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Quercetin and Astragaloside IV Mitigate the Developmental Abnormalities Induced by Gestational Exposure to Zinc Oxide Nanoparticles

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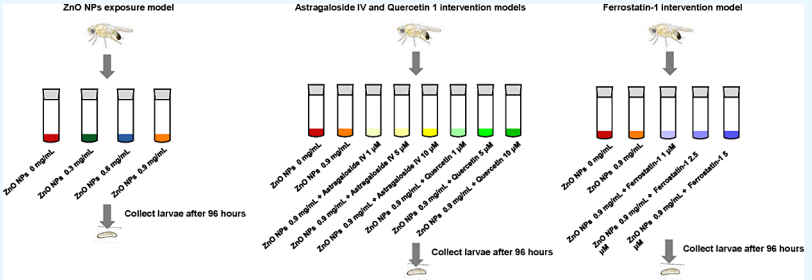

ABSTRACT: Zinc oxide (ZnO) nanoparticles (NPs) are extensively utilized in the commercial and biomedical sectors, posing heightened risks of potential cytotoxicity through various mechanisms. Nonetheless, the regulatory framework governing the gestational toxicity of ZnO NPs and the corresponding intervention strategies remain largely obscure. In this study, using the *Drosophila* model, we observed that gestational exposure to ZnO NPs led to growth and developmental anomalies in a dose-dependent manner when compared with the control (no ZnO NP exposure). Subsequent dietary administration of Quercetin and Astragaloside IV resulted in effective mitigation of the developmental toxicity induced by exposure to ZnO NPs. Moreover, the latter also triggered activation of the ferroptosis pathway. The associated parameters were successfully ameliorated by the administration of Quercetin and Astragaloside IV. Notably, treatment with Ferrostatin-1 also alleviated developmental disorders arising from exposure to ZnO NPs. In conclusion, our investigation demonstrated that exposure to ZnO NPs during gestation interfered with growth and development via the ferroptosis pathway, underscoring the significance of dietary supplementation with Quercetin and Astragaloside IV for protection against developmental toxicity.

1. INTRODUCTION

Zinc oxide (ZnO) nanoparticles (NPs) are extensively utilized in cosmetics, medical supplies, food additives, gas sensors, UV radiation blockers, paints, sunscreens, and ceramics.[1,2] The yearly output of ZnO NPs is approximated at 5500 tons, leading to a significant level of environmental emission.[3] Despite being comparatively less toxic than other semiconductor NPs, an escalating amount of untreated ZnO NPs is being deposited in soil and could potentially be released into the environment, raising concerns about their toxicity.[4,5] It is reported that ZnO NPs extensively accumulate in sediments, natural and urban soil, as well as in landfills.[5] Previous studies have suggested that the toxic effects of ZnO NPs can result in oxidative DNA damage and mitochondria-mediated apoptosis triggered by reactive oxygen species (ROS).[3,6] In addition, ZnO NPs have the potential to induce adverse toxicological effects on multiple systems, such as hepatic, renal, cardiac, neural, immune, and reproductive systems.[7] Studies in vivo have found that ZnO NP exposure induced toxic effects on the ovaries, impairing the female reproductive system.[8,9] Nevertheless, the question of whether ZnO NPs induce developmental toxicity remains debatable.

Drosophila melanogaster stands out as a highly efficient model organism since it offers numerous advantages, including cost-effectiveness, easy handling and maintenance, substantial offspring production, relatively short life cycle, a relatively low number of paralogous genes, and the presence of gene sequences that are conserved between *Drosophila* and humans.[10] *Drosophila* has been reported to be a dynamic in

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vivo model organism for examining the toxicity of diverse environmental contaminants and toxic substances.[11] Unlike mammals, *Drosophila* development is a metamorphic process, passing through the fertilized egg, larva (L1, L2, and L3), pupa, and adult, typically taking around 9−10 days at 25 °C.[12,13] Third instar larvae are the hallmark stage in the study of development.[14] Previously, Kumar et al. utilized third instar larvae in *Drosophila* to assess the developmental toxicity of rotenone.[13] Moreover, a recent study adeptly employed the *Drosophila* model to study the effect of exposure to metals during pregnancy.[15] Wang et al. observed a notable decrease in the size, weight, and quantity of third instar larvae after exposure to metal antimony (0, 0.3, 0.6, and 1.2 mg/mL), thereby shedding light on its role in modulating developmental disorders.[15] A prior investigation effectively leveraged *Drosophila* as an in vivo model to explore the biological impacts of ZnO NP exposure.[16] The flies exposed to ZnO NPs displayed discernible phenotypic alterations, including malformed segmented thoraxes and singular or distorted wings, which were heritably transmitted to subsequent generations.[17] Additionally, ZnO NPs induced lethality by significantly disrupting the oxidative stress equilibrium, with ROS generation serving as a direct instigator of ZnO NP-induced toxicity in *Drosophila*.[18]

