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**METHYLGLYOXAL INFLUENCES DEVELOPMENT OF *CAENORHABDITIS*
ELEGANS VIA HETEROCHRONIC PATHWAY**

A Thesis Presented

By

JIAYING WANG

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

MASTER OF SCIENCE

May 2017

FOOD SCIENCE

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ABSTRACT

METHYLGLYOXAL INFLUENCES DEVELOPMENT OF *CAENORHABDITIS*

ELEGANS VIA HETEROCHRONIC PATHWAY

May 2017

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Methylglyoxal is a highly reactive dicarbonyl compound, which is widely distributed in food products and beverages, and is particularly high in Manuka honey. In addition to its antibacterial effects, methylglyoxal is also known as a major precursor of advanced glycation end products (AGEs), that produces altered macromolecules (such as proteins and DNA), leading to abnormal physiological changes. However, the effects of methylglyoxal on development is unclear. Thus, this study aimed to determine the role of methylglyoxal in this aspect using *Caenorhabditis elegans* (*C. elegans*). Treatment of methylglyoxal at 0.1 mM and 1 mM for 48 h significantly inhibited development of *C. elegans* and reduced pumping rate. Activity, measured by moving speed, was increased with 0.1 mM methylglyoxal, but reduced with 1 mM methylglyoxal. Lifespan of *C. elegans* was not influenced by methylglyoxal at 0.1 mM, but was shortened at 1 mM. Treatment methylglyoxal on the mutant, *lin-41*, which has a precocious phenotype, could alleviate the implication on wild-type worms. These results suggested that methylglyoxal significantly influenced the development of *C. elegans* through the heterochronic

pathway.

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CHAPTER 1

INTRODUCTION

Methylglyoxal is the natural active component of Manuka honey, well known for its antibacterial function (Degen et al., 2012). However, it is also reported to be associated with diseases including diabetes, atherosclerosis, and neurological disorders (Vistoli et al., 2013). Previous *in vitro* research found that methylglyoxal exhibited an autoxidative character, which could generate reactive oxygen species (ROS) in the mitochondria, and it is susceptible to alter the structure and functions of macromolecules, such as protein and nucleotides (Lo et al., 1994; Singh et al., 2001). Animal studies further investigated the effects of methylglyoxal in biological system. Although there were inconsistent results of no effects or anti-cancer effects of methylglyoxal *in vivo*, the majority of results concluded that methylglyoxal could induce weight-loss, inactivity, certain types of cancers, or even death in a dose-dependent manner (Dakin and Dudley, 1913; Sjolemma and Seekles, 1926; Nagao et al., 1986; Kalapos et al., 1991; Choudhary et al., 1997; Golej et al., 1998). In addition, aging and neurological disorders induced by methylglyoxal are the most investigated problems and several pathways in biological system regulated by methylglyoxal metabolism were previously identified (Bonifati et al., 2003; Morcos et al., 2008; Lee et al., 2012). However, to our best knowledge, there are no reports on effects of methylglyoxal in the animal development. Therefore, we explored the effect of methylglyoxal on development and determined the underlying mechanism.

Caenorhabditis elegans is an animal model with a short lifespan of about 21 days (Leung et al., 2008). The development time of *C. elegans* only requires 48 h after hatching at 20°C (Leung et al., 2008). *C. elegans* is the first animal to have its genome

completely sequenced, and more than 65% of the genes associated with human disease are conserved (Kaletta and Hengartner, 2006). Thus, we choose this model to conduct the current project.

CHAPTER 2

LITERATURE REVIEW

2.1. Methylglyoxal

2.1.1. Introduction

Methylglyoxal is a naturally occurring component of Manuka honey. It is a commercial factor used for classification of premium honey products as the so-called Unique Manuka Factor (UMF), based on *in vitro* anti-microbial assays (Mavric et al., 2008). It is also known to be one of the most important components in the formation of advanced glycation end products (AGEs) (Shinohara et al., 1998), which is associated with diseases, such as diabetes, atherosclerosis, and neurological disorders (Vistoli et al., 2013). Methylglyoxal is a yellowish, clear, and water-soluble liquid at room temperature, and it is also known as pyruvaldehyde, acetyl formaldehyde, or propanedione (Figure 1). It contains two carbonyl groups (C=O): an aldehyde and a ketone. Therefore, it is reactive because aldehyde is readily oxidized to carboxylic acids (Corey et al., 1968).

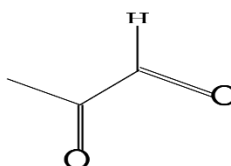


Figure 1. Methylglyoxal chemical structure

2.1.2. Methylglyoxal formation

The origin of methylglyoxal can be separated into exogenous, from food and beverages, and endogenous, from enzyme-catalyzed and spontaneous reaction. Overall levels of methylglyoxal are a balance between exogenous and endogenous formation and elimination of methylglyoxal in the biological system. There are a great number of publications reporting the effects of methylglyoxal in the biological system in past

decades.

2.1.2.1. Exogenous sources

Except for honey, its only natural source, methylglyoxal can be formed in many food products and beverages derived from carbohydrates, amino acids, lipids, and microorganisms during food processing, cooking, and storage. Average levels of methylglyoxal in food are summarized in Table 1.

It is known that the retro-aldol condensation and autoxidation of sugar results in methylglyoxal, with higher levels found in monosaccharides than disaccharides. As mentioned, honey (Table 1) is a great source of methylglyoxal (0.4 – 5.4 mg/kg) due to simple sugar degradation during storage (Weigel et al., 2004). In addition, the Maillard reaction, which is a reaction between amino acids and reducing sugars, can produce methylglyoxal (Martins et al., 2003). As known in the Maillard reaction, temperature contributes significantly to methylglyoxal formation (Martins et al., 2003). Thirdly, decomposition of different lipids during food storage and processing could generate methylglyoxal in food samples (Fujioka and Shibamoto, 2004). Furthermore, photodegradation of lipids and related compounds yield methylglyoxal as a degradation product (Niyati-Shirkhodae and Shibamoto, 1993). Lastly, methylglyoxal can be formed by microorganisms during fermentation processing, such as alcoholic drinks and dairy products. Methylglyoxal is synthesized by *Saccharomyces cerevisiae* during alcoholic fermentation as well as by *Oenococcus oeni* (*Leuconostocoenos*) during malolactic fermentation (Yadav et al., 2005).

2.1.2.2. Endogenous source

In addition to food, methylglyoxal can be formed in biological systems

endogenously. Both enzymatic and non-enzymatic pathways have been identified for endogenous methylglyoxal formation (Vander Jagt and Hunsaker, 2003). Enzymatic pathways include reactions involved in glycolytic bypass, acetone metabolism, and amino acid breakdown (Cooper, 1984; Kalapos, 1999; Ko et al., 2005). Non-enzymatic pathways include acetol autoxidation, lipid peroxidation and others (Nemet et al., 2006).

