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ORIGINAL ARTICLE

Uncoupling Healthspan and Lifespan in Long-lived *Daf-2*, *Pmk-3* and *Sgk-1* Knockdown *Caenorhabditis elegans*

SITI BAZILAH Z, GEETHA G, GOON JA*

Department of Biochemistry, Faculty of Medicine, Universiti Kebangsaan Malaysia, Jalan Yaacob Latiff, Bandar Tun Razak, 56000, Cheras Kuala Lumpur, Malaysia

ABSTRAK

Modulasi pengekspresan gen dalam tapak jalan isyarat berupaya melanjutkan jangka hayat Caenorhabditis elegans (C. elegans) namun kesannya terhadap kesihatan masih tidak jelas. Tujuan kajian ini adalah untuk menentukan tahap kesihatan nematoda mutan daf-2, pmk-3 dan sgk-1. Tahap kesihatan nematoda yang dikultur dalam media pertumbuhan nematoda yang mengandungi Escherichia coli telah dinilai pada hari ke-5, ke-10 dan ke-15 kedewasaan. Kerintangan terhadap haba ditentukan dengan menganalisis kadar jangka hayat nematoda pada suhu 37°C. Kerintangan terhadap tekanan oksidatif pula diukur melalui daya tahan nematoda setelah terdedah kepada 10mM hidrogen peroksida. Kapasiti pergerakan dianalisis dengan mengira jarak yang dilalui pada media pepejal dan jumlah bilangan lenturan badan dalam media cecair. Hasil kajian ini menunjukkan bahawa nematoda mutan dapat bertahan lebih lama daripada nematoda jenis liar. Mutan daf-2 dan pmk-3 menunjukkan peningkatan pada kapasiti pergerakan pada hari ke-5 dan ke-10 dalam media pepejal dan cecair tetapi menunjukkan hasil yang serupa dengan nematoda jenis liar pada hari ke-15. Mutan sgk-1 turut menunjukkan peningkatan pada kapasiti pergerakan, namun hanya pada hari ke-5 bagi kedua-dua media. Peningkatan ketahanan nematoda mutan terhadap tekanan haba dan oksidatif adalah ketara. Kesimpulannya, mutasi gen daf-2, pmk-3 dan sgk-1 dapat meningkatkan daya tahan terhadap tekanan haba dan oksidatif tetapi tidak meningkatkan pergerakan semasa usia lanjut dalam C. elegans.

Kata kunci: C. elegans, jangka hayat, kesihatan, pergerakan, stress

ABSTRACT

Address for correspondence and reprint requests: Goon Jo Aan. Department of Biochemistry, Faculty of Medicine, Universiti Kebangsaan Malaysia, Jalan Yaacob Latiff, Bandar Tun Razak, 56000, Cheras Kuala Lumpur, Malaysia. Tel: +603-91549558 Email: joaan@ukm.edu.my

Modulation of genes expression in signaling pathways promotes longevity in *Caenorhabditis elegans* (*C. elegans*) but it is unclear whether this will improve healthspan. This study aimed to determine the healthspan of *daf-2*, *pmk-3* and *sgk-1* knocked-down worms. Health measures were determined at day 5, day 10 and day 15 of adult worms which were cultured in nematode growth media containing *Escherichia coli*. Heat resistance was determined by analysing the survival of worms at 37°C. Oxidative stress resistance was measured after exposure to 10mM hydrogen peroxide to analyse its survival capacity. Movement capacity was analysed by computing the distance travelled on solid media and the number of thrashing in liquid media. Results showed that the gene knockdown worms survived longer than wildtype worms. *Daf-2* and *pmk-3* knockdown increased movement capacity at day 5 and 10 in solid and liquid media but resulted in similar capacities to wildtype worms at day 15. *Sgk-1* knockdown increased movement capacity only at day 5 in both media. The ability of gene knocked-down worms to withstand heat and oxidative stress significantly increased. In conclusion, knockdown of *daf-2*, *pmk-3* and *sgk-1* improved resistance to heat and oxidative stress but did not modulate movement incompetency at advanced ages *C. elegans.*

Keywords: *C. elegans*, healthspan, lifespan, movement, stress

INTRODUCTION

Healthy aging is one of the chief concern of many people as we strive towards enhancement of life quality. As age progresses, our physiological systems decline in function due to disruptions of homeostasis which lead to disabilities, diseases and subsequently death (Sohal et al. 2002). Healthspan is the period of functional capacity before declination begins (Herndon et al. 2002). In the past decade, countless studies had been carried out on aging specifically towards the aim of life extension or longevity with the assumption that longer lifespan entail prolonged healthspan (Selman & Withers 2011).