While previous studies have reported the in vivo evaluation of the toxic and genotoxic effects of exposure to ZnO NPs, further in vivo investigations are imperative to elucidate the mechanism of action. In addition, a comprehensive look at strategies for mitigating and ameliorating these toxicities is required. This study employs the *Drosophila* model of gestational exposure to ZnO NPs to investigate the latter's influence on growth and development. We further examined the roles of Quercetin and Astragaloside IV in the alleviation of developmental toxicity in order to establish a theoretical framework for preventing and intervening in gestational ZnO NP poisoning. Taken together, this study deepens our understanding of ZnO NP-induced developmental toxicity and provides promising therapy targets.

2. RESULTS

2.1. Characteristics of ZnO NPs. The morphology and particle size distribution of the ZnO NPs were examined using scanning electron microscopy (SEM) in order to elucidate their properties (Figure 1A). It showed the average NP size to be within the nanoscale range. Subsequently, the mean zeta potential value of ZnO NPs in water was determined to be 21.23 mV (Figure 1B). Furthermore, as shown by the UV−vis absorption spectrum, the characteristic absorption peak of ZnO NPs was around 370 nm (Figure 1C). The analyses of ZnO NPs laid the groundwork for further functional experiments.

2.2. Role of Gestational ZnO NP Exposure in Developmental Disorders in a Simulated *Drosophila* Model. A *Drosophila* model was utilized to investigate the effect of gestational exposure to ZnO NPs on larval development. Our results indicated a significant reduction in the size of third instar larvae following gestational ZnO NP exposure (0.3, 0.6, 0.9 mg/mL) in a dose-dependent manner compared with the control (no ZnO NP exposure) (Figure 2A). Additionally, compared with the control (no ZnO NP exposure), exposure to ZnO NPs resulted in a dose-dependent decrease in the number of third instar larvae (Figure 2B). A prior investigation has demonstrated that the toxicity in

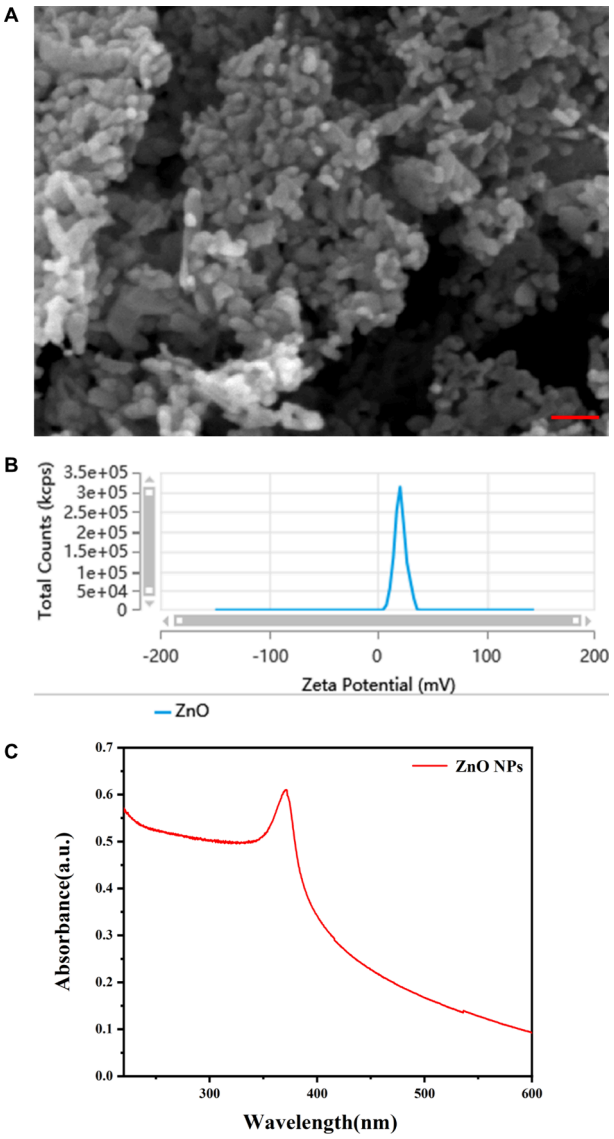

Figure 1. Characteristics of ZnO NPs. (A) SEM image of ZnO NPs. (B) Zeta potential (mV) of ZnO NPs measured in water. (C) UV−vis absorption of ZnO NPs. Scale bar is 100 nm.

zebrafish is exacerbated by ZnO NPs themselves rather than the released Zn^{2+} ions from these NPs.[19] In this study, we also incorporated $ZnSO_4$ to evaluate the toxicological impact of Zn^{2+} ions. Our findings revealed that gestational exposure to $ZnSO_4$ did not elicit a dose-dependent toxic effect on the number of third instar larvae compared with the control (no $ZnSO_4$ exposure) (Figure S1). Moreover, high-dose exposure to ZnO NPs led to a notable reduction in larval body length and weight when compared to the control group (Figure 2C,D), thereby highlighting the developmental toxicity induced by gestational ZnO NP exposure.

