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The negative effect of the PI3K inhibitor 3-methyladenine on planarian regeneration via the autophagy signalling pathway

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Abstract

As an important PI3K (VPS34) inhibitor, 3-methyladenine (3-MA) can block the formation of autophagic vesicles in animals. Most toxicological studies using 3-MA have shown that 3-MA leads to serious disorders via autophagy suppression in mammals. However, no toxicological research on 3-MA has been performed on individuals undergoing regeneration. The freshwater planarian has powerful regenerative capability, and it can regenerate a new brain in 5 days and undergo complete adult individual remodelling in approximately 14 days. Moreover, it is also an excellent model organism for studies on environmental toxicology due to its high chemical sensitivity and extensive distribution. Here, *Dugesia japonica* planarians were treated with 3-MA, and the results showed that autophagy was inhibited and *Djvps34* expression levels were down-regulated. After exposure to 10 mM 3-MA for 18 h, all the controls showed normal phenotypes, while one-half of the planarians treated with 3-MA showed morphological defects. In most cases, an ulcer appeared in the middle of the body, and a normal phenotype was restored 7 days following 3-MA exposure. During regeneration, disproportionate blastemas with tissue regression were observed. Furthermore, 3-MA treatment suppressed stem cell proliferation in intact and regenerating worms. These findings demonstrate that autophagy is indispensable for tissue homeostasis and regeneration in planarians and that 3-MA treatment is detrimental to planarian regeneration via its effect on the autophagy pathway.

Keywords 3-MA · Autophagy inhibitor · Toxicological research · Planarian · PI3K signaling pathway

Introduction

Autophagy is a lysosome-mediated degradation process by which aged proteins, misfolded proteins and damaged organelles are delivered to lysosomes for bulk degradation (Gonzalez-Estevez and Salo 2010; Kang et al. 2019a). Simultaneously, the function of autophagy is to control protein quality and maintain cellular homeostasis; the dysregulation of autophagy may lead to serious disorders in humans (Kang et al. 2019a; Zhang et al. 2020). In the heart, autophagy occurs constitutively at a basal level but is enhanced under pathological conditions (Lavandero et al. 2015; Zhang et al. 2020). Many signalling pathways, including the phosphatidylinositol-3,4,5-triphosphate kinase (PI3K, VPS34) pathway, have been shown to be involved in the regulation of autophagy (Ding et al. 2018). The PI3K pathway has been found to regulate the formation of autophagosomes and autophagic vacuoles (Ding et al. 2018). As a PI3K inhibitor, 3-methyladenine (3-MA) has been reported to inhibit the activity of PI3K and block the formation of autophagosomes and autophagic vacuoles (Zhao et al. 2019). Christian et al. (2014) reported that autophagy is inhibited in the HTC11 human colon cancer cell line after 5 mM 3-MA exposure for 48 h. Autophagy is inhibited in the nerve cells of mouse embryos after treatment with 30 mM 3-MA for 6 h (Hou et al. 2016). In zebrafish, a large number of cells died after exposure to 5 mM 3-MA for 24 h due to autophagy inhibition (Pang et al. 2019).

Environmental exposure to autophagy inhibition has significantly increased in recent decades, mostly because of increased environmental pollution and antibiotic residue (Jiang et al. 2012; Zhang et al. 2019; Avila-Rojas et al. 2019). For example, tetracycline is a widely-used and well-tolerated

