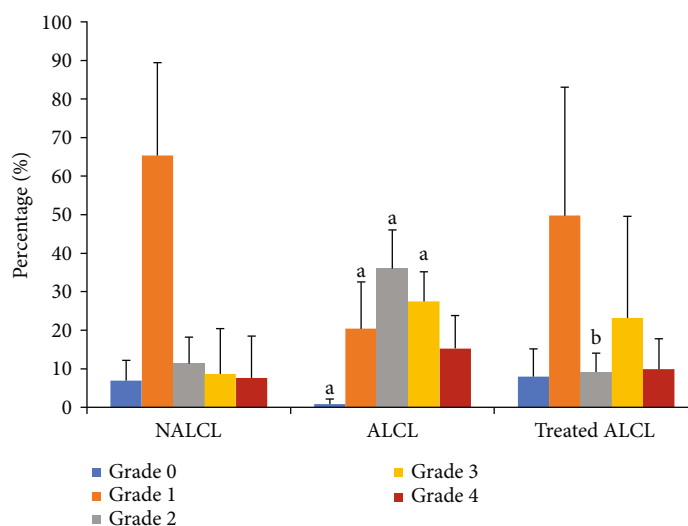


FIGURE 5: Percentage of DNA damage in different groups of cells, normal (NALCL), autism (ALCL), and KH-treated ALCL. Results are expressed as mean \pm SD. ^aDenotes $p < 0.05$ compared to NALCL. ^bDenotes $p < 0.05$ compared to ALCL.

higher oxidative stress than a normal person. However, the treatment with KH showed no significant difference in the MDA level between treated ALCL and the untreated group. We hypothesized that the significant effect could be accomplished with the adjustment of incubation time and doses of KH.

Other than lipids, oxidative damage occurs on nucleic acids which caused destructions on DNA strands such as breaks, protein crosslinking, and mutations [4]. Our data disclosed a significantly more severe DNA damage of Grades 2 and 3, indicating higher levels of DNA damage occurring in the autism cells than the normal cells. These results were congruent with the previous findings that reported the incidence of DNA oxidation damage and a deficit in antioxidant capacity in the plasma of ASD children compared to nonaffected siblings [22]. Interestingly, KH treatment successfully decreased the DNA damage (Grade 2) in ALCL after 24 h of incubation time.

Studies showed that manuka honey (MH) treatment protects macrophages induced with LPS (lipopolysaccharides) from oxidative damages [23]. ROS and nitrite production were inhibited; inflammatory markers were suppressed; pro-

teins, lipids, and DNA of the cells were preserved; and antioxidant enzyme activities were restored in the macrophages [23]. Other data on MH also reported that supplementation with MH reduced DNA damage and MDA in the liver of young and middle-aged rats [12]. However, up to the present, no report was found concerning the effect of honey treatment especially stingless bee honey (KH) on the autism cells pertaining to oxidative stress and DNA damage.

Gelam honey (GH) is also reported to modulate endogenous antioxidant activities in rats [24]. GH increased the erythrocyte CAT and cardiac SOD activities in young and aged groups. DNA damage and MDA level, which increased in the aged rats, were reduced with GH supplementation [24]. Several studies on different types of honey treatment revealed the protective effects of honey on cisplatin- (CP-) induced oxidative damages. Oxidative stress markers (MDA and iNOS) were reduced, while antioxidant enzymes (SOD, GSH, and CAT) were increased with honey treatment on CP-induced oxidative stress [25].

Oxidative stress, which occurs from the cumulative impact of multifactor, can lead to neuronal damage and possibly play a critical role in the pathogenesis of ASD. A

significantly lower concentration of GPx and SOD in the ASD group may indicate a reduced capacity of H₂O₂ neutralization and another ROS. This condition subsequently causes severe damage to the macromolecules. Reduced enzyme activities could result from (1) deficient production and/or (2) excessive consumption during the process to neutralize free radicals. Meanwhile, the greater oxidative damage occurring in autism is most likely due to (1) excessive oxidative stress and/or (2) the failure of restoring the endogenous antioxidant enzyme activity in response to stress. These collective impacts will probably contribute to the pathogenesis of autism.

Treatment with KH on autism cells effectively reduced the oxidative stress by modulating the increment of the endogenous enzymes, SOD and GPx activities, while reducing the oxidative stress and damages indicated by the decline of Grade 2 DNA damage. The increased endogenous enzyme activities exhibited with KH treatment could be due to the restoring effects of antioxidant enzymes in autism cells to neutralize H₂O₂ and accumulated ROS, thus protecting the cells from further oxidative damages to molecules.

5. Conclusions

KH, an antioxidant-rich natural product, has a high potential in alleviating oxidative stress, reducing the oxidative damage, and may help to improve the symptoms and problems of ASD.

6. Limitations and Future Directions

In vitro study is subject to certain limitations, and one of them is the inability to assess behavior and the variations in ASD characteristics. Another technical limitation is the limited number of cell lines used. These drawbacks limit the study for nonpersonalized treatment.

Significant findings from this study will be a reasonable justification for expanding the work to elucidate the protective effects of KH on cells under the ROS-challenged environment. ROS generation and the possible involvement of mitochondria would be the impactful parameters to be tackled in the future.

Data Availability

All data underlying the findings of the study are attached in this manuscript.

