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Current Measures of Healthspan in Aging

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Review Article

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Abstract

Progressive physiological decline in function occurs as age advances, which increases vulnerability to diseases and subsequently leads to disabilities in aging process. Modulation of genes and pathways has been reported to promote longevity in various animal model and its findings has been used in efforts to help maximize lifespan of human. Lifespan defines the number of survival days of an organism before death while healthspan is the period where an organism can function optimally and live free from diseases until the end of life. However, it remains unclear whether its benefit in prolonging lifespan also helps to improve healthspan since extended lifespan could also indicate a longer frailty period. The purpose of this review is to discuss healthspan assays used in various aging models. Healthspan analysis were found to be different depending on the advancement of biological functions. Domains of health commonly measured were accumulation of metabolites, physiological function and capacities. These measures generally declined with age. Interventions and treatments that promoted lifespan in different aging models improved the health measures although comparative analysis between treatment and control groups were limited to the normal lifespan of animals. Further determination of health measures focused on the extended period of life as a result of interventions and treatments in aging models would provide a better picture of their mechanistic action and validate the reported findings.

Keywords: Aging; Healthspan; Lifespan; Bone; Strength; Muscle; Cognitive; Memory

Abbreviations: AD: Alzheimer's Disease; PD: Parkinson's Disease; MOCA: Montreal Cognitive Assessment; MMSE: Mini-Mental State Exam; CT: Computed Tomography; MRI: Magnetic Resonance Imaging; DEXA: Dual-Energy X-Ray Absorptiometry; BMD: Bone Mineral Density; CRP: C-Reactive Protein; CSF: Cerebrospinal Fluid; LDI: Low Density Lipoproteins; LRP: Lipoprotein Receptor-Related Protein; OFBT: Open Field Behavioural Test; ORT: Object Recognition Task; LTT: Lymphocyte Transformation Test; PMN: Polymorph Nuclear; CNS: Central Nervous System; HFD: High Fat Diet; Ach: Acetylcholine; CSF: Cerebrospinal Fluid; AMMSCs: Amniotic Membrane-Derived Mesenchymal Stem Cells; ADMSCs: Adipose Tissue-Derived Mesenchymal Stem Cells; CHAT: Choline Acetyltransferase; DHE: Dihydroethidium Fluorescent Dye; SOCS: Suppressor Of Cytokine Signaling; IRS: Insulin Receptor Substrate.

Introduction

Aging is often associated with several forms of dysfunctions either cardiovascular, musculoskeletal or neurological which can lead to death. Degenerative diseases are associated with function and structure impairment of tissues and organs over time. Examples of degenerative

diseases include Alzheimer's disease (AD), Parkinson's disease (PD), atherosclerosis, osteoporosis, cardiovascular disease, and hypertension [1]. These health problems result in financial drawbacks of the health and social protection systems [2].

In the past century, studies of interventions in aging were focused on lifespan as the main indicator of success. Although people are living longer, later phases of age are often accompanied by age-related diseases [3]. Therefore, healthy aging is the chief concern of many as we strive towards enhancement in quality of life. Healthy aging in this context involves the process of evolving and sustaining functional ability that permits well-being in older age [4].

Elderlies with physical, social and mental capacities are able to be an active part of the society besides living an independent life. With this in mind, healthspan has been the cornerstone of aging studies in recent years. There are several determinants of health in aging. These factors include health-care accessibility, environment, employment, lifestyle and the use of medications [5,6]. The collective effect of these factors results in the general wellbeing of people at late adulthood.

Animal models are preferable in documenting changes with age because human research is complicated by social, biological and environmental diversities [7]. Aging models have genomic and transcriptomic data that are easily accessible besides their versatility to experimental manipulation [8]. Common interventions used in a research include genetic, nutritional or environmental manipulations [9]. Short-lived organisms such as yeasts (*Saccharomyces cerevisiae*), worms (*Caenorhabditis elegans*), flies *(Drosophila melanogaster*), rat, mice and rabbit are suitable aging models because they have genes and signalling pathways regulating lifespan that are highly conserved in different species [10-13].

Improvements in all aspects of health are rarely seen in anti-aging studies because functional decline in aging reduces the ability to respond and recover. Most reported studies are able to measure improvements in one or several measures of function with intervention or treatment [14]. A variety of healthspan assays will contribute to a more comprehensive evaluation of the effects of therapies in anti-aging studies.

Measures of Healthspan in Human Research

Cognitive Function

Cognitive capacity has often been measured to elucidate the mental health of humans during aging. Cognitive test such as Montreal Cognitive Assessment (MoCA), Mini-Mental State Exam (MMSE) and Mini-Cog are frequently performed to detect initial cognitive impairment [15,16]. These tests evaluate mental functions through a series of questions or execution of simple tasks. MoCA determines the ability to memorize a short list of words, identify pictures of an animal and replicate pictures of a shape or object. MMSE involves naming the current date, counting backward and identifying common objects while Mini-Cog test includes recollection of a list of objects and drawing the clock [17,18].

Score range (based on time spent on questions) of the tests are used to determine the severity of cognitive impairments. However, these tests are not sufficient to make diagnoses without a comprehensive clinical evaluation such as computed tomography (CT) and magnetic resonance imaging (MRI) for brain imaging [19]. In a study involving adults aged between 20 to 87 years with median 61 years that attended and had consecutive referrals to cognitive function clinic, the study showed MoCA was rapid and easy to execute and resulted a sensitive detection of mild cognitive impairments among patients [20]. However, an extended evaluation is much needed for confirmation since MoCA showed to resulted a sensitive outcome of scores but less specific, which might give a false positive result. Also, combination of tests showed to have no effects or improvements of detection sensitivity or specificity but the tests are decent for screening purpose [20].