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antibiotic in the clinic that can inhibit autophagy in the ischaemic brain in rats that underwent a stroke (Jiang et al. 2012). Moreover, recent studies have shown that heavy metals also enhance cell damage by suppressing autophagic flux (Zhang et al. 2019). In addition, pesticide residues are important sources of environmental pollution, and their toxic effects are exerted via the mechanism of autophagy (Avila-Rojas et al., 2019). Simultaneously, autophagy is one of the key degradation systems in organisms, and the dysregulation of autophagy may lead to serious disorders in humans (Kang et al. 2019a; Zhang et al. 2020). Moreover, toxicological studies show that dysregulation of autophagy may lead to disorders in cell homeostasis and regenerative ability (Li et al. 2018; Zhang et al. 2020; Nieuwenhuis et al. 2020). Furthermore, the regenerative ability and homeostasis disruptions caused by dysregulated autophagy increase the risk of diseases, and the correlation between tissue regeneration and autophagy inhibition has been confirmed by many studies (Saera-Vila et al. 2016; Xu et al. 2020; Lee et al. 2019). Autophagy inhibition can effectively reduce muscle regeneration in a zebrafish model (Saera-Vila et al. 2016), and autophagy inhibition might suppress regeneration in ageing mouse livers (Xu et al. 2020). Additionally, autophagy plays critical roles in skeletal muscle homeostasis, regeneration and ageing in humans because muscle stem cell senescence is associated with suppression of autophagy during regeneration (Lee et al., 2019). However, the correlation between autophagy inhibition and individual regeneration is still not clear.

Freshwater planarians, belonging to the phyla Platyhelminthes, order Tricladida and class Turbellaria, are among the most abundant predators in aquatic ecosystems (Gonzalez-Estevez 2009). Moreover, they have powerful regenerative capability and can regenerate a new brain in 5 days and undergo complete adult individual remodelling in 14 days. Therefore, it is an excellent model animal for study in the field of regenerative medicine and toxicity (Zhang et al. 2017; Zhang et al. 2018; Kang et al. 2019b). The freshwater planarian *Dugesia japonica* is widely distributed in East Asia and is an excellent freshwater species for studying regenerative medicine and assessing toxicity (Ross et al. 2017; Kang et al. 2019b; Danielle et al. 2020). Therefore, to elucidate the role of autophagy inhibition in planarian regeneration, our study focused on the regulatory effects of 3-MA and investigated the relationship between 3-MA effects and individual *D. japonica* regeneration.

Materials and methods

Animals and experimental design

The animals used in these experiments were the freshwater planarian *D. japonica* collected from Shilaogong, Henan Province, China. They were cultured in autoclaved tap water and incubated in the dark at 20 °C. Planarians were fed beef liver once a week until sexual maturity was reached. The worms (0.8–1 cm in length and weighing 18–20 mg) were selected and starved for 1 week in the dark at 20 °C before all experiments (Dong et al. 2017; Kang et al. 2019b).

A P13K inhibitor, 3-MA (Sigma-Aldrich, St. Louis, MO, USA) is widely used as an inhibitor of autophagy. Therefore, this study aimed to determine whether autophagy was inhibited in planarians after 3-MA exposure for different concentrations and for different lengths of time; to this end TEM was performed. On the basis of the literature (Christian et al. 2014; Hou et al. 2016; Pang et al. 2019), 5 mM and 10 mM concentrations were tested. Several different exposure times (6 and 18 h) were also selected. The preliminary experimental results revealed no autophagic vesicles in *D. japonica* after 10 mM 3-MA exposure for 18 h but autophagic vesicles were observed in the controls. Therefore, it was decided that an 18-hour exposure of *D. japonica* to a 10 mM 3-MA dose was appropriate for this study.

3-MA treatment

To produce a 0.5 M 3-MA solvent solution, 7.5 mg of 3-MA was added to 0.1 ml of sterilized double steaming water and mixed. Then, the solution was place in a refrigerator set at 4 °C. The animals were exposed to 10 mM 3-MA for 18 h. Subsequently, they were rinsed three times and cultured in autoclaved tap water for 7 days. Images were captured with a Leica digital camera attached to a compound stereomicroscope (M165C, Germany). To analyse the effects of 3-MA during *D. japonica* regeneration, 20 normal worms (Fig. 1B) were selected after 10 mM 3-MA exposure for 18 h and compared with the control worms (Fig. 1A). Then, the worms were cut at the prepharyngeal and post-auricle levels to produce two fragments (Fig. 2B). The head and tail fragments were separately incubated in autoclaved tap water for 14 days, and images were captured following their amputation and separation.

