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Effects of Berberine on Development in *Caenorhabditis elegans*

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**EFFECTS OF BERBERINE ON DEVELOPMENT IN *CAENORHABDITIS*
*ELEGANS***

A Thesis Presented

by

ZHUOJIA QIAN

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

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FOOD SCIENCE

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ABSTRACT

EFFECTS OF BERBERINE ON DEVELOPMENT IN *CAENORHABDITIS*

ELEGANS

SEPTEMBER 2020

ZHUOJIA QIAN, B.E., HUAZHONG AGRICULTURAL UNIVERSITY

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Directed by: Professor Yeonhwa Park

Berberine is an isoquinoline alkaloid found in some plants and has many bioactivities including anti-microbial, lipid- and glucose-lowering, anti-cancer, anti-inflammatory, etc. However, there is limited knowledge about berberine's effects on development and locomotive activity. Herein, *in vivo* studies were conducted to determine these effects of berberine using *Caenorhabditis elegans* as an *in vivo* model. Treatment of berberine at 50 μ M starting at L1 stage significantly retarded the growth rate of nematodes, and reduced the length, width and moving speed of worms by 19%, 12% and 29%, respectively, compared to the control. In addition, triglycerides (TG) and protein content in worms was reduced by 23% and 28%, respectively, after berberine treatment from L1 stage compared with the control group. However, no significance was observed when berberine was treated from young adult stage. These findings suggest that berberine has effects on development in *C. elegans*.

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CHAPTER 1

INTRODUCTION

Berberine is an isoquinoline alkaloid found in several plants such as *Berberis vulgaris* (barberry), *Berberis aristata* (tree turmeric), and *Coptis chinensis* (Chinese goldthread)¹. There are also dietary supplements containing berberine processed from these plants. It has been proven that berberine has health benefits, including anti-microbial, lipid- and glucose-lowering, anti-cancer, anti-inflammatory, reducing cardiovascular diseases, and improving nervous system, etc²⁻¹⁰. There are limited reports of the effects of berberine on locomotion, including its protective role for morphine-, cocaine- and ethanol-induced adverse effects and its stimulation effects on locomotor activity in *Drosophila melanogaster*¹¹⁻¹⁵. However, studies on the effects of berberine on growth and locomotive activity, especially *in vivo* studies, are still inadequate.

Caenorhabditis elegans is a small free-living nematode found in temperate soil environments, which has been widely applied in many scientific researches focusing on obesity, aging, and development¹⁶⁻¹⁹. This animal model has many advantages, such as a small body size, short life span, large brood size, and low maintenance cost²⁰. In addition to its rapid life cycle²⁰, every stage in its life cycle has easily distinguishable features²⁰. Locomotion behavior of *C. elegans*, as an indicator of activity, can be measured with a tracking system²¹. Furthermore, it has many development- and locomotion-related genes, which are orthologs of human

genes²¹⁻²⁴. Thus, *C. elegans* was utilized as an *in vivo* model in the current study to investigate the effects of berberine on growth and locomotion.

CHAPTER 2

LITERATURE REVIEW

2.1 *Caenorhabditis elegans*

2.1.1 Introduction

C. elegans is a small free-living nematode found in temperate soil environments worldwide^{16,17}. It was first introduced as a genetic model to study developmental biology and neurobiology by Sydney Brenne in 1960s^{16,17}. *C. elegans* is now widely applied in research involving obesity, aging, development, locomotion behavior, and neurodegenerative disorders^{19,25}.

Compared with traditional animal models, this eukaryotic and multi-organ animal model possesses many advantages. It has a small body size, short life span, and large brood size²⁰. At the same time, *C. elegans* is inexpensive and convenient to maintain²⁰. Furthermore, it has a completely sequenced and well-annotated genome, which encodes over 65% of characterized human disease-related genes²⁵. Moreover, it has many mutants available, which could be easily utilized for mechanistic studies²⁶. And research using *C. elegans* does not require approval from the Institutional Animal Care and Use Committees²⁵.

