



Guilingji Protects Against Spermatogenesis Dysfunction From Oxidative Stress via Regulation of MAPK and Apoptotic Signaling Pathways in *Immp2l* Mutant Mice

Zhenqing Wang^{1†}, Yun Xie^{1†}, Haicheng Chen^{2†}, Jiahui Yao¹, Linyan Lv², Yanqing Li², Chunhua Deng¹, Min Zhang^{1*}, Xiangzhou Sun^{1*} and Guihua Liu^{2*}

OPEN ACCESS

Edited by:

Karl Tsim,
Hong Kong University of Science and
Technology, Hong Kong SAR, China

Reviewed by:

Alaaeldin Ahmed Hamza,
National Organization for Drug Control
and Research (NODCAR), Egypt
Weicheng Hu,
Huaiyin Normal University, China
Attapon Cheepsattayakorn,
Western University, Thailand

*Correspondence:

Guihua Liu
liuguihua@mail.sysu.edu.cn
Xiangzhou Sun
sunxzh@mail.sysu.edu.cn
Min Zhang
zhangm287@mail.sysu.edu.cn

[†]These authors have contributed
equally to this work

Specialty section:

This article was submitted to
Ethnopharmacology,
a section of the journal
Frontiers in Pharmacology

Received: 06 September 2021

Accepted: 23 December 2021

Published: 13 January 2022

Citation:

Wang Z, Xie Y, Chen H, Yao J, Lv L,
Li Y, Deng C, Zhang M, Sun X and
Liu G (2022) Guilingji Protects Against
Spermatogenesis Dysfunction From
Oxidative Stress via Regulation of
MAPK and Apoptotic Signaling
Pathways in *Immp2l* Mutant Mice.
Front. Pharmacol. 12:771161.
doi: 10.3389/fphar.2021.771161

¹Department of Urology, The First Affiliated Hospital of Sun Yat-sen University, Guangzhou, China, ²Reproductive Centre, The Sixth Affiliated Hospital, Sun Yat-sen University, Guangzhou, China

Male infertility is a major health issue with an estimated prevalence of 4.2% of male infertility worldwide. Oxidative stress (OS) is one of the main causes of male infertility, which is characterized by excessive reactive oxygen species (ROS) or lack of antioxidants. Meanwhile, it is reported that oxidative stress plays an important role in the spermatogenic impairment in Inner mitochondrial membrane peptidase 2-like (*Immp2l*) mutant mice. In this study, we focused on the potential mechanism of Guilingji in protecting the spermatogenic functions in *Immp2l* mutant mice. The results revealed that *Immp2l* mutant mice exhibit impaired spermatogenesis and histology shows seminiferous tubules with reduced spermatogenic cells. After administration of Guilingji [150 mg/kg per day intragastric gavage], however, alleviated spermatogenesis impairment and reversed testis histopathological damage and reduced apoptosis. What's more, western blotting and the levels of redox classic markers revealed that Guilingji can markedly reduce reactive oxygen species. Moreover, Guilingji treatment led to inhibition of the phosphorylation of mitogen-activated protein kinase (MAPK), regulated apoptosis in the cells. In summary, Guilingji can improve spermatogenesis in *Immp2l* mutant mice by regulating oxidation-antioxidant balance and MAPK pathway. Our data suggests that Guilingji may be a promising and effective antioxidant candidate for the treatment of male infertility.

Keywords: oxidative stress, spermatogenesis, guilingji, *Immp2l* mutant mice, apoptosis

INTRODUCTION

Infertility is defined as having regular and unprotected sex with the same partner and not getting pregnant after 1 year (Niederberger et al., 2001). Half of infertility cases are male reproductive dysfunction (Holden et al., 2005), and idiopathic oligasthenospermia is the clinical phenotype (Irvine, 1998). There are many factors affecting sperm quality, including endogenous and exogenous factors. While, many studies have shown that oxidative stress should be considered as a reasonable cause of idiopathic male infertility (Sheweita et al., 2005). Oxidative stress can cause loss of membrane integrity, increased cell permeability, enzyme inactivation, DNA structure damage and cell death, which may be related to decreased sperm count and motility (Atig et al., 2012;

TABLE 1 | The main Chinese medicinal herbs contained in Guilingji.

Chinese name	Latin name	English name	Use part	Properties
Hong Shen	<i>Radix ginseng rubra</i> (<i>Panax ginseng</i> C.A.Mey.)	Red ginseng	Root	Warm; mild; bitter
Lu Rong	<i>Cervi cornu pantotrichum</i>	Hairy antler	Non-ossifying young horn of male deer or stag	Warm; sweet; salty
Hai Ma	<i>Hippocampus</i>	Sea-horse	Hippocampus	Warm; sweet; salty
Gou Qi Zi	<i>Fructus lycii</i> (<i>Lycium barbarum</i> L.)	Fruit of Chinese wolfberry	Fruit	Mild; sweet
Ding Xiang	<i>Flos caryophylli</i> (<i>Syzygium aromaticum</i> (L.) Merr. & L.M.Perry)	Clove	Flower bud	Warm; pungent
Chuan Shan Jia	<i>Manis pentadactyla</i> (<i>Manis pentadactyla</i> Linnaeus)	Pangolin	Scale	Minor cold; salty
Niu Xi	<i>Radix achyranthis bidentatae</i> (<i>Achyranthes bidentata</i> Blume)	Root of twotooth achyranthes	Root	Mild; sweet; sour; bitter
Suo Yang	<i>Cynomorium songaricum</i> (<i>Cynomorium cocci-neum</i> L.)	Songaria cynomorium	Fleshy stem	Warm; sweet
Shu Di Huang	<i>Radix rehmanniae preparata</i> (<i>Rehmannia glutinosa</i> (Gaertn.) DC.)	Prepared rehmannia root	Steamed and sundried root	Minor warm; sweet
Bu Gu Zhi	<i>Psoralea corylifolia</i> (<i>Cullen corylifolium</i> (L.) Medik.)	Malaytea scurfpea	Fruit	Extreme warm; pungent; bitter
Tu Si Zi	<i>Semen cuseutae</i> (<i>Cuscuta chinensis</i> Lam.)	Dodder seed	Seed	Mild; pungent; sweet
Du Zhong	<i>Cortex eucommiae</i> (<i>Eucommia ulmoides</i> Oliv.)	Eucommia bark	Bark	Warm; sweet
Rou Cong Rong	<i>Herba cistanches</i> (<i>Cistanche deserticola</i> Ma)	Desertliving cistanche	Fleshy stem	Warm; sweet; salty
Gan Cao	<i>Radix glycyrrhizae</i> (<i>Glycyrrhiza inflata</i> Batalin)	Root of ural licorice	Root and rhizome	Mild; sweet
Tian Dong	<i>Radix asparagi</i> (<i>Asparagus cochinchinensis</i> (Lour.) Merr.)	Cochinchinese asparagus root	Root	Cold; sweet; bitter
Sha Ren	<i>Fructus amomi</i> (<i>Amomum villosum</i> Lour.)	Villous amomum fruit	Ripe fruit or seed	Warm; pungent
Fu Zi	<i>Radix aconiti lateralis</i> (<i>Aconitum carmichaeli</i> Debeaux)	Common monkshood daughter root	Daughter root	Extreme hot; pungent; sweet
Di Gu Pi	<i>Cortex lycii radices</i>	Root - bark of Chinese wolfberry	Velamen	Cold; sweet

Agarwal et al., 2014). At present, the treatment of infertility, especially idiopathic oligasthenospermia, is still mainly drug therapy. Traditional Chinese medicine (TCM) has been attracted more and more attention in recent years for its mild and lasting effects in regulating physiological functions (Duan et al., 2017; Xiao et al., 2019).