2.3. Mitigation of Developmental Toxicity Caused by Gestational Exposure to ZnO NPs through Dietary Supplementation with Quercetin and Astragaloside IV. To address the developmental toxicity caused by gestational ZnO NP exposure, dietary supplementation rescue assays were conducted in vivo with varying doses of Quercetin (1, 5, and 10 μM) or Astragaloside IV (1, 5, and 10 μM), alongside ZnO NPs (0.9 mg/mL). Remarkably, both Quercetin and Astragaloside IV treatments effectively restored the size of the third

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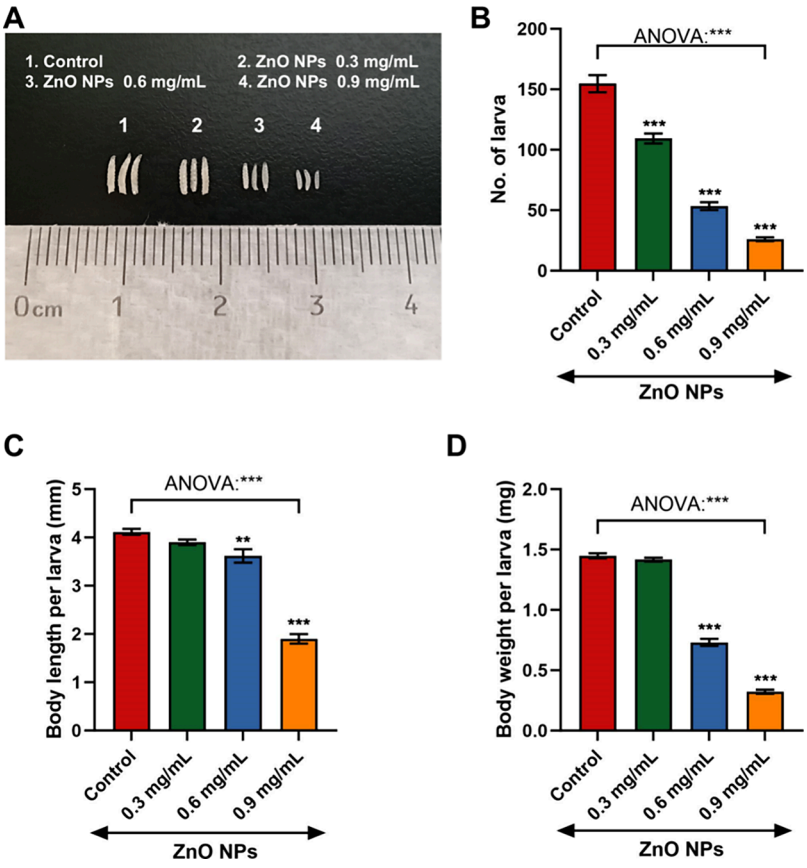

Figure 2. Gestational ZnO NPs caused developmental defects in *Drosophila* larvae. In the control (no ZnO NP exposure) and different ZnO NP exposure (0.3 0.6, 0.9 mg/mL) groups, the larval size (n = 3) (A), number of larvae (n = 3) (B), larval body length (n = 6) (C), and larval body weight (n = 6) (D) were measured and recorded at 96 h after laying eggs. Statistical differences were computed based on comparison with the control group (no ZnO NP exposure). $^{**}P < 0.01$, $^{***}P < 0.001$.

instar larvae exposed to ZnO NPs in a dose-dependent manner (Figure 3A,B). Similarly, Quercetin and Astragaloside IV dose-dependently mitigated the reduction in the number of larvae, which occurred as a result of gestational exposure to ZnO NPs (Figure 3C). Notably, both compounds had a similar dose-dependent recovery effect against developmental defects on the body length and weight of third instar larvae (Figure 3D,E). In addition, our study found that the combined effect of Quercetin and Astragaloside IV was also effective in restoring the phenotypes of third instar larvae caused by gestational ZnO NP exposure, including the size, quantity, body length, and weight (Figure S2A−D). However, it seemed that the combination of Quercetin and Astragaloside IV did not work better than their independent effects. These results demonstrated their potential as crucial compounds for preventing and treating developmental toxicity associated with gestational ZnO NP exposure.