Conflicts of Interest

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

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Review Article

The Gut Microbiota and Oxidative Stress in Autism Spectrum Disorders (ASD)

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Autism spectrum disorders (ASDs) are a kind of neurodevelopmental disorder with rapidly increasing morbidity. In recent years, many studies have proposed a possible link between ASD and multiple environmental as well as genetic risk factors; nevertheless, recent studies have still failed to identify the specific pathogenesis. An analysis of the literature showed that oxidative stress and redox imbalance caused by high levels of reactive oxygen species (ROS) are thought to be integral parts of ASD pathophysiology. On the one hand, this review aims to elucidate the communications between oxidative stress, as a risk factor, and ASD. As such, there is also evidence to suggest that early assessment and treatment of antioxidant status are likely to result in improved long-term prognosis by disturbing oxidative stress in the brain to avoid additional irreversible brain damage. Accordingly, we will also discuss the possibility of novel therapies regarding oxidative stress as a target according to recent literature. On the other hand, this review suggests a definite relationship between ASD and an unbalanced gastrointestinal tract (GIT) microbiota (i.e., GIT dysbiosis). A variety of studies have concluded that the intestinal microbiota influences many aspects of human health, including metabolism, the immune and nervous systems, and the mucosal barrier. Additionally, the oxidative stress and GIT dysfunction in autistic children have both been reported to be related to mitochondrial dysfunction. What is the connection between them? Moreover, specific changes in the GIT microbiota are clearly observed in most autistic children, and the related mechanisms and the connection among ASD, the GIT microbiota, and oxidative stress are also discussed, providing a theory and molecular strategies for clinical practice as well as further studies.

1. Introduction

ASD, a loose umbrella term that includes a series of life-long heterogeneous clusters of neurodevelopmental disorders, is characterized by stereotyped behavior and deficits in social communication, interaction, and perception [1]. It is well documented that ASD occurs in all racial, ethnic, and socioeconomic groups. Last updated on July 11, 2016, the US Centers for Disease Control and Prevention (CDC <https://www.cdc.gov/>) reported an incidence of 1 in 54 children worldwide; in addition, studies in Asia, Europe, and North America have identified individuals with ASD and shown an average prevalence of between 1% and 2% (see Table 1). Additionally, it is conservatively estimated that the preva-

lence of ASD in China is 1%, among which more than 2 million children aged 0-14 years have the disease [2]. Undoubtedly, ASD, considered a hidden disability, creates an enormous burden on individuals, families, and society [3]. Regrettably, there is no practical and targeted treatment for ASD, which has become a major worldwide health problem [4].

Although the causes of ASD remain unclear, many studies have pointed to the possible link between ASD and multiple environmental as well as genetic risk factors [5]. Of note, much evidence indicates that oxidative stress plays a vital role in the pathophysiology of nervous and mental diseases, particularly in ASD [6–9]. Anecdotal reports have suggested that oxidative stress response is crucially important

TABLE 1: Summary of the Prevalence of ASD in different areas.

Country	Age range studied	Number of children in population	Criteria used	Methodology used	ASD prevalence (CI)
USA	8	346,978	DSM-IV	Case enumeration and record review	14.6 (8.2-24.6)
Faroe Islands	7 to 16	7122	DSM-IV, ICD-10	Screening and direct exam	5.6
Denmark	N/A	404,816	DSM-IV	Case enumeration	6.9 (6.5-7.2)
Oman	0 to 14	798,913	DSM-IV	Case enumeration	0.1 (0.1-0.2)
Taiwan	0 to 18	372,642	ICD-9	Case enumeration	2.9
South Korea	7 to 12	55,266	DSM-IV	Case enumeration from survey and direct exam	26.4 (19.1-33.7)
Western Australia	N/A	152,060	DSM-IV	Case enumeration	5.1 (4.7-5.5)

Note: data are from the US Center for Disease Control and Prevention (CDC <https://www.cdc.gov/>). N/A: not applicable, i.e., the lack of data in a form or table.

to the neuroinflammatory response, and in a sense, the neuroinflammatory response has always been regarded as one of the pathogenic factors of ASD [10]. A sibling control study by Shannon et al. suggested that autistic children are more susceptible to oxidative stress on account of an imbalance in glutathione levels inside or outside of cells and a decrease in glutathione (GSH) storage capacity [11].

In recent years, as research continues, the research focus of the intestinal microbiota has been rapidly shifting from the abundance and diversity of microbial members to functional aspects. A variety of studies have concluded that the GIT microbiota influences many aspects of human health, including metabolism, the immune and nervous systems, and the mucosal barrier. In addition, the intestinal microbial fermentation of dietary fibres and resistant starch produces short-chain fatty acids (SCFAs), especially butyrate, propionate, and acetate [12]. Notably, propionate can result in GIT metabolic disturbance, reversible behavioral and signalling changes, neuroinflammation, etc. [13]. Therefore, not surprisingly, increasing evidence supports that autistic children are more likely to experience problems related to GIT, including food allergies (FAs), dysbiosis, inflammatory bowel disease (IBD), and indigestion [14, 15]. The GIT dysfunction in autistic children is related to mitochondrial redox imbalance, i.e., mitochondrial dysfunction, and there is an interaction between oxidative stress and mitochondrial dysfunction [16, 17]. Additionally, some connections exist between these factors (see Figure 1). The specific associations are discussed below.

In this review, many aspects of the role of oxidative stress and GIT microbiota in ASD are described. Furthermore, we will discuss, in the context of the most recent literature, the connections among oxidative stress, ASD, and GIT microbiota. Finally, the possibility of oxidative stress and GIT microbiota as therapeutic targets will be discussed, which will provide theoretical basis and novel strategies for clinical practice and future studies.