2.1.2 Life Cycle

The life cycle of *C. elegans* consists of four larval stages (L1, L2, L3, and L4 stage) and adulthood²⁷. *C. elegans* embryogenesis takes around 16 hours at 20 °C. L1 larvae are hatched out from eggs and take around another 16 hours to develop into

the L2 stage. L2, L3 and L4 stages last around 12 hours long, respectively²⁰. Each stage ends with a period called lethargus, during which a new cuticle of nematodes is made²⁸. Lethargus ends with molting of the old cuticle. Approximately 12 hours after molting of nematodes at the L4 stage²⁰, *C. elegans* at adulthood begins to produce progeny, which will last 5-6 days. However, when L2 larvae lack food or are crowded, they will develop into an alternative life cycle²⁹ and molt into an alternative L3 stage called the “dauer” stage³⁰. At this stage, the dauer larvae have enhanced resistance against environmental stresses, can survive for several months, and continue to develop as slightly different L4 larvae if they are provided with enough food²⁰.

2.1.3 Locomotion Behavior

There are 302 neurons of 959 somatic cells in nematodes. *C. elegans* shares highly conserved neurotransmitter receptors, neurotransmitter synthesis and release pathways, heterotrimeric guanosine-5'-triphosphate (GTP) binding proteins coupled second messenger pathways, and major genes involved with mammals^{23,31}. Therefore, this animal model has been widely applied in research to study basic mechanisms underlying drug-induced behaviors by investigating its locomotion behavior^{23,24}. 75 motoneurons of 302 neurons control body wall muscles, which provide thrust for *C. elegans* during locomotion³². The regular contraction and relaxation of muscle cells innervated by motoneurons in *C. elegans* lead to its movement^{20,32}.

C. elegans locomotes in an undulatory way^{33,34}. It generates thrust by propagating the bending of its body in the direction of locomotion³⁴. When nematodes move on the surface of the agar, the routes they crawled through will produce typical S shapes. Without the consideration of steering, the locomotion behavior of *C. elegans* could be generally divided into four patterns: forward locomotion, backward locomotion, dwelling (nondirectional body bends), and quiescence^{28,35-37}. Each of these patterns could appear randomly and persist for a while during nematodes' locomotion³⁸⁻⁴⁰. Such locomotion behavior can be recorded and analyzed with a tracking system²¹.

2.1.4 Uptake of Compounds into *C. elegans*

C. elegans is actively utilized as a research model to determine activities of various compounds. There are mainly three ways to deliver compounds to *C. elegans*: [1] mix compounds with nematodes' bacterial food source, [2] directly spread compounds onto the surface of nematode growth medium (NGM) agar plates, and [3] add compounds into nematodes' liquid medium⁴¹. However, it is worth noting that the final concentration of dimethyl sulfoxide (DMSO) solution in nematodes' medium higher than 0.6% (v/v) will shorten the life span of *C. elegans*⁴².

Compounds can be taken up by *C. elegans* via three ways: ingestion, through the cuticle, and exposed sensory neuronal cilia⁴³. During ingestion, appropriate food sources will be recognized and chosen by chemosensory neurons and then consumed via aspiration by pharynx of *C. elegans*. Then compounds will be absorbed by intestinal cells and distributed throughout the worm body⁴³. The

cuticle of *C. elegans* can also allow diffusion of some compounds into and out of nematodes through a permeability barrier established by hypodermis⁴⁴. Lastly, compounds may be directly taken up via exposed sensory neuronal cilia of nematodes⁴⁵.

2.2 Berberine

2.2.1 Introduction

Berberine is an isoquinoline alkaloid found in plants such as *Berberis vulgaris* (barberry), *Berberis aristata* (tree turmeric), and *Coptis chinensis* (Chinese goldthread)¹. The main biological properties of berberine reported are anti-microbial, lipid- and glucose-lowering, anti-cancer, and anti-inflammatory effects^{1,9,46}. In addition, berberine has been reported to have beneficial effects on cardiovascular system¹ and nervous system².

There are reports of berberine's effects at the cellular level, particularly in hepatocytes, macrophages, endothelial cells, adipocytes, and myocytes⁴⁶. Berberine mainly protected them from the adverse effects of atherosclerosis⁴⁷⁻⁵⁰ and improved glucose utilization in adipocytes and myocytes⁵¹.