Table 1 Methylglyoxal concentration of some food

Food or beverage	Methylglyoxal concentration (mg/kg)	Reference
Sweetened cola (sucrose-sweetened)	0.007 to 0.32	(Tan et al., 2008)
Sweetened cola (high fructose corn syrup-sweetened)	0.23 to 1.4	(Tan et al., 2008)
Fruit juice	0.05	(Degen et al., 2012)
Fermented beverages (yogurt, alcoholic beverages, and soy sauce)	0.02-7.6	(Degen et al., 2012)
Jams, jellies and sweeteners	4	(Degen et al., 2012)
Coffee	23	(Degen et al., 2012)
Bread and cookies	210	(Degen et al., 2012)
Commercial honey	0.4-5.4	(Degen et al., 2012)
Manuka honey	740	(Mavric et al., 2008)

2.1.2.2.1. Glycolytic Bypass

Glycolytic bypass is the major pathway of methylglyoxal formation endogenously, and is catalyzed by methylglyoxal synthase (Chakraborty et al., 2014). If there is too much sugar, one of the intermediates of glycolysis, glyceraldehyde-3-phosphate will be converted to dihydroxyacetone phosphate by triosephosphate isomerase, and then subsequently converted to methylglyoxal by methylglyoxal synthase (Ahmed et al., 2003a). It has been proven that increased concentration of dihydroxyacetone phosphate is

associated with increased levels of methylglyoxal (Ahmed et al., 2003a).

2.1.2.2.2. Acetone Metabolism

Diabetes is known to have high levels of ketone bodies and among them, acetoacetate is regarded as a characteristic feature of diabetes (Kupari et al., 1995; Veech, 2004). Acetoacetate is converted to acetone, then subsequently to hydroxyacetone by Cytochrome P450 and acetol monooxygenase, respectively. The latter is oxidized to methylglyoxal (Rabbani et al., 2016). Thus, it is known that diabetes has higher levels of methylglyoxal.

2.1.2.2.3. Amino Acid Breakdown

Another non-glycolytic formation of methylglyoxal is from amino acid breakdown, particularly from L-threonine and L-glycine. L-Threonine is initially oxidized by threonine dehydrogenase into oxobuturate, then to aminoacetone (Shimizu et al., 2005). L-Glycine is converted to aminoacetone by acetyl aminoacetone synthase with CoA. Methylglyoxal is the end product of aminoacetone degradation, which is catalyzed by amino oxidase (Shimizu et al., 2005).

2.1.3. Methylglyoxal elimination

Methylglyoxal can be eliminated by four enzymes: the glyoxalase system, aldose reductase (ALRs), aldehyde dehydrogenase (ADHs), and 2-oxoaldehyde dehydrogenase (2-ODHs) (Nemet et al., 2006).

The glyoxalase system comprises two enzymes; glyoxalase I and glyoxalase II (Thornalley, 1990). This is the major enzyme system for the elimination of methylglyoxal. Methylglyoxal is non-enzymatically converted to hemithioacetal with glutathione and subsequently catalyzed by glyoxalase I to *S*-D-Lactoylglutathione (Thornalley, 1995). *S*-

D-lactoylglutathione is converted to D-lactate by glyoxalase II, and further metabolized to pyruvate by 2-hydroxyacid dehydrogenase at the mitochondria (Thornalley, 1996).

Apart from the glyoxalase system, aldose reductases could convert methylglyoxal into acetol and subsequently reduce it to propanediol (Vander Jagt and Hunsaker, 2003). In addition, betaine aldehyde dehydrogenase and 2-oxoaldehyde dehydrogenases are known to catalyze methylglyoxal to pyruvate (Vander Jagt and Hunsaker, 2003).

2.1.4. Methylglyoxal research *in vitro*

(Kisch, 1932) reported that methylglyoxal decreased oxygen consumption in Jensen-sarcoma cells. (Thornalley et al., 1984) found that methylglyoxal was autoxidative, generated reactive oxygen species (ROS), modified mitochondrial proteins, and led to apoptosis of rat osteoblasts. However, methylglyoxal was cytotoxic to rat hepatocytes even under anaerobic conditions, which suggested that the autoxidation of methylglyoxal cannot explain its toxicity completely (Kalapos et al., 1993; Chan et al., 2007).

Methylglyoxal is susceptible to react irreversibly with protein, nucleotides, and basic phospholipids, which is verified in *in vitro* experiments (Thornalley, 1995; Ahmed et al., 2003b). This process is called dicarbonyl glycation and the adducts formed are AGEs (advanced glycation end-products). AGEs are known to have altered macromolecule structure and functions, and to be associated with complications of diabetes, atherosclerosis, and altered immune response (Chakraborty et al., 2014; Thornalley et al., 1999).

Methylglyoxal targets arginine residues, forming hydroimidazolone adducts (MG-H1), which is one of the major AGEs in physiological systems (Rabbani and Thornalley, 2012). There are also minor lysine-derived AGEs: such as CEL [N ϵ -(1-carboxyethyl)

lysine], argpyrimidine, and trace levels of MOLD (MG-derived lysine dimer cross-link) (Rabbani and Thornalley, 2012). Protein adducts bind specifically to cell-surface receptors, which is a specific receptor for AGEs, to activate cell dysfunction (Bierhaus et al., 2005). Methylglyoxal also forms DNA adducts in physiological systems. The major adduct is an imidazopurinone MGdG {3-(2-deoxyribose)-6,7-dihydro-6,7-dihydroxy-6/7-methylimidazo-[2,3-b] purine-9(8) one} and a minor adduct CEEdG [N2-(1-carboxyethyl)-deoxyguanosine], which were associated with DNA instability and resulted in mutagenesis (Thornalley and Rabbani, 2010).

2.1.5. Methylglyoxal research *in vivo*

The research of methylglyoxal *in vivo* is summarized in Table 2. The first *in vivo* experiment of methylglyoxal reported that the subcutaneous injection of this compound had no effects on rabbits (Dakin and Dudley, 1913). (Apple and Greenberg, 1968) demonstrated methylglyoxal had an anti-cancer effect in mice. However, the reduced psychomotoric activity of methylglyoxal was first reported in 1926 as the adverse effect of methylglyoxal in rabbits (Sjolemma and Seekles, 1926). Others reported that methylglyoxal injected rats were found to develop skin cancers (Nagao et al., 1986). The acute lethal dose of methylglyoxal was 800 mg/kg body weight, while a lower dose of methylglyoxal led to a decrease of liver weight, inactive and/or irregular muscle movement (Kalapos et al., 1991; Choudhary et al., 1997). Oral administration of methylglyoxal targeted kidneys, accumulating methylglyoxal collagen that thickened glomerular basement membrane with an obligatory morphological change (Golej et al., 1998).