Mechanisms that regulate aging have been explored and established using short-lived organisms such as yeasts, worms and flies. There was comparable mechanisms observed in mammals which indicated similarity in molecular regulators of lifespan are similar across different species (Uno & Nishida 2016). Among the studied model organisms, *Caenorhabditis elegans* (*C. elegans)* is preferred in most aging studies, primarily because of its well-characterised genome which has 60-80% homologous to human, equivalent functions of organs and tissues to humans, ease of maintenance as well as short lifespan (Lai et al. 2000; Leung et al. 2008).

Interventions in signalling pathways and environmental conditions have been shown to delay the aging process and prolong life expectancy by enhancing stress resistance and

reducing oxidative damage by macromolecules (Gems & Partridge 2013; Goon et al. 2017). However, most of these interventions focused on the measurement of life expectancy as an outcome. More recent studies suggested that life expectancy may not able to measure the level of health precisely (Bansal et al. 2015; Dues et al. 2019). More studies had since evolved to determine changes in health capacities including movement, cognitive function and behaviour as a measurement of intervention efficacy (Tan et al. 2021; Yahya et al. 2017). Some had even claimed that increased longevity may prolong the period of disability in aging stage (Palliyaguru et al. 2019; Rockwood et al. 2011). Thus, the aim of this study was to determine the healthspan of long-lived *C. elegans* with knocked down expressions of *daf-2*, *pmk-3* and *sgk-1* during aging. *Daf-2* is a homolog of the insulin/insulin-like growth factor-1 receptor in humans. Knockdown of *daf-2* results in the inhibition of insulin/insulin-like growth factor-1 signaling (IIS). Depleted IIS activity causes the translocation of *daf-16* into the nucleus to stimulate the transcription of stress resistance proteins such as heat-shock proteins, superoxide dismutase and catalase as well as increase metabolism and autophagy (Murphy et al. 2003). Elsewhere, *pmk-3* is one of three *pmk* genes (*pmk-1, pmk-2, pmk-3*) that encodes p38 mitogen-activated protein kinase (MAPK) in *C. elegans*. The MAPK is an immune-signaling pathway that is highly conserved between *C. elegans* and humans. MAPK responds to extracellular signals and transduces the signal to regulate the expression of genes that involved in apoptosis, cell cycle, cell differentiation, cell development and inflammation (Zarubin & Jiahuai 2005). Knockdown of *pmk-3* enhances the expression of *pmk-1* which is pivotal in upregulating immune defenses in *C. elegans* through MAPK and stimulating neuronal functions (Irazoqui et al. 2008). Meanwhile, *sgk-1* encodes serine-threonine kinase which is homologous to Akt/protein kinase B (PKB) in humans. This gene modulates survival growth and metabolism through signaling cascades involving AGE-1/ PI3K (Blackwell et al. 2019).

Generally, these genes increase the life expectancy of the worms through signalling pathways and reduction of reactive oxygen species (ROS) to prevent accumulation of oxidative damage and cellular dysfunction with age (Hayyan et al. 2016). The ROS is a product of aerobic respiration that oxidises macromolecules which in turn leads to cell death (Scheibye-Knudsen et al. 2015). Reactive oxygen species will cause lipid peroxidation that interferes fluidity and plasticity of cell membrane as well as disruption of cell integrity (Ristow & Schmeisser 2011). When oxidative damage is not completely repaired or eliminated, accumulation of damaged proteins will further deteriorate functions of aging related cells. These phenotypic changes increase the potential for diseases such as cancer, nerve degeneration, atherosclerosis, osteoporosis and inflammation (Ahmad et al. 2017; Ardeljan & Chan 2013).

MATERIALS AND METHODS

General Methods and Strains

Nematodes were grown on Nematode Growth Medium (NGM) seeded with *Escherichia coli* strain OP50 (*E. coli* OP50) bacteria at 20°C (Brenner 1974). Strains used were WT (N2 bristol), CB1370 *daf-2(e1370), BS3383 pmk-3(ok169)* and *VC345 sgk-1(ok538)*. The N2 strain was obtained from Univeristi Kebangsaan Malaysia (UKM) Medical Molecular Biology Institute (UMBI) and the mutant strains were obtained from *Caenorhabditis Genetics Center* (CGC) (University of Minnesota, USA).

Lifespan Analysis

The lifespan assay was conducted based on previously described studies (Bansal et al. 2015; Lee et al. 2017). Lifespan assays were done in triplicates with 30 worms per replicate. A synchronised population of approximately 30 larvae stage 1 (L1) for each strain was transferred onto new plates with lawn of *E. coli* OP50 supplemented with 5-fluorodeoxyuridine (FuDR). The number of surviving worms were counted daily under a light microscope by gently tapping the worms with a platinum wire. Non-responsive worms were recognised as dead. The following healthspan measures were performed at day 5, 10 and 15 of adult worms.