Musculoskeletal and Body Composition

To determine bone health, dual-energy x-ray absorptiometry (DEXA) is commonly used to measure bone mineral density (BMD). In a previous study, strength training was reported to increase BMD of elderly men of 68±6 years. Strength training regulated bone formation by decreasing the expression of sclerostin and secretion which inhibits bone formation. Increased in serum insulin growth factor-1 (IGF-1) also helped to maintained bone mass and skeletal growth. Interestingly, the study also found that training-induced surge of total lean body mass and muscle thickness was significantly lower with vitamin C and E supplementation in elderly men [21]. This could be due to the higher leptin levels in control group compared to supplemented group after the training period. Hormones produced by the adipocytes such as leptin are involved in the regulation of fat storage and calories burned. Leptin regulates energy balance by controlling food intake and energy outflow. When fat mass is reduced, leptin levels is low, appetite will be increased to enhanced food uptake until fat mass is restored [22,23]. Hence, the higher increase in lean mass was observed in the control group. Leptin administration has shown to have beneficial skeletal effects. However, leptin concentrations

in humans are inconsistent and contradictory, thus it is still uncertain of its mechanism [24].

Inflammatory Biomarkers

C-reactive protein (CRP) is an acute inflammatory protein in which its level rises in response to infection or inflammation. CRP is a protein synthesized by the liver, smooth muscle cells, macrophages, endothelial cells, lymphocytes and adipocytes. Studies have shown that CRP expression could potentially be induced by interleukin-6 (IL-6) with help from IL-1 [25]. Inflammation and host responses to infection mediated by CRP involves apoptosis, phagocytosis, release of nitric oxide and cytokine production [26]. Myostatin (MSTN) or known as growth and differentiation factor-8, is a protein produced in the muscle tissues. It is a negative regulator of the skeletal muscle growth where inactivation can induce skeletal muscle hypertrophy and overexpression will causes muscle atrophy [27,28]. Follistatin-like 1 (FSTL1) is a glycoprotein secreted from different tissues such as skeletal muscle and liver that represent expression changes in the regulation of muscle mass where FSTL1 would bind and neutralize MSTN prevents the atrophic action of MSTN [29]. study found that MSTN concentration significantly decreased while FST concentration increased significantly in the group of resistance training. This indicate that changes of growth factor profiles at baseline have an important role in quadriceps hypertrophy in elderly men which shows that lower MSTN expression and higher FST levels after exercise is positive for muscle growth [30].

Cognition Biomarkers

Blood-based biomarkers for predictive diagnosis of cognitive impairments and its progression that are frequently used are accumulation of metals (Cu, Al, Zn, Pb, Mn, Cad), total-tau and amyloid levels [31,32]. Study done by Iqbal, et al. 2018 show that higher levels of Al, Cu and Pb were found in cognitively impaired patients [33]. Cu is required for normal brain function however, dysregulation of Cu metabolism during aging indirectly contributes to oxidative stress/damage [34]. Tau proteins play important role in stabilizing microtubules. It is found in the central nervous system (CNS) and is mainly active in the distal portion of axons. Higher levels of total-tau and phosphorylated tau in the cerebrospinal fluid (CSF) indicate a possibility of cognitive dysfunction and progression of dementias [35]. Amyloid-beta is one of several breakdown products of a larger protein called amyloid precursor protein. Its accumulation between neurons in the form of plaques disrupts cell functioning. Amyloid-beta plaques were identified as a histopathological hallmark of AD. Increased amyloid levels seem to indicate early AD [36].

Healthspan in Rabbit Models

Strains and Age Selections

Rabbits have several strains that were used for specific research purposes comprise of the Californian white, Dutch-belted, New Zealand red, New Zealand white and Polish [37]. *Oryctolagus cuniculus* (also known as New Zealand white strain) comes from Leporidae family is one of the mostly used model in research because it is the most easy to locate surface veins and arteries for blood collection [38]. Rabbits are frequently used in research involving reproduction, aging and development because of ease of handling. Rabbits' short lifespan also makes them valuable for studies in reproduction, aging and development. However, the biological age equivalence of rabbits to humans varies across different strains. For instance, Sengupta and Dutta (2020) reported that one human year is equivalent to 56.77 days for New Zealand White (NZW) and New Zealand Red (NZR) rabbits, 71.01 days for Dutch belted and Polish rabbits and 85.28 days for Californian rabbits [39]. Hence, researchers tend to use ranges of age of rabbits to represent the biological aging of humans. Wu et al. (1999) used rabbits between 5-7 months old to represent young adult, 29-31 months as middle-aged, 35-37 months as upper middle-aged and 58-62.5 months as aged groups in their research [40]. Meanwhile Bonomo, et al. (2000) used 36-40 months old rabbits as an aged group and 3-6 months as a young group [41]. In another study, 70 days old rabbits were considered to be equivalent to pre-adolescent humans [42].