2.4. Ferroptosis Induced by Gestational ZnO NP Exposure and Its Inhibition by Quercetin and Astragaloside IV. The induction of ferroptosis by gestational ZnO NP exposure was investigated by assessing the activity levels of malondialdehyde (MDA), glutathione (GSH), and superoxide dismutase (SOD) in third instar larvae. Following gestational ZnO NP exposure, the third instar larvae showed a significant decrease in GSH level and SOD activity, along with an increase in MDA level, when compared with the control (no ZnO NP exposure) (Figure 4). Importantly, gestational ZnO NP exposure induced the activation of the ferroptosis pathway, which could be counteracted by independent or combined treatments of Quercetin and Astragaloside IV (Figures 4 and S2E−G), underscoring the potential of these compounds in mitigating the ferroptotic response triggered by exposure to ZnO NPs.

2.5. Reversal of ZnO NP-Induced Developmental Disorders by Ferrostatin-1 Treatment. The relationship between gestational ZnO NP exposure and ferroptosis was explored through dietary supplementation with the ferroptosis inhibitor, Ferrostatin-1. A ferroptosis rescue assay was conducted in vivo with varying doses of Ferrostatin-1 (1 μM, 2.5 μM, and 5 μM) administered alongside ZnO NPs (0.9 mg/mL). Remarkably, Ferrostatin-1 treatment partially restored the size, quantity, body length, and weight of third instar larvae exposed to gestational ZnO NPs (Figure 5). The findings imply that ferroptosis activation plays a crucial role in the developmental toxicity triggered by gestational exposure to ZnO NPs in *Drosophila* larvae.

3. DISCUSSION

ZnO NPs have garnered considerable attention among metal oxide NPs due to their versatility and resilience in harsh environments.[20] A study had shown that exposure to ZnO NPs induced antioxidant responses in mouse follicular ovarian cells, leading to changes in the expression of antioxidant molecules and a decrease in the levels of ROS.[9] Similarly, ZnO NPs were observed to adversely affect ovarian tissue in rats.[8] Moreover, ZnO NPs have been found to exhibit toxicity in the

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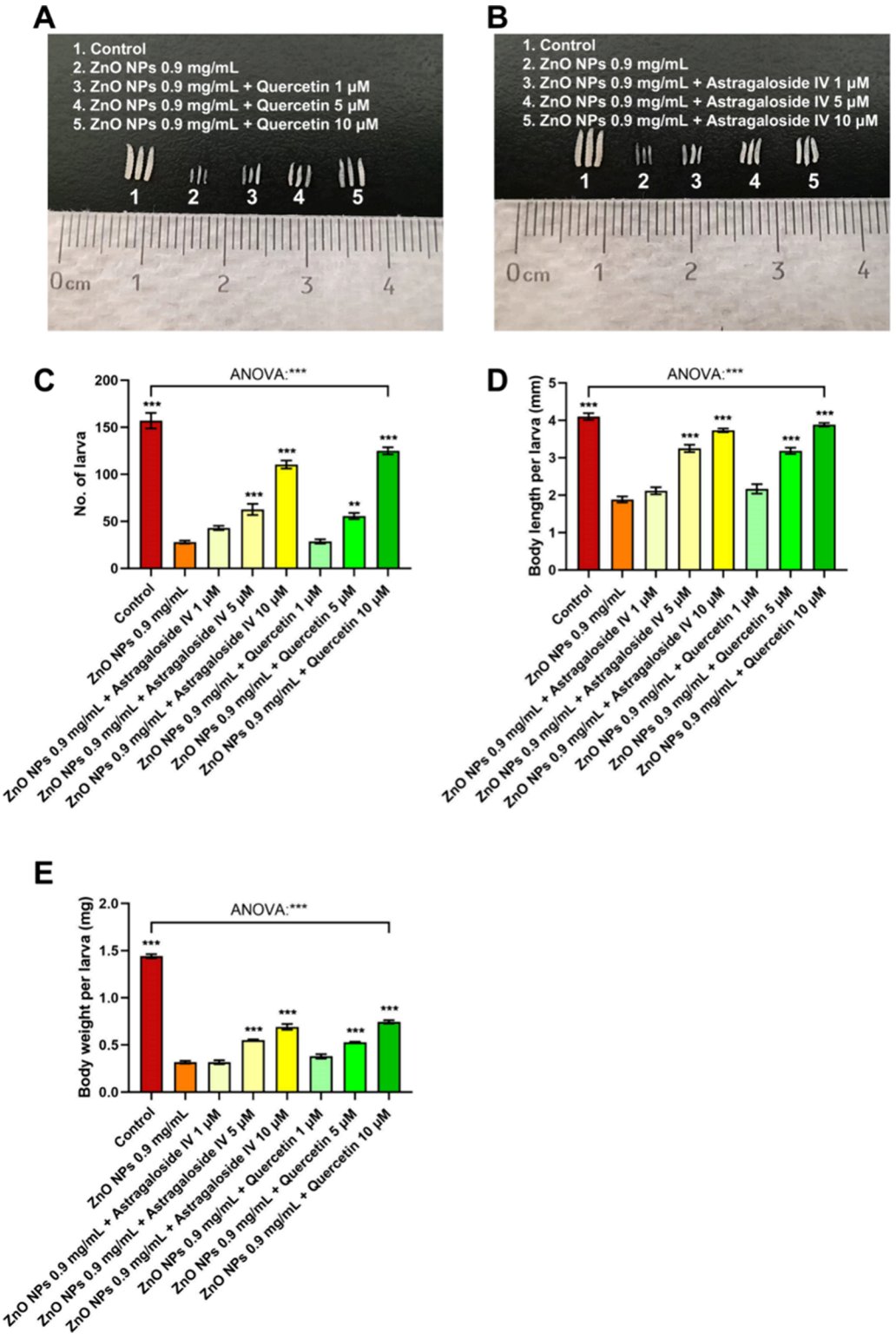