Guilingji is a TCM from the Ming Dynasty. According to the 2015 edition of the Chinese Pharmacopoeia, one of the confidential products of national brands of TCM with Red ginseng as the main raw material (Zhao et al., 2017). It has been used for lasting good health and longevity with processing various bioactivities, including anti-aging (Zhao et al., 2020), epilepsy (Liu et al., 1990), testicular dysfunction (Zhao et al., 2019). However, the mechanism of action that produces the above effects is still unclear. We presume that it is related to the balance of oxidative stress during spermatogenesis.

In order to test this hypothesis, we selected *Imp21* mutant mice as our animal model. Inner mitochondrial membrane peptidase 2-like (IMMP2L) is a functional domain of mitochondrial intima proteina that plays a crucial role in cleaving space-sorting signal peptide sequences of cytochrome c1 (CYC1) and mitochondrial glycerol phosphate dehydrogenase 2 (GPD2) (Lu et al., 2008; Yang et al., 2016; Du et al., 2020). Therefore, it is an oxidative stress state in the *Imp21* mutant mice. Some studies have shown that oxidative stress mediates age-dependent spermatogenesis damage in *Imp21* mutant mice (Yang et al., 2016; Du et al., 2020). Thus, we employed the

Imp21 mutant mice to investigate the protective effects of Guilingji on oligospermia induced by oxidative stress.

In this study, *Imp21* mutant mice were used as the animal model of oligosthenospermia, and Guilingji was selected as the treatment drug by using the unique characteristics of traditional Chinese medicine to explore the influence on these specific antioxidative pathway on oligosthenospermia in mice. This research will provide valuable insights for understanding the pathological changes and treatment mechanisms of spermatogenic dysfunction caused by oxidative stress.

MATERIALS AND METHODS

Medicine and Reagents

The main components of Guilingji (Shanxi Guangyuyuan Medicine Co., Ltd., Lot No. 20151108) are presented in **Table 1**. Guilingji meets the standard requirements as described in the Chinese Pharmacopoeia (Version 2015) (Zhao et al., 2019). Before the experiment, Guilingji was dissolved in 0.5% sodium carboxymethyl cellulose (CMC-NA) suspension to prepare 30 mg/ml suspension.

Animals and Experimental Design

Imp21 mutant mice have been described previously (Lu et al., 2008). Mice were raised in isolation cages with light/dark cycles of 12 h. The genotype is determined by coat color. Homozygous

TABLE 2 | Primary and secondary antibodies used for Western blotting and Immunofluorescence.

Antibodies (Species, IgG type)	Dilution	Distributor
Primary antibody:		
anti-JNK	1:1,000	Cell signaling technology
anti-p-JNK	1:1,000	Cell signaling technology
anti-ERK1/2	1:1,000	Cell signaling technology
anti-p-ERK1/2	1:1,000	Cell signaling technology
anti-p-38	1:1,000	Cell signaling technology
anti-p-p38	1:1,000	Cell signaling technology
DDX4	1:100	Abcam
Secondary antibody:		
anti-GAPDH	1:10,000	Cell signaling technology
Cy3	1:500	Beyotime

normal mice became albino mice due to FVB/NJ males (FVB) background. Heterozygous mice were slightly pigmented. Homozygous mutant mice had dark fur color. The animal experiments were carried out in accordance with the Helsinki Declaration and approved by the Laboratory Animal Welfare and Ethics Committee of Sun Yat-sen University.

A total of 40 animals were included in this experiment, including 20 *Immp21^{+/+}* mice and 20 *Immp21^{-/-}* mice. There were 4 groups in this experiment: *Immp21^{+/+}* group; *Immp21^{+/+}*-Gulingji group; *Immp21^{-/-}* group; *Immp21^{-/-}*-Gulingji group. And there were 10 mice in each group. *Immp21^{-/-}* and *Immp21^{+/+}* mice were administered intragastrically with Gulingji for 30 days, respectively. The appropriate therapeutic doses of Gulingji is 150 mg/kg per day (Du et al., 2020; Zhao et al., 2020). Mice that received the 0.5% CMC-NA suspension were used as untreated controls. The mice were euthanized by the inhalation of an overdose of isoflurane after treatment. All the mice were weighed each 3 days, that was aimed to reflect the fundamental survival state of the mice.

Analysis of Sperm Motility and Concentration

After euthanasia of mice, the caudal epididymis of the mice was shredded and suspended in the sperm incubation solution at 37°C for 10–20 min. Then, the sperm suspension was placed on the counting chamber. Sperm count, concentration and motility were measured using a computer assisted sperm analysis system. Then, we place the sperm suspension on the counting chamber, and use computer assisted sperm analysis system to measure sperm count, concentration and motility (SPERM CLASS ANALYZER). a, b, c, d grade respectively represents different motility states of sperm. Sperm motility is quantitatively evaluated by motile sperm (grade a + b)/all sperm*100.

Testicular Histology

After the mice were euthanized, fresh testicular tissue was taken and cleaned with cold PBS (pH 7.4). Then, fixed in formalin for 48 h and embedded in paraffin after conventional treatment. The wax block was cut into 5 µm thin slices with a microtome, and the

slices were stained with hematoxylin and eosin, which were scanned by a light microscope.

Western Blotting Analysis

Fresh testicular tissue was taken, Radio Immunoprecipitation Assay (RIPA) lysis buffer containing phosphatase inhibitors and protease was added, and total protein was extracted from the tissue after centrifugation. Western blotting analysis was based on previous reports (Yang et al., 2016). Concisely, the denatured proteins were added to the polyacrylamide gel (PAGE) for electrophoresis, and then electrically transferred to polyvinylidene difluoride (PVDF) membranes. This membrane was blocked use non-fat milk at room temperature for 1 h. Next, the membrane was incubated overnight with primary antibody (Table 2) at 4°C. After that, the membrane was rinsed with Tris Buffered Saline with Tween (TBST) and incubated with horseradish peroxidase linked secondary antibody (1:2,000) at room temperature for 1 h. Finally, the membrane was incubated with enhanced chemiluminescence substrate to observe the protein bands. To analysis the protein expression level, ImageJ software was used to relatively quantify protein bands from western blot films. Western bolt was replicated for three parallel experiments.