Locomotion Assay

Locomotion assay was done on solid and liquid media (Bansal et al. 2015). A total of 15 worms were randomly picked and placed onto individual NGM (solid media) plates, where tracks were observed under a light microscope equipped with camera. After 5 minutes, the worms were removed and pictures of the tracks was taken. The average distance travelled was measured and calculated using the software ImageJ (NIH, Bethesda, Maryland, USA). For the liquid media assay, 15 worms were transferred into individual wells of 24-well plates. Each well was added with 1 mL of M9 solution (3 g KH_2PO_4, 6 g Na_2HPO_4, 5 g NaCl, 1 ml 1M $MgSO_4$ in 1 L H_2O). The worms were left to acclimatise for 30 seconds. Then the number of thrashing was counted for 1 minute under a light microscope.

Resistance to Heat Stress

Worms were removed from 20°C and transferred to a 37°C incubator (Dues et al. 2019). Every 2 hours, the worms were tapped with a platinum wire and those that did not respond were scored as dead. The percentage of survival and mean survival was then calculated using the following formula:

$$\frac{\text{number of worms alive}}{\text{number of worms alive and dead}} \times 100\%$$

Assays were done in triplicate with 30 worms per replicate.

Resistance of Oxidative Stress

Worms were removed from a 20°C incubator and transferred into a microcentrifuge tube. 10 mM hydrogen peroxide (H_2O_2) solution was added into the microcentrifuge tube containing

approximately 30 worms and incubated for 2 hours in a dark orbital shaker. The optimal concentration of H_2O_2 was determined based on 50% worm survival after exposing to a range of H_2O_2 concentration (0.25-14 mM) in a similar manner. The tube was centrifuged at 2000 rpm for 1 minute and rinsed to remove excess H_2O_2. The worms were transferred into a plate with NGM and incubated at 20°C. The number of surviving worms were determined daily.

Statistical Analysis

Results were expressed as mean ± S.D on graphs and statistical analysis were generated with GraphPad Prism 7.0 (GraphPad Software Inc., La Jolla, CA, USA). Lifespan, heat and oxidative stress resistance assays were analysed using log rank test. Repeated measures ANOVA and Tukey's post hoc test were used to analyse the locomotion and comparison between two groups to assess its statistical significance. $p<0.01$ was considered statistically significant.

RESULT AND DISCUSSION

Lifespan of *C.elegans*

C. elegans is frequently used as a model organism in aging studies because of its short lifespan which has average span of 2-3 weeks. This characteristic enables observation of aging until the end life of an organism. Results of the study showed that *daf-2* mutants exhibited a significantly longer lifespan compared to the wildtype (Figure 1). *C. elegans* has detection ability which is regulated by the sensory organs (amphids) and a variety of isolated sensory neurons located along the body of the nematode. *C. elegans* feeds on bacteria, which it detects using a pair of sensory cells known as ADF neurons. Once bacteria is detected, *C. elegans* releases serotonin which stimulates the consumption of food by pumping them into the pharynx

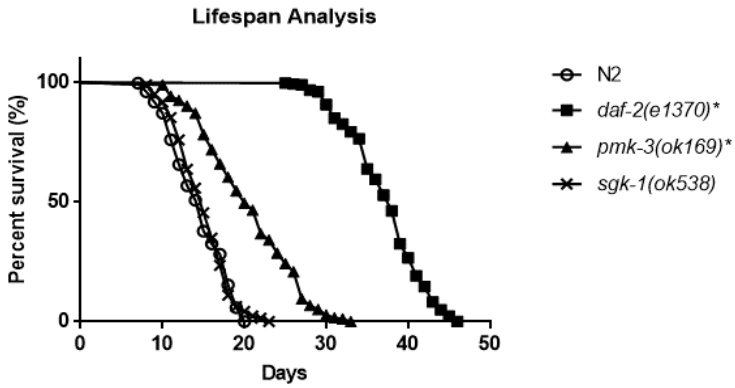

Figure 1: Lifespan assay of *C. elegans* strain (N2), mutant strain CB1370 *daf-2* (e1370), BS3383 *pmk-3 (ok169)* and VC345 *sgk-1 (ok538)*; wild strains (circle point), *daf-2 (e1370)*(square point), *pmk-3 (ok169)*(triangle point) and *sgk-1(ok538)*("X" point). The *daf-2* and *pmk-3* strains had significantly higher mean and maximum lifespan values than that on N2 (control) strain nematides. Data are shown as mean ± SD (N=3), *(p<0.0001)

and transport them to the intestine (Song et al. 2013). The IIS pathway is then activated by the binding of insulin receptor substrate to *daf-2* which is a receptor for IIS. This initiates a series of phosphorylation that in turn deactivates a FOXO transcription factor known as *daf-16* (Altintas et al. 2016). Thus, inhibition or mutation of *daf-2* deters phosphorylation of *daf-16* and promotes translocation of *daf-16* into the nucleus. *Daf-16* is known as a master regulator because it regulates the development, metabolism and stress response towards various environmental stimuli (Van Der Horst & Burgering 2007).