Transmission electron microscopy (TEM)

Planarian individuals were placed in on the inside wall of a 100-ml glass beaker and sacrificed with 2% dilute hydrochloric acid (HCl). Then, they were fixed with 2.5% glutaraldehyde at room temperature for 2 h. Then, the fixed samples were sent to the TEM Laboratory Center of Xinxiang Medical College, where they were washed repeatedly with buffer solution, fixed with osmium acid, dehydrated, embedded with epoxy resin, sliced into ultrathin sections, stained with uranium acetate and lead citrate, and observed and imaged by a professional technician (Abdel et al. 2014; Kang et al. 2019b).

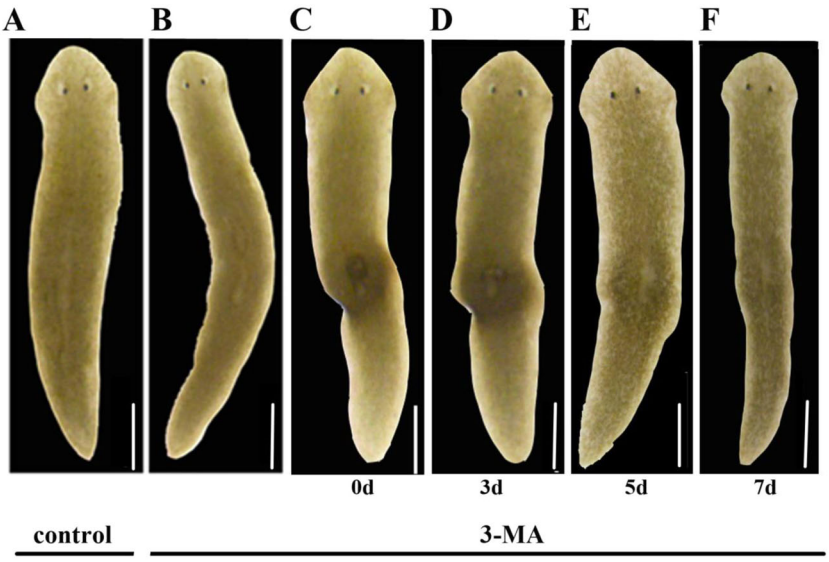

Fig. 1 Effects of 3-MA treatment on intact planarians ($n = 20$ animals for each treatment). **A** The control planarian is normal; **B** Half of planarians are normal after 3-MA treatment; **C–E** Planarians show ulcer in the middle of the body for 0, 3, 5 d after 3-MA treatment; **F** Planarians are normal for 7 d after 3-MA treatment. Scale bar, 500 μm

Real-time PCR (RT-PCR)

After exposure to 10 mM 3-MA for 18 h, worm RNA was extracted from 6 worms using RNAiso plus (TaKaRa, China) and then subjected to agar electrophoresis. In addition, the concentration of RNA was measured with a spectrophotometer (Thermo, United States), and the amount of RNA template was calculated according to the concentration. Then, reagents were added according to the instructions of a cDNA reverse transcription kit (TaKaRa, China) and mixed. The mixture was placed in a thermal cycler for reverse transcription of the mRNA to cDNA. Real-time PCR primers were mixed with the cDNA template and SYBR Green Master Mix reagent for real-time PCR detection (Dong et al. 2017). The following *Djvps34* RT-PCR primers were used: F-5′ CGTAGTTTGGCTGGTTA TTGTGTC 3′ and R-5′ GTAAAGGTTTAGGATCATT GCCC 3′. The *Djβ-actin* gene (accession number: AB292462) was used as the housekeeping gene in the experiment (Dong et al. 2017; Dong et al. 2015; Kang et al. 2019a, 2019b). The expression ratios were determined using the $2^{-\Delta\Delta CT}$ method, which was described by Kang et al. (2019a, 2019b). The statistical analyses were performed with one-way analysis of variance (ANOVA) using SPSS 13.0 software (Dong et al. 2017). $P < 0.05$ was considered significant, and $P < 0.01$ was considered extremely significant.

Whole-mount immunofluorescence (WIF)

After 10 mM 3-MA exposure for 18 h, 40 normal worms were selected (Fig. 1B). Then, 20 worms were cultured in autoclaved tap water for the intact immunofluorescence experiment. In addition, other worms were cut as described, and the head and tail fragments were separately incubated in autoclaved tap water for observation in the regenerative immunofluorescence experiment. Cell proliferation and nervous system phenotypes of the intact and regenerating worms were examined by whole-mount immunofluorescence as described previously (Dong et al. 2019).