Immunofluorescence Analysis

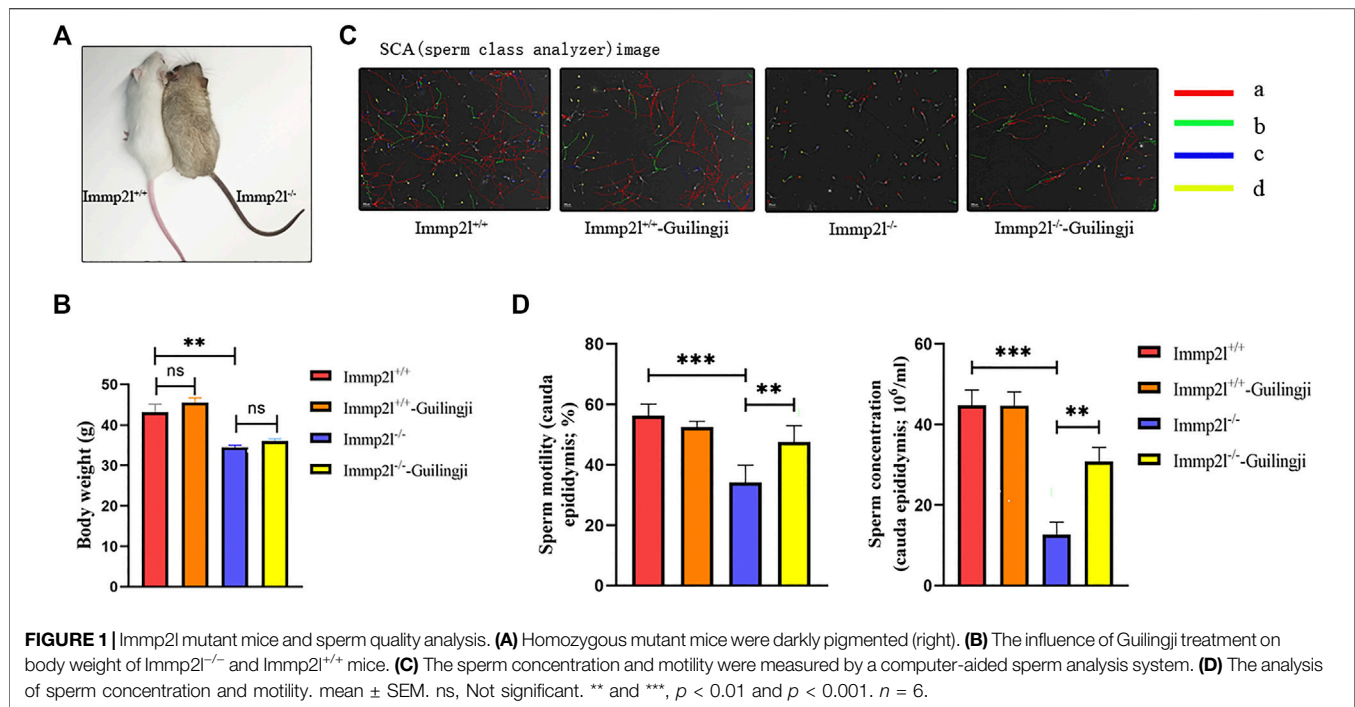
For immunofluorescence analysis, the paraffin sections were dewaxed, dehydrated, and incubated overnight at 4°C with primary antibodies. Then, the tissues were incubated with secondary antibodies for 1 h in the dark. Finally, the nuclei were counterstained with DAPI for 10 min. Immunofluorescence signals were visualized and recorded using a laser scanning confocal microscope (TCS SP8, Leica, Germany). The primary and secondary antibodies used in this study are listed in Table 2.

Measurement of SOD, MDA and GSH Levels in Testicular Tissues

After the mice were euthanized, the tissues were homogenized, then placed in cold sterile phosphate buffered saline (PBS). The homogenate was centrifuged at 2,500 rpm/min at 4°C for 10 min, and the supernatant was extracted for further analysis of oxidative stress level. Assays of Malondialdehyde (MDA), Superoxide dismutase (SOD) and Glutathione (GSH) levels in testicular tissues were performed using commercial kits (Nanjing Jian cheng Bioengineering Institute, China). We further analyzed the test data according to the instructions.

Transferase dUTP Nick End-Labeling Analysis

The TUNEL assay was executed by using a One-Step TUNEL Apoptosis Assay Kit. First, paraffin-embedded sections were placed in 65°C drying oven, followed by deparaffinized in xylene, then treated with a range of different concentrations of alcohol [100, 95, 85, 70 and 50% ethanol (v/v)] and rinsed with PBS at pH 7.5. The tissue was then treated with protease K solution and refixed with 4% paraformaldehyde solution. The



prepared TUNEL reaction mixture was dropped onto the tissue and incubated at 37°C for 1 h in a dark environment. The nuclei were stained with DAPI. TUNEL positive cells were counted under fluorescence microscope.

Data Downloading and Functional Enrichment Analysis

Traditional Chinese medicine (TCM) prescriptions can effectively delay the progression of disease through the coordination of various compounds and targets (Li et al., 2021). Therefore, it is necessary not only to identify the potential active ingredients of Guilingji, but also to further explore its therapeutic targets. Gene expression data for TCM herbs/ingredients were downloaded from HERB, which is a high-throughput experiment- and reference-guided database of TCM, traditional with its Chinese name as BenCaoZuJian (<http://herb.ac.cn>) (Fang et al., 2021). Different molecular mechanisms and involved pathways were explored through Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) analyses. First, we downloaded the gene expression data from HERB's website (<http://herb.ac.cn>). Then, using Oncomine (<https://www.oncomine.org>) for Go and KEGG analysis. Go analysis can reveal the main enrichment terms in Biological Process (BP), Cellular Component (CC) and Molecular Function (MF). KEGG analysis can reveal the major enriched signaling pathways. Pathway with enrichment $p < 0.01$ was considered as significant.

Statistical Analysis

All experiments were performed at least three times and the data are expressed as the mean ± SEM. When the data were normally distributed, one-way ANOVA followed by Tukey test or two-way ANOVA followed by Bonferroni's multiple comparison test. If these

data were not normal distributed, Kruskal-Wallis test was used. GraphPad Prism 7.0 Software (GraphPad Software Inc., CA, United States) was used for Statistical analysis chart. The differences between groups were considered statistically significant when $p < 0.05$.

RESULTS

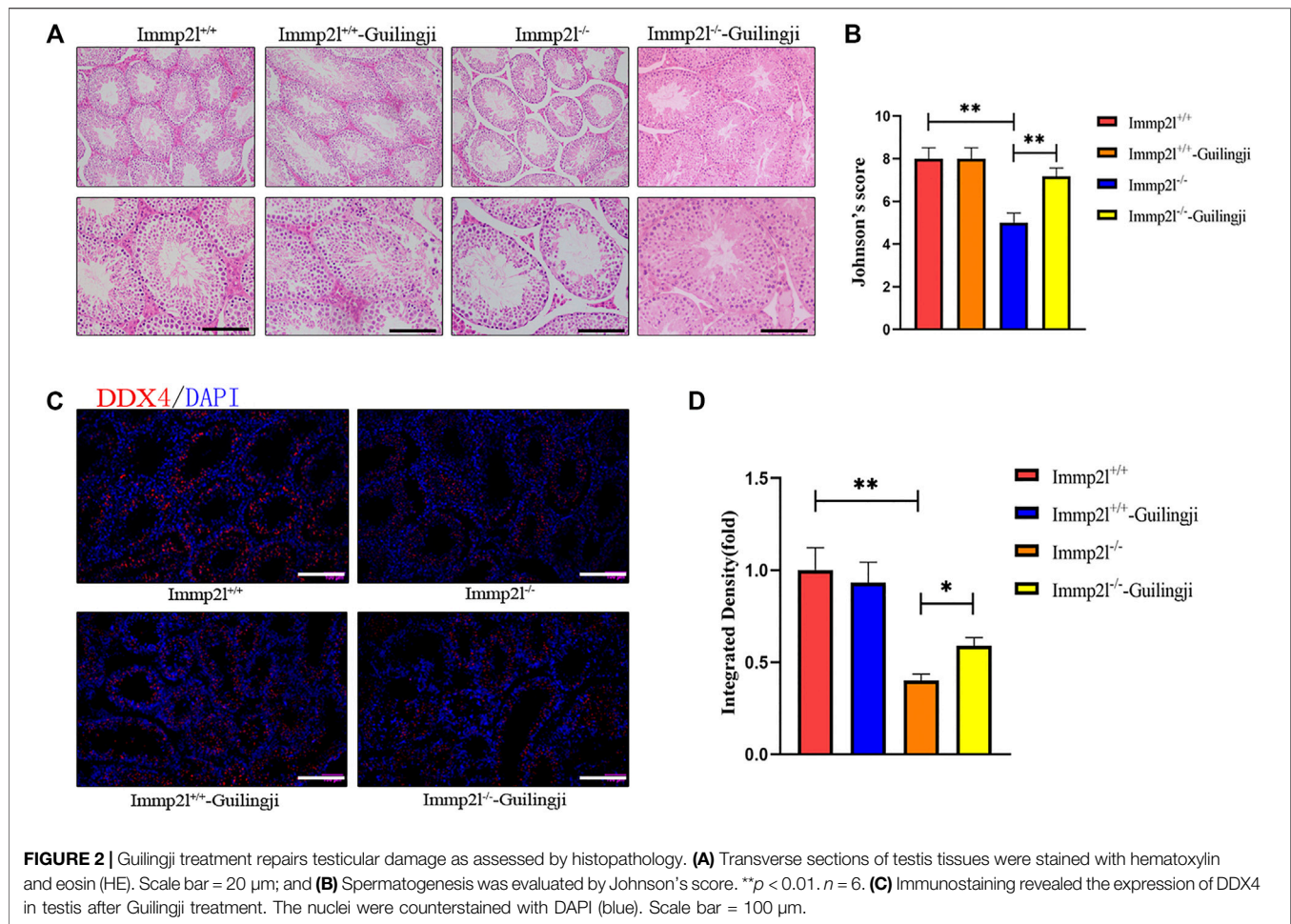
Guilingji Treatment Improves Sperm Quality and Testicular Index in Immp2l Mutant Mice