Figure 3. Effect of Quercetin and Astragaloside IV treatment on developmental toxicity caused by gestational ZnO NP exposure. (A) Larval size at 96 h after laying eggs in the control (no ZnO NP exposure), ZnO NP exposure (0.9 mg/mL), ZnO NPs (0.9 mg/mL) + Quercetin (1 μM), ZnO NPs (0.9 mg/mL) + Quercetin (5 μM), and ZnO NPs (0.9 mg/mL) + Quercetin (10 μM) groups; $n = 3$. (B) Larval size at 96 h after laying eggs in the control (no ZnO NP exposure), ZnO NP exposure (0.9 mg/mL), ZnO NPs (0.9 mg/mL) + Astragaloside IV (1 μM), ZnO NPs (0.9 mg/mL) + Astragaloside IV (5 μM), and ZnO NPs (0.9 mg/mL) + Astragaloside IV (10 μM) groups; $n = 3$. (C) Number of larvae at 96 h after laying eggs in the control (no ZnO NP exposure), different ZnO NP exposure, and Quercetin/Astragaloside IV intervention treatment groups; $n = 3$. (D) Body length per larva in the control (no ZnO NP exposure), different ZnO NP exposure, and Quercetin/Astragaloside IV intervention treatment groups; $n = 6$. (E) Body weight per larva in the control (no ZnO NP exposure), different ZnO NP exposure, and Quercetin/Astragaloside IV intervention treatment groups; $n = 6$. Statistical differences were compared to the 0.9 mg/mL ZnO NP exposure group. $^{**}P < 0.01$, $^{***}P < 0.001$.

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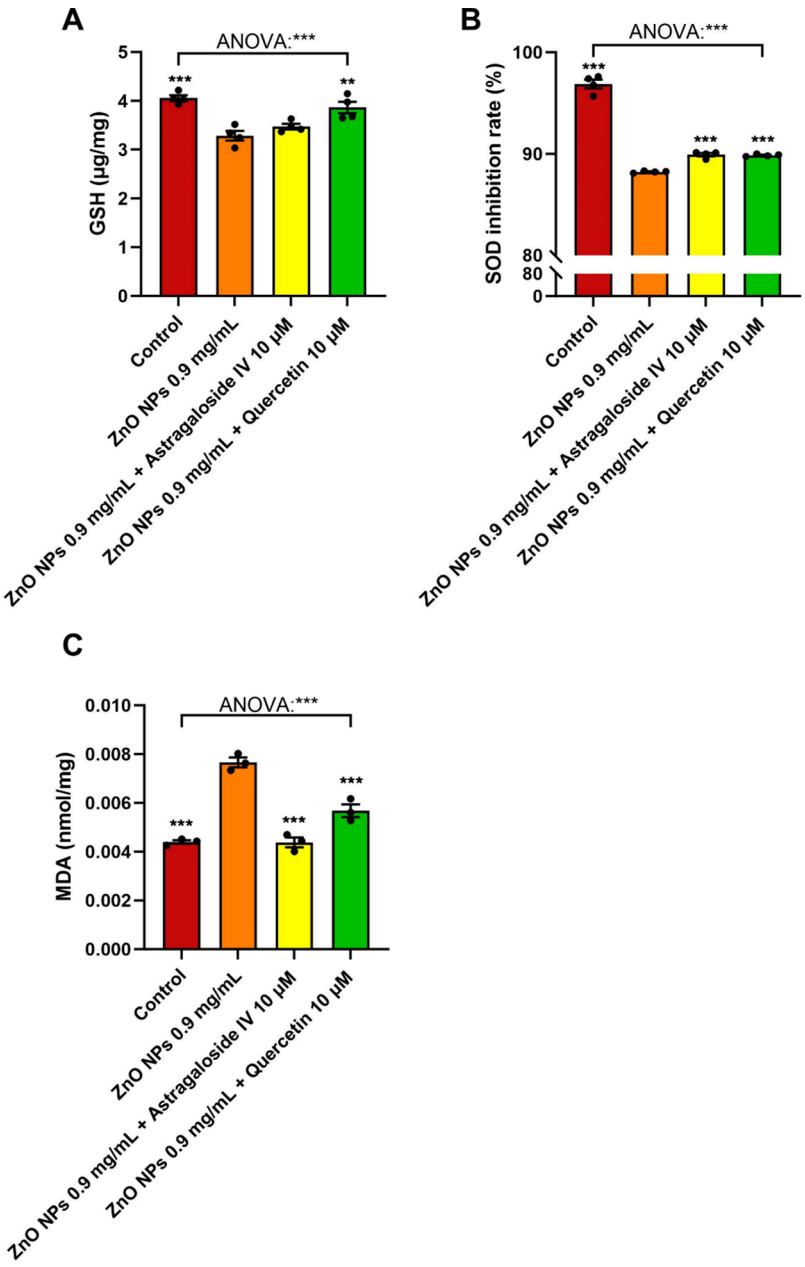