The *sgk-1* genes encode serine–threonine kinase that is homologous to Akt/protein kinase B (PKB). Akt/PKB and SGK modulate lifespan, metabolism, growth and many other processes through signalling pathways (Pearce et al. 2010). Activated *sgk-1* binds to Akt-1 and Akt-2 to form a complex that transduces AGE-1/PI3K signals to phosphorylate DAF-16 (Blackwell et al. 2019). Similar extended lifespans were seen on *daf-2* and *sgk-1* mutants because Akt-1/Akt-2/sgk-1 complex is involved in *daf-2* mediated pathway. Besides that, *sgk-1* is believed to regulate *C. elegans* lifespan by inhibiting the FOXO transcription factor *daf-16,* similar to that resulted by Akt-1 and Akt-2 (Li et al. 2008). However, a study suggested that the interaction between SGK and FOXO transcription factors were more complex than merely subcellular localisation of *daf-16*/FOXO alone (Chen et al. 2013). The activation of *daf-16* is dependent on post-translational modifications and nuclear/cytoplasmic translocations. In addition, nuclear *daf-16* activation also requires co-factors (Li et al. 2008; Lin et al. 2001).

Daf-16 encodes the transcription of protective genes such as antimicrobial, heat shock protein (HSPs), antioxidants and others that act in a collective way to influence longevity and stress response (Altintas et al. 2016; Girard et al. 2007; Murphy et al. 2003). Antioxidant such as *sod-3* gene, which encodes Mn-superoxide dismutase (SOD), is highly expressed in *daf-2* and *sgk-1* mutants than in the wildtype (Honda & Honda 1999; Honda et al. 2010). Superoxide dismutase is an important enzyme that catalyses the removal of O_2, a major ROS that generates many other toxic ROS (Kowaltowski et al. 2009). Heat shock proteins function as molecular chaperones assisting in protein folding and degradation of damaged proteins which are important in maintaining cellular homeostasis (Koga et al. 2011; Murphy et al. 2003). *Daf-2* and *sgk-1* mutants reduces the level of ROS and cellular oxidative damages thus increasing life maintenance ability and lifespan (Barsyte et al. 2001; Braeckman & Vanfleteren 2007; Chiang et al. 2012).

Results showed that mutants of *daf-2* had maximum lifespans of 46 which were significantly longer compared to wildtype worms (Figure 1). In previous studies, *daf-2* mutants had been shown to have longer lifespan compared to wildtype (Chen et al. 2018; Kumar et al. 2016; Zhao et al. 2017). These mutants had been reported to develop higher expression of antimicrobial genes that resulted in lower bacterial proliferation

in the intestine and prolonged lifespan (Hahm et al. 2011; Murphy et al. 2003). The claim was supported by other findings which showed that the death in aging stage might be related to bacteria accumulation and proliferation in the nematode (Youngman et al. 2011).

Previous study had shown that *sgk-1* null mutant had longer lifespan compared to wildtype (Hertweck et al. 2004). In line with previous study, our results showed the maximum lifespan of *sgk-1 (ok538)* mutants was 23 days in average which was slightly higher than wildtype worms but not significant (Figure 1). Recent finding reported that *sgk-1* was needed for longevity in nematodes (Chen et al. 2013). Although *sgk-1* mutants had similar lifespan with wild type in this study, they showed enhanced resistance towards heat and oxidative stress. The *sgk-1* expression is regulated by Target of Rapamycin (TOR) complex-2 (TORC2) (Ruf et al. 2013). Inhibition of TOR signalling, which mimics nutrient and energy deficiency conditions, has been shown to increase the lifespan of *C. elegans* in a similar mechanism to *daf-16* (Uno & Nishida 2016). A previous study indicated that *sgk-1* regulated TORC2 pathway indirectly (Jones et al. 2009). TOR complexes are important for nutrient and energy sensing as well as the release of growth factors and signals that regulates development, reproduction, metabolism, stress responses and aging (Zoncu et al. 2011). Because *sgk*-1 mutants were found to have higher resistance towards heat and oxidative stress in this study, this gene was suggested to deactivate TORC2 pathway that resulted in degeneration and deficiencies in aging of wildtype worms.