Mice were monitored for weight loss according to our animal protocol; Immp2l^{-/-} mice weighed less than Immp2l^{+/+} mice (Figure 1B), which is consistent with the results of previous studies (Han et al., 2013). Body weights in Guilingji-treated mice (including Immp2l^{+/+} and Immp2l^{-/-} mice) were not different than in saline-treated mice of the same genotype (Figure 1B).

The detection of sperm motility by Computer-assisted sperm analysis (CASA) was used to evaluate whether Guilingji affected sperm motility and concentration. Results in Figure 1C show the semen picture of Immp2l^{-/-} mice, which revealed that Immp2l^{-/-} mice had lower sperm concentration, motility, and viability. Importantly, the Immp2l^{-/-}-Guilingji group displayed a significant increase ($p < 0.05$) in sperm motility percentage and sperm concentration, when compared to the Immp2l^{-/-} group, as presented in Figure 1D.

Guilingji Treatment Reverses Testis Histopathological Damage in Immp2l Mutant Mice

As shown in Figure 2A, spermatogenesis was normal and spermatogenic epithelial cells were arranged regularly in



normal mice. While compared with normal mice, all untreated Immp21 mutant mice showed impaired spermatogenesis, disorganized and vacuolated seminiferous tubules, consistent with oligospermia. In addition, testicular spermatogenesis was evaluated using the Johnsen scores, which is a numerical score from 1 to 10, with 10 indicating complete spermatogenesis. The Immp21^{-/-}-Guilingji group mice showed thicker seminiferous epithelium, and more tubules with the higher Johnsen scores (*p* < 0.05, **Figure 2B**). It indicated that the testicular structure of the treatment group was less damaged and the function recovered. Furthermore, DDX4 is specific to germ cells, which is necessary for germ cell development. The increased quantity of DDX4-positive cells demonstrated that the spermatogenesis of the experiment animals was improved after the Guilingji administration. As shown in **Figure 2C**, more DDX4-positive cells were present in the testes of the Immp21^{-/-}-Guilingji group than those in the Immp21^{-/-} group testes. Summarized data are given in **Figure 2D**.

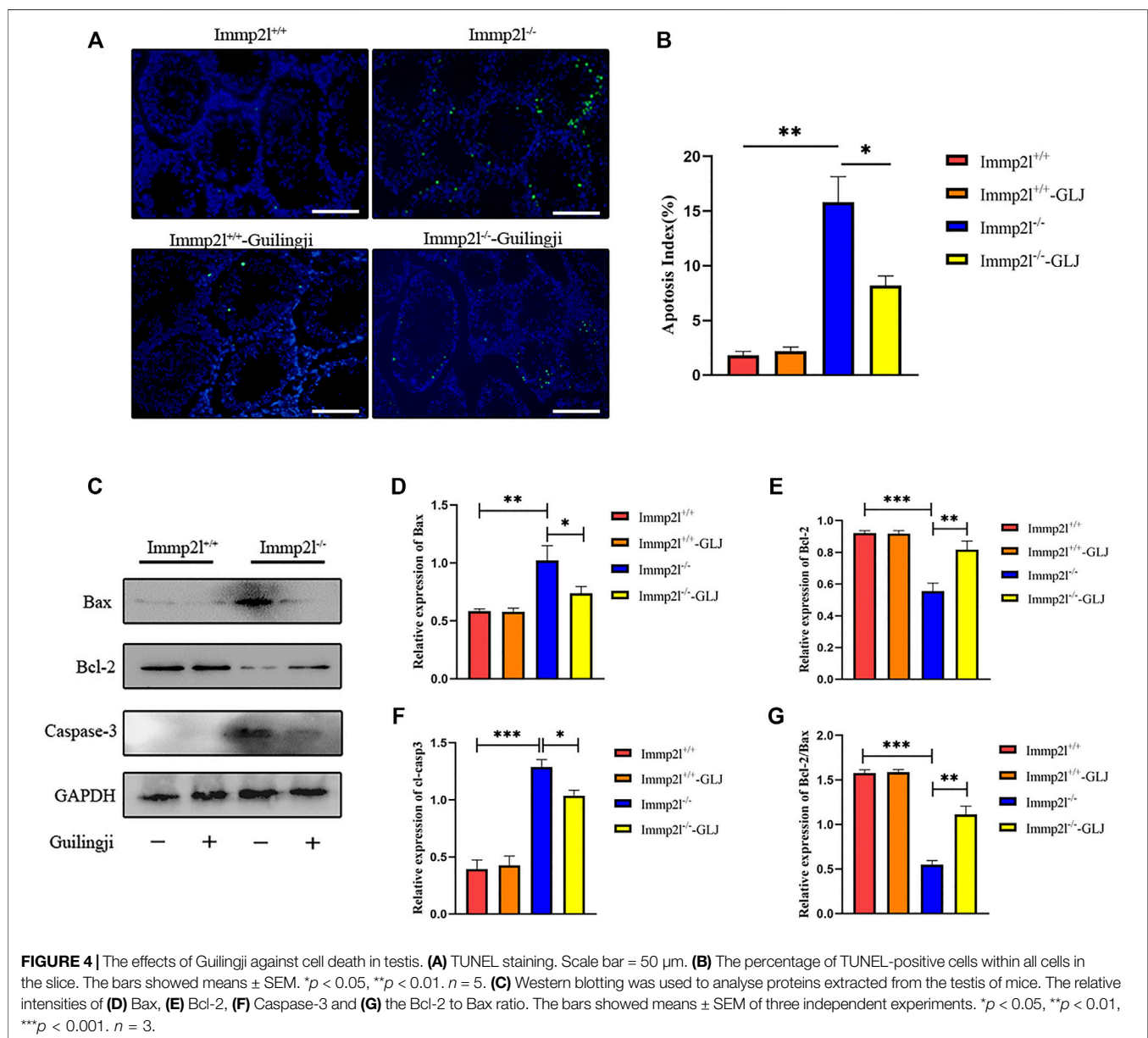
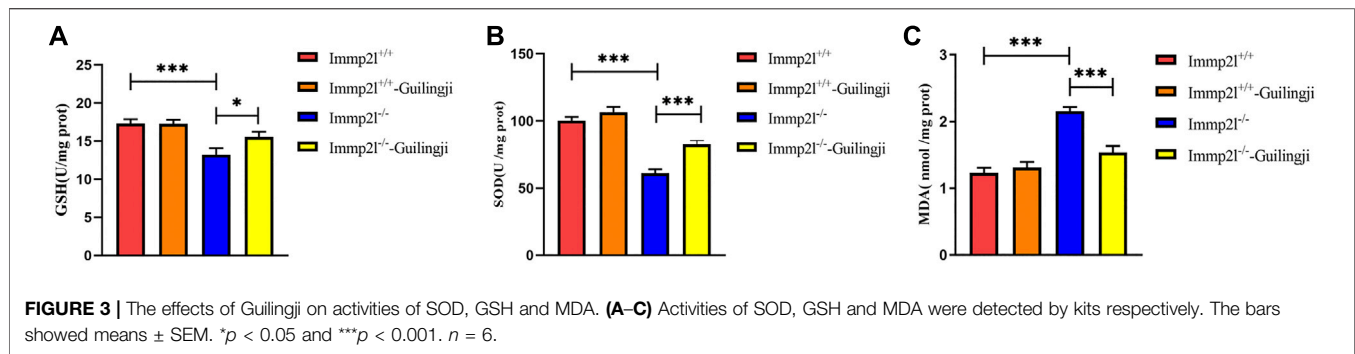
Guilingji Reduces Oxidative Stress in the Testes of Immp21 Mutant Mice

To assess testicular antioxidant status, we measured the levels of classic markers of redox. As shown in **Figure 3**, compared with

Immp21^{+/+} mice group, the level of MDA in Immp21^{-/-} mice group was significantly increased, while glutathione levels and testicular tissue of the superoxide dismutase (SOD) activity were decreased. On the contrary, Guilingji administration can significantly reduce the above effects. The results showed that Guilingji alleviated oxidative stress by balancing oxidation and anti-oxidation.