Transgenic rabbits generated by pronuclear microinjection of transgenic DNA into fertilized embryos provided opportunities to explore gene functions associated with human disease through genetic modification to suits each research purpose [43]. Information on rabbit transcriptome and genome information can be accessed on NCBI database or http://www.picb.ac.cn/RabGTD/ that was established by the Chinese Academy of Sciences in Shanghai [44]. For example, Song et al (2013) has generated two knockout (KO) rabbits using TALEN technology. They are able to produce an immune deficient KO rabbit with deficiency of Rag1 and Rag2 genes which RAG-deficient rabbits would be without mature B and T cells. These KO rabbits would be valuable for studies of allografts, xenografts, tumours, vaccine development and infectious diseases [45].

Most recent technology used to produce transgenic strains of rabbits is by using CRISPR-Cas9 technology. ApoE KO rabbits were produced at University of Michigan using CRISPR-Cas9 [46]. ApoE is a ligand for both low density lipoproteins (LDL) receptor and low-density lipoprotein receptor-related protein (LRP) that is important

for catabolism of remnant lipoproteins in the liver and genetic deficiency of apoE is a cause of human type III hyperlipoproteinemia. This makes the apoE KO rabbit as potential model for human hyperlipidemia because apoE KO rabbits fed with cholesterol diet shown to develop more prominent hypercholesterolemia than wildtype rabbits, which caused by the remarkable accumulation of intestinally-derived remnant lipoproteins, β-VLDLs [47].

Physical, Cognition and Biomarkers Measures

Physiological and behavioral changes that are usually assessed in rabbits are heart rate, arterial blood pressure, serum corticosterone concentrations, facial expression and home pen behaviours. Observation of facial expression changes were done to develop the Rabbit Grimace Scale in order to evaluate acute pain by observing five facial action units (FAUs); orbital tightening, cheek flattening, nose shape, whisker position and ear position [48].

Open Field behavioural Test (OFBT) and Object Recognition Task (ORT) are also among the assays done in rabbit research [49]. OFBT is an experimental test used to evaluate general locomotor activity levels, anxiety and willingness to explore in animals. ORT is a frequently used behavioral assay for the study of various aspects of learning, cognition and recognition memory in rabbits or rodents based on spontaneous tendency to spend more time exploring a novel object over a familiar one. Reduced exploratory behavior indicates poor memory and learning in rabbit [50]. There is also dark-light box test which is used to test the unconditioned anxiety response of animals. It is based on the innate light dislike and the spontaneous exploratory behavior of animals in response to mild stressors; the novel environment and the light/open space [51]. Tests such as Comet assay for observation of DNA fragment migration patterns using fluorescent microscope, applied Lymphocyte Transformation Test (LTT), Polymorph nuclear (PMN) cells and ELISA (IL-6, TNF-α and IL-12) were also done through blood samples [52].

Healthspan in Rat and Mice Models

Strains and Age Selections

Rodents such as rat and mice are frequently used as animal models in aging research due to their similarity in terms of anatomical, physiological and genetic to humans. Advantages of rodents include their small sizes, ease of maintenance, short life cycles and abundance of genetic resources [53].

Details such as age, sex, diet, randomizations and strain used varied depending on the objective of each study [54]. Rodents aged between 8 to 12 weeks are often used to represent the young (20 to 30 years old in human age) while 18 to 24 months-old are considered as old which is equivalent to human age of 56 to 69 years old [55]. Nonetheless, mice and humans have different lifespan and does not linearly correlate to be compare accurately however, there is consistency in disease pathogenesis making a possible implementation between both in research [56]. Age group is the most important factor in aging studies as the presence of senescence need to be accurate to ensure proper assessment of physiological and biological biomarkers [57]. Studies showed that the muscle mass, fiber size, fiber number, fiber type distribution and muscle specific tension in rodents differ between species as well as within species, but qualitatively show the same pattern as that observed in human [58,59].

Advantages of Rodents as an Aging Model

The physiological changes in rodents are easily monitored and consistent with humans. Rodents' physiological systems in learning and memory have been so extensively studied. Rats are more intelligent than mouse and are capable of learning a wide variety of tasks that are important for cognitive research [60]. Rats are the best model for breast cancer research because they are anatomically superior to mice and they are responsive to hormones such that their histopathology and premalignant stages closely resemble the human disease [61,62]. Rats are the primary model for mechanistic studies of human reproduction. In models of diabetes, the rat model behaves more like the human disease in important ways, including the ability of environmental agents (toxins, stress, diet and vaccination) to modify the disease [63]. Large sizes of veins in rats also eases serial blood draws for pharmacology research [64].

Rats and mice have been the foremost used model in biomedical research aside from zebrafish, fruit flies and roundworms. Rats are also much easier to handle than mice and less easily stressed by human contact. In fact, repeated handling of rats prior to a behavioural experiment does not induce stress in the animal as opposed to mice [65,66]. Differences between mice and rats could impact neuroscience research involving social, addictive, impulsive and cognitive behaviours. The greater size of rats provides a number of practical advantages, especially in relation to surgical procedures and in studies of spinal cord injury. Rats are also more suitable to be used in studies that involve implantation, to allow drug self-administration [67,68]. Drug administration implemented into a specific brain region of rats, allows observation of the specific brain region regulating behavioural phenotype. The larger size of the rat brain not only makes surgery easier, but damages caused by cannula insertion tend to cause less damage, thus increasing the sensitivity of the treatment [69].

Mice, on the other hand, is smaller in size and has advantages for certain techniques, such as in optogenetics. This method is used for precise stimulation or inhibition of neuronal pathways in freely moving animals, and is based on the transfection of a specific set of neurons with specific light-sensitive proteins that can subsequently be activated by illumination. The smaller brain of mice makes it easier for light to pass through and reach deeper brain regions therefore optogenetics is commonly used in mice [70].