Table 2. Methylglyoxal research *in vivo*

Time	Animal models	Treatment	Methylglyoxal dose	Conclusion	Reference
1913	Rabbits	Subcutaneous injection	Unknown	No effects	(Dakin and Dudley, 1913)
1926	Rabbits	Subcutaneous injection	Unknown	Without appetite and depressed	(Sjolemma and Seekles, 1926)
1968	Male swiss albino mice	Unknown	Unknown	Anti-cancer effects on variety of cancers	(Apple and Greenberg, 1968)
1986	Male & female fischer 344 rats	Skin injection	2 mg/animal	Developed cancers	(Nagao et al., 1986)
1991	Male CFLP mice	Intraperitoneal injection	Unknown	Slowed movement and ataxy	(Kalapos et al., 1991)
1991, 1997	Male swiss albino mice	Intraperitoneal injection	800 mg/kg body weight	Lethal within 4 h	(Kalapos et al., 1991; Choudhary et al., 1997)
1991, 1997	Male swiss albino mice	Intraperitoneal injection	Less than 800 mg/kg body weight	Decreased liver weight after 24 h	(Kalapos et al., 1991; Choudhary et al., 1997)
1991	Male swiss albino mice	Intraperitoneal injection	400 mg/kg body weight	Slight histological changes in livers (vacuole formation in the centrilobular areas)	(Kalapos et al., 1991; Choudhary et al., 1997)
1998	Female OF-1 mice	Oral administration	50 mg/kg body weight	Collagen accumulation in kidneys; glomerular basement membrane thickening	(Golej et al., 1998)

2.1.6. Strategies for minimizing methylglyoxal

Based on the information above, strategies for prevention of methylglyoxal toxicity could be by: (a) prevention of methylglyoxal formation, (b) scavenging of methylglyoxal directly, and (c) enhancing the expression of enzymes of methylglyoxal elimination, particularly glyoxalase-1 (Rabbani et al., 2016). First, the formation of methylglyoxal can be reduced by a high dose of thiamine supplementation by decreasing the activity of triosephosphate isomerase, a key enzyme for methylglyoxal production. Secondly, scavengers, such as aminoguanidine, phenacylthiazolium derivatives, and arginine-rich peptides, can be used to scavenge methylglyoxal directly. However, it is not clear that these products are without any toxic effects (Rabbani et al., 2016). Lastly, enhancing glyoxalase-1, ALRs, and ADHs, which are the key enzymes for eliminating methylglyoxal, can reduce methylglyoxal levels. Based on reports that the transcription factor, Nrf2 (nuclear factor-erythroid 2-related factor-2), regulates the above enzymes and is downregulated during aging, it is possible that by targeting Nrf2, elimination of methylglyoxal can be enhanced (Rabbani et al., 2016).

2.2. *Caenorhabditis elegans*

2.2.1. General characteristics of *C. elegans*

C. elegans is a small (1~1.5 mm as an adult), eukaryotic, multi-organ, and free-living nematode found worldwide. *C. elegans* is easily cultivated on agar plates and typically feeds on non-pathogenic *Escherichia coli* (*E. coli*). The body and neural circuitry is anatomically simple, with approximately 1,000 somatic cells and 300 neurons

(Giles and Rankin, 2009; Dimitriadi and Hart, 2010). The transparent body of this worm makes it easy to view the tissue in all stages of development under an optical microscope. Normally, a wild-type *C. elegans* has a short lifespan of about 21 days, and reaches adulthood within 38~46 h after hatching at 20°C. *C. elegans* produces large broods of 300 progenies and each hermaphrodite has two ovaries, oviducts, a cavity for storing sperm (spermatheca), and a uterus (L'Hernault, 2009). Most importantly, *C. elegans* is the first animal to have its genome completely sequenced, and more than 65% of the genes associated with human disease are conserved in the worm (Greer et al., 2008). In addition, *C. elegans* exhibits very complicated functions varying from feeding to locomotion suggesting further application for *in vivo* research.

2.2.2. *C. elegans* as an animal model for development research

C. elegans has been used in studies for cell biology, genetics, and neurobiology since 1965. Between 1970 and 1980, the complete cell lineage of this worm, from the fertilized egg to adult, was characterized by laser ablation and microscopy (Sulston et al., 1983). (White et al., 1986) constructed the entire nervous system by electron microscopy and serial sectioning, and (Coulson et al., 1991) started to work on genetic and genomic data collection. Bioassays to assess the impact of inherent or environmental changes on *C. elegans* can be carried out through different endpoints. These endpoints can be grouped according to their effects on biological parameters, such as lethality, growth, locomotion, reproduction, etc. It is also possible to use molecular markers to determine oxidative stress, changes in gene or protein expression, DNA damage, or green fluorescence protein (GFP) expression (Tejeda-Benitez and Olivero-Verbel, 2016).

2.2.2.1. Physiological parameters of *C. elegans*

Among worms' development parameters, body length of synchronous worms before and after exposure can be used to evaluate the effect of a compound in the growth of the nematode. The developmental stage of *C. elegans* can be determined by: L1, four or fewer gonad cells, L2, over four gonad cells which have begun to spread along the length of the animal, L3, extension of the gonad, and vulvae morphogenesis has started, L4, a dorsal rotation of the gonad, and adults, observable eggs (Helmcke et al., 2009). The deterioration of the neural system during development can be evaluated by locomotion, which includes head thrash, body bend frequency, and basic movements (Yu et al., 2013). A head thrash is defined as a change in the direction of bending in the body, observed as a change in direction of the upper pharynx along the Y-axis, assuming that the nematodes are moving along the X-axis. The body bend frequency refers to the frequency of oscillations between adjacent body segments. The basic movement is measured as average speed of worm crawling or swimming (Yu et al., 2013). Brood size is the endpoint of development and is used to evaluate the reproduction of the nematodes. Moreover, the gonad size, obtained by image analysis under a microscope, has been utilized to evaluate the effects on reproductive organs and the rate of egg laying, which means the number of eggs laid in 1 h is a biomarker of reproduction (Jadhav and Rajini, 2009; Shashikumar and Rajini, 2010; Wu et al., 2011). The lifespan is defined as the period between the L4 larval stage and death. At 20°C, the wild type *C. elegans* has an average lifespan of around 2–3 weeks (Yunhui et al., 2009; Shen et al., 2009; Wang et al., 2010; Zhuang et al., 2014).