Guilingji Protects Testis Tissue Against Apoptosis

TdT-mediated dUTP-biotin nick end labeling (TUNEL) is an assay for the detection of apoptosis. As shown in **Figure 4A,B**, compared with non-mutant mice, the testicular germ cell apoptosis of Immp21^{-/-} mice was dramatically increased. After Guilingji treatment, the level of apoptosis of germ cells were considerably strangled in Immp21^{-/-} mice. Meanwhile, Guilingji treatment had no vital effect on the germ cell apoptosis in Immp21^{+/+} mice. To further confirm this result, we also detected apoptosis-associated proteins active-Casp3, Bcl-2, and Bax expression by western blotting, and found that Guilingji inhibited cleaved Casp3, Bax expression and promote Bcl-2 expression (*p* < 0.05, **Figures 4C-F**).



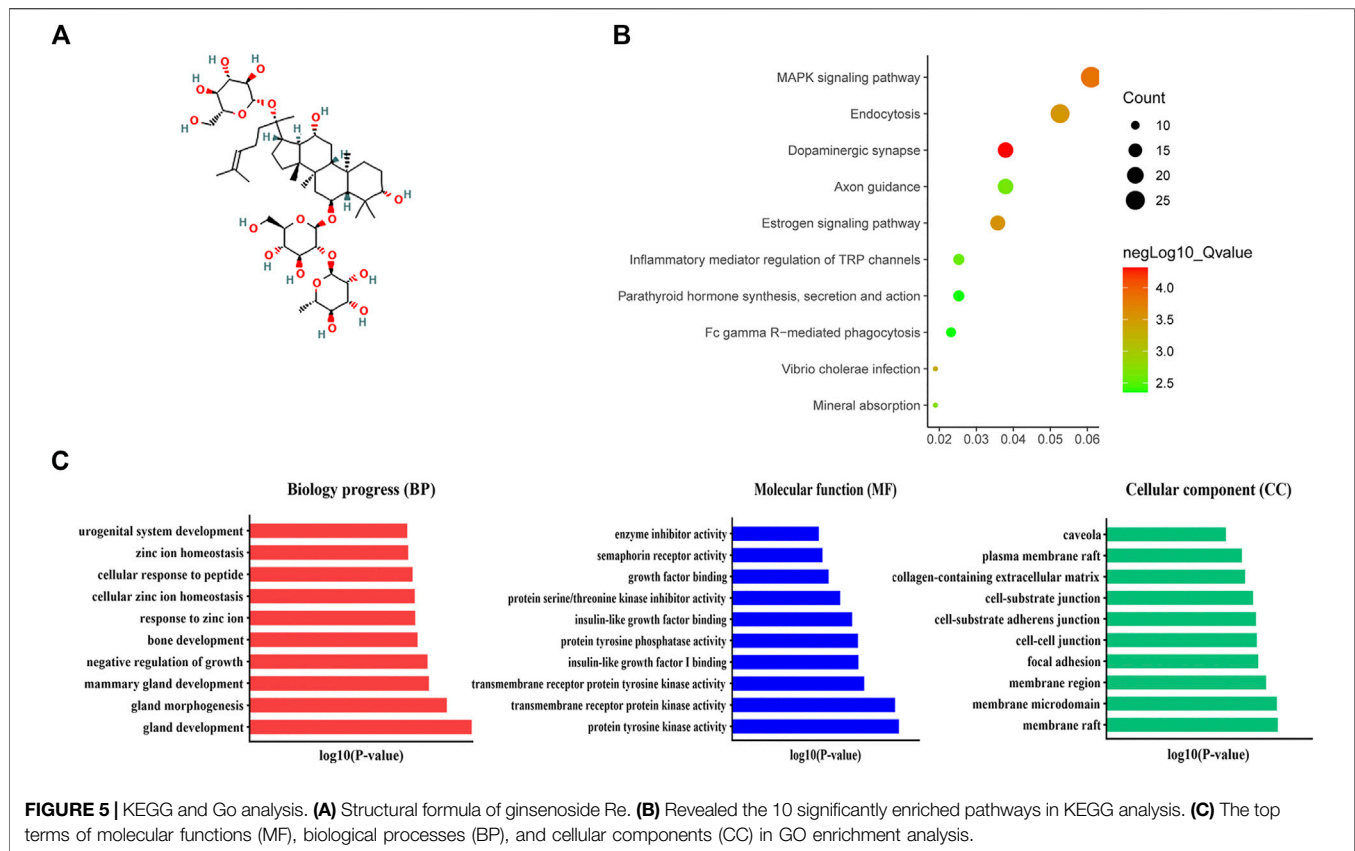


FIGURE 5 | KEGG and Go analysis. **(A)** Structural formula of ginsenoside Re. **(B)** Revealed the 10 significantly enriched pathways in KEGG analysis. **(C)** The top terms of molecular functions (MF), biological processes (BP), and cellular components (CC) in GO enrichment analysis.

Functional Enrichment Analysis of Ginsenoside Re, the Main Ingredient of Guilingji

Functional Enrichment Analysis of Ginsenoside Re, the Main Ingredient of Guilingji The structural formula of ginsenoside Re is shown in **Figure 5A**. Evaluation of drug efficacy and discovery of new drug targets are the characteristics of pharmacotranscriptomics (Fang et al., 2021). Therefore, it is necessary not only to identify the active ingredients, but also to further explore their therapeutic targets. Hence, GO analysis and KEGG were performed to assess key molecules and pathways. As shown in **Figure 5B**, MAPK signaling pathway, endocytosis and dopaminergic synapse were mainly enriched. GO analysis revealed that gland development, gland morphogenesis, mammary gland development, and negative regulation of growth were the primary terms involved in BP; membrane microdomain, membrane raft, and membrane region were significantly enriched in CC; protein tyrosine kinase activity, transmembrane receptor protein tyrosine kinase activity, transmembrane receptor protein kinase activity, and insulin-like growth factor I binding were top enriched in MF (**Figure 5C**).