Different genes that are expressed in mouse and rat brains were investigated using the Sprague Dawley rat and C57BL/6 mice [71]. Microarray-based analysis showed that 4713 out of 10,833 genes were differentially expressed in the dendrites of hippocampal neurons between rats and mice. Only 54 genes were differentially expressed between two different but often used, mouse strains (C57BL/6 and Balb/c). Since hippocampus is very important in behavior (especially memory) evaluation, this finding is relevant in understanding the species differences in many cognitive tests. Hence, the nervous and endocrine systems of rats and mice tend to show different responses in studies of human diseases, particularly behavioral and neuropsychiatric disorders [72].

Mice and rats also have different sensitivity to the effects of toxic and medicinal substances because substance absorption efficiency differs within animal models used. Therefore, dosage of treatment used in mice model should differ from that used in the rat model [73]. Rats are more sensitive to many substances, and they need a lower dose to get a significant biological effect than mice [74]. The differences in drug metabolism between mice and rats have been attributed to the variances in expression of cytochrome isoforms which plays a critical role in pharmacokinetic properties of a compound [75,76].

Neuromuscular Function

Besides determination of the effect of treatments on lifespan, evaluation of functional capacities enables overall evaluation of its efficacy. Examples of parameters that are most widely used in behavioural neuroscience for evaluating psychological processes and neural mechanisms of spatial learning and memory are the Morris water maze, open field test, rotarod test and Y-maze test. Morris water maze test relies on distal cues to navigate from a start location around the perimeter of an open swimming arena to a submerged escape platform. This measure has been widely used to determine neuroprotective effects of treatments in rat models [77,78].

Open field and Y-maze test have mutual purposes which is to evaluate exploration habits in rodents, hence, mostly used in observation related with central nervous system (CNS) disorders. Open field maze consists of a wall-enclosed area built to prevent escape that are usually circular, rectangular or square in shape with an area large enough for the model to move around in open space while Y-maze is similar to that except that it is in a Y-shape maze. Distance moved, rearing and time-spent in zones within the maze reflects on the willingness of exploratory behaviour in the model [79]. Short term application of high fat diet (HFD) mice shows less anxiety-like behaviour while in the Y-maze which suggested HFD could enhance working memory and concurrently reduce anxiety-like behaviour [80]. However, the underlying mechanism involved is still unclear and should be explored in the future.

The rotarod test is widely used to evaluate the motor coordination and cerebellar dysfunction in rodents. This test consists of a horizontal rotating rod and observation time of the animal to remain upright and not fall off as speed are accelerated. The rotarod provides an easy way to test the effects of drugs, brain damage or diseases on motor coordination or fatigue resistance in rodents. The test successfully showed that 24-month old mice have shorter latency time as compared to 3-month old mice which suggest reduction in neuromuscular coordination with age [81]. In the same study, treatment with icariin was found to improve the motor skills of aged mice besides enhancement of bone density, biomechanical properties and bone microstructure [81]. This treatment has previously been reported to extend healthspan by increasing antioxidant properties such as SOD activities and reducing oxidative stress [82].

Learning and memory functions of AD model have been shown to increase when transplanted with human amniotic membrane-derived mesenchymal stem cells (AMMSCs) or adipose tissue-derived mesenchymal stem cells (ADMSCs). The production of choline acetyltransferase (ChAT) protein and acetylcholine (ACh) in cerebrospinal fluid (CSF) were increased in rats after AMMSCs or ADMSCs transplantation [83,84]. ChAT is an establish marker of motor neurons [85]. Repeated transplantation of AMMSCs or ADMSCs markedly recovered the decreased levels of microtubule-associated protein 2 (MAP2) as well as cholinergic and dopaminergic nervous system markers in aged rats which indicate structural restoration of brain integrity. Evaluation with rotarod test showed improvements of cognitive and motor functions after stem cell transplantation because of increased ACh levels in the brain and muscles besides restoration of aged rat cholinergic neurons [86].

Muscle strength of rodents has also been measured to determine the effect of age on muscle function and motor coordination. Bitto et al. (2016) used forelimb grip strength and rotarod performance to investigate delaying or reverse

effects in cognitive decline after treated with rapamycin [87]. Muscle strength relates to the movement ability and also contributes to reduce risk of injuries in elderlies due to lose of stability and flexibility. This is because muscle strength and bone strength are equally related. Study show myokines are released during muscle contraction are beneficial for bone where a condition culture myotube show to stimulate preosteoblast viability and migration hence enhanced bone formation [88].

Oxidative Stress

Oxidative stress properties in rodents have been measured using dihydroethidium fluorescent dye (DHE) where fluorescence intensity of stained liver and pancreatic tissues were observed. Increased superoxide anions in pancreatic, hepatic tissue and mesenteric artery of short-term HFD rats exhibited development of oxidative stress combined with metabolic syndrome [89,90]. Metabolic syndrome is a combination of several metabolic abnormalities such as impaired glucose tolerance or insulin resistance, impaired blood pressure, dyslipidemia (increase in triglycerides and high-density lipoprotein cholesterol), cardiovascular alterations and central obesity [91].