Figure 4. Ferroptosis-associated indexes in larvae. (A) GSH level in larvae of the control (no ZnO NP exposure), ZnO NP exposure (0.9 mg/mL), ZnO NPs (0.9 mg/mL) + Astragaloside IV (10 μM), and ZnO NPs (0.9 mg/mL) + Quercetin (10 μM) groups; $n = 4$. (B) Relative SOD activity in the larvae of corresponding groups; $n = 4$. (C) MDA level in the larvae of corresponding groups; $n = 3$. Statistical differences were compared to the 0.9 mg/mL ZnO NP exposure group. $^{**}P < 0.01$, $^{***}P < 0.001$.

development of mouse oocyte and preimplantation embryo.[3] In this investigation, we simulated the gestational exposure of ZnO NPs using the *Drosophila* model and observed a robust developmental arrest phenotype in the larvae following exposure to the NPs. ZnO NPs were found to elevate the intracellular levels of ROS, thereby leading to genotoxicity and cell demise.[6,21] A previous study showed that ZnO NPs were internalized by human epidermal keratinocytes and led to cytotoxic and genotoxic responses.[22] Exposure to ZnO NPs in human epidermal cells resulted in cell cycle arrest, ROS generation, mitochondrial dysfunction, apoptosis, and disordered glucose metabolism.[23] Additionally, ZnO NPs were found to induce DNA damage through LPO and oxidative stress.[24] In this study, GSH and SOD were utilized for evaluating the antioxidant status, whereas MDA was employed for assessing the LPO level. Our study revealed a reduction in GSH level, decreased SOD activity, and an increased MDA level in third instar larvae post gestational ZnO NP exposure, thus highlighting the crucial role played by the ferroptosis pathway.

Ferroptosis, an iron-dependent form of programmed cell death triggered by excessive LPO accumulation,[25] is regarded as a promising target for disease prevention and intervention.[26] Ferrostatin-1, a specific inhibitor of ferroptosis, may play a pivotal role in mitigating cellular damage.[27] The developmental arrest phenotype was partially alleviated by dietary supplementation with Ferrostatin-1 of *Drosophila* food exposed to gestational ZnO NPs. The present study focused on the

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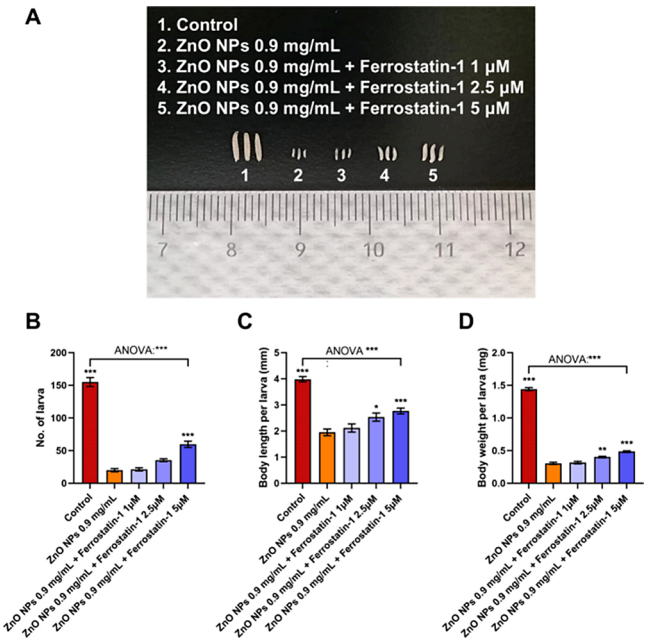