We evaluated nerve development levels by immunofluorescence (antibody: anti-SYNAPSIN, diluted 1:200) on the 5th day of regeneration (Gonzalez-Estevez et al. 2012). Fluorescence signals were detected with a stereo fluorescence microscope (Axio Zoom. V16, Germany). For each experiment, we selected 20 animals and repeated the observations three times. More than 80% of the changes were statistically significant.

To analyse the relationship between cell proliferation and 3-MA treatment in tissue turnover, we performed immunofluorescence using phospho-H3P antibody (diluted 1:2000, Gonzalez-Estevez et al. 2007). Fluorescence signals of intact and regenerating worms were detected with a stereo fluorescence microscope (Axio Zoom. V16, Germany). The numbers of H3P + cells/mm^2 were counted and quantified in all groups. The statistical analyses were performed with one-way analysis of variance (ANOVA) using SPSS 13.0 software (Dong et al. 2017). $P < 0.05$ was considered significant, and $P < 0.01$ was considered extremely significant.

Statistical analysis

All experiments were biologically repeated three times. The data are the means ± standard deviation (SD), and statistical analyses were performed by Student's t-test for group pairs and one-way analysis of variance (ANOVA) for multiple groups using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered significant, while $P < 0.01$ was considered extremely significant (Dong et al. 2015).