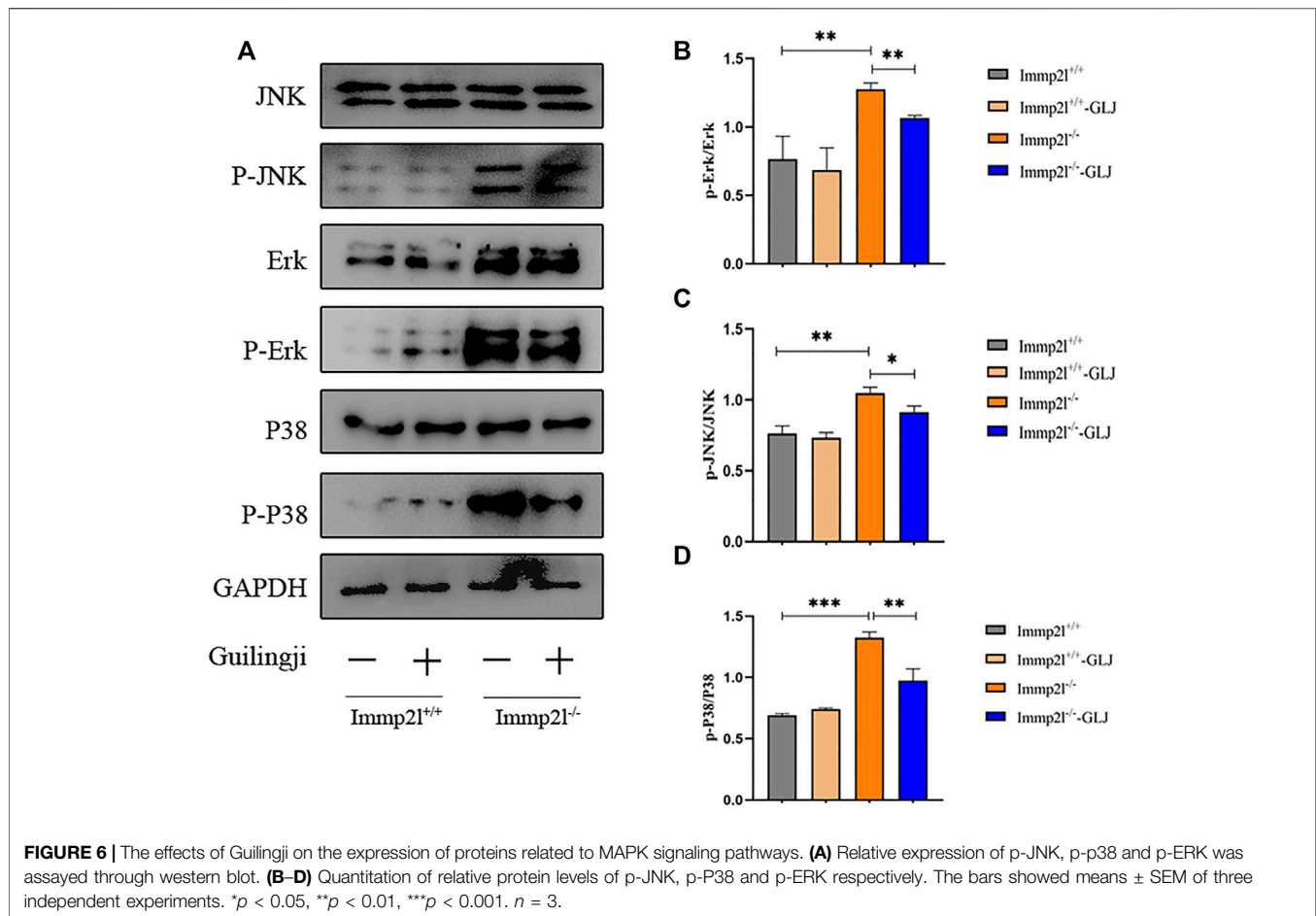
Guilingji Inhibits the Phosphorylation of MAPK Pathway Proteins in the Testes of *Imp21* Mutant Mice

Previous studies have shown that activation of MAPK pathways is closely related to oxidative stress-related apoptosis (Wang et al.,

2019). Pathway enrichment analysis suggested that MAPK signaling pathway may play a role in the administration of Guilingji. We hypothesized that Guilingji might play an antioxidative role through the inhibition of the MAPK signaling pathways. In this study, the expressions of p38, p-p38, ERK, p-ERK, JNK, and p-JNK in the MAPK subfamily were detected by western blotting. As shown in **Figure 6**, the expression level of phosphorylated proteins in the MAPK pathways was remarkably increased in *Imp21*^{-/-} mice. However, compared with the untreated group, the levels of phosphorylated proteins in MAPK pathway were decreased in the treatment group ($p < 0.05$). In conclusion, these results suggested that Guilingji may play a protective role in spermatogenesis by inhibiting the above signal pathway.

DISCUSSION

In this study, we showed that the level of oxidative stress in *Imp21* mutant mice testis was higher compared with wild-type mice, leading to spermatogenic impairment. We revealed that the Guilingji administration reduced ROS level, inhibited germ cell apoptosis and improved spermatogenic function. Importantly, we found that the therapeutic effects of Guilingji was depended on MAPK pathways. In summary, we elaborated the therapeutic effects of Guilingji administration in oligospermia animal models, which was depended on the ant-oxidative capacities.



As we know, oxidative stress refers to the imbalance between the oxidative system and the antioxidant system, which is the main factor in the pathology of infertility in 30–80% of infertile men (Aitken and Fisher, 1994; Agarwal et al., 2006; Barati et al., 2020). Compared with fertile men, infertile men show higher levels of semen oxidative stress and increased apoptosis. Poor sperm condensed chromatin (due to elevated ROS levels and high levels of oxidative stress) entering the apoptotic cascade, a lot of apoptotic germ cells in testis or Phosphatidylserine externalized cell, high levels of caspase activation and DNA damage, it is considered to be the main causes of male infertility (Bisht et al., 2017). In our study, *Immp21* mutant mice exhibit the same phenotype, with high oxidative stress level, increased apoptosis level and spermatogenic dysfunction in testis. Some studies have already demonstrated that mitochondrial superoxide level was increased in *Immp21* mutant mice, which lead to spermatogenic damage and infertility (George et al., 2012). These evidences suggest that oxidative stress is an important mediator of infertility and *Immp21*^{-/-} mice act as one of the ideal animal models of infertility, alleviating oxidative stress is conducive to ameliorating spermatogenesis of *Immp21* mutant mice.

Previous studies have shown that red ginseng and ginsenosides can decrease and/or eliminate the production of free radicals by

modulating the activity of antioxidase (Shi et al., 2013). While red ginseng and ginsenosides are the main herbs contained in Gulingji. And the total amount of ginsenoside Re (C48H82O18) is more than 100 ug in Gulingji capsule (Zhao et al., 2020). The multiple pharmacological effects of Gulingji have been confirmed by other study groups, including antioxidation, anti-inflammation, anti-aging and so on (Zhao et al., 2019; Du et al., 2020; Zhao et al., 2020). Accordingly, we speculate that Gulingji can protect the testis from apoptosis under oxidative stress.

Intracellular antioxidants such as glutathione (GSH), superoxide dismutase (SOD) protect cells against oxidative damage by eliminating cytoplasmic ROS (Nakamura et al., 2010). Gulingji obviously increased the activities of above enzymes, which may reduce the accumulation of ROS and subsequently reduce the oxidative damage to biological macromolecules. Malondialdehyde (MDA) is an important lipid peroxidation product, which can be used as an index to detect the oxidative damage of cell membrane. And it is a measure of peroxidation damage to spermatozoa under the condition of oxidative stress (Aitken et al., 2013; Dong et al., 2015). We found a decrease of MDA levels in the Gulingji-treatment compared with that control group, indicating the

reduced function on lipid peroxidation that occurred in the *Immp2l* mutant mice.

The apoptotic cascade is caused by excessive production of ROS and oxidative stress, which hinder the basic functions of sperm, thereby making sperm inactive and immotile. Therefore, the development of therapeutic methods to improve or inhibit sperm cell apoptosis induced by oxidative stress is an urgent requirement (Greco et al., 2005). In this study, there is evidence that when oxidative stress is inhibited, apoptosis can be significantly reduced. The result of western blot shown that the expression of pro-apoptotic proteins, including cleaved caspase-3, and Bax, were increased. while the anti-apoptotic protein Bcl-2 was down-regulated. We believe that Guilingji can protect against mitochondrial dysfunction by regulating oxidative stress, thereby reducing the activation of caspase and inhibiting cell apoptosis.