2.2.2.2. Development pathway of *C. elegans*

After embryogenesis, *C. elegans* develops through four larval stages (L1–L4) before reaching sexual maturity. The proper temporal control of stage-specific events of cell division, cell behavior, and differentiation through four larval stages are controlled by the heterochronic pathway, which is comprised by a number of microRNA and protein regulators (Figure 2). The precise scheduling for the stage-specific events and temporal controls of cell division, differentiation, and morphogenesis is regulated by a complex but organized schedule of heterochronic gene activities for animal normal development. Adult stage is also regulated by the heterochronic pathway to exit the cell cycle, fuse with neighboring hypodermal seam cells, and generate cuticle alae (Sulston and Horvitz, 1977; Reinhart et al., 2000). However, improper development can result in either retarded or precocious development, by repeating early developmental events or by expressing late developmental events at early stages, respectively.

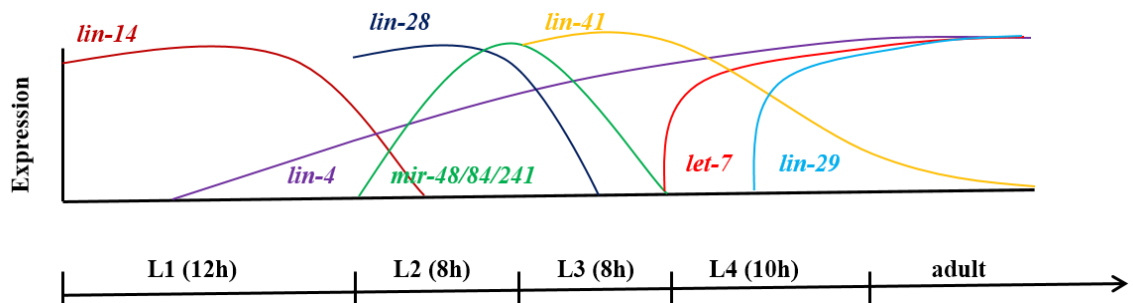


Figure 2. Expression of some heterochronic genes during larval development (Lee et al., 1993; Rougvie and Ambros, 1995; Slack et al., 2000).

2.2.2.2.1. *lin-14*

The gene *lin-14* encodes a novel protein, LIN-14, in *C. elegans*, whose activity is required for the timings of vulva cell group division during postembryonic development (Lee et al., 1993). The normal L1 stage requires a high level of *lin-14* activity and the execution of L1 stage to later stages depends essentially on the decrease of *lin-14* expression. In addition, *lin-14* acts with *lin-4* to govern L1 cell fates, and then interacts

with *lin-28* to regulate L2 developmental events (Ambros and Horvitz, 1984). The *lin-14* null (0) mutant misses the expression of L1 specific events, thus becoming precocious compared to the normal developmental program. In the contrary, *lin-14* gain-of-function mutant displays a retarded phenotype (Ambros and Horvitz, 1984).

2.2.2.2.2. *lin-4*

The *lin-4* encodes a small non-coding temporal RNA (stRNA) and is a critical gene in the early stages of development in diverse cell types of *C. elegans* (Lee et al., 1993). Expression of *lin-4* begins at the end of the L1 larval stage and increases to highest levels in adult animals (Ambros and Horvitz, 1984). Repression translation of the *lin-14* and execution L1 stage is the function of *lin-4* (Ambros and Horvitz, 1984). Thus, *lin-4* loss-of-function mutation, *lin-4(e972)*, displays reiterations of L1 events at late developmental stages (Ambros and Horvitz, 1984).

2.2.2.2.3. *lin-28*

For L2 stage specific developmental events, the heterochronic gene, *lin-28*, plays a critical role. In addition, LIN-28 is an RNA binding protein, best known for regulating the maturation of the microRNA *let-7* (Rougvie and Ambros, 1995;Reinhart et al., 2000;Pepper et al., 2004). However, *lin-28* expression was not detectable after the L3 stage. Several researches confirmed that the function of *lin-28* at the L2 stage was a premise for larva-to-adult transition, which means *lin-28* interacted with other heterochronic genes to affect the larval-to-adult transition (Rougvie and Ambros, 1995;Reinhart et al., 2000;Pepper et al., 2004). Thus, *lin-28* loss-of-function mutant not only skips the L2 stage seam cell proliferative program, but also causes precocious execution of the larva-to-adult transition, which presents the phenotype of lateral

hypodermal seam cells ceasing division, differentiation, and adult alae production (Ambros and Horvitz, 1984).

2.2.2.2.4. *let-7* family microRNAs

The *let-7* family microRNAs comprise *let-7*, a critical regulator of developmental timing events at the larval-to-adult transition, and three other microRNA genes: *mir-48*, *mir-84*, and *mir-241*, which regulate L2-to-L3 developmental timing decisions. The retarded behaviors, like extra seam cell divisions, could be found in diverse cell types from the L2 to L3 transition in double mutants *mir-48: mir-241*, *mir-48: mir-84*, and triple mutants (Abbott et al., 2005). However, normal phenotype was observed in the singly mutant worms with deletions of *mir-48*, *mir-84*, or *mir-241* and the double mutant *mir-84: mir-241*.

The *let-7* functions as a temporal switch between larval and adult fates since the expression of *let-7* is only detected at L4 and adult stages. Thus, the *let-7* loss-of-function mutant reiterates larval patterns of hypodermal cell division during the L4-to-adult transition. Besides, it undergoes a supernumerary molt to an extra stage, L5, before adult. In contrast, the overexpression of *let-7* causes precocious adult fates during larval stages, which means hypodermal cells exit the cell cycle ahead of time.

2.2.2.2.6. *lin-41*

The *lin-41* encodes a RING-finger B-box Coiled-coil (RBCC) protein and is negatively regulated by the *let-7* microRNA during L4 and adult stages (Solomon et al., 2008). In addition, *lin-41* represses *lin-29* during late larval stages, which prevents terminal differentiation of hypodermal seam cells. Thus, *lin-41* loss-of-function mutants display premature phenotypes from the L3 larval stage that seam cells adopt the adult fate

to secrete alae ahead of time, but does not affect L1 and L2 stage fates (Reinhart et al., 2000; Solomon et al., 2008).

2.2.2.2.7. *lin-29*

The *lin-29*, a zinc-finger transcription factor, which regulates adult-specific patterns of cell lineage and cell differentiation, acts as a downstream of heterochronic genes, *let-7* and *lin-41* (Rougvie and Ambros, 1995; Reinhart et al., 2000). Furthermore, *lin-29* is the most direct known regulator of the larval to adult transition in *C. elegans* (Rougvie and Ambros, 1995). Thus, *lin-29* activity is essentially required for the execution of almost all aspects of the larval to adult transition, particularly for the terminal differentiation of the lateral hypodermal seam cells and the formation of the adult cuticle (Rougvie and Ambros, 1995). In the *lin-29* loss-of function mutant, the seam cells continue to synthesize a larval-specific cuticle lacking adult-specific alae, which means the animal undergoes supernumerary molts (Ambros and Horvitz, 1984). Interestingly, both the *lin-29* loss-of-function mutant and the *let-7* loss-of-function mutant display a retarded phenotype, but the former repeats the cell cycles in the L4-stage indefinitely, while *let-7* loss-of-function mutants only repeat once, which indicates *lin-29* displays a stronger function in the heterochronic pathway than *let-7* (Slack et al., 2000).