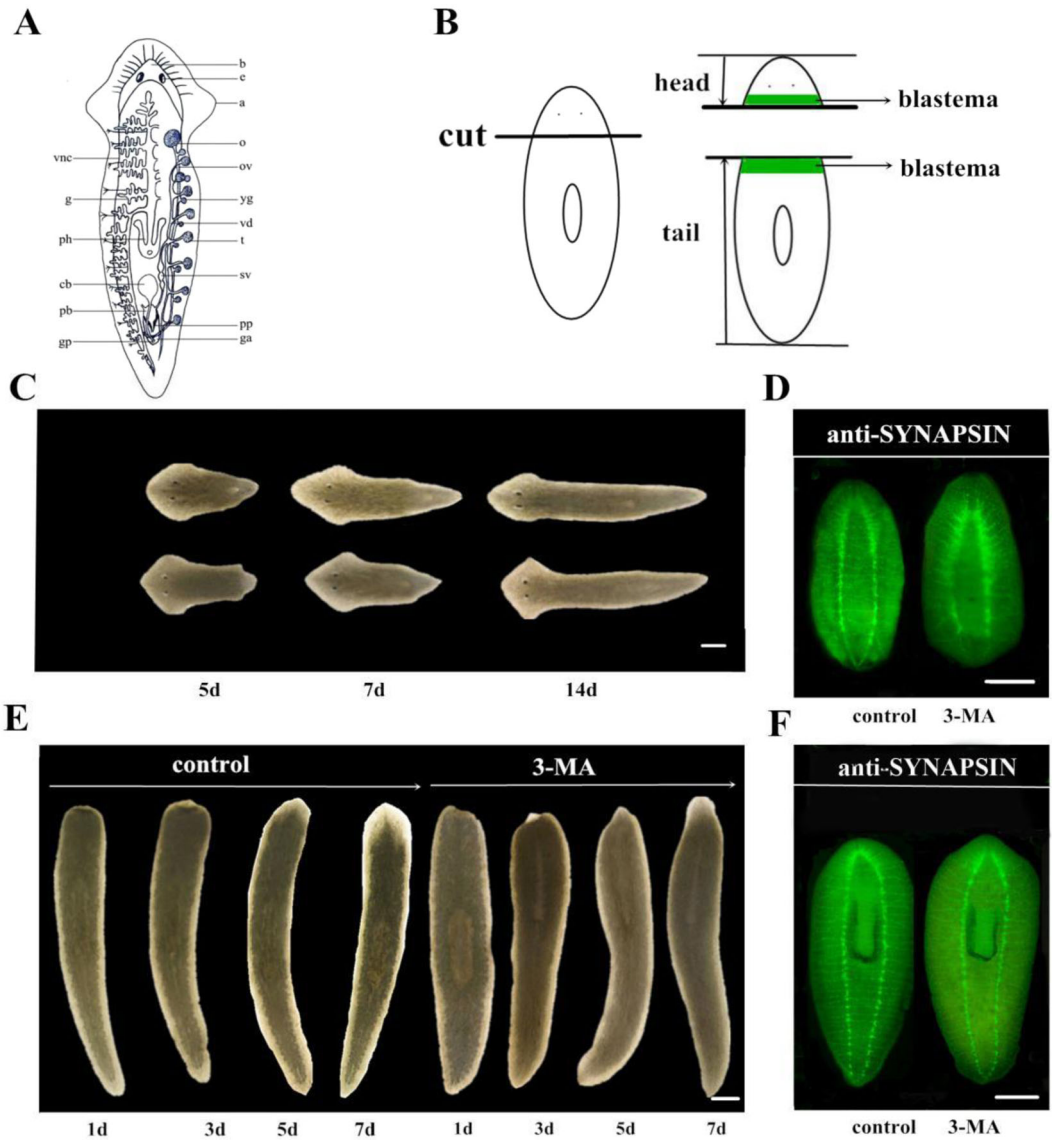

Fig. 2 Effects of 3-MA treatment in regeneration planarians ($n = 20$ animals for each treatment). **A** Schematic draw in sexual maturity individuals of *D. japonica* (Dong et al. 2015) (vnc ventral nerve cords, g gut, ph pharynx, cb copulatory bursa, pb penis bulb, Gp genital pore, b brain, e eye, a auricle, o ovary, ov oviduct, yg yolk gland, Vd vas deferens, t testis, sv seminal vesicle, pp penis papilla, ga genital atrium, Ca common atrium); **B** Schematic illustration of amputation (Green areas: the blastemas); **C** Head regeneration for 5, 7 and 14 d after 3-MA treatment; **D** Head regeneration of the CNS for 5 d by whole-mount immunofluorescence (antibody: anti-SYNAPSIN) after 3-MA treatment; **E** Tail regeneration for 1, 3, 5 and 7 d with disproportionate blastemas after 3-MA treatment; **F** Tail regeneration of the CNS for 5 d by whole-mount immunofluorescence (antibody: anti-SYNAPSIN) after 3-MA treatment. Scale bar, 500 μm

Results

3-MA treatment induced ulcers, which returned to normal in intact worms by the 7th day of treatment

The intact worms were exposed to 3-MA for 18 h, and the results showed that 50% of the worms presented ulcers in the middle of the body (Fig. 1C). The ulcers had stopped getting larger by the 3rd day (Fig. 1D), and gradually, healthy tissue was growing on the 5th day of treatment (Fig. 1E). Finally, the worms recovered and were normal intact worms by the 7th day of treatment (Fig. 1F).

3-MA treatment induced disproportionate blastemas with tissue regression during planarian regeneration

After 3-MA treatment for 18 h, the worms were amputated. In the morphological observation experiments, more than 50% of the changes were statistically significant. Then,

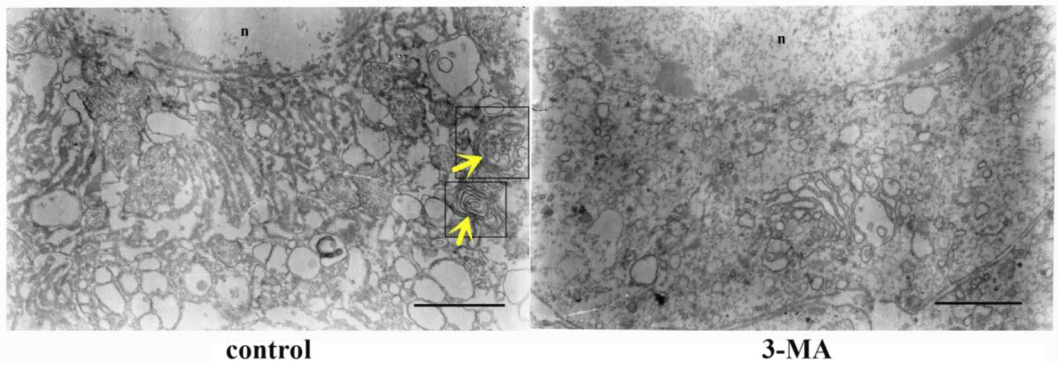

Fig. 3 Ultrastructural changes in *D. japonica* induced by 3-MA treatment ($n = 3$ animals for each treatment). Yellow arrows, autophagic vesicles (AVs); scale bars, 1 μm