C. elegans originally is found in soil and commonly exposes to various threats from bacteria and fungi. Therefore, the nematode has a unique set of immune genes that is activated when exposed to different types of pathogens (Wan et al. 2021). Similar to humans, MAPK in nematodes is important to protect against stress, regulate cell differentiation, apoptosis and autophagy (Chen et al. 2016; Liu & Zhou 2017). *Nsy-1, sek-1 and pmk-1* kinases are part of the p38-MAPK pathway which regulate the expression of immune response genes (Kim et al. 2002; Troemel et al. 2006). The p38-MAPK pathway is highly conserved between humans and *C. elegans* (Cuenda & Rousseau 2007). The homologs that encode p38 MAPKs in *C. elegans* are *pmk-1*, *pmk-2* and *pmk-3.* Although each homolog functions differently in aging, they are regulated by a similar promoter (Shivers et al. 2010). Activation of immune effectors maintain the basal level of immune function for lifespan extension (Irazoqui et al. 2010; Kwon et al. 2016). The p38-MAPK and insulin signalling pathways have been found to be involved in pathogenic response in a similar manner (Mondoux et al. 2010).

Increased expression of *nsy-1*, *sek-1* and *pmk*-1 stimulate SKN-1 to synthesise ROS detoxification enzymes. Overexpression of these genes in *pmk-3* mutants could help to reduce the level of ROS and cellular damages (Liu et al. 2011; Van Der Hoeven et al. 2011). Previously, *pmk-3(ok169)* mutants had been reported

to develop longer lifespans compared to wildtype worms (Rahman et al. 2010; Uno et al. 2013). Since *pmk-3* mutants had higher expression of *pmk-1*, this could possibly contribute to lifespan extension of the mutants. *Pmk-1* controls the immune defence of *C. elegans* for resistance towards pathogens (Kim et al. 2002; Pujol et al. 2008). It activates transcription factor ATF-7 which stimulates the expression of intestinal genes encoding defence proteins such as C-type lectins, lysozymes and antimicrobial peptides (Shivers et al. 2010; Shivers et al. 2008). Similar to the ability of *daf-2* mutants, the ability to minimise bacterial proliferation in the intestine is also seen in *pmk-3* mutants. Loss of *pmk-1* or *sek-1* had been found to inhibit the ability of *daf-2* mutants to resist *Pseudomonas aeruginosa* infection (Troemel et al. 2006). *C. elegans* requires *daf-16* and *pmk-1* proteins for survival because they regulate behavioural and stress responses which enable the nematode to segregate food sources from pathogens (Irazoqui et al. 2008; Schulenburg & Ewbank 2007). All findings indicates that *daf-2/daf-16* insulin-signalling and p38-MAPK pathway were closely related.

Locomotion Assay

C. elegans detects environmental signals by receptors and channel proteins. Sensory organelle which is known as sensory cilium, extends from the dendritic endings of neurons, is essential for chemosensation in *C. elegans* (Inglis et al. 2018; Sassa et al. 2013). Cilia of the amphid channel neurons are found in the middle and a distal segments of *C. elegans* (Avasthi & Marshall 2012). *Dlk-1, sek-1* and *pmk-3* are essential for the formation of axons and synapses as well as the regeneration of axons (Nix et al. 2011; Van Der Vaart et al. 2015). Interestingly, *pmk-3 (ok169)* mutants were found to have significantly better locomotion capacity than wildtype at day 5 and 10 of adulthood but dropped significantly as compared to wildtype at day 15 in both types of media (Figure 2a & 2b). These results indicated that *pmk-3* mutants had better locomotion as they matured to a certain age. This may be due to improved neuron regeneration which is negatively regulated by *pmk-3*. According to previous reports, absence of *pmk-3* resulted in the stimulation of p38-MAPK pathway that enhanced microtubule stabilisation and endocytosis (Cavalli et al. 2001; Tedeschi & Bradke 2013).

Healthspan can be defined by a few measurable physiological changes such as mobility, pharyngeal pumping, and accumulation of lipofuscin (Leiser et al. 2011). Healthspan was determined through multiple assays at different ages of the nematodes in this study to elucidate the changes of capabilities and capacities during aging. Locomotion of *C. elegans* has been used as a measure for neuromuscular function (Pierce-Shimomura et al. 2008). This is because locomotion or movement are mainly due to muscle contraction along the body which differ depending on the viscosity of environment (Izquierdo & Beer 2018). Consistent with previous studies, a significant declined in