Reduced insulin/IGF-1 levels and NF-E2–related factor 2 (Nrf2) corelates with each other in terms of expressed similar antioxidants genes through evolutionarily conserved mechanisms to extend lifespan by reducing oxidative stress, increasing oxidative stress resistance and preventing age associated diseases. Nrf2 signalling is activated when insulin signalling is low and functions parallel with each other. A study found that peripubertal growth hormone (GH) treatment in Lewis dwarf rats increased cellular resistance to diverse oxidative stressors (including H2O2, rotenone, paraquat, and LPS) because expression of antioxidant genes regulated by the transcription factor Nrf2, a key regulator of the adaptive antioxidant defence response, was decreased [92,93]. In line with this, the expression of Nrf2 and Nrf2 target genes were found to be increased in parenchymal tissues of Snell dwarf mice treated with arsenite, an inducer of Nrf2 activity [94].

Regulation of GH and IGF-1 are interrelated in terms of insulin sensitivity. Which then affects process such as lipid metabolism and energy homeostasis. GH is secreted, synthesized, and stored by the anterior pituitary gland. The GH receptor (GHR) belongs to the type I cytokine receptor family. Similar to insulin, GH signaling also involves cascades of tyrosine-phosphorylation of multiple intracellular proteins [95]. GH have same feedback loop as IGF-1 in terms of maintaining hormone balance where in reduced condition such as hypopituitary and hypophysectomized (removal of the pituitary gland) subjects, GH is able to stimulate glucose and amino acid transport, hence induced insulin secretion, lipolysis, and gluconeogenesis [96]. GH induced the phosphorylation of the insulin receptor substrate (IRS), and phosphorylation of STAT-family transcription factors that translocate into the nucleus and activate the transcription of suppressor of cytokine signaling (SOCS) proteins. However, the insulin-like effects of GH also have a negative feedback loop. When SOCS-3 is increased, GH will inhibit GH-induced GHR/JAK2/IRS-1-IRS-2 phosphorylation that will block GH secretion. Activation of STAT proteins can lead to diabetogenic effects of GH through the Janus kinase (JAK-2) but the IRS proteins will not be phosphorylated or induce insulin-like effects [97]. Hence shown that there is cross interaction of receptor for pathways activation between GH and IGF-1. Nevertheless, information regarding its mechanisms and contribution to increased longevity, therapeutic potential, and perils in humans are yet to be unraveled.

Research Limitations

The efficacy of interventions and treatments are often measured on physiological systems and biological markers of rats and mice to represent healthspan during aging. The effects of treatments were determined by comparing changes in neuromuscular function, cognition and blood biochemistry between treated and non-treated animals. These animals were comparable because they were housed and maintained in control environments. Response to treatments was measurable between 4 to 8 weeks because the physiology and biology of rodents changes rapidly. However, the long-term effects of treatments were mostly unknown because the lifespan of rodents was not measured.

Healthspan in Drosophila

Genus of Drosophila particularly fruit fly or *Drosophila melanogaster*, is also one of the most commonly used model organisms for biological researches. Aside from its low cost and rapid generation time, excellent genetic tools have made the fly crucial for basic research. Drosophila has been used as a model organism for studies involving human disease, dissection of cellular morphogenesis, behaviour and aging [98]. This fruit fly has also been used as a model for more complex studies such as development of cognitive behavior and memory as well as wound repair and infection prevention [99,100].

Drosophila has been used to investigate evolutionary conservation between genders. Its genome is 60% homologous to that of humans. In addition, more than one homolog of the genes responsible for human diseases are present in Drosophila [101]. Previous studies have

measured the nervous system, neuromuscular junction electrophysiology, giant-fiber-system recordings, axonal degeneration and regeneration, cardiovascular function, body composition and nephrocyte function of the fly [101,102].

Advantages of Drosophila as an Aging Model

Main advantages of this animal model are its short life cycle and minimal culturing requirements. An embryo emerges within 24 hours of egg fertilization. The embryo then goes through three different larval stages that eventually matures into an adult Drosophila. Development of an adult fly only takes 10 days from fertilization. The female fly can produce up to 1500 eggs in its lifetime thereby providing a constant supply of new Drosophila for genetic studies. Drosophila genes can be mapped easily to investigate genetic transmission. The entire genome of Drosophila has been sequenced and 60% of its gene is homologous to the human genome [103].

Drosophila has anatomical features (such as wings and eyes) which allow for easy characterization. These genetic markers can be easily identified under a microscope. Behaviours such as eating, mating and sleeping that are observed in humans are also seen in Drosophila. Therefore, the possible effect of genetics upon human behaviour can also be assessed. However, the anatomy of the brain and other major organs within the fly are very different from humans [104].

The nervous system in Drosophila is required for sensing and processing information related to vision, hearing, olfaction, proprioception and taste. Similar to humans, this information is conveyed to the central nervous system and processed to provide a motor output. Drosophila and humans share numerous conserved genetic, cellular, electrophysiological and chemical properties. As in vertebrates, many different types of neurons are required to process information in fruit flies. For example, in the visual system, about 115 different types of neurons have been identified in Drosophila. It is very similar number to what has been estimated in vertebrates hence it has been used as model in research related to human neurological disease [105]. Drosophila nervous system permits thorough valuation of the function of genes and neuronal networks. Many different assays have been developed to assess neuronal function which include hearing, flight, learning and memory, diurnal rhythmicity assays and behavioural assays [106,107]. Hence this animal model is suitable for the determination of neurological dysfunction, including neurodegeneration, epilepsy, dementias, stroke, traumatic brain injury and brain tumours.