The separation of active ingredients from TMC and further analysis of their mechanism of action are of great significance for discovering drug targets and evaluating drug efficacy (Fang et al., 2021). Therefore, we downloaded data from HERB database, and the pathway enrichment analysis found that MAPK signaling pathway was mainly enriched. Furthermore, there is also the mechanism of apoptosis may be related to the activation of p38/MAPK and JNK/MAPK signal transduction pathways (Ashwell, 2006; Liu et al., 2016). MAPK is composed of three main subgroups: extracellular signal-regulated kinase (ERK), p38-MAPK, and c-Jun N-terminal kinase (JNK) (Yue and López, 2020). Accumulating evidence has identified MAPK pathways are activated by oxidative stress in cells and tissues. Importantly, MAPK signaling pathways are involved in regulating reproductive functions in the testis (Sinha Hikim et al., 2003; Johnson et al., 2008; Li et al., 2009). For example, Liu B et al. found that PM2.5 disrupted spermatogenesis via ROS-mediated MAPK pathways, while antioxidants (vitamin C and E) can inhibit the MAPK pathways and improve testicular spermatogenesis (Liu et al., 2019). Li M et al. mentioned that it can alleviate the damage of BTB and spermatogenesis caused by cadmium through inhibiting the unnecessary activation of MAPK pathways in Sertoli cells (Li et al., 2009). What's more, some studies revealed that administration of antioxidants can alleviate oxidative stress and injury via MAPK pathways (Li et al., 2020; Ye et al., 2020). In our research, Guilingji treatment reduced the high phosphorylation levels of p-38, JNK and ERK1/2 in *Immp2l*^{-/-} mice, demonstrate that Guilingji strongly inhibit the MAPK signal transduction pathways. All in all, our experiment revealed that Guilingji could attenuated oxidative stress and apoptosis in *Immp2l* mutant mice via restrain activation of the MAPK signaling pathways.

REFERENCES

- Agarwal, A., Prabakaran, S., and Allamaneni, S. (2006). What an Andrologist/urologist Should Know about Free Radicals and Why. *Urology* 67, 2–8. doi:10.1016/j.urology.2005.07.012
- Agarwal, A., Virk, G., Ong, C., and du Plessis, S. S. (2014). Effect of Oxidative Stress on Male Reproduction. *World J. Mens Health* 32, 1–17. doi:10.5534/wjmh.2014.32.1.1

CONCLUSION

In short, the results of this study systematically proved that Guilingji can attenuate ROS, oxidative stress and apoptosis by inhibiting MAPK-mediated signal pathways in the *immp2l* mutant mouse model. This study provides evidence that Guilingji may be effective in the clinical treatment of male infertility. However, the use of a single dose of Guilingji is deficiency of our experiment, which will be improved in future studies. In addition, the regulation mechanism of Guilingji on metabolites needs further experimental verification.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by The animal experiments in our study were conducted in accordance with the Helsinki Declaration and approved by the Laboratory Animal Welfare and Ethics Committee of Sun Yat-sen University.

AUTHOR CONTRIBUTIONS

ZW, YX and HC: Collection and assembly of data, data analysis and interpretation, and manuscript writing; CD: Conception and design and obtaining financial support; GL, XS and MZ: Conception and design, manuscript writing, and obtaining financial support; JY, LL and YL: Collection and assembly of data. All authors have read and agreed to the published version of the manuscript.

FUNDING

This research was supported by the National Natural Science Fund (81971759, GL; 81873829, MZ; 81971314, CD). Fund (K0113144).

ACKNOWLEDGMENTS

We thank Prof. Baisong Lu of Wake Forest University Health Sciences for providing *Immp2l* mutant mice and his suggestions.

- Aitken, J., and Fisher, H. (1994). Reactive Oxygen Species Generation and Human Spermatozoa: the Balance of Benefit and Risk. *Bioessays* 16, 259–267. doi:10.1002/bies.950160409
- Aitken, R. J., Smith, T. B., Lord, T., Kuczera, L., Koppers, A. J., Naumovski, N., et al. (2013). On Methods for the Detection of Reactive Oxygen Species Generation by Human Spermatozoa: Analysis of the Cellular Responses to Catechol Oestrogen, Lipid Aldehyde, Menadione and Arachidonic Acid. *Andrology* 1, 192–205. doi:10.1111/j.2047-2927.2012.00056.x