2. Oxidative Stress and ASD

2.1. The Definition of Oxidative Stress and the Importance/Role of Reactive Oxygen Species (ROS). Oxidative stress, considered

an out-of-balance state between antioxidants and antioxidants, could lead to the cellular damage caused by reactive ROS or reactive nitrogen species (RNS) [18]. ROS, a kind of signalling molecule, may contribute to cell viability and tissue oxygen metabolism or inhibit the expression of related genes, including antioxidants [19, 20]. When the level of oxidation exceeds the antioxidant defenses, oxidative stress occurs; in addition, different conditions may result in different levels of oxidative stress [21, 22]. On the one hand, when ROS levels are relatively low, cells can appropriately respond to oxidative stress, a condition called “mild oxidative stress” or eustress. On the other hand, the condition that is more reactive to oxidative stress is termed distress, which is reported to be one of the principal causes of neuroinflammation and damage to astrocyte crosstalk resulting in ASD [23, 24].

In light of published references, under normal circumstances, there is a dynamic balance between the production of ROS and the antioxidant capacity of cells [25]. Furthermore, ROS are intermediate products and by-products that are produced in the electron transport in mitochondria [26, 27]. Notably, many enzymes, including superoxide dismutase (SOD) and glutathione peroxidase (GPx), have a high ROS scavenging capacity [8]. However, the specific role of ROS in ASD is still far from understood.

2.2. Blood Oxidative Stress Markers in Autistic Children. An increasing amount of evidence has shown that the pathogenesis of ASD is related to the accumulation of oxidized products and the disorder of antioxidant metabolism [28–30]. The role of oxidative stress in the development of ASD has been studied for decades, and a very large number of markers, as revealed by comparing blood levels of oxidative stress markers in autistic patients to those of healthy individuals, have been the focal point of clinical practice (see Table 2) [31–38]. As shown, the blood levels of oxidative stress markers were all decreased or increased to varying degrees in autistic patients compared with healthy controls, indicating that these markers may be used not only to diagnose ASD but also to provide indicators to monitor and guide individual therapy in the clinic [28]. However, the ability to implement these measures as soon as possible is still far from

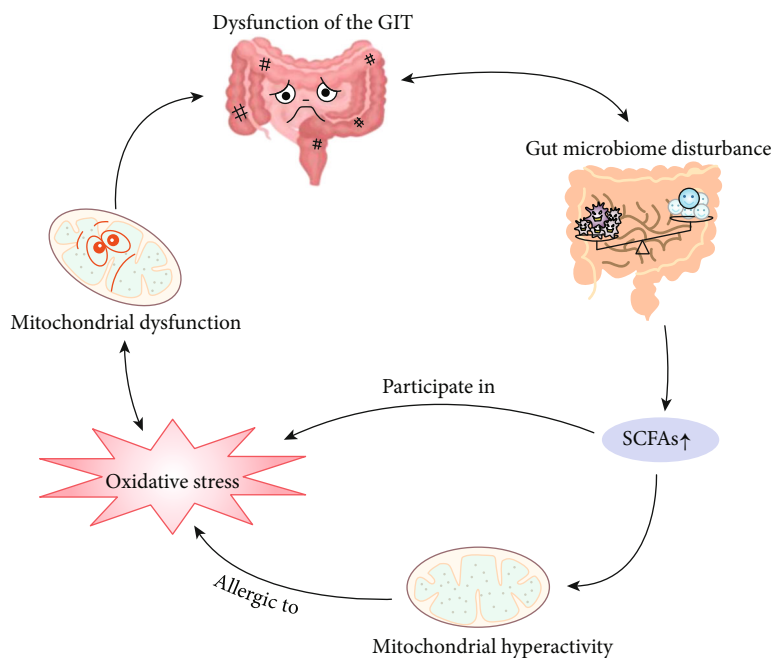


FIGURE 1: The connections among oxidative stress, mitochondrial dysfunction, and dysfunction of GIT in autistic children. The dysfunction of GIT in autistic children is related to mitochondrial dysfunction, and there is an interaction between oxidative stress and mitochondrial dysfunction. SCFAs, metabolites of the GIT microbiota, not only participate in the reaction process of oxidative stress but also can result in mitochondrial hyperactivity and further make mitochondria allergic to the oxidative stress.

TABLE 2: Blood levels of oxidative stress markers in autistic patients.

Marker/specimen	Units	Values ASD		p value	Reference
		Autistic children	Controls		
Lipid hydroperoxide (LOOH) in the temporal cortex	mmol/mg protein	About 21 [#]	About 15 [#]	<0.05	[31]
Plasma malondialdehyde (MDA)	nmol/mL (mean ± SD)	4.16 ± 1.67	1.49 ± 0.58	<0.001	[32]
Serum malondialdehyde (MDA)	nmol/mL (mean ± SD)	8.6 ± 0.5	1.76 ± 0.33	≤0.001	[33]
RBC thiobarbituric acid reactive substances (TBARS)	mmol/g Hb (mean ± SD)	0.032 ± 0.0077	0.015 ± 0.0033	<0.001	[32]
Plasma protein carbonyl	nmol/mL (mean ± SD)	4.202 ± 0.3912	2.256 ± 0.148	<0.0001	[34]
Serum 8OHdG	ng/mL (mean ± SD)	13.134 ± 1.33	1.46 ± 0.326	≤0.001	[33]
Plasma glutathione peroxidase (GPx)	U/L (mean ± SD)	40.9 ± 11.3	24.2	<0.0001	[35]
Serum catalase (CAT)	UAE/L (mean ± SD)	2.836 ± 0.479	0.689 ± 0.157	≤0.001	[33]
RBC catalase (CAT)	k/g Hb (mean ± SD)	209.31 ± 61.92	515.77 ± 127.9	<0.001	[32]
RBC superoxide dismutase (SOD)	U/g Hb (mean ± SD)	2123.59 ± 543.53	971.31 ± 239.14	<0.001	[32]
Plasma reduced glutathione (GSH)	μmol/L (mean ± SD)	3.1 ± 0.53	4.2 ± 0.72	<0.0001	[36]
Plasma glutathione (GSH)	μmol/L (mean ± SD)	3.14 ± 0.56	4.2 ± 0.72	<0.0001	[37]
Plasma oxidized glutathione (GSSG)	nmol/L (mean ± SD)	0.48 ± 0.16	0.35 ± 0.05	<0.001	[37]
MT-1A expression in blood	N/A	Higher (no data available)	N/A	≤0.001	[38]

Note: the data are from references 31–38. RBC: C red blood cell; SD: standard deviation. [#]Values were estimated from the figure. N/A: not applicable, i.e., the lack of data in a form or table.