Neurological Function

Different assays have been used to study neurological disorders in Drosophila. These assays help to gain different perceptions into pathogenic mechanisms and might help to provide new therapeutic strategies for different neurological diseases [108]. Identification of required genes for axonal degeneration and regeneration and its regulatory processes that are involved in spinal cord and nerve injuries can be gain through the wing injury assay. Mechanosensory and chemosensory neurons reside in the wing margin of the fly and project their axons toward the thoracic ganglion. Severing these axons using scissors or lasers causes degeneration of the distal portions of the axons [108]. After about 7 days, the proximal portion of the injured axons regenerates by extending sprouts toward the lesion site where the axons regrow by invading veins of another wing vein [109]. These processes can be visualized by expressing a cytoplasmic green fluorescent protein (GFP) marker under the control of a neuronal GAL4 driver.

Cardiovascular System

Cardiovascular assays have also been used to measure heart development and function of the fly [110]. The Drosophila heart, called the dorsal vessel, differs from the human heart in that it is an open circulatory system consisting of a hollow, muscular tube closed at the posterior end. The vessel runs longitudinally from the posterior abdomen (heart proper) into the thorax (aorta). Similar to the human heart, which consists of distinct chambers, the fly heart is also divided into four chambers that are separated by small valve-like openings through which blood, or rather the analogous fluid in insects, hemolymph, enters the heart [107]. Each chamber consists of six myocardial cells to facilitate the flow of hemolymph through the dorsal vessel. The aorta, which is made up of myocardial cells that do not contract very much, is a tube that facilitates the transport of hemolymph to the head, from where it flows into the body cavity [111]. Through this assay, information applicable to human heart development and physiology can be obtained.

Other than that, heartbeat measurement of Drosophila is an important assay to determine heart function. Measurements of the heartbeat, either in the dissected dorsal vessel of the larva or the adult abdomen, are based on visual recordings. These methods rely on the optical intensity of light passing through the heart while it beats [112]. Similar to mammals, the fly heartbeat consists of a cardiac cycle that includes diastolic and systolic periods. Interestingly, the cardiac cycle in adult flies is composed of alternating anterograde and retrograde beats, leading to a periodic change in the flow of hemolymph. As these animals age,

the contraction becomes arrhythmic, analogous to cardiac arrhythmias observed in elderly humans [113].

Optical coherence tomography (OCT), which is comparable to echocardiography in humans allows non-invasive characterization of the Drosophila's heartbeat [113]. OCT uses laser beams to scan the entire tissue and subsequently uses the scattered light to produce subsurface images. However, this method is relatively slow and inconvenient to measure heart rhythmicity [114].

Neuromuscular Function

The neuromuscular junction (NMJ) is the connection (synapse) between motor neuron and muscle. Disorders of the NMJ span can be affected by genetic or environmental factors [115]. The Drosophila NMJ is used as reference to study neuromuscular diseases because it allows detailed analyses of structural connections between the neuron and muscle as well as their electrophysiological properties. The fly larval NMJ consists of arrays of overlapping striated muscle fibers that are innervated by motor neurons that form synaptic boutons [116]. NMJ of the fly is a large glutamatergic synapse that is easy to access, thereby permitting a detailed characterization of the properties of synaptic transmission, including assessment of excitatory junctional potentials (EJPs), spontaneous miniature EJPs, synaptic plasticity, transmission electron microscopy imaging and pre- versus postsynaptic phenotypic analysis. Since NMJs of the fly are glutamatergic, comparative analysis to mammalian CNS synapses can provide insights on pathogenic mechanisms associated with neurological diseases, such as amyotrophic lateral sclerosis (ALS), spinal muscular atrophy and encephalopathies [117,118].

Giant-fiber-system (GFS) recordings are a useful tool to study synaptic development and transmission over time. Gradual changes in neuronal function along aging can be observed through this assay and particularly beneficial for the study of neurodegenerative disorders. When flies detect a change in luminescence, the giant-fiber neurons in the brain signal to the thoracic ganglion to activate flight muscles [dorsal longitudinal muscles (DLMs)] and jump muscles [tergotrochanteral muscles (TTMs)]. Electrophysiological recordings of the GFS can be made by stimulating the eyes while recording the depolarization in DLMs and TTMs [119]. The GFS assay has been widely used to study features of epilepsy and seizures by high-frequency stimulation in the eye and detection of seizure-like electrical activity in the muscles [120]. Lower insulin-growth-factor signalling (IIS) and proteasomal activity helps in reducing decline in neuronal function of aging Drosophila. Aging caused decreased of gap junction proteins in flies thus reducing the speed of neuronal transmission. In aging Drosophila, impairments of the neural transmission caused decline in behaviour such as flight and visual in flies. Speed of signal transmission measured through the GFS by recording response latencies from the downstream muscles (TTM and DLM) shows increased in response latency which indicates slower transmission and reduced circuit function in old flies. Augustin, et al. results shows that mutants with overexpression of Rpn1 (a component of the proteasomal regulatory subunit) and reduced IIS shows reduction in age-related functional decline of aging flies by suppressing the accumulation of damaged and misfolded proteins in the fly brain, by promoting proteasomal activity and increased antioxidant capacity in aging flies [121].

Lipid Accumulation

Lipid-droplet accumulation has been used to asses storage of fat and oenocyte function in flies in response to differential nutritional conditions. Dyes such as Oil Red-O or BODIPY were used to observed lipids in oenocytes and fat accumulation in the body of flies [122]. Lipid-droplet presence have distinct effects depending on the accumulation location. It is suggested that maybe phytochemicals acted through the hormetic mechanism and activation of adaptive stress response signalling pathways such as Nrf2/Keap1 and Sirtuin-FOXO. Study done involving flavonoids treatment show lipid-droplet accumulation in adipocytes is reduced when treated with flavonoids (xanthohumol and isoquercetin) but increased in muscles of treated flies. Increased lipid-droplet in adipocytes caused pathological malfunction while an increase in nervous system and muscles gives a protective effect [123]. However, mechanism of how flavonoids improve health needed further studies.