In addition, *in vivo* and clinical studies of berberine have reported on its effects on hypercholesterolemia, diabetes, and obesity⁴⁶. However, based on the evidence available to date, additional well-designed trials to confirm the safety and efficacy of berberine are still needed⁴⁶.

2.2.2 Effects of Berberine on Cytotoxicity

Research on the cytotoxicity of berberine generally used bacterial colony and mammalian cell lines. Several studies concluded that berberine could inhibit the growth of different species of bacteria and change their structures^{3,4}, which proves that berberine has anti-microbial effects. Others reported that berberine inhibited the growth of many cancer cells, originated from prostate⁵, neuroblastoma⁵², lung⁶, cervix⁷, breast⁸, and others¹⁰. This also supports the anti-cancer properties of berberine.

2.2.3 Effects of Berberine on Locomotion

Previously it was reported that berberine could prolong life span and stimulate locomotor activities of *Drosophila melanogaster*¹¹. In this study, the vertical climbing assay was used to measure the ability of *Drosophila* to climb the wall of a vial when startled¹¹, assessing ability to complete a strenuous activity⁵³. Berberine stimulated vertical climbing of flies by 39% over the control in the climbing height indicator¹¹.

In addition, the effects of berberine on locomotion were investigated when studying its inhibition of morphine-, cocaine- and ethanol-induced adverse effects. Berberine could attenuate the expression of sensitization to locomotor stimulant effects of morphine, cocaine, and ethanol¹²⁻¹⁵. However, research focusing on the influence of berberine on animals' locomotion still needs further study.

2.2.4 Effects of Berberine on Lipid Metabolism

Berberine has been studied on its lipid-lowering effect both in cellular and animal models^{50,54-60}. Treatment with berberine reduced plasma total cholesterol and non-HDL (high-density lipoprotein) cholesterol levels in atherogenic diet-fed rats and high-fat diet-fed mice^{56,57,59,60}. Oral administration of berberine in hypercholesterolemic patients also reduced serum cholesterol, triglycerides, and LDL (low-density lipoprotein) cholesterol levels⁵⁰. Berberine also inhibited hepatic fat accumulation in mice and attenuated hepatic steatosis in hamsters^{54,59}. It has been concluded that berberine acts in the liver to regulate lipid utilization⁵⁴. These findings support that berberine has therapeutic potential to treat metabolic dysfunctions under nutritional overloads, such as fatty liver diseases and type 2 diabetes, and is suggested to be a new hypolipidemic drug.

2.2.5 The Role of AMP-activated Protein Kinase in Berberine's Effects

A number of studies reported that the major cellular process in which berberine is effective involves AMP-activated protein kinase (AMPK)⁶¹, which plays a critical role in cellular energy homeostasis⁶¹. The activation of AMPK by berberine led to its antidiabetic properties⁶²⁻⁶⁵. In addition, this can also demonstrate that berberine's potential in inhibiting aging-related diseases via AMPK cellular kinase activation⁶⁶⁻⁶⁸. AMPK also possesses significance in physical activity^{69,70}, lysosomal damage, and inflammatory diseases^{71,72}.

2.3 Conclusion

C. elegans is a well-established *in vivo* model for studying the beneficial effects of food bioactive compounds. Berberine has shown to possess anti-microbial, lipid- and glucose-lowering, anti-cancer, and anti-inflammatory properties. However, research on its effects on exercise and growth is not comprehensive yet. Therefore, the goal of the current research was to determine the role of berberine on growth and locomotive activity using *C. elegans* as an animal model.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

Berberine chloride (97%), commercial kits for triglycerides (Infinity™ Triglycerides Liquid Stable Reagent), and protein (Pierce™ BCA Protein Assay Kit) quantification were purchased from Thermo Fisher Scientific Chemicals, Inc. (Waltham, MA). Chemicals used for *Caenorhabditis elegans* maintenance including agar, peptone, and LB broth were purchased from Fisher Scientific, Inc. (Pittsburgh, PA). Ampicillin, carbenicillin, and fluorodeoxyuridine (FUdR) were purchased from Sigma-Aldrich Co. (St. Louis, MO). *Escherichia coli* (*E. coli*) OP50 and *C. elegans* wild isolate (N2) were obtained from Caenorhabditis Genetics Center (Minneapolis, MN).