Figure 5. Ferrostatin-1 treatment effects for ZnO NP exposure. (A) Larval size at 96 h after laying eggs in the control (no ZnO NP exposure), ZnO NP exposure (0.9 mg/mL), ZnO NPs (0.9 mg/mL) + Ferrostatin-1 (1 μM), ZnO NPs (0.9 mg/mL) + Ferrostatin-1 (2.5 μM), and ZnO NPs (0.9 mg/mL) + Ferrostatin-1 (5 μM) groups; n = 3. (B) Number of larvae at 96 h after laying eggs in the control (no ZnO NP exposure), different ZnO NP exposure, and Ferrostatin-1 intervention treatment groups; n = 3. (C) Larval body length in the control (no ZnO NP exposure), different ZnO NP exposure, and Ferrostatin-1 intervention treatment groups; n = 6. (D) Larval body weight in the control (no ZnO NP exposure), different ZnO NP exposure, and Ferrostatin-1 intervention treatment groups; n = 6. Statistical differences were compared to the 0.9 mg/mL ZnO NP exposure group. *P < 0.05, **P < 0.01, ***P < 0.001.

processes associated with ferroptosis in order to identify compounds capable of alleviating the cytotoxic effects of gestational ZnO NP exposure.

Our study revealed that two chemical compounds, Quercetin and Astragaloside IV, are capable of ameliorating developmental disorders and injuries triggered by gestational exposure to ZnO NPs in *Drosophila*. Astragaloside IV is a bioactive constituent extracted from the traditional Chinese herb *Astragalus membranaceus*.[28] Astragaloside IV has a wealth of pharmacological functions in clinical applications, including antioxidant and anti-inflammatory properties, which are protective against a wide range of diseases.[29] It has been shown to have a protective effect against cellular damage induced by xenobiotic compounds through antiferroptotic mechanisms by bolstering Nrf2 signaling.[30−32] Quercetin, a major flavonoid present in several medicinal plants, has been shown to exert beneficial effects in several ailments, including alcoholic hepatitis, renal ischemia-reperfusion injury, and cancer.[33,34] Notably, Quercetin has been shown to possess pharmacological impacts on ischemic injury alongside antiferroptotic mechanisms.[35] It inhibits ferroptosis in pancreatic β cells in type 2 diabetes by reducing MDA levels and elevating GSH levels.[36] Furthermore, quercetin enhances cell viability, migration, and angiogenesis while inhibiting apoptosis. It does so by the activation of Keap1-Nrf2 signals and a decrease in the expression of ATF6/GRP78 in human brain microvascular endothelial cells.[37] Previous studies had also shown that both astragaloside IV and quercetin mitigated PM2.5-induced lung damage by targeting ferroptosis through the Nrf2 signaling pathway in mice.[38,39] Collectively, our findings provide a potential therapeutic target for developmental disorders caused by ZnO NPs.

There are certain unavoidable limitations that should be addressed. First, *Drosophila* serves as an important model for understanding developmental toxicology due to the parallels in their neurological and developmental pathways with those of vertebrates.[40] However, *Drosophila* experience metamorphosis, leading to the delineation of discrete developmental stages, while vertebrates undergo internal development characterized by embryonic, fetal, and postnatal phases.[41] In this study, we systematically evaluated the developmental toxicity and mechanism of ZnO NPs in the *Drosophila* model. However, the toxicity of ZnO NPs at specific developmental stages might need to be further evaluated. Second, the toxicity of nanomaterials was influenced by several factors, including physicochemical properties and exposure patterns.[42,43] Hence, a combination of additional studies was needed to comprehensively assess the toxicity of ZnO NPs. Meanwhile, the actual intake of different individuals may also contribute to the experimental error. In this study, we assessed the toxicity of exposure of ZnO NPs to a fly population, minimizing the individual differences. Third, we found that both the individual and combined effects of Quercetin and Astragaloside alleviated the toxicity of ZnO NPs. However, the possible mechanism of Quercetin and Astragaloside requires further investigation in the future.

4. CONCLUSIONS

In conclusion, our findings demonstrate that gestational exposure to ZnO NPs induces growth and developmental toxicity in *Drosophila* through the activation of ferroptosis. Furthermore, Quercetin and Astragaloside IV have been identified as promising chemical compounds for the mitigation of the toxicity induced by gestational ZnO NP exposure, thereby offering a theoretical foundation for the prevention and intervention of fetal developmental arrest caused by exogenous metal interferents.

5. MATERIALS AND METHODS

5.1. Fly Strains and Culture. The w^{1118} strain was employed for an in vivo analysis. The flies were kept at 25 °C under a photoperiod of 12 h day/night cycle, with the relative humidity ranging from 40 to 60%. All flies were raised in vials filled with a standard cornmeal molasses agar medium.