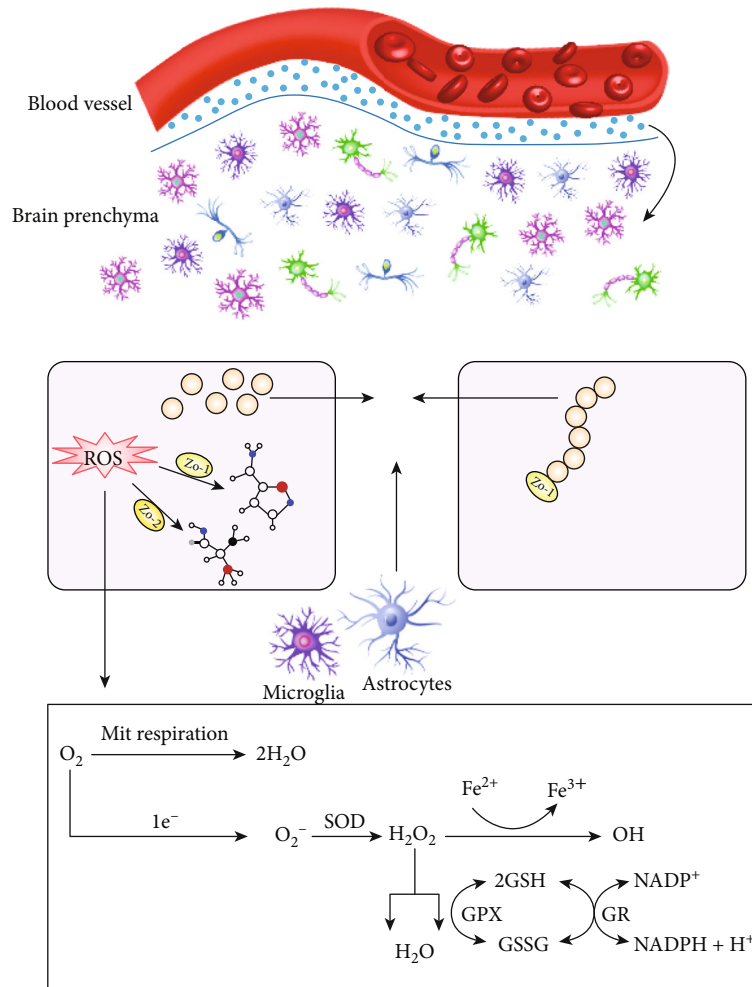


FIGURE 2: Schematic representation of oxidative stress in the brain.

fully accomplished and requires further insightful evidence, clinical reports, and research studies.

2.3. Oxidative Stress in the Brain of Autistic Children. It is well documented that oxidative stress is a primary potential cause of neuroinflammatory disorders and damage to the blood-brain barrier (BBB), a highly selective boundary that separates circulating blood from the brain and extracellular fluid from the central nervous system (CNS) [39–41]. In healthy individuals, the BBB is formed by tight junctions between tight junction proteins and the endothelial cells of adjacent brain capillaries; then, the tight junction proteins are fixed in the endothelial cells by scaffolding proteins such as ZO-1 and ZO-2 [42]. When oxidative stress occurs in endothelial cells in autistic children, the BBB may be damaged, resulting in varied diffusion and transport [43]. Under normal conditions, ROS from various sources, including mitochondria, microglia, and astrocytes, accumulate. When excessive ROS are not properly scavenged, tight junctions can be altered, leading to further oxidative stress (see Figure 2) [44]. To combat this condition, some mechanisms are required to detoxify or neutralize the oxygen/nitrogen free radicals in the cell (see Figure 2). Devasagayam et al. found that super-

oxide (O_2^-) could be produced as a by-product of normal metabolism; nevertheless, its accumulation may lead to the injury of cell structures and subsequently to oxidative stress [45]. As a result, superoxide enzymes called superoxide dismutase (SODs) immediately convert superoxide to hydrogen peroxide (H_2O_2). Moreover, H_2O_2 is likely to be toxic to the cells as it can pass through cell membranes, thus damaging DNA. For this reason, scavenging hydrogen peroxide can be a target to disturb oxidative stress and may be a therapeutic intervention for ASD [46, 47]. According to the study by Popa et al., catalase and GPx are considered the most vital enzymes that have the ability to convert H_2O_2 to H_2O . In addition, the tripeptide glutathione, an important antioxidant, plays a vital role in eliminating ROS [46]. Under a reaction catalysed by glutathione peroxidase (GPx), glutathione provides an electron to H_2O_2 and is then converted to an oxidized state. By making use of NAD(P)H as the electron donor, glutathione (GSH) could be reproduced again by glutathione reductase. Additionally, glutathione can also obliterate toxic substances from the cells in its role as a cofactor for GSH transferase [46, 48]. In general, the brain is very sensitive to the accumulation of radicals such as ROS on account of the relatively weak protective mechanisms [49, 50].