- Ashwell, J. D. (2006). The many Paths to P38 Mitogen-Activated Protein Kinase Activation in the Immune System. *Nat. Rev. Immunol.* 6, 532–540. doi:10.1038/nri1865
- Atig, F., Raffa, M., Ali, H. B., Abdelhamid, K., Saad, A., and Ajina, M. (2012). Altered Antioxidant Status and Increased Lipid Per-Oxidation in Seminal Plasma of Tunisian Infertile Men. *Int. J. Biol. Sci.* 8, 139–149. doi:10.7150/ijbs.8.139
- Barati, E., Nikzad, H., and Karimian, M. (2020). Oxidative Stress and Male Infertility: Current Knowledge of Pathophysiology and Role of Antioxidant Therapy in Disease Management. *Cell Mol Life Sci* 77, 93–113. doi:10.1007/s00018-019-03253-8
- Bisht, S., Faiq, M., Tolahunase, M., and Dada, R. (2017). Oxidative Stress and Male Infertility. *Nat. Rev. Urol.* 14, 470–485. doi:10.1038/nrurol.2017.69
- Dong, G., Wei, D., Wang, J., Guo, P., Li, M., Yang, M., et al. (2015). Study of the Cardiotoxicity of Venenum Bufonis in Rats Using an 1H NMR-Based Metabolomics Approach. *PLoS one* 10, e0119515. doi:10.1371/journal.pone.0119515
- Du, K., Gao, X. X., Feng, Y., Li, J., Wang, H., Lv, S. L., et al. (2020). Integrated Adrenal and Testicular Metabolomics Revealed the Protective Effects of Gulingji on the Kidney-Yang Deficiency Syndrome Rats. *J. Ethnopharmacol* 255, 112734. doi:10.1016/j.jep.2020.112734
- Duan, D. D., Wang, K. X., Zhou, Y. Z., Qin, X. M., Gao, L., and Du, G. H. (2017). Baicalein Exerts Beneficial Effects in D-Galactose-Induced Aging Rats through Attenuation of Inflammation and Metabolic Dysfunction. *Rejuvenation Res.* 20, 506–516. doi:10.1089/rej.2017.1919
- Fang, S., Dong, L., Liu, L., Guo, J., Zhao, L., Zhang, J., et al. (2021). HERB: a High-Throughput experiment- and Reference-Guided Database of Traditional Chinese Medicine. *Nucleic Acids Res.* 49, D1197–D1206. doi:10.1093/nar/gkaa1063
- George, S. K., Jiao, Y., Bishop, C. E., and Lu, B. (2012). Oxidative Stress Is Involved in Age-dependent Spermatogenic Damage of Imp21 Mutant Mice. *Free Radic. Biol. Med.* 52, 2223–2233. doi:10.1016/j.freeradbiomed.2012.04.003
- Greco, E., Iacobelli, M., Rienzi, L., Ubaldi, F., Ferrero, S., and Tesarik, J. (2005). Reduction of the Incidence of Sperm DNA Fragmentation by Oral Antioxidant Treatment. *J. Androl.* 26, 349–353. doi:10.2164/jandrol.04146
- Han, C., Zhao, Q., and Lu, B. (2013). The Role of Nitric Oxide Signaling in Food Intake; Insights from the Inner Mitochondrial Membrane Peptidase 2 Mutant Mice. *Redox Biol.* 1, 498–507. doi:10.1016/j.redox.2013.10.003
- Holden, C. A., McLachlan, R. I., Pitts, M., Cumming, R., Wittert, G., Agius, P. A., et al. (2005). Men in Australia Telephone Survey (MATES): a National Survey of the Reproductive Health and Concerns of Middle-Aged and Older Australian Men. *Lancet* 366, 218–224. doi:10.1016/S0140-6736(05)66911-5
- Irvine, D. S. (1998). Epidemiology and Aetiology of Male Infertility. *Hum. Reprod.* 13 (Suppl. 1), 33–44. doi:10.1093/humrep/13.suppl_1.33
- Johnson, C., Jia, Y., Wang, C., Lue, Y. H., Swerdloff, R. S., Zhang, X. S., et al. (2008). Role of Caspase 2 in Apoptotic Signaling in Primate and Murine Germ Cells. *Biol. Reprod.* 79, 806–814. doi:10.1095/biolreprod.108.068833
- Li, M. W., Mruk, D. D., and Cheng, C. Y. (2009). Mitogen-activated Protein Kinases in Male Reproductive Function. *Trends Mol. Med.* 15, 159–168. doi:10.1016/j.molmed.2009.02.002
- Li, X., Tian, G., Xu, L., Sun, L., Tao, R., Zhang, S., et al. (2021). Wenxin Keli for the Treatment of Arrhythmia—Systems Pharmacology and *In Vivo* Pharmacological Assessment. *Front. Pharmacol.* 12. doi:10.3389/fphar.2021.704622
- Li, X., Yao, Z., Yang, D., Jiang, X., Sun, J., Tian, L., et al. (2020). Cyanidin-3-O-glucoside Restores Spermatogenic Dysfunction in Cadmium-Exposed Pubertal Mice via Histone Ubiquitination and Mitigating Oxidative Damage. *J. Hazard. Mater.* 387, 121706. doi:10.1016/j.jhazmat.2019.121706
- Liu, B., Wu, S. D., Shen, L. J., Zhao, T. X., Wei, Y., Tang, X. L., et al. (2019). Spermatogenesis Dysfunction Induced by PM2.5 from Automobile Exhaust via the ROS-Mediated MAPK Signaling Pathway. *Ecotoxicol Environ. Saf.* 167, 161–168. doi:10.1016/j.ecoenv.2018.09.118
- Liu, J., Edamatsu, R., Kabuto, H., and Mori, A. (1990). Antioxidant Action of Gulingji in the Brain of Rats with FeCl3-Induced Epilepsy. *Free Radic. Biol. Med.* 9, 451–454. doi:10.1016/0891-5849(90)90023-c
- Liu, Q., Tao, B., Liu, G., Chen, G., Zhu, Q., Yu, Y., et al. (2016). Thromboxane A2 Receptor Inhibition Suppresses Multiple Myeloma Cell Proliferation by Inducing P38/c-Jun N-Terminal Kinase (JNK) Mitogen-Activated Protein Kinase (MAPK)-mediated G2/M Progression Delay and Cell Apoptosis. *J. Biol. Chem.* 291, 4779–4792. doi:10.1074/jbc.M115.683052
- Lu, B., Poirier, C., Gaspar, T., Gratzke, C., Harrison, W., Busija, D., et al. (2008). A Mutation in the Inner Mitochondrial Membrane Peptidase 2-like Gene (Imp21) Affects Mitochondrial Function and Impairs Fertility in Mice. *Biol. Reprod.* 78, 601–610. doi:10.1095/biolreprod.107.065987
- Nakamura, B. N., Lawson, G., Chan, J. Y., Banuelos, J., Cortés, M. M., Hoang, Y. D., et al. (2010). Knockout of the Transcription Factor NRF2 Disrupts Spermatogenesis in an Age-dependent Manner. *Free Radic. Biol. Med.* 49, 1368–1379. doi:10.1016/j.freeradbiomed.2010.07.019
- Niederberger, C., Rowe, P. J., Comhaire, F. H., Hargreave, T. B., and Mahmoud, A. M. A. (2001). WHO Manual for the Standardized Investigation, Diagnosis and Management of the Infertile Male. *Urology* 57, P208. doi:10.1016/S0090-4295(00)00803-7
- Shewetta, S. A., Tilmisany, A. M., and Al-Sawaf, H. (2005). Mechanisms of Male Infertility: Role of Antioxidants. *Curr. Drug Metab.* 6, 495–501. doi:10.2174/138920005774330594
- Shi, J., Xue, W., Zhao, W. J., and Li, K. X. (2013). Pharmacokinetics and Dopamine/acetylcholine Releasing Effects of Ginsenoside Re in hippocampus and mPFC of Freely Moving Rats. *Acta Pharmacol. Sin.* 34, 214–220. doi:10.1038/aps.2012.147
- Sinha Hikim, A. P., Lue, Y., Diaz-Romero, M., Yen, P. H., Wang, C., and Swerdloff, R. S. (2003). Deciphering the Pathways of Germ Cell Apoptosis in the Testis. *J. Steroid Biochem. Mol. Biol.* 85, 175–182. doi:10.1016/s0960-0760(03)00193-6
- Wang, K., Chen, B., Yin, T., Zhan, Y., Lu, Y., Zhang, Y., et al. (2019). N-methylparoxetine Blocked Autophagic Flux and Induced Apoptosis by Activating ROS-MAPK Pathway in Non-small Cell Lung Cancer Cells. *Int. J. Mol. Sci.* 20, 3415. doi:10.3390/ijms20143415
- Xiao, Y., Xiong, T., Meng, X., Yu, D., Xiao, Z., and Song, L. (2019). Different Influences on Mitochondrial Function, Oxidative Stress and Cytotoxicity of Antibiotics on Primary Human Neuron and Cell Lines. *J. Biochem. Mol. Toxicol.* 33, e22277. doi:10.1002/jbt.22277
- Yang, Q., Chen, X., Zheng, T., Han, D., Zhang, H., Shi, Y., et al. (2016). Transplantation of Human Urine-Derived Stem Cells Transfected with Pigment Epithelium-Derived Factor to Protect Erectile Function in a Rat Model of Cavernous Nerve Injury. *Cel Transpl.* 25, 1987–2001. doi:10.3727/096368916X691448
- Ye, J., Wang, Y., Xu, Y., Wang, Z., Liu, L., Wang, M., et al. (2020). Interleukin-22 Deficiency Alleviates Doxorubicin-Induced Oxidative Stress and Cardiac Injury via the P38 MAPK/macrophage/Fizz3 axis in Mice. *Redox Biol.* 36, 101636. doi:10.1016/j.redox.2020.101636
- Yue, J., and López, J. M. (2020). Understanding MAPK Signaling Pathways in Apoptosis. *Int. J. Mol. Sci.* 21, 2346. doi:10.3390/ijms21072346
- Zhao, S. J., Liu, X. J., Tian, J. S., Gao, X. X., Liu, H. L., Du, G. H., et al. (2020). Effects of Gulingji on Aging Rats and its Underlying Mechanisms. *Rejuvenation Res.* 23, 138–149. doi:10.1089/rej.2018.2118
- Zhao, S. J., Tian, J. S., Tai, G., Gao, X. X., Liu, H. L., Du, G. H., et al. (2019). 1H NMR-Based Metabolomics Revealed the Protective Effects of Gulingji on the Testicular Dysfunction of Aging Rats. *J. Ethnopharmacol* 238, 111839. doi:10.1016/j.jep.2019.111839
- Zhao, X. Z., Zhao, S. J., Tian, J. S., Gao, X. X., and Du, G. H. (2017). Research Progress on Material Basis of Gulingji Based on Pharmacodynamical Effect. *Chin. Traditional Herbal Drugs* 48, 1424–1431.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2022 Wang, Xie, Chen, Yao, Lv, Li, Deng, Zhang, Sun and Liu. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.