5.2. Characteristics of ZnO NPs. ZnO NPs were procured from Beijing Deke Daojin Science and Technology Co., Ltd. Briefly, the hydrothermal method was used for the synthesis of ZnO NPs.[44] The morphology of ZnO NPs was assessed using SEM. The zeta potential of ZnO NPs was determined by dynamic light scattering using a Malvern Zetasizer Nano ZS 90. The UV−vis adsorption spectra were recorded using the UV−vis spectrophotometer (UV-2600, Shimadzu, Japan).

5.3. Exposure Methods. The ZnO NPs or $ZnSO_4$ powders were dispersed in 1× phosphate-buffered saline, which was followed by 5 min of ultrasonic treatment and 10 min of mechanical vibration. Subsequently, the solutions containing ZnO NPs or $ZnSO_4$ were incorporated into a standard fly food to achieve the desired exposure concen-

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trations. The control group consisted of flies without exposure to ZnO NPs or $ZnSO_4$.

Mated female flies, aged 2−3 days, were exposed to the specified concentrations of ZnO NPs (0, 0.3, 0.6, and 0.9 mg/mL). Each group consisted of at least three identical vials, with each vial containing 20 mated females. The embryos were exposed to the environment with ZnO NPs, and they traversed the complete life cycle of flies from embryos to emerging adults. Various parameters including size, body length, body weight, and the quantity of third instar larvae in each vial were measured and documented for comparative analysis.

5.4. Methods of Chemical Compound Treatment. A series of experiments involving concurrent exposure to ZnO NPs and Quercetin (HY-18085, MedChemExpress)/Astragaloside IV (B20564, Shanghai Yuanye Biotechnology)/Ferrostatin-1 (HY-100579, MedChemExpress) were performed. Different concentrations of Quercetin/Astragaloside IV/Ferrostatin-1 were coadministered with ZnO NPs and subsequently incorporated into standard corn syrup food for the flies. The experimental groups were structured as follows: (1) control (no ZnO NP exposure); (2) ZnO NPs 0.9 mg/mL; (3) ZnO NPs 0.9 mg/mL + Quercetin 1 μM; (4) ZnO NPs 0.9 mg/mL + Quercetin 5 μM; (5) ZnO NPs 0.9 mg/mL + Quercetin 10 μM; (6) ZnO NPs 0.9 mg/mL + Astragaloside IV 1 μM; (7) ZnO NPs 0.9 mg/mL + Astragaloside IV 5 μM; (8) ZnO NPs 0.9 mg/mL + Astragaloside IV 10 μM; (9) ZnO NPs 0.9 mg/mL + Ferrostatin-1 1 μM; (10) ZnO NPs 0.9 mg/mL + Ferrostatin-1 2.5 μM; and (11) ZnO NPs 0.9 mg/mL + Ferrostatin-1 5 μM. The dimensions, body length, body weight, and quantity of third instar larvae in each feeding tube were monitored and documented.

5.5. Oxidative Stress Assays. Ferroptosis was assessed using the MDA assay (BC5245, Solarbio), the SOD assay (S311, Dojindo Molecular Technologies), and the GSH assay (BC1175, Solarbio). Larval tissues from different experimental groups were extracted and homogenized in ice-cold saline using an ultrasonic homogenizer, followed by centrifugation at 12000*g* for 10 min. The resulting tissue supernatants were gathered for the quantification of MDA and GSH levels as well as the relative SOD activity. As per the manufacturer's guidelines, the supernatants and detection reagents were transferred to 96-well plates and mixed completely. The absorbance of different experiments (MDA: $\lambda_{532\ nm}$; GSH: $\lambda_{412\ nm}$; SOD: $\lambda_{450\ nm}$) was measured using a FlexStation 3 Multi-Mode Microplate Reader. All experiments were repeated a minimum of three times, and the data were normalized according to the larval tissue quality.

5.6. Statistical Analysis. The quantitative results were depicted as the mean ± standard error of the mean and analyzed with GraphPad Prism 8.0 (GraphPad Software, CA, USA). One-way analysis of variance was utilized for comparing multiple groups with Dunnett's multiple comparison test. *P < 0.05; **P < 0.01; ***P < 0.001.

■ ASSOCIATED CONTENT

Data Availability Statement

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

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c08235.

Gestational $ZnSO_4$ exposure; and combined effect of Quercetin and Astragaloside IV (PDF)

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Author Contributions

J.Y., Y.Q., X.Z., and X.W. initiated the project, designed the study, coordinated the experiment, and wrote the manuscript. L.J., Q.H., Y.Q., Z.W., X.K., X.Z., and B.Y. performed the experiments and provided conceptual inputs for the paper. J.L., X.H., X.D., X.C., H.Y., Y.S., and Z.L. analyzed the data. All

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authors read and approved the final manuscript. L.J., Q.H., and Y.Q. contributed equally to this work.

Notes

The authors declare no competing financial interest.

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