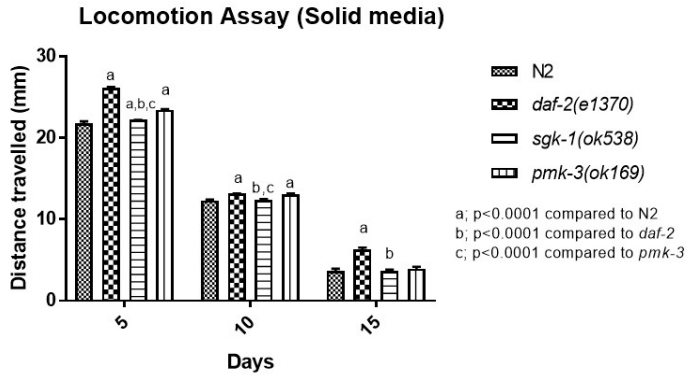

Figure 2a: The mobility capacity of nematodes was measured with the mean of the distances produced on solid media and the significant differences at p<0.0001 between the study group and N2 strain nematodes (control) were labelled. All strains had different travelled distances and showed differences value compared to wild strains. Data were shown as mean ± SD (N=3).

movement ability was noted at day 5, 10 and 15 in all mutant and wildtype worms (Figure 2a & 2b) which showed the locomotion in all worm groups decreased as they aged (Bansal et al. 2015; Podshivalova et al. 2017). This indicated reduced neuromuscular function in aged worms. During aging, the nervous system of nematodes undergo structural regrowth and synaptic deterioration (Toth et al. 2012). As opposed to wildtype nematodes, *daf-2* and *pmk-3* mutants were found to have significantly increased locomotion at early adulthood in the present study. This may be due to the increased expression of protective genes such as *daf-16* and *hsf-1* in the mutants. These neuroprotective genes prevent structural changes during aging by maintaining protein homeostasis (Toth et al. 2012). Endocytosis regulation which is regulated by *dlk-1* and *sek-1,* ensure transportation of the components needed for regeneration of axon and synapse are balance, where an imbalance will cause shortening or unwanted alteration

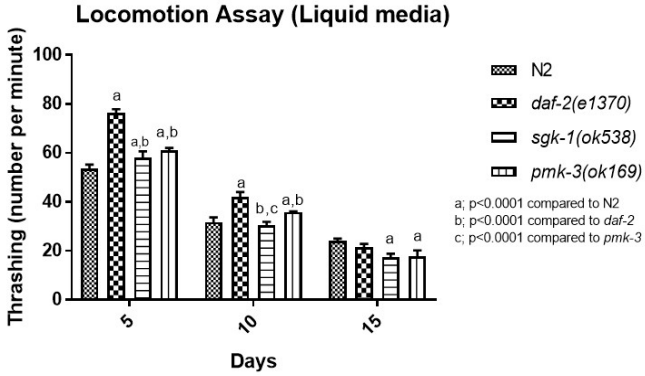

Figure 2b: Number of body bending or thrashing of nematodes in liquid media for 1 minute for *C. elegans* strain (N2). mutant strain CB1370 *daf-2 (e1370),* BS3383 *pmk-3 (ok169)* and VC345 *sgk-1 (ok538).* Data were shown as mean ± SD (N=3).

hence functional decline (Kaplan et al. 2012; Molla-Herman et al. 2010). Abnormalities of aging neurons such as outgrowth formation of dendrites in *C. elegans* could be due to the accumulation of protein aggregates (Cohen et al. 2006). Protein aggregates in dendrites induce cytoskeletal rearrangements, alterations of synapses and neuromuscular deterioration in *C. elegans* (Pan et al. 2011; Tank et al. 2011).

Increased ROS levels during aging also reduce levels of dopamine by nerve cells (Rodriguez et al. 2013). Dopamine plays a role in the sensing of food, bacteria and also locomotion in *C. elegans* (Flames & Hobert 2009; Sawin et al. 2000). Disruption of dopamine homeostasis in *C. elegans* leads to neuron degeneration or neurite outgrowth that correlates with human Parkinson disease (Cao et al. 2010). Mechanistic action of *daf-16* is suggested to contribute to higher locomotion capacity in *daf-2* mutants during aging because *daf-16* stimulates the transcription of antioxidants, such as SOD-3 in the nucleus (Duangjan et al. 2019; Peixoto et al. 2016).

Resistance to Heat Stress

C. elegans daf-2, *pmk-3* and *sgk-1* mutants were found to have higher survival capacity to heat stress during aging (Figure 3). Homeostasis restoration in organisms is a crucial ability for survival and also a healthy life. Different stimuli or stress from external or internal environment will activate transcription of different protective and adaptive genes. Thermal adaptation in nematodes is controlled by a subset of sensory neurons that activate the heat shock response by promoting transcription of heat shock genes in the intestine, muscle and other tissues (Douglas et al. 2015). *C. elegans* has thermosensory neurons that is very sensitive to temperature changes even within 0.01°C (Goodman & Sengupta 2018). However, nematodes which were cultivated at 20°C only responded to temperature changes above 17°C (Clark et al. 2007).