2.3. Methylglyoxal Research in *C. elegans*

The activity of glyoxalase-1 is markedly reduced with age. However, in aging, methylglyoxal level, methylglyoxal-derived adducts, and oxidative stress markers, such as carboxymethyllysine (CML) and 3-nitrotyrosine, increase dramatically (Morcos et al., 2008). As reported, over-expression of the glyoxalase-1 ortholog, CeGly 1, could prolong *C. elegans* lifespan by decreasing modification of mitochondrial protein and ROS

production (Morcos et al., 2008).

In humans, protein deglycase, DJ-1, is expressed to protect cells from oxidative and exert neuroprotection against Parkinson's Disease (Bonifati et al., 2003; Lee et al., 2012). In *C. elegans*, cDJR-1.1, a DJ-1 ortholog expressed primarily in the intestine, when lacking decreased 71% of the enzyme activity for methylglyoxal elimination; cDJR-1.2, another DJ-1 ortholog, expressed primarily in the head region and leading to the significant degeneration of the dopaminergic neurons, when lacking decreased 28% of the enzyme activity for methylglyoxal elimination (Bonifati et al., 2003; Lee et al., 2012). Thus, these findings imply that cDJR-1.1 and cDJR-1.2 are important in methylglyoxal elimination, which could protect worms from methylglyoxal.

At present, the methylglyoxal research in *C. elegans* is limited to aging and neurological disorders. As an ideal model for development study, *C. elegans* is a great tool to investigate the interaction between methylglyoxal and development.

2.4. Conclusion

Methylglyoxal, as an active carbonyl compound, can be produced inherently or elevated by exogenous sources. In order to minimize its toxicity on aging, development, and the nervous system, there are endogenous elimination systems to keep the normal metabolic activity. According to previous studies, glyoxalase-1 and DJ-1 are reported as essential pathways in methylglyoxal regulation with aging and neurological issues, respectively. As for development, *C. elegans* could be a great model to be used for its completed understood genome sequence and heterochronic system. This review elucidates the mechanism of methylglyoxal in development of *C. elegans*.

CHAPTER 3

MATERIALS AND METHODS

3.1. Strains

The *C. elegans* strain N2 was used as the wild-type worm. The N2 strain, mutant *lin-41(ma104) I* and *Escherichia coli* (*E. coli*) OP50 were obtained from the Caenorhabditis Genetics Center (CGC), University of Minnesota, USA.

3.2. Chemicals

Methylglyoxal aqueous solution was purchased from MP Biomedicals, LLC (Solon, OH). 5-fluoro-2'-deoxyuridine (FUdR) was purchased from Sigma-Aldrich Co. (St. Louis, MO). Household bleach for synchronization of worms was purchased from a local store (The Clorox company, Oakland, CA). Other chemicals were purchased from Thermo Fisher Scientific Inc. (Pittsburgh, PA).

3.3. Worm maintenance

Synchronized worms were prepared by placing adult worms on a nematode growth media (NGM) plate containing a lawn of *E. coli* OP50. After removing the worm bodies using freshly prepared bleach solution, each synchronized egg was transferred to a S-complete solution in 15 ml conical tubes. When the eggs hatched, concentrated *E. coli* OP50 was added with 0.1 mM or 1 mM methylglyoxal, respectively. All *C. elegans* strains were raised at 25°C.

3.4. Growth rate assay

For each treatment group, 50 worms were selected randomly and paralyzed with 10 mM NaN₃. The worms were transferred onto NGM plates without a bacterial lawn and the developmental stages were identified under an optical microscope. The growth rates

were measured as the population ratio of worms at different developmental stages.

3.5. Pumping rate assay

For each treatment, 12 worms were randomly selected and placed on NGM plates with a thin layer of *E. coli* OP50. The number of nematodes' pharynx contractions was counted for 1 min using an optical microscope. Experiments were carried out in triplicates.

3.6. Body size and activity determination

For each treatment, 50 worms were randomly selected and placed on low-peptone NGM plates. *E. coli* OP50 were seeded 5 min before tracking. Then worms were allowed to acclimate and move for 15 min before video recording. A 1-min recording (7.99 frames per second) was captured by WormLab Video Capture System (Version 3.1.0, MBF Bioscience, Williston, VT, USA). The average moving speed and the body size (length and width) were analyzed by WormLab (Version 3.1.0, MBF Bioscience, Williston, VT, USA).

3.7. Lifespan assay

Lifespan analysis was carried out at 25°C. Worms were treated with methylglyoxal at 0.1 mM and 1 mM from the L4 stage, at which 120 µM 5-Fluoro-2'-deoxyuridine (FUdR) was added in HTS Transwell®-96 well permeable plates (Corning Inc., NY). The medium was changed twice a week. Plates were shaken for 2 min before counting and worm viability was scored every other day until all the worms died.

3.8. Statistical analysis

All data were expressed as mean ± standard error (S.E.). Mean differences between treated groups and controls were determined by one-way analysis of variance (ANOVA),

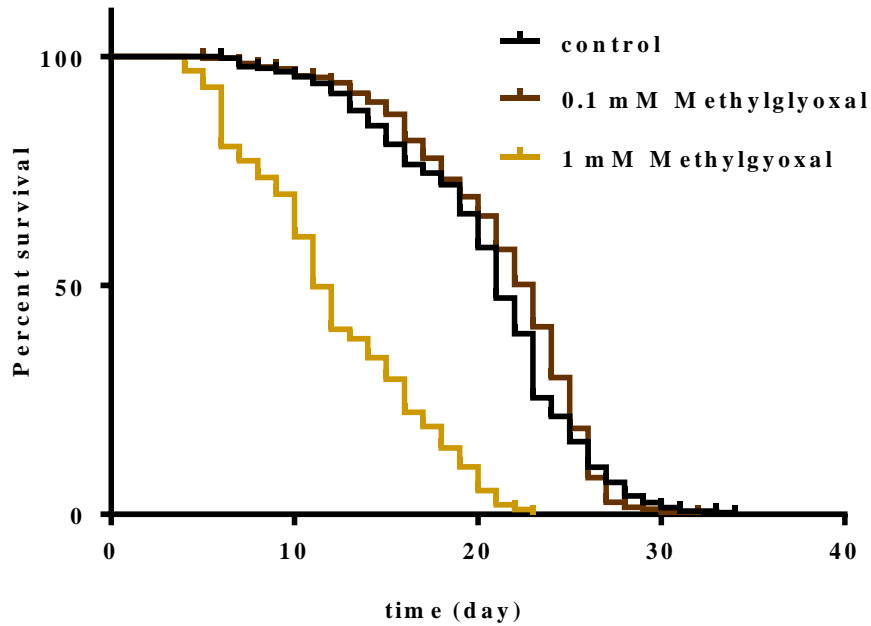
followed by Dunnett's test using the Statistical Analysis System (Version 9.4, SAS Institute, Cary, NC). A *P*-value of less than 0.05 was considered significant.