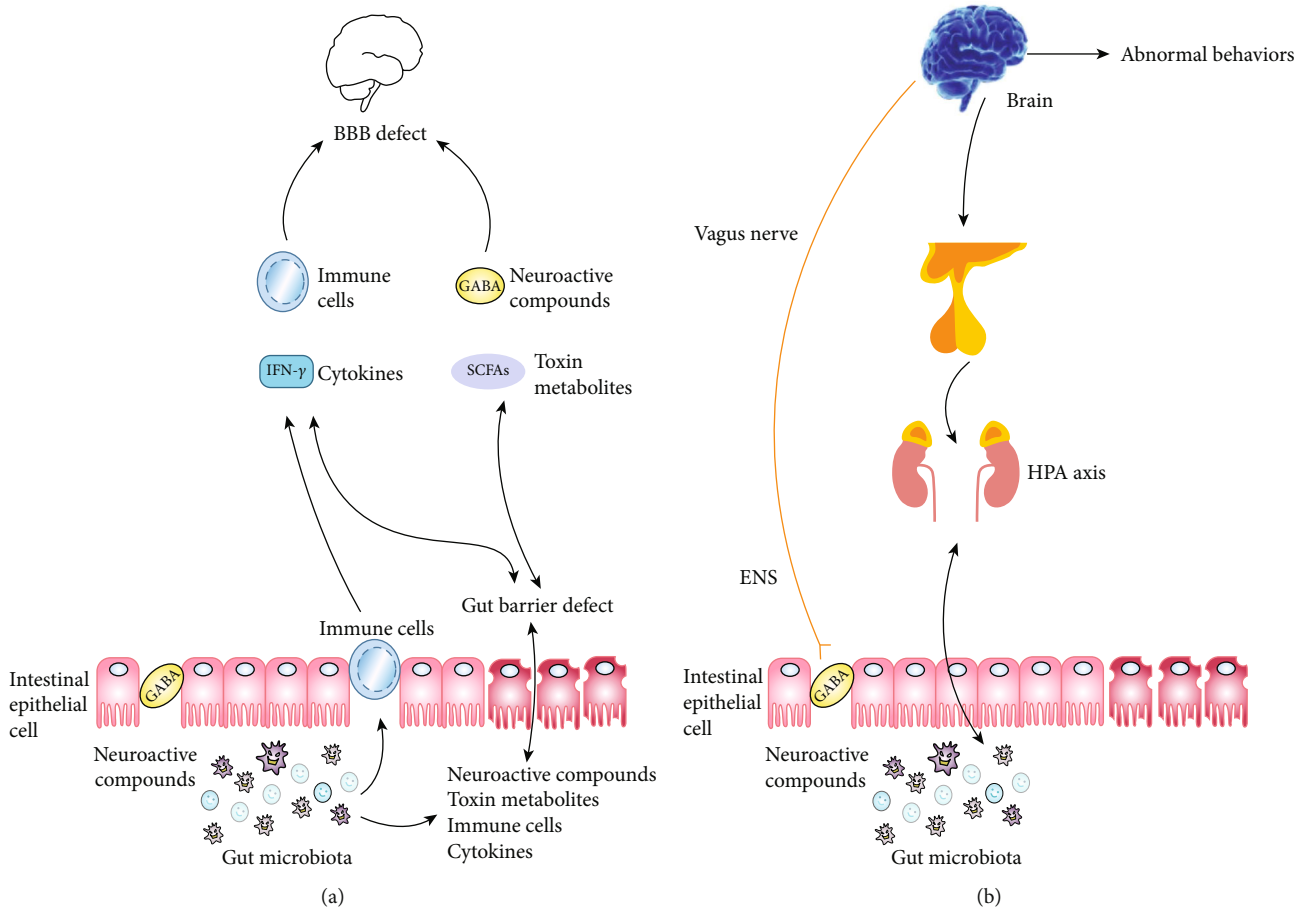


FIGURE 3: Relationships between the GIT microbiota and ASD (the microbiota-gut-brain-axis). Note: BBB: blood-brain barrier; ENS: enteric nervous system; GABA: γ -aminobutyric acid; HPA: hypothalamic-pituitary-adrenal; SCFAs: short-chain fatty acids.

3. Gastrointestinal Tract (GIT) Microbiota and ASD

3.1. GIT Microbiota and Factors That Affect the GIT Microbiota. In recent years, as research continues, the research focus of the GIT microbiota has been rapidly shifting from the abundance and diversity of the microbial members to functional aspects. A variety of studies have concluded that the intestinal microbiota influences many aspects of human health, including metabolism, the immune and nervous systems, and the mucosal barrier. The intestinal microbial fermentation of dietary fibres and resistant starch produces short-chain fatty acids (SCFAs), especially butyrate, propionate, and acetate [12]. Butyrate is a key energy substrate for colonocytes [51] and can drive the energy metabolism of colonocytes towards β -oxidation by stimulating PPAR- γ signalling and limiting the luminal bioavailability of oxygen, maintaining homeostasis, and preventing gut microbiota dysbiosis [52]. Propionate regulates satiety signalling and gluconeogenesis in the liver, protecting the host from diet-induced obesity and associated glucose intolerance [53]. Other metabolites are produced by intestinal microbiota, and additional clinical evidence is needed to fully investigate their functions in physiology and pathophysiology. Examples include indole propionic acid, which seems to

improve mucosal integrity in the gut [54], and ethylphenyl sulfate, which is connected to the exacerbation of autistic behaviour in a mouse model [55]. In addition, the intestinal microbiota has also been shown to have a positive impact on glycaemic control [56], energy homeostasis [57], lipid metabolism [58], and protein metabolism [59]. For the immune and nervous systems, the intestinal microbiota modulates the maturation and function of tissue-resident immune cells in the central nervous system (CNS), including microglia and astrocytes; they also influence the activation of peripheral immune cells, which regulate responses to neuroinflammation, brain injury, autoimmunity, and neurogenesis [60]. In addition, the healthy gut microbiota not only plays a dominant role in reinforcing the immunologic barrier [61] but also maintains the structural integrity of the intestinal mucosal barrier [62]. In summary, the GIT microbiota plays vital roles in human physiology and pathology.