Research Limitations

Collectively, Drosophila provides prevailing insights on functional changes during aging and diseases in humans. Phenotypic and functional measures that have been established can be used to validate the effectiveness of treatment or therapy on health besides longevity. However, future studies should embark on comparing the functional changes from young until the end of life of Drosophila to validate the long-term effects of treatments to healthspan.

Healthspan in *C. elegans*

Caenorhabditis elegans or also known as *C. elegans* has unique characteristics that are distinctive compared to other organisms. *C. elegans* is a small -sized nematode (~ 1 millimeter long and 50μm wide when adult) that can be found in soil and lives with bacteria as its food source. *C. elegans* exists in two sexes namely male and hermaphrodite. Hermaphrodites can produce male and female gametes

which in turn reproduce through self -fertilization while male nematodes need hermaphrodites for fertilization [124]. About one in a thousand nematodes are male while the rest are hermaphrodites [125].

Advantages of *C. elegans* as an Aging Model

In addition to its small size, the nematode is easily handled and reproduced in large sample sizes over a short period of time. The short average lifespan of the nematode allows rapid data collection at different stages of life. These nematodes have a translucent body which allows observations involving internal activities to be made [126]. Other unique characteristic of *C. elegans* is that the hermaphrodites have 302 nerve cells while the male nematodes have 391 nerve cells where it is considered as a simpler nervous system compared to other animal models. Hence, genetic screening to identify genes required for specific neuron functions and behaviours is more possible and makes *C. elegans* a prime model organism for neuronal studies [127]. These features of *C. elegans* ease understanding of basic biological processes that occur in living organisms. A variety of molecular genetic studies such as random mutation induction as well as single gene inactivation, to elucidate factors and genes that control life expectancy can be easily conducted since the genome of the nematode has been completely sequenced [128].

Neurological Function

Complete mapping of *C. elegans* nervous system has allowed researchers to identify individual neurons involved in detecting environmental stimuli including mechanosensation, chemosensation and thermosensation [129,130]. Detection and response to sensory stimuli including UV light, O2 and CO2 concentrations, electric fields and others have been used to dissect neuron function within an intact *C. elegans* neural circuit [131]. Through this, neurons that release specific neurotransmitters or express particular receptors are able to be identified and associated with environmental stimuli. To reflect the overall nervous system functions at the organismal level, behavioural measures are determined [132].

Locomotion

Locomotion is one of the most important features that reflect the physical ability of nematodes in aging [133]. The swimming ability of nematodes which is age-dependent, can be measured as wave initiation rate, activity index, brush stroke and body wave number. Wave initiation rate reflects the number of body waves per minute, which indicates speed of movements, whereas body wave number determines the waviness of the body at each time point. Activity index is an indicator of vigorousness of bending over time while brush stroke reflects the depth of movement [134].

Lipid Accumulation

One of the most measured healthspan parameter in *C. elegans* is the accumulation of lipofuscin. This auto-fluorescent material builds up over time in cells and tissues of *C. elegans*. The transparent nature of *C. elegans* enables observation of this auto-fluorescent material in living nematodes [135]. It has been suggested that this autofluorescence material, located mainly in the intestine of *C. elegans*, reflects the aging status of the nematodes [136]. Accumulation of lipofuscin is associated with increased oxidative stress or reduced lifespan in *C. elegans*. Hence, reduced lipofuscin indicate reduced internal oxidative stress and intracellular ROS in *C. elegans* [137].

Resistance to Stress

Stress resistance in *C. elegans* has been tested by exposing the nematodes to various stressors such as heat, UV, oxidative stress, pathogen and heavy metals (eg; copper, zinc, cadmium and chromium), abnormally low and high oxygen levels. Hypoxia can be simulated using physical or chemical methods. Worms are usually placed in a sealed hypoxic chamber with 60% oxygen to induce hypoxia or treated with sodium azide to inhibit mitochondrial respiration complex IV. Hypoxic and hyperoxic tests in *C. elegans* have led to the characterization of key genetic factors, including hypoxia inducible factor 1 (HIF-1) in living organisms [138].

Heat stress is done because high temperatures cause cellular disorders, such as neuronal degeneration and heat induced necrotic cell death. *C. elegans* is equipped with systems that protect cells from the deleterious effects of high temperatures (eg: HSF-1, FOXO, DAF-16). Hyperosmotic shock induces water efflux and protein aggregation, leading to cellular deterioration and disruption of protein homeostasis (proteostasis). Osmotic stress resistance assays are usually performed on agar gels containing various concentrations of NaCl (between 50 mM to 500 mM) using L4 larval or young adult worms kept at 20°C [139]. Ultraviolet radiation (between 100 to 400 nm) is used to induce specific DNA modifications in exposed *C. elegans* grown on solid media. Ultraviolet light has been found to have a dose-dependent effect on the health and survival of *C. elegans*. UV irradiation at 10 J/m2/min reportedly induced a mild stress response in the nematodes [140].