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Methylglyoxal treatment significantly reduced the lifespan of *C. elegans*

The result (Figure 1) showed that the 1 mM methylglyoxal treatment group had a significantly reduced maximum lifespan of 21 days, compared to the control (34 days). Methylglyoxal at 0.1 mM did not influence the lifespan of wild-type worms. The median lifespan was 21, 23, and 11 days in the control, 0.1 mM, and 1 mM treatment groups, respectively. These indicated that 1 mM methylglyoxal, but not 0.1 mM, significantly accelerated aging compared to control.



concentration	0.1 mM versus control	1 mM versus control	1 mM versus 0.1 mM
<i>P</i> -value	Not significant	< 0.0001	< 0.001

Figure 3. Effects of methylglyoxal on survival of *C. elegans*. Age-synchronized L4 *C. elegans* were cultured in 96-well plates with 0, 0.1 mM, and 1 mM methylglyoxal from the first day of adulthood. The number of survivals were recorded every other day. Around 100 worms were raised for each group.

4.2. Methylglyoxal treatment significantly delayed the development of *C. elegans*

To determine whether methylglyoxal had any effects on the development of the nematodes, the growth rate was analyzed. As shown in Figure 2, after 48 h of treatment with methylglyoxal, 28-34% of the worms were at the L3 stage in the control and 0.1 mM treatment groups, while this number significantly increased to 77% in the 1 mM treatment group. In the control and 0.1 mM treatment group, 56-59% of worms reached the L4 stage, but it was 19% in the 1 mM treatment group. These suggested that 1 mM methylglyoxal delayed *C. elegans*' development from the L3 stage.

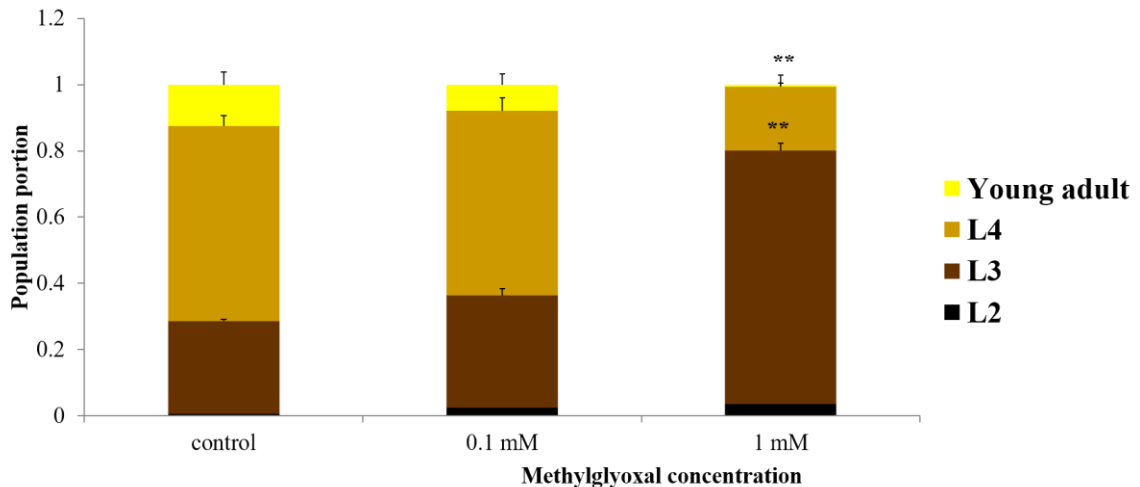


Figure 4. Effect of methylglyoxal on the development of *C. elegans*. Age-synchronized L1 *C. elegans* were cultured on NGM plates with 0, 0.1, and 1 mM methylglyoxal. The number of worms at each developmental stage after 48 h was counted. Values represent means \pm S.E. (n=50). ** in the L3 stage means significantly different when 1 mM compared with the control and 0.1 mM treatment group ($P<0.01$). ** in the L4 stage means significantly different when 1 mM compared with the control and 0.1 mM treatment group ($P<0.01$).

4.3. Methylglyoxal treatment significantly reduced body size of *C. elegans*

There was a significant decrease in body length when comparing the treatment group to the control (Figure 5). The body width showed consistent results with body length, specifically that methylglyoxal treated worms decreased worm width compared to the control. Both 0.1 mM and 1 mM methylglyoxal reduced the body size of *C. elegans*.

Importantly, 0.1 mM methylglyoxal exerted this effect on worms without inhibiting their growth rate (Figure 4).

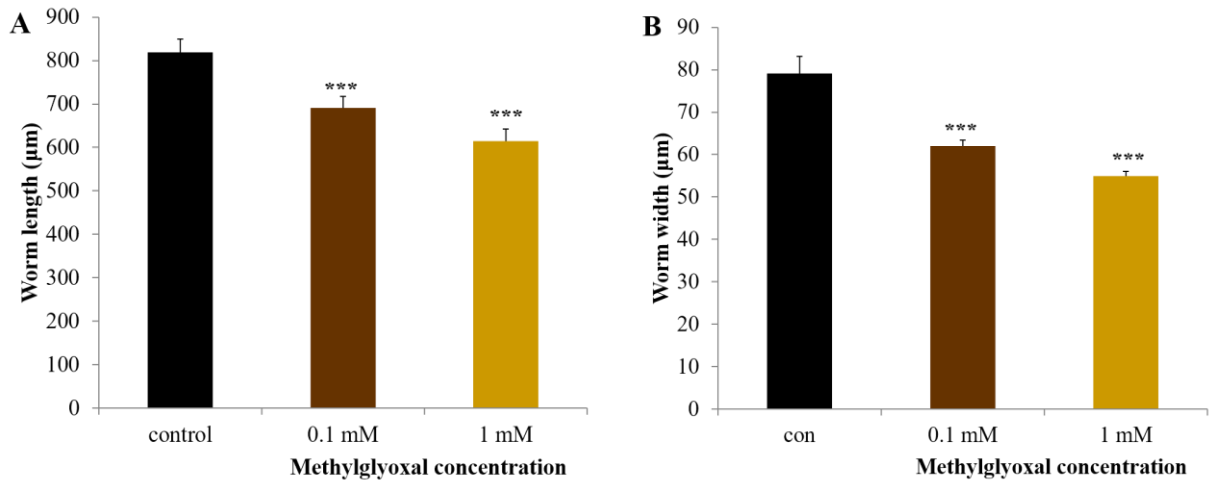


Figure 5. Effect of methylglyoxal on the body size of *C. elegans*. A: Worm length and B: worm width was measured by WormLab software after 48 h treatments. Values represent means \pm S.E.(n=50). *** means significantly different from control ($P < 0.001$)

4.4. Methylglyoxal treatment significantly altered locomotion behaviors of *C. elegans*

After exposure to methylglyoxal for 48 h, 0.1 mM of methylglyoxal led to a significant increase in the mean speed, while the 1 mM treatment group significantly slowed down compared to the control (Figure 6A). However, both 0.1 mM and 1 mM treatment groups decreased pharyngeal pumping rates significantly compared to the control (Figure 6B). The reduction was 7% and 8% in 0.1 mM and 1 mM methylglyoxal, respectively.