As the GIT microbiota is a popular area in therapeutic research, several factors that contribute to the shaping of the normal GIT microbiota have been demonstrated. The mode of delivery (vaginal or caesarean), the local environment (i.e., mother and hospital), and the type of feeding (breast or formula) are significant factors that impact GIT microbiota composition during the neonatal period, resulting in changes that persist until infancy [63–65]. Age also

TABLE 3: Literatures on the treatments of ASD linked to GIT microbiota.

Model	Behavior tests	Treatments	Dosages	Time	Effects	Limitations	Year	References
10 autistic children, 9 nonautistic siblings, 10 control	CARS and ADI	Probiotic including <i>Lactobacillus</i> , <i>Bifidobacteria</i> and <i>Streptococci</i>	One pill three times a day	4 months	Increased abundance of the <i>Bacteroidetes/Firmicutes</i> , normalized the amount of Bifidobacterium and Lactobacillus can decrease the TNF α levels in the autistic children.	No follow-up was performed after treatment	2015	[90]
A 12-years-old boy with ASD, severe cognitive disability	ADOS-2	Probiotic (<i>lyophilized bifidobacteria, lactobacilli, and Streptococci</i>)	9 – 20 \times 10 ¹⁰	4 weeks	Reduced GIT symptoms and improved in dominating autistic symptoms	More well-designed studies with a larger sample size are needed to offer more proofs supporting the feasibility of it.	2016	[91]
3 autistic child, 3 nonautistic children	N/A	Prebiotic: galactooligosaccharide and B-GOS	2 g	Everyday	Increased abundance of <i>Bifidobacterium spp.</i> , acetate, and butyrate	It is in an in vitro gut model system	2017	[92]
18 autistic children	PGI-III and CARS	Microbiota transfer therapy (MTT)	Vancomycin (40 mg/kg per day)	2 weeks	Improved both GIT and ASD-related symptoms; normalized the microbiota of autistic children	No placebo controlled, blinded or randomized	2017	[93]
ASD animal model	Self-grooming evaluation, three chambers social test	Ketogenic diet	N/A	2 weeks	Prevention of autism symptoms	The ketosis and glucose levels were not measured	2016	[94]
C57BL/6 and BTBR mice	Three-chamber sociability test et al.	Ketogenic diet	N/A	2 weeks	Decreased all host bacterial abundance in cecal and fecal matter	N/A	2016	[95]

Note: N/A: not applicable, i.e., the lack of data in a form or table. The data are from the references 90–95. CARS: The Childhood Autism Rating Scale; ADI: Autism Diagnostic Interview; ADOS-2: Autism Diagnostic Observation Schedule-2; GI-III: The Parent Global Impressions III.