regenerating fragments were cultured in autoclaved tap water and incubated in the dark at 20 °C. The fragments displayed slower regeneration speed compared with the control worms and exhibited normal phenotypes after 14 days of regeneration (Fig. 2C). In the head fragments, a new pharynx (Fig. 2C) and full ventral nerve cords (Fig. 2D) were not observed on day 5 of regeneration. Smaller tails were found between 5 and 7 days of regeneration compared with the controls (Fig. 2C). Correspondingly, 70% of the tail fragments regenerated more slowly and had smaller blastemas from day 1 to day 5 (Fig. 2E) than the controls. On the 7th day, a disproportionate head had taken shape (Fig. 2E). Fluorescence microscopy showed that there was an immature central nervous system (CNS) (Fig. 2F) and ventral nerve cord (VNC) (Fig. 2D) on the 5th day of worm regeneration. More than 80% of the changes were statistically significant.

3-MA treatment inhibited autophagy vesicle formation and *Djvps34* expression during planarian tissue regeneration

TEM is an effective method of detecting autophagic vesicles (Gonzalez-Estevez et al. 2007). Therefore, TEM was used to detect whether autophagy was inhibited in planarians after 3-MA exposure at different concentrations and exposure times. The results showed that the number of autophagic vesicles (yellow arrows) decreased in the treated animals after 10 mM 3-MA exposure for 18 h (Fig. 3). In these images, more than 80% of the changes were statistically significant. As an inhibitor of autophagy, 3-MA has the ability to suppress *Vps34* expression in mammals (Ding et al. 2018). Here, RT-PCR was performed in *D. japonica* after 3-MA treatment. We found that *Djvps34* expression was significantly down-regulated in parallel with autophagy being inhibited in the planarians (Fig. 4). This result indicated that 3-MA may inhibit the formation of autophagy in *D. japonica* through the PI3K pathway.

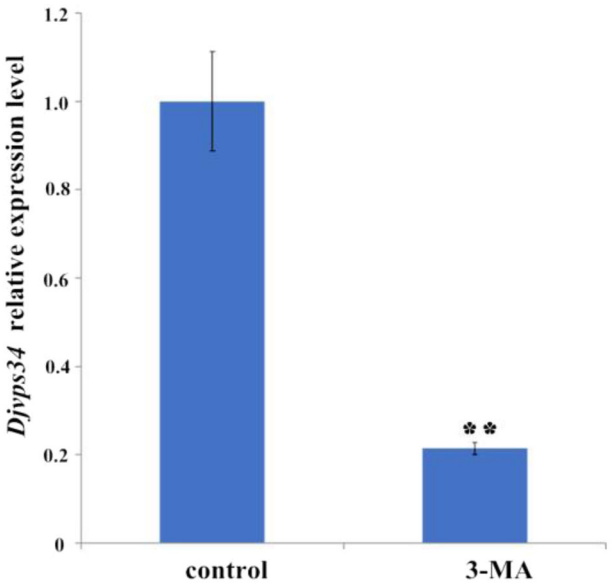

Fig. 4 Quantitative PCR (qPCR) showing the relative expression level of *Djvps34* in 3-MA treatment animals ($n = 6$ animals). Asterisks indicate statistical significance ($^{**}P < 0.01$). Samples were collected at 8th d after feeding

3-MA treatment suppressed stem cell proliferation in planarians

Neoblasts (stem cells) are the only proliferative cells in planarians, and their mitotic activities were evaluated by H3P immunofluorescence (Dong et al. 2019). To analyse the relationship between stem cell proliferation and 3-MA treatment, we performed immunofluorescence using a phospho-H3P antibody in intact and regenerating worms following 3-MA treatment. The results showed decreased mitotic activity in the intact worms after 3-MA treatment compared to the controls (Fig. 5A, B). Following planarian amputation, 3-MA treatment suppressed stem cell proliferation as evidenced by observations made at the 6th hour and 48th hour of regeneration (Fig. 5C, D).