3.2 *C. elegans* Maintenance and Treatment

C. elegans was maintained as previously reported²⁶. *E. coli* OP50 was prepared as a food source. Berberine stock solutions were prepared in dimethyl sulfoxide ($\geq 99.9\%$, DMSO). For treatment starting from the L1 stage, synchronized L1 *C. elegans* was treated with DMSO (0.1% v/v final) as vehicle or 50 mM berberine stock solutions (0.1% v/v final) in S Medium for 48 hours at 20 °C. For treatment starting from the young adulthood stage, firstly synchronized L4 *C. elegans* was treated with 6 mM FUdR solution (2% v/v final). And then, worms on the first day

of adulthood were treated with DMSO (0.1% v/v final) as vehicle or 50 mM berberine stock solutions (0.1% v/v final) in S Medium for 48 hours at 20 °C.

3.3 Growth Rate, Body Size and Locomotive Activity Assay

For the growth rate assay, numbers of *C. elegans* at different larval stages were counted after 48-hour treatment. Results were presented as percentages of *C. elegans* numbers at different larval stages to the total.

Body size and locomotive activity of *C. elegans* were measured with a WormLab tracking system⁷³ (MBF Bioscience, Williston, VT). *C. elegans* were transferred onto a low-peptone NGM (nematode growth medium) plate freshly seeded with *E. coli* OP50 solution in LB broth. The worms were allowed to move around freely for 10 minutes and become acclimated to light for another 10 minutes before recording. Each video recording of *C. elegans* for 1 minute was analyzed with a WormLab software (MBF Bioscience version 3.1.0, Williston, VT) for average moving speed, worm length, and worm width.

3.4 Triglyceride and Protein Quantification

After the 48-hour treatment, *C. elegans* was collected and washed five times with Milli-Q water to eliminate *E. coli* and berberine. *C. elegans* was then dissolved in Tween 20 (0.05% v/v), sonicated, and measured for triglycerides (TG) and protein content with the commercial kits as described before⁷³. Protein content was used to normalize TG levels.

3.5 Statistical Analysis

Statistical analysis was performed through Chi-square (and Fisher's exact) test and t-tests (and nonparametric tests) using GraphPad Prism (GraphPad Software version 8.4.2, San Diego, CA) or one-way ANOVA (Tukey's test for multiple comparisons) using Statistical Analysis System (SAS Institute version 9.4, Cary, NC). Differences between groups were considered significant at $p < 0.05$.

CHAPTER 4

RESULTS

4.1 Berberine Inhibited Growth of *Caenorhabditis elegans*

There were 91% of the worms at the L4 stage, 3% at the L3 stage, 5% at the L2 stage, and 1% at the L1 stage in the control group after 48 hours of incubation (Figure 4.1). However, only 67% of the worms reached the L4 stage, 19% at the L3 stage and 14% at the L2 stage in the 50 μ M berberine group. This suggests that the growth rate was significantly slowed after 48 hours of treatment of berberine at 50 μ M compared to the control ($p = 0.0001$).