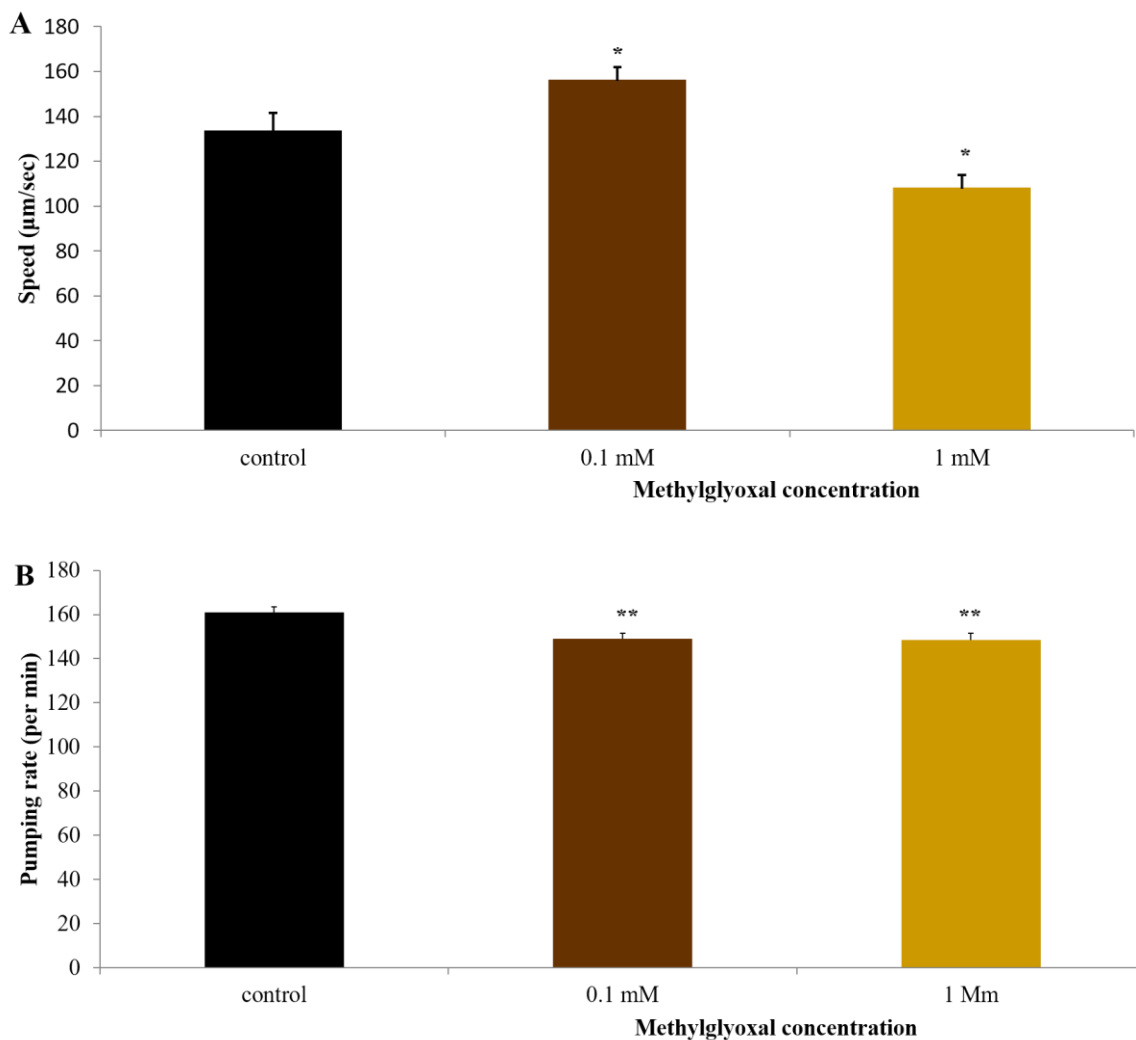


Figure 6. Effects of methylglyoxal on the locomotion behavior of *C. elegans*. After treated to methylglyoxal for 48 h from L1, A: the average moving speed was measured by WormLab software, and B: pharyngeal pumping rate was measured under the optical microscope. Values represent means \pm S.E.(n=50). * in A means significantly different from control ($P<0.05$). There is a significant difference between 0.1 mM and 1 Mm treatment group in A ($P<0.001$). ** in B means significantly different from control ($P<0.01$).

4.5. Heterochronic pathway related gene, *lin-41*, is involved in the effect of methylglyoxal in *C. elegans*

In the *lin-41* mutants, we found that the developmental period (L1 to adult) was reduced to approximately 40 h, while the wild-type worms were approximately 48 h. It might be because *lin-41* mutant displayed their premature behaviors from the L3 stage

(Figure 7A). Besides, the worm length reduced by 45% (Figure 7B), the average speed reduced by 59% (Figure 7C), and the pumping rate decreased by 30% (Figure 7D) compared to the wild-type worms. The results shown in Figure 7 indicated that there were no significant differences on development, body size, and locomotion behaviors among the different methylglyoxal treatment groups in the *lin-41* mutants.