Heat stress resistance mediated longevity in *C. elegans* is suggested to be negatively regulated by *daf-2*, *pmk-3* and *sgk-1* because null mutants of these genes were found to have increased survival capacity after heat stress (Figure 3). When *C. elegans* exposed to heat, activated AFD neurons in these mutants stimulates higher expression of heat shock transcription factor through induction of chaperone molecules. This enables protection against heat stress and extends lifespan of the nematodes (Prahlad et al. 2008). Heat causes protein aggregation and denaturation, DNA and RNA damage, alteration in fluidity and permeability of membranes (Dayalan Naidu et al. 2016). SEK-1 has been reported to promote DAF-16 nuclear translocation that consequently modulates the expression of its downstream genes (Mertenskötter et al. 2013). In addition, activation of DAF-16 together with HSF-1 also upregulates heat shock proteins such as *hsp-12.6* and *sip-1* mRNA expression through p38-MAPK pathway (Sugawara & Sakamoto 2020). Thus, results from heat stress assay in the present study suggested that *daf-2*,

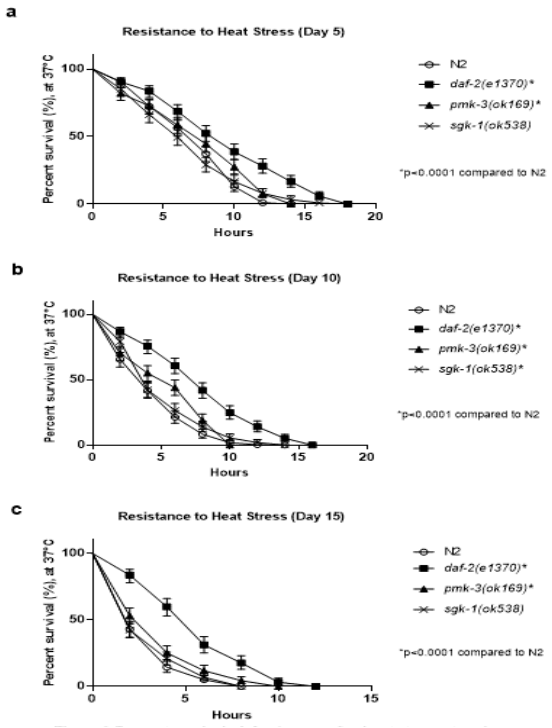

Figure 3: Percent survival of *C. elegans* after heat stress at a. Day 5, b. Day 10 and c. Day 15 of maturity. All strains showed differences compared to N2 (control) strains. Data are shown as mean ± SD (N=3)

pmk-3 and *sgk-1* were involved in the down regulation of heat shock proteins expression.

Resistance of Oxidative Stress

In this study, H_2O_2 was used as an oxidative stress agent to determine the level of stree resistance in worms at different ages. The optimum concentration of H_2O_2 used was determined based on LC50 principle where the concentration that caused 50% mortality in worms was used for further analysis (Figure 4a). Oxidative damage is known to affect proteostasis, lipids and DNA. Although high levels of the $O_2^{\cdot}$ are harmful, long term exposures to low concentrations of these oxidants may prolong lifespan (Yang & Hekimi 2010). In line with this, exposure to low doses of juglone, paraquat or hydrogen peroxide have been found to enhance the levels of glutathione, superoxide dismutase, catalase as well as expressions of *sod-3* and *hsp-16.2* (Heidler et al. 2010). According to Schulz et al. (2007), ROS can promote longevity through stress responses that are mediated by *daf-16*, SIR-2.1 and SKN-1. This phenomenon is known as hormesis or mitohormesis.

Inhibition of *daf-2* reduces glucose uptake and induces a temporary decline in ATP levels that eventually activates AAK-2. This protein then facilitates the production of a brief ROS signal that enhances oxidative phosphorylation

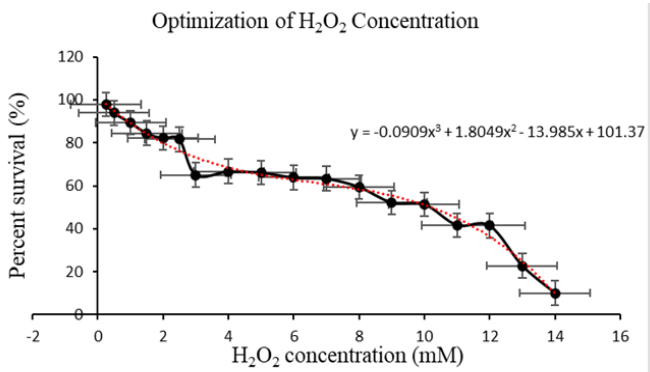

Figure 4a: The optimum H_2O_2 concentration was determined for oxidative stress assay. Wildtype strain nematodes, N2 were exposed at concentration of 0.25 to 14 mM H_2O_2 and perecent survival of *C. elegans* was recorded. The optimum concentration was obtained based on the linear equation. Data were shown as mean ± SD (N=3).