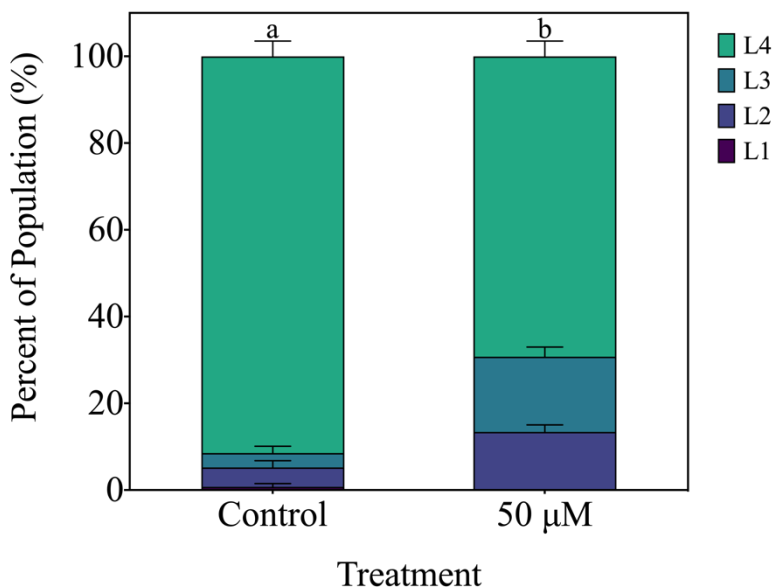


Figure 4.1 Berberine significantly slowed the growth rate of *C. elegans*. Synchronized L1 worms were treated with control (0.1% DMSO) or berberine (50 μ M) for 48 hours at 20 °C. The growth rate was scored as percentages of worms' numbers at different developmental stages to the total. Results are presented as means \pm S.E. (n = 4 plates, each plate contained \approx 50 worms). ^{a, b} Means with different letters are significantly different ($p < 0.05$).

4.2 Effects of Berberine on Body Size and Locomotive Activity

When berberine was treated from L1 stage, the length of worms was significantly decreased by 19% compared to the control ($p < 0.0001$, Figure 4.2A). Similarly, the width of the worms in the 50 μM berberine treated group was also decreased significantly by 12% compared with the control group ($p < 0.0001$, Figure 4.2B). These are consistent to the result of retarded growth rate by berberine in Figure 4.1.

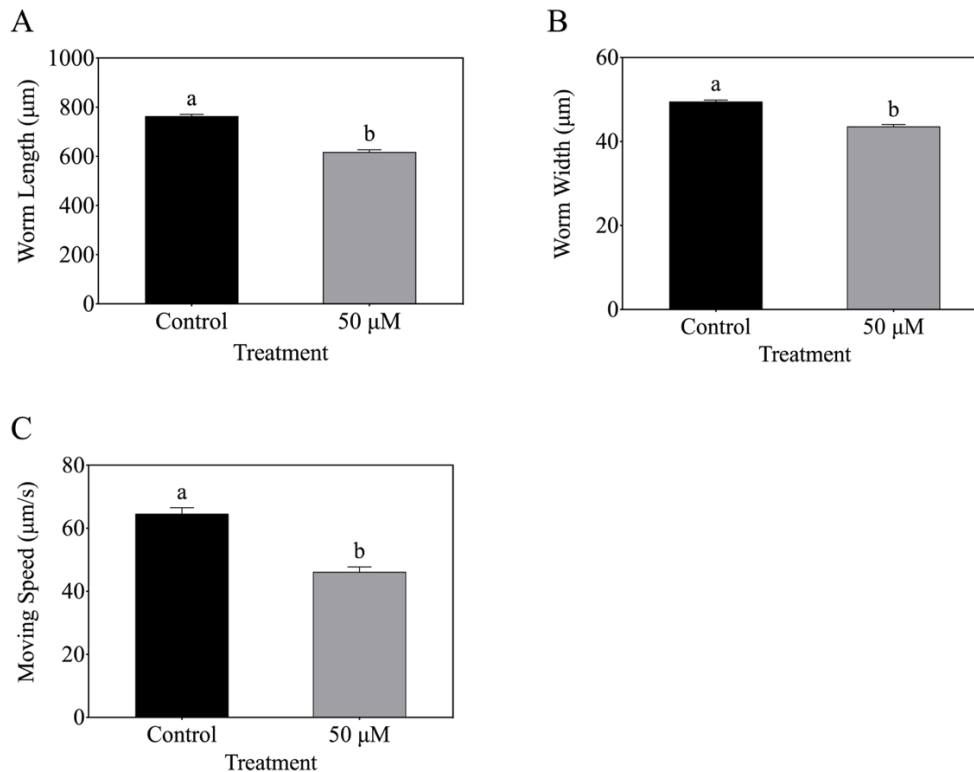


Figure 4.2 Effects of berberine treatment during development on body size and locomotive activity of *C. elegans*. Synchronized L1 worms were treated with control (0.1% DMSO) or berberine (50 μM) for 48 hours at 20 $^{\circ}\text{C}$. Body size, including worm length (A) and width (B), and locomotive activity, presented as average moving speed (C), were measured with the WormLab tracking system. Results are presented as means \pm S.E. ($n = 350\text{-}400$ worms). ^{a, b} Means with different letters are significantly different ($p < 0.05$).