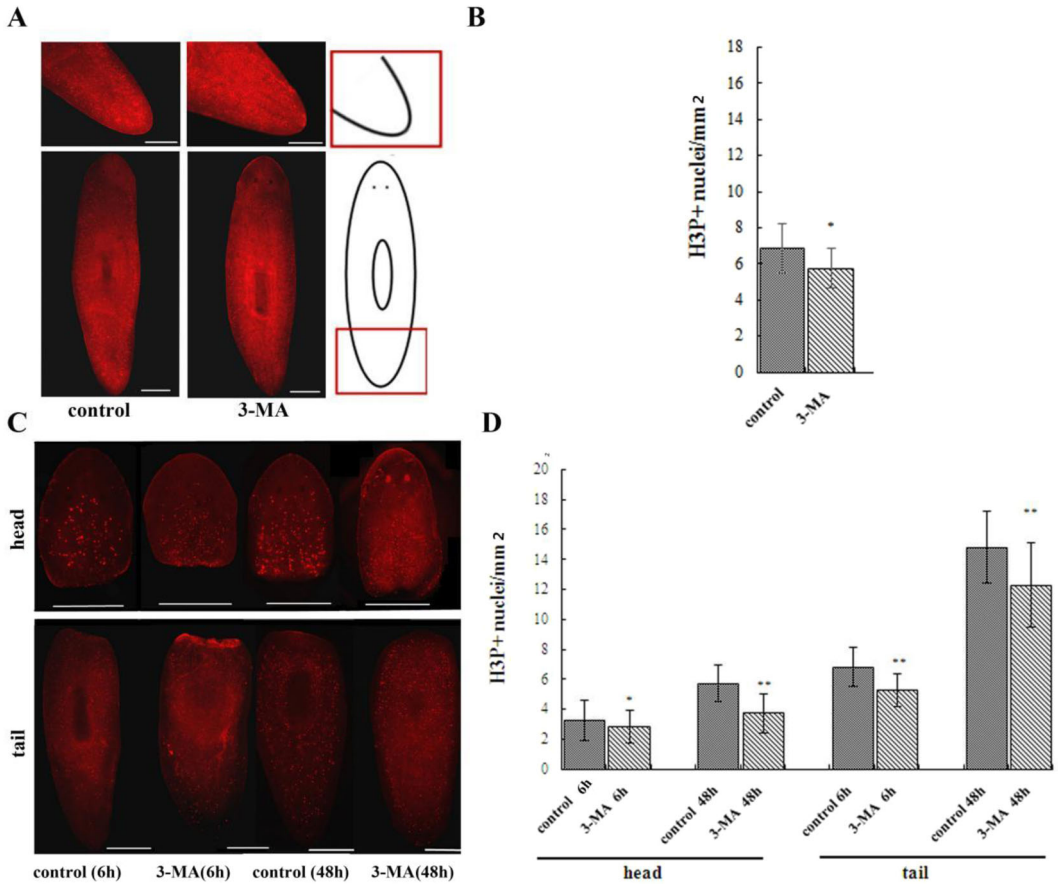

Fig. 5 Whole-mount immunofluorescence using anti-phospho histone3 after 3-MA treatment ($n = 20$ animals for each treatment) **A** Immunofluorescence with anti-H3P antibody following 3-MA treatment in intact worms; **B** Mitotic density in intact planarian ($^*P < 0.05$); **C** Immunofluorescence with an anti-H3P antibody following 3-MA treatment (Scale bar, 500 μm) in regeneration worms; **D** Mitotic density in regeneration planarians ($^{**}P < 0.01$, $^*P < 0.05$)