(Ristow & Zarse 2010). Increased ROS levels stimulate adaptive responses that are partially mediated by the Nrf/SKN-1 transcription factor. These responses result in stress resistance and extended lifespan in *daf-2* mutants whereby current study demonstrated similar outcome (Zarse et al. 2012).

Mutants of *sgk-1* may exhibit higher thermotolerance compared to wildtype because of the overexpression of SKN-1 which is an ortholog of the mammalian Nrf protein. Activation of SKN-1 is required for prolongation of life expectancy (Blackwell et al. 2015). SKN-1/Nrf mediates the expression of genes involved in stress resistance, protein homeostasis and metabolism (Deng et al. 2020; Ewald et al. 2015). Lifespan extension resulted from increased expression of SKN-1 had been found to have overlapping functions with daf-16/FOXO (Zhao et al. 2016). SKN-1 that is activated by *pmk-1* translocates into the nucleus and activates *daf-16* (Ding et al. 2017).

Results of this study showed that mutants of *daf-2*, *pmk-3* and *sgk-1* had better oxidative stress resistance at ages of 5, 10 and 15 days old compared to wildtype (Figure 4b). These indicated that the absence of *daf-2, pmk-3* and *sgk-1* genes enhanced expressions of redox-dependent transcription factors that increased survival capacity in oxidative stress condition from early to late adulthood of the worms. Since aging and degenerative diseases in humans are associated with increased oxidative stress, inhibiting the expression of the homolog genes in human IIS, MAPK and PKB pathways may serve as potential therapies in the future. However, *in vivo* studies involving mammalian models are necessary to validate the effect of knockdown of these genes especially on growth and regeneration since the chronological ages of *C. elegans* have yet to be mapped against humans.

CONCLUSION

The knockdown of *daf-2, sgk-1 and pmk-3* genes prolonged lifespan and improved resistance to heat and

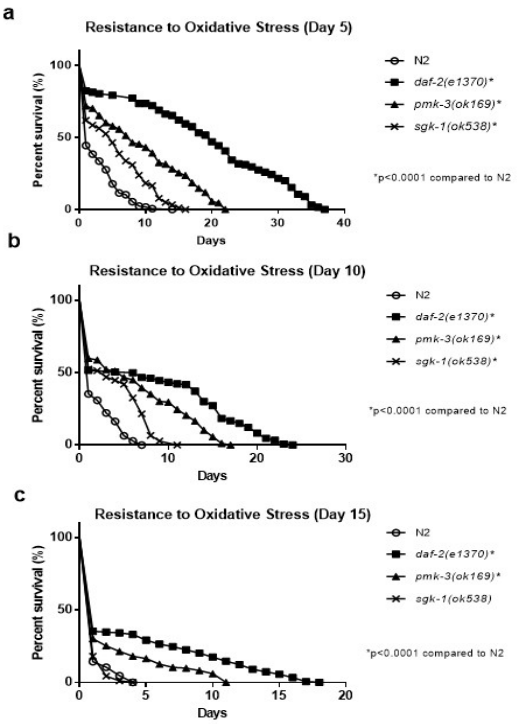

Figure 4b: Percent survival of *C. elegans* after exposure to H_2O_2 at a. Day 5, b. Day 10 and c. Day 15 of maturity. All strains showed differences compared to N2 (control) strains. Data are shown as mean ± SD (N=3)

oxidative stress in aging *C. elegans*. Although movement capacity of mutants was improved at early days of adulthood, this ability declined at later stages of life, similar to that of wildtype worms. These indicated that absence of *daf-2, sgk-1 and pmk-3* caused biological adjustments that resulted in improved stress resistance and to a certain extent of movement ability, which was later compromised by age. To understand the mechanisms involved, proper evaluation of the genes involved in the regulation of neuromuscular activity, heat and stress resistance was necessary. Consistency in molecular changes and exhibition of phenotypes will validate the variations during aging. Since mutant worms had a longer mean lifespan than wildtype worms, future studies should investigate the changes in healthspan beyond 20 days in mutants to determine whether the extended duration of life will be correlated with improvement of functional capacities.

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