Treatment of 50 μM berberine significantly reduced the moving speed of the worms by 29% compared to the control ($p < 0.0001$, Figure 4.2C), which may also be due to the retarded growth rate by the berberine treatment. Taken together, it is suggested that both the body size and moving speed of worms were significantly reduced after 48 hours of treatment of berberine at 50 μM .

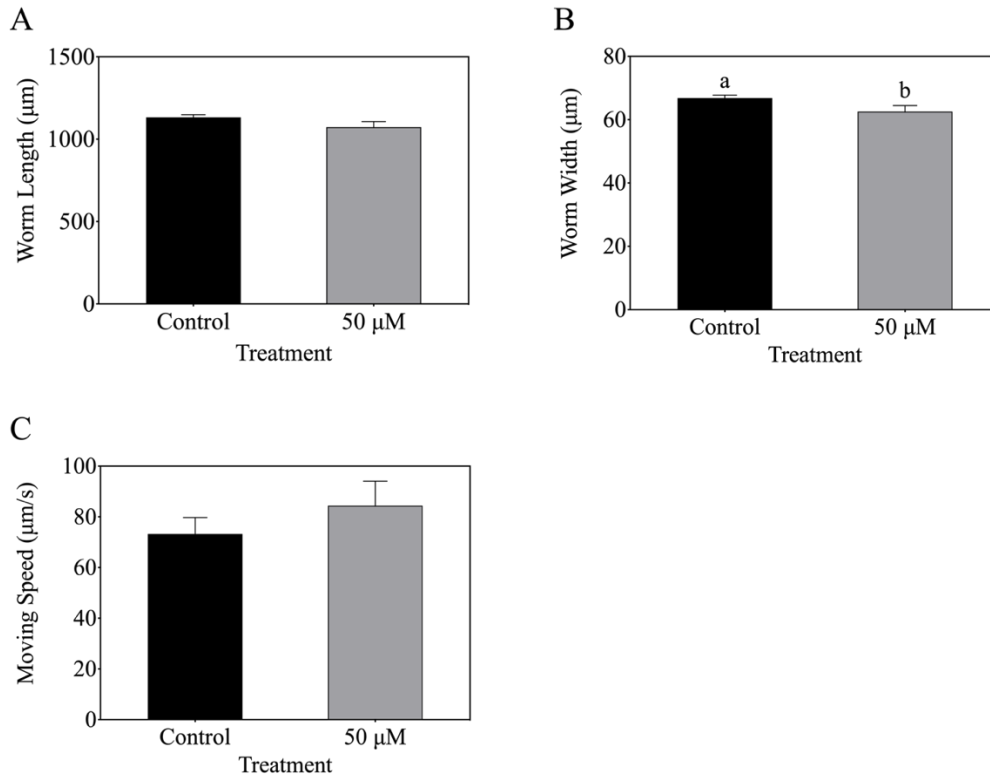


Figure 4.3 Effects of berberine on body size and locomotive activity treated from young adulthood in *C. elegans*. Synchronized worms on the first day of adulthood were treated with control (0.1% DMSO) or berberine (50 μM) for 48 hours at 20 $^{\circ}\text{C}$. Body size, including worm length (A) and width (B), and locomotive activity, presented as average moving speed (C), were measured with the WormLab tracking system. Results are presented as means \pm S.E. ($n = 40\text{-}80$ worms).

Next, we treated berberine after nematodes were fully grown. There was no significant difference in worm length between worms of the control and berberine groups (Figure 4.3A). However, there was a significant reduction of body width by

berberine treatment over the control, 6% reduction with $p = 0.0199$ (Figure 4.3B). This suggests that berberine has small, but significant effects on body size of *C. elegans* at the young adulthood stage. There was no difference in moving speed between treatments (Figure 4.3C).