Discussion

Autophagy is regulated by multiple pathways, among which the PI3K pathway is the most important pathway in the initial stage of autophagosome formation (Zhao et al. 2019). Furthermore, the synthesis of autophagosomes can proceed smoothly when the pI3K-III complex is successfully activated in yeast and mammals (Zhang et al. 2020). VPS34 is a catalytic unit of the class III phosphatidylinositol 3-kinase complex (PI3K-III) and is the progenitor of the phosphoinositide 3-kinase (PI3K) family (Xia et al. 2014). VPS34 can phosphorylate phosphatidylinositol to produce PtdIns3P, which is important in autophagy and endocytic pathways (Ohashi et al. 2018). 3-MA has been established as an autophagy inhibitor with effects realized via the PI3K pathway (Ding et al. 2018). In *D. japonica*, 3-MA efficiently suppressed the formation of autophagosomes and inhibited *Djvps34* expression. These results indicate that 3-MA treatment caused autophagy inhibition via the PI3K pathway in freshwater planarians, similar to that previously observed in vertebrates (Zhang et al. 2020).

Autophagy is an evolutionarily conserved mechanism that continuously maintains the homeostasis of all tissues (Kang et al. 2019a; Gonzalez-Estevez and Salo 2010). Further, it has been reported that autophagy also plays a key role in the antioxidant repair system (Kang et al. 2019a; Gonzalez-Estevez and Salo 2010). In the present study, we treated planarians with 3-MA to assess its toxicity. 3-MA exposure led to serious disorders in *D. japonica*, and most worms formed ulcers in the middle of the body and healthy tissue was restored by the 7th day of treatment. Moreover, 3-MA treatment induced disproportionate blastemas with tissue regression during planarian regeneration, and the number of blastemas was restored by the 14th day of regeneration. These findings suggested that 3-MA treatment caused tissue homeostasis disorder in both regenerating and intact worms by inhibiting autophagy. However, autophagy slowly returned to normal levels following exposure, and homeostasis also slowly returned to normal levels in *D. japonica*.

Planarians continuously adapt their body (morphallaxis) to different environmental stresses, which involves a cell

proliferation process (Gonzalez-Estevez and Salo 2010). Moreover, they are equipped with cellular mechanisms that enable them to modulate the balance between cell proliferation and autophagy during tissue turnover (Kimberly et al. 2012). In addition, autophagy is tightly coupled to cell proliferation during stress-induced events (Gonzalez-Estevez and Salo 2010). To analyse the relationship between cell proliferation and autophagy suppression in *D. japonica*, we performed immunofluorescence using an anti-H3P antibody to detect the cell proliferation rate after 3-MA treatment. The results showed a reduction in mitotic activity in planarians after 3-MA treatment. We suspected that the planarians showed a reduced cell proliferation rate in response to the inhibition of autophagy. Because of cell proliferation, much energy was required, and autophagy was activated in the control group. However, autophagy in the experimental group was inhibited after treatment with 3-MA, and the proliferation of the cells was also inhibited, and the energy supply and demand reached a relative balance. In mammals, cells can survive through autophagy when tissues need energy to respond to stress (Sahani et al. 2014; Dong et al. 2019). We speculated that autophagy was also strictly controlled and regulated at the energy base line in planarians.

In this work, exposure to 10 mM 3-MA for 18 h led to serious disorders in *D. japonica*. Moreover, the number of autophagic vesicles and *Djvps34* expression decreased after 3-MA treatment. After amputation, disproportionate blastemas with tissue regression were observed during regeneration. Furthermore, the immunofluorescence results indicated that 3-MA treatment suppressed cell proliferation during tissue regeneration. These results revealed that treatment with the autophagy inhibitor 3-MA might induce negative effects on planarian homeostasis via the PI3K pathway.

Data availability

All data, models, and code generated or used during the study appear in the submitted article.

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Author contributions JK performed TEM, 3-MA treatment,morphological observation and quantitative real-time PCR. JZC performed immunofluorescence. GWC and ZMD conceived of the study, participated in its design and coordination and helped to draft the manuscript. DZL had been involved in revising the manuscript. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

Informed consent Informed consent was obtained from all individual participants included in the study.

Research involving human participants and/or animals' note This study do not involve endangered or protected species, and the collection of specimens is approved by the Forestry Department of Wild Animal Protection, Henan Province, China.

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