Resistance to Infection

The pathogenesis of fungal infection in *C. elegans* showed that the virulence factor of fungus is similar to that in mammals [141]. Pseudomonas aeruginosa, a ubiquitous Gram-negative

bacterium that causes opportunistic infections, is widely used in *C. elegans* pathogen resistance assays. P. aeruginosa kills the nematode by generating multiple virulence factors or secretion of lethal toxins. Survival of nematodes has to be analysed daily because dead bodies are lysed quickly by exoenzymes produced by P. aeruginosa [142]. Resistance to fungal infections has also been tested in *C. elegans* using opportunistic fungal pathogens, such as Cryptococcus neoformans, Candida albicans, and Drechmeria coniospora that are capable of infecting and killing the nematode.

Thermotolerance

Temperature affects the survivability of *C. elegans* [143]. The nematode has a shorter lifespan when kept at 25°C, compared to 20°C. The average lifespan of the nematode is also longer at temperature as low as 15°C, because the growth and development of *C. elegans* are altered at different temperatures [144].

C. elegans has thermosensory neurons such as AFD sensory neurons in its head that detect temperature changes of 0.01°C or less [145]. Temperature of 20°C is often used in studies as it results in average median lifespan of approximately 21 days. Nematodes generally grow approximately 10 days longer at 15°C and 10 days shorter at 25°C [146]. A previous study reported that pre-exposure of higher temperatures resulted in increased activity, locomotion velocity and chemotaxis index (CI) in nematodes but these measures changed during the recovery period [147].

Thermotolerance assays of *C. elegans* are mostly performed at 35°C or higher temperatures. However, extended exposure to high temperatures can cause lethality in two hours [148]. High environmental temperatures cause protein destabilization and degradation, halting of transcription and translation, or transition into the state of dauer [149]. To determine genetic factors and pathways playing a role in healthspan, precise and consistent control of environmental temperature thus becomes absolutely necessary.

Research Limitations

C. elegans is a useful alternative for in vivo studies to determine changes in healthspan during aging. Measures of lipid accumulation, neuromuscular functions, locomotion, survivability in heat and stress environments enable quantification of healthspan in a short duration of time. Since the *C. elegans* has a short lifespan, future studies should embark on determining the changes in healthspan measures until the end of life of the nematode, more so in the extended duration of life as a result of treatment to establish the long-term effects.

Healthspan in Yeast

Yeasts from the Saccharomyces genus play an important role in research. The species *S. cerevisiae* or also known as baker's yeast that is a unicellular fungus that shares common cellular and genetic characteristics with mammals were commonly used for research purpose. Information from this simple organism can be further studied in understanding multicellular organism as well. Recent studies done using yeast has uphold information such as presence of similar genes in both yeast and mammals that corresponds to decline aging and its resistance to oxidative stress, which leads to identification of several signalling pathways. Half of the essential proteins in yeast has human orthologs [150]. Apart from that, genomics, transcriptomics and morphological data of yeast are well established [151].

Advantages of *S. cerevisiae* as an Aging Model

S. cerevisiae is used in aging research because of its physiology characteristics and fully sequenced genome. One out of every four of its genes are orthologous to humans hence facilitate a variety of genomic engineering applications using *S. cerevisiae* [152]. Aging in yeast is assessed by measuring replicative or chronological lifespan. Replicative lifespan analysis is typically performed by measuring the number of daughter cells produced by mother cells before they cease division. This assay enables identification of conserved pathways regulating viability of cell division such as stem cells in humans [153]. Chronological lifespan involves monitoring survival of cells over time in a quiescent-like stationary phase culture [154,155].

Unicellular eukaryote *S. cerevisiae* has been an exceptional model for revealing large networks of genes critical to the survival of cells in diverse types of environmental stressors [156,157]. Environmental factors, including radiation and the presence of redox-cycling agents, are sources of oxidative stress. Reactive oxygen species (ROS) produced causes oxidative damage to proteins, lipids, and nucleic acids [158]. Aerobic respiration also generates low levels of endogenous ROS from leakage of electrons from the electron transport chain, and mitochondrial dysfunction. Increased ROS levels triggers the induction of cellular defence mechanisms such as activated expression of antioxidant enzymes [158].

Viability of Stress Induction

In a study, yeast was exposed to hydrogen peroxide (H2O2) and resistance to H2O2 was measured as viability

of cells. The antioxidant capacities of natural plants such as vegetables, fruits, and tea mainly said because of their abundance bioactive flavonols and pharmaceutical properties that helps to counteract the oxidative stress in cells hence reduce the rate of diseases related to oxidative damage [159]. Results obtained showed that myricetin (flavonol), provided higher resistance to H2O2 compared to phloroglucinol because the latter contains pyrogallol structure, which enables donation of hydrogen to free radicals [160]. Consistently, protective effect of hyperoside which is part of flavonoid group against H2O2-induced stress may be due to catalase, where it contributes to conversion of H2O2 to generate H2O and O2 hence improve tissue injury done by oxidative stress [161].

The lifespan and health measures of aging models are dependent on the concentration of studied compounds used in each assay. For example, low concentrations of *R. rosea* extract was found to prevent cell death and increased reproductive potential of aged yeast cells, while high concentrations of *R. rosea* extract decreased cell viability. In line with this, similar dose-dependent action of *R. rosea* extract on the lifespan of fruit flies was observed [162]. Regulation of stress-responsive genes in yeast is critically dependent on the regulation of Msn2/Msn4 from the cytosol to the nucleus. In response to environmental stresses or depletion of glucose results in predominant nuclear localization of Msn