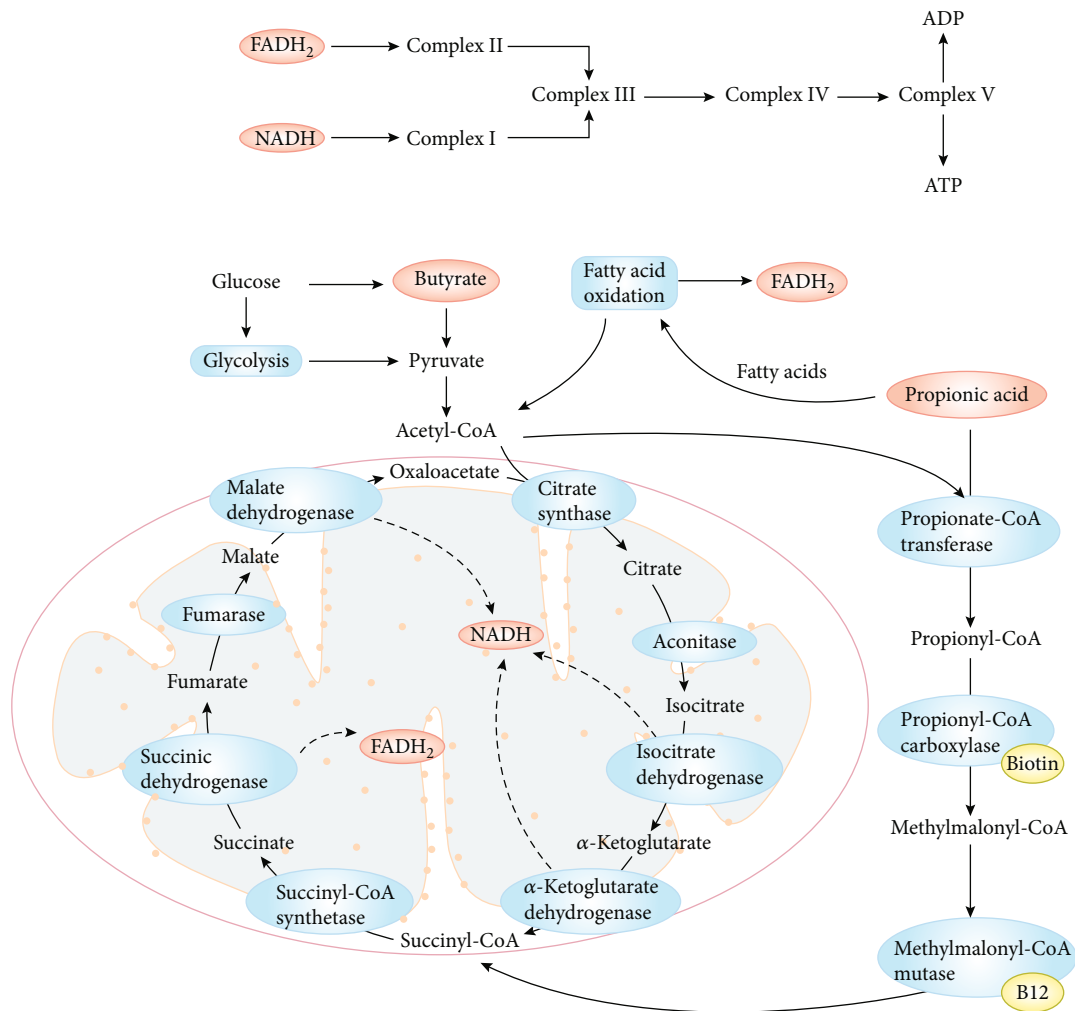


FIGURE 4: Mitochondrial pathways involved in SCFAs as substrates. There are two different starting points in the electron cycle chain, i.e., Complex I and Complex II, which have their exclusive fuel sources. Notably, Complexes III, IV, and IV are all involved in the abovementioned reactions; furthermore, butyrate and propionic acid enter into mitochondria to participate in related reaction via two crossed and overlapped pathways. Butyrate which resembles the glucose commonly enters into the citric (TCA) cycle via Acetyl-CoA, a key reaction substance. The TCA cycle mainly generates a kind of substrate of Complex I called Nicotinamide adenine dinucleotide (NADH). FADH₂, as the substrate of Complex II, can be massively produced in two varied metabolic pathways which propionic acid participates in. Equally, propionic acid can produce some substrates of oxidative stress such as SCFAs et al. to be involved in related responses.

plays an important role in shaping the gut microbiota. It is widely believed that mammals are first exposed to the microbiota in utero and that the microbiota expands rapidly after birth [66]. Studies have also shown that young children and adolescents have a significantly higher abundance of *Clostridium* and *Bifidobacterium* than adults [67, 68]. Diet can also flexibly modulate gut microbiota composition. Just a four-day administration of entirely animal-based or plant-based diet is sufficient to lead to significant shifts in the human gut microbiota [69]. In general, the intake of a diet rich in fruits, vegetables, and fibres is associated with a higher richness and diversity of the gut microbiota [70]. While antibiotics are usually used for saving lives in the fight against infectious disease, many studies have shown their effect on gut bacterial ecology in recent years. The major changes in the GIT microbiota in response to antibiotics include diminished taxonomic diversity and persistence of the changes in a

substantial proportion of individuals [71, 72]. In addition to the factors mentioned above, the gut microbiota configuration of individuals is affected by many other factors, including the genotype of the host, ethnicity, and sex [73–75].

3.2. Relationship between the GIT Microbiota and ASD. The study by Wang et al. showed that GIT symptoms, such as constipation (20%) and diarrhoea (19%), are more common in autistic children than in healthy children [76], similar to the results of meta-analyses by Coury et al. and McElhanon et al. [77, 78]. In addition, Buie et al. found that autistic children with GIT symptoms may display more apparent behavioral manifestations including anxiety, automatization, and aggression [79]. An increasing amount of evidence has shown that the GIT microbiota is directly or indirectly related to the symptoms of autistic children, mostly by affecting the mucosal immune system and human

TABLE 4: The novel therapies of ASD regarding the oxidative stress as a target.

Drugs	Pesticide effect	References
Leukotrienes	Inhibition of the expression or activity of 5-LOX; ameliorate neuroinflammation; restore normal synaptic plasticity; Improve learning and memory function in depressed rats	[105]
Docosahexaenoic acid (DHA)	Be good for the growth and development of the brain and effective at improving cognitive function	[106]
ω -3	Be needed for the appropriate growth and development of the brain, proper synapse formation, and to improve cognitive function	[107]
Vit. B12	Normalization of the Hcy level and amelioration of impaired lipid metabolism	[108]

Note: the information are from references 105–108.

metabolism [3, 80]. According to a study on laboratory animals published online by the journal *Cell*, GIT barrier defects and GIT microbial disorder occur in mouse models of ASD; additionally, the abundance of *Porphyromonadaceae*, *Prevotellaceae*, *Bacteroidales*, and *Lachnospiraceae* in the offspring of mothers with maternal immune activation (MIA) was greater than that of the controls. Notably, the abundance of *Ruminococcaceae*, *Erysipelotrichaceae*, and *Alcaligenaceae* was greater in the controls [81]. The evidence shown by Finegold et al. demonstrates that the GIT microbiota of autistic children has a greater abundance of *Lactobacillus*, *Clostridium*, *Bacteroidetes Desulfovibrio*, *Caloramator*, and *Sarcina* as well as lower levels of *Bifidobacterium* and *Firmicutes* than the GIT microbiota of nonautistic children [82]. Furthermore, autistic children with GIT symptoms show lower levels of *Prevotella*, *Coprococcus*, and *Veillonellaceae* than autistic children without the abovementioned symptoms [83]. As stated, numerous studies have demonstrated the changes in the GIT microbiota of autistic children, but the relationship between GIT microbiota and ASD is relatively unexplored.

In recent years, as study continues, the microbiota-gut-brain-axis has been considered a bidirectional physiological communication between the brain, the GIT microbiota, and the GIT; not surprisingly, accumulating evidence demonstrates that this axis is related to the aetiology and pathogenesis of ASD [84–86]. As shown in Figures 3(a) and 3(b), the metabolites produced by the GIT microbiota, especially SCFAs, can pass through enterocytes (ECs) to have an impact on the function of the brain. In addition, some kinds of GIT microbiota can generate neuroactive substances such as 5-HT and GABA, which can also pass through the EC, affect the function of the brain, and further lead to unexpected behaviors [87]. On the one hand, neuroactive substances, some GIT microbiota, and metabolic products could activate neurons in the brain and act on the function of the brain via vagus nerves. On the other hand, among the abovementioned substances, the neuroactive substances that directly affect the hypothalamic-pituitary-adrenal (HPA) axis can ultimately increase circulating levels of cortisol. Additionally, some GIT microbiota and metabolic products could also activate and induce GIT-immunizing cells to liberate cytokines (CKs) to play a corresponding role in the body's circulation [88, 89].