4.3 Effects of Berberine on Triglycerides and Protein Content

The triglycerides (TG) level in worms treated with 50 μM berberine from L1 stage was significantly reduced by 23% compared with the control group ($p = 0.0004$, Figure 4.4A). Similarly, the content of protein was also significantly reduced by berberine treatment, 28%, compared to the control ($p < 0.0001$, Figure 4.4B). The lower level of the TG and protein by berberine treatment seen in Figure 4.4 may be due to the retarded growth rate in *C. elegans*^{74,75}.

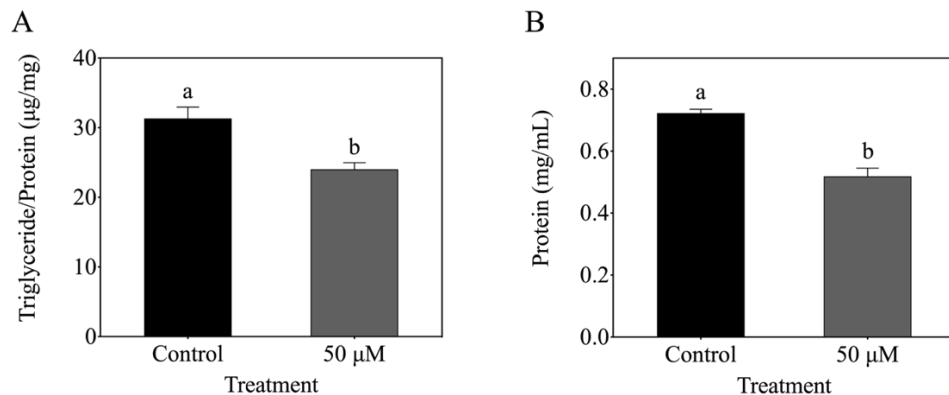


Figure 4.4 Effects of berberine on triglycerides (TG, A) and protein (B) content treated from L1 stage in *C. elegans*. Synchronized L1 worms were treated with control (0.1% DMSO) or berberine (50 μM) for 48 hours at 20 $^{\circ}\text{C}$. TG content was measured and normalized by protein levels. Results are presented as means \pm S.E. (n = 5 plates, each plate contained \sim 1,000 worms). ^{a, b} Means with different letters are significantly different ($p < 0.05$).

When nematodes were treated with berberine from young adulthood, there was no significant difference in TG content between control and berberine groups (Figure 4.5). And no significant difference was observed in protein levels between two groups as well. This suggests that berberine has no effect on fat accumulation in *C. elegans*.

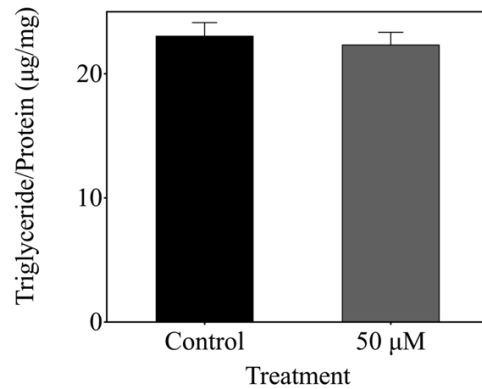


Figure 4.5 Effects of berberine on triglycerides (TG) content treated from young adulthood in *C. elegans*. Synchronized worms on the first day of adulthood were treated with control (0.1% DMSO) or berberine (50 µM) for 48 hours at 20 °C. TG content was measured and normalized by protein levels. Results are presented as means ± S.E. (n = 6 plates, each plate contained ~ 1,000 worms).

CHAPTER 5

DISCUSSION

Berberine, an isoquinoline alkaloid found in several plants, has been reported to have many beneficial effects^{1,2,9,46}. In the current study, treatment of berberine during growth period significantly retarded the growth of *Caenorhabditis elegans*, accompanied with reduced body size, reduced moving speed, and reduced both triglycerides (TG) and protein content of worms, while no significant effects were observed when berberine was treated after worms were fully grown.

Normally, L1 larvae will take around 40 hours to develop into the L4 stage. When facing unfavorable environmental condition, larvae of nematodes at the L2 stage can arrest development themselves by forming dauer larvae^{29,30}. In addition, *C. elegans* larvae were reported to be able to reversibly arrest development at the L1 stage, which was called “L1 arrest”⁷⁶. These are two kinds of naturally occurring growth inhibition without any treatment, which are similar to the results observed in the current study.