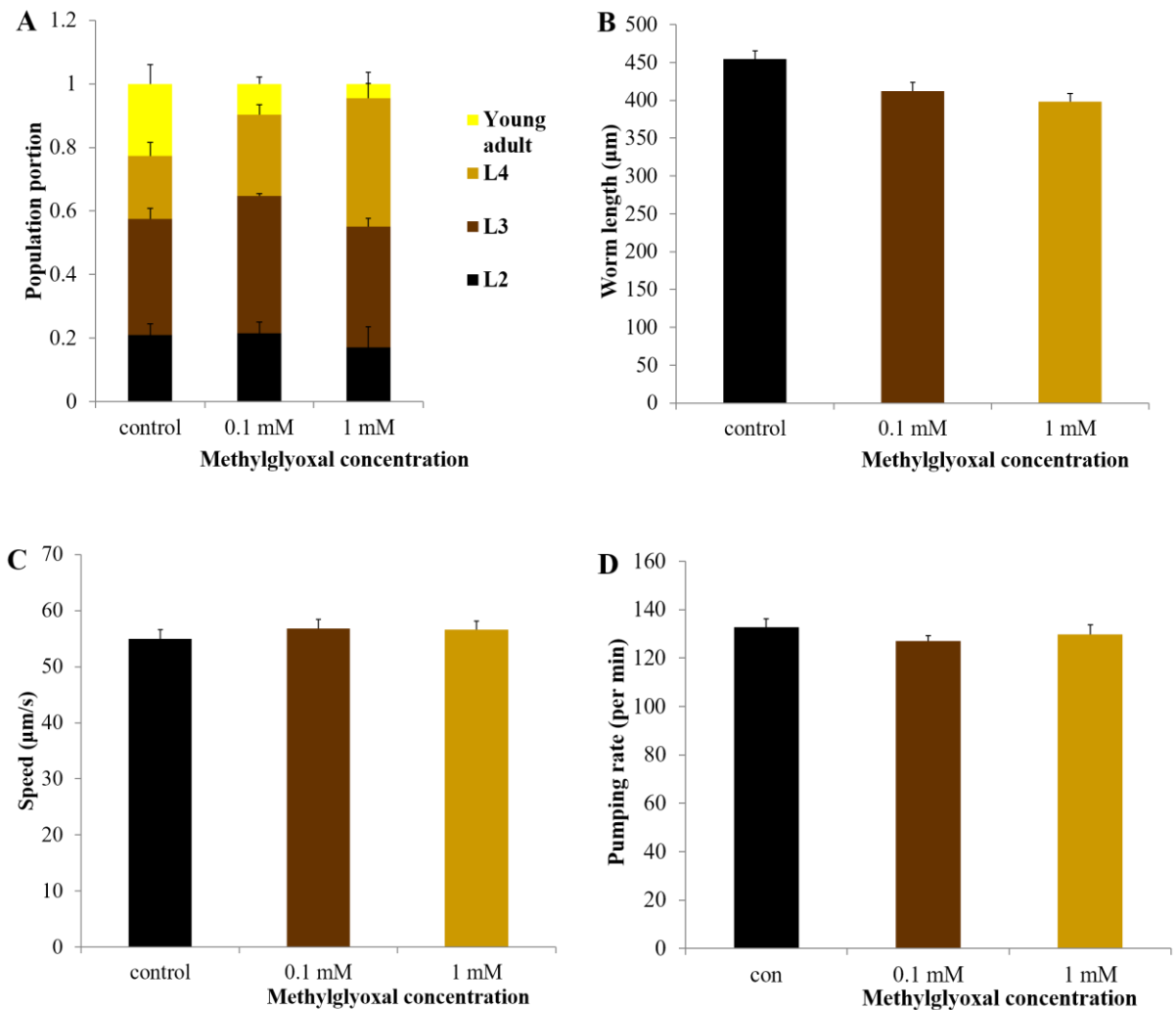


Figure 7. Effects of methylglyoxal on development in the *lin-41* mutants. Age-synchronized L1 *lin-41* worms were cultured on NGM plates with 0, 0.1, and 1 mM methylglyoxal. A: The number of worms at each developmental stage after 40 h was counted to calculate population percentages of each larval stage. B: worm length and C: moving speed of *lin-41* mutants were measured by WormLab software when the control group worms reached L4/young adult after 40 h treatments. D: Pharyngeal pumping rate was counted under optical microscope. Values represents means \pm S.E. (n=50 in A, B, & C, n=12 in D).

4.6. Discussion

In the present research, methylglyoxal at 1 mM significantly decreased lifespan and delayed development of *C. elegans*. Furthermore, methylglyoxal at 0.1 mM displayed effects on worm body size and locomotion behaviors. The *lin-41* mutant abolished the effects of methylglyoxal on development, worm body size, and locomotion behaviors, at both 0.1 mM and 1 mM, suggesting that this heterochronic pathway is a target of methylglyoxal's effect.

In previous studies, *Drosophila melanogaster* upon methylglyoxal treatment significantly reduced its maximum lifespan in a dose-dependent manner from 5 mM to 50 mM, where 10 mM of methylglyoxal reduced 48% of the median lifespan (Guelinckx et al., 2008). However, the current study showed that 1 mM methylglyoxal reduced 33% of the maximum lifespan of *C. elegans* compared to the control group, which suggested that *C. elegans* is more susceptible to methylglyoxal than *Drosophila melanogaster*.

Locomotion is a natural behavior that the worm's movement exhibits as an undulatory motion produced by waves of muscular contraction and relaxation regulated by the nervous system (Burr et al., 2004). The results of this research indicated that the average moving speeds of methylglyoxal-treated worms depends on concentrations of the compound. This type of response was similar to those observed in Parkinson's disease, which include periods of abnormally high (rigidity) and then low (bradykinesia) motor activity. Thus, it would be important to investigate effects of methylglyoxal in the nervous system in the future.

The control of pharyngeal pumping rate is a complex process that involves both the pharyngeal muscle cells and neurons that innervate the pharynx. The obvious reduction

of pumping rate by methylglyoxal could be a cause for retarded development. However, in this research, the significant reduction of pumping rate was not consistent with the developmental delay at 0.1 and 1 mM, which suggests that there would be at least two distinctive pathways involved in methylglyoxal's effects on these.

Growth rate under microscopic observation is the most direct parameter of development due to specific developmental events: the L1/L2 molt and the start of oogenesis in young adult *C. elegans*. Corresponding with lifespan data, growth was delayed significantly when the concentration of methylglyoxal was increased to 1 mM. The current results showed both 0.1 mM and 1 mM of methylglyoxal causing significant reductions in worm length and width. Since most of the worms (77 %) were at the L3 stage upon 1 mM treatment, compared to 28-34 % of worms at the L4 stage of control and 0.1 mM treatment, the reduced worm sizes may result from the retarded development stage. However, without interfering by development delayed, low dose at 0.1 mM methylglyoxal still exerted its effect on body sizes.

In *C. elegans*, *lin-41* encodes a RING-finger B-box Coiled-coil-containing protein and is involved in the heterochronic pathway, which temporally control the timing of each larval stage (Solomon et al., 2008). The *let-7* microRNAs, which takes effect on the L2-to-L3 transition, regulates *lin-41* negatively. More importantly, *lin-41* represses *lin-29*, the most direct regulator of the larval to adult transition in *C. elegans*, to prevent terminal differentiation of hypodermal seam cells (Reinhart et al., 2000). Thus, *lin-41* loss-of-function mutants would display premature phenotypes at the L3 larval stage and secrete adult alae ahead of time, but would have normal L1 and L2 stage fates (Reinhart et al., 2000; Solomon et al., 2008). The current study showed that *lin-41* mutants abolished all

the effects of methylglyoxal on development, suggesting that methylglyoxal delays development through the heterochronic pathway, particularly *lin-41*.

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