An increasing amount of evidence suggests that modulation of the GIT microbiota is a potential therapy in autistic children by elucidating the relationship between the

GIT microbiota and ASD, including faecal microbiota transplantation (FMT), probiotics, and dietotherapy. The literature on ASD treatments linked to the GIT microbiota in the past five years is summarized and analysed in Table 3 [90–95]. First, probiotics are considered to prevent intestinal diseases through functions such as regulating and controlling the blood-brain barrier (BBB) and gap-associated proteins [96]. The recently developed approach FMT is an intervention in which the faecal microorganisms from healthy persons are delivered to patients with bad GIT microbiota [97]. However, although many scholars and experts have speculated about the safety of FMT, but no final conclusions have been reached on this matter. Of course, the other abovementioned therapies also have their own limitations (see Table 3). In my opinion, additional well-designed studies with a larger sample size are needed to offer further evidence supporting the feasibility of these treatments.

4. The Relationship among GIT Microbiota, Oxidative Stress, and ASD

As discussed above, the relationship between GIT symptoms and ASD via mitochondrial dysfunction is quite convincing and worthy of study, as it is well documented that ASD is linked to GIT symptoms and mitochondrial dysfunction; additionally, the latter two are strongly related [98]. According to authoritative reports, the GIT, as an available site, can induce the production of SCFAs such as PPA and BUT [99]. Additionally, GIT dysfunction is observed in autistic children, such as the increased abundance of *Clostridia* spp., which produce PPA and BUT. There is no doubt that both PPA and BUT can regulate metabolism, including acting as mitochondrial fuels [100], despite entering into the mitochondrial energy pathways at slightly different sites (see Figure 4) [101]. In addition, SCFAs impair the physiological function of cells, resulting in further GIT symptoms related to ASD, including nonspecific inflammation [79, 93, 102].

To verify whether a connection among the GIT microbiota, oxidative stress, and ASD truly exists, Shannon et al. performed a blinded case-control study and unsurprisingly found differences in the function of mitochondria during several enzymatic reactions in autistic children compared to control children, indicating that differences actually exist in mitochondria rather than in certain enzymes.

These researchers also found that the mitochondrial physiology of the GIT in autistic children differs from that in healthy children; notably, the discrepancies are particularly prominent in the caecum [17]. We speculate that autistic children might have different mitochondrial parameters, particularly in the caecum.

Mitochondria are characterized by energy generation; in addition, approximately 5% of autistic children exhibited impaired energy generation to make energy, and more than 30% of autistic children exhibit elevated biological markers. Therefore, we hypothesized that autistic children may have nontraditional mitochondrial diseases [103]. Similarly, a sibling control study found that lymphoblastoid cell lines (LCLs) from autistic children are active in the mitochondrial respiration process, which further leads to a greater sensitivity to oxidative stress [11]. Indeed, PPA and BUT are also abundant in the caecum, which indicates a role for the GIT microbiota in mitochondrial dysfunction in autistic children [104]. In general, the abovementioned correlated responses could result in a clearer understanding of the pathological relationship linked to GIT dysfunction and oxidative stress in ASD, which provides a theoretical basis as well as molecular strategies for new treatment paradigms.

5. Novel Therapeutic Approaches for Oxidative Stress in ASD

Based on the relationship discussed above, we will next discuss, in the context of the most recent literature, the possibility of novel therapies regarding oxidative stress as a target (Table 4) [105–108]. It is well documented that COX-2, as a vital enzyme that is overexpressed in tissues under oxidative stress, affects the metabolism of polyunsaturated acid (PUFA). ω -3 is also considered to be connected with the high expression of COX-2. Leukotrienes are reported to decrease the expression or activity of an iron-containing dioxygenase named 5-LOX, ameliorating neuroinflammation, restoring normal synaptic plasticity, and improving learning ability and memory [11]. Additionally, docosahexaenoic acid (DHA) and ω -3 are both needed for the appropriate growth and development of the brain, for proper synapse formation and to improve cognitive function [104, 105]. Notably, the use of vitamin B12 as a novel therapeutic approach that has recently aroused public attention has been shown to be used for the treatment of ASD; however, its efficacy is unclear [105].

6. Concluding Remarks

Many reports have highlighted the relationship among the GIT microbiota, oxidative stress, and ASD. A number of studies emphasize the important role of oxidative stress, which is connected to mitochondrial dysfunction, GIT microbiota disturbance, and thus the production of various metabolites. This review has summarized the latest research on the related mechanisms, but most of the authors have only concentrated on individual pathogenic mechanisms or metabolites; thus, there is an urgent need for studies considering broader biochemical pathways. Additionally, among all the novel ASD therapies mentioned above, the potential use-

fulness of most have been investigated. Although the results are promising, these therapies are still thought to have limitations and lack safety testing. In general, the author thinks that additional well-designed studies with a larger sample size are needed to offer further evidence supporting the feasibility of these treatments. In addition, the appropriate dose and timing of therapy also require further study.

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contributions

TH drafted the manuscript, YD generated the figure, CH and YD performed the background research, and MZ and QH edited the manuscript. All authors have read and approved the content of the manuscript. Tingting Hu and Yinmiao Dong contributed equally to this study.

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