The most direct reason for the above two phenomena is the starvation of worms^{29,30,76}. High density and temperature are also common reasons for dauer arrest⁷⁶. As for our study, because worms of both the control and treatment groups were provided with almost the same nematode density and temperature, one of the possible reasons is that berberine suppressed the worms’ food intake.

What’s more, insulin-like signaling, phosphatase and tensin homolog (PTEN), liver kinase B1 (LKB1), AMPK, and fatty acid biosynthesis in *C. elegans*

have already been reported to be involved in development^{22,76}. It was reported that reduced insulin-like signaling would induce developmental quiescence⁷⁷. At the same time, AMPK would interact with insulin-like signaling^{76,78}. In *C. elegans*, mutation of *aak-2*, which is a homologue of AMPK and evolutionarily conserved in both *C. elegans* and mammals⁷⁹, was proved to result in an L1 arrest-defective phenotype^{76,80}. Besides, *C. elegans* orthologues of LKB1 and AMPK could cooperate to establish cell cycle quiescence in the germline stem cell population as well when insulin-like signaling was reduced²². Combined with the reports that AMPK is critical in many properties of berberine mentioned before⁶¹, AMPK could also play an important role in the retarded growth rate caused by berberine here.

Accompanied with the retarded growth rate, worms' body size, moving speed, and both TG and protein content were reduced after treatment of berberine. The reduction of body size was consistent with the developmental delay in *C. elegans*. And it is known that worms deposit TG after they are grown^{18,81}. Thus, the lower TG content was accompanied with retarded growth rate. In addition, the lower level of protein in worms may be due to the influence of berberine on the metabolic activities in *C. elegans*^{74,75}.

One previous study using *Drosophila melanogaster* as a model reported the stimulation effects of berberine on locomotor activities¹¹. However, we did not observe any significant changes of activity by berberine treatment, which means that 50 μ M berberine cannot stimulate locomotive activity in *C. elegans*. It is possible that the dosage of berberine treated in this study was lower than the

Drosophila study (1 mM)¹¹. Higher doses of berberine could be utilized to conduct locomotive activity studies in *C. elegans* in the future.

Berberine has been reported to have lipid-lowering effect^{54,56,57,59,60}. However, the reduction of TG content in the current study was observed when berberine was treated during worms' growth period only, but not after worms were fully grown. Based on our preliminary experiment using fat models (worms treated with glucose from L4 stage) of *C. elegans*⁸², berberine still have no effect on TG content. In addition, berberine at higher concentration (100, 200, and 300 μ M) significantly reduced protein content without changing the TG content. This suggests that berberine has no effect on lipid metabolism in this model. Possible reasons may be the different stages of the life cycle of nematodes used in this study and the comparatively lower dosage of berberine treated compared to other *in vivo* studies^{54,56,57,59,60}.

To conclude, the current results suggest the inhibitory effect of berberine on the growth of *C. elegans*. Its body size and moving speed were significantly reduced when berberine was treated during worms' growth period. This is the first study to demonstrate the influence of berberine on the development of *C. elegans*, which could provide important information and foundation for future studies of berberine.

CHAPTER 6

FUTURE RESEARCH

In this current research, berberine has effects in *C. elegans* on development as seen in growth rate, body size, locomotion, protein, and triglycerides. However, the mechanisms of these effects have not been determined. Further researches could be conducted to provide more comprehensive evidence for berberine's effects.

To complement current research, future work could be carried out from three aspects. First, the food intake assay of *C. elegans* at the L1 stage treated by berberine could be conducted. This could be one possible reason leading to the retard growth rate^{29,30,76}. Secondly, AMPK is a potential critical pathway of berberine's effects on development of *C. elegans*^{22,61,76}, thus using different mutants with gene knocked out²⁶, such as *aak-2* (homolog of AMPK), can be utilized to determine its effects. Lastly, the effects of berberine at higher doses, particularly on the locomotive activity in *C. elegans*, need to be determined.

In conclusion, the current research showed that berberine could significantly delay the development in *C. elegans*. These findings could provide important foundation for future studies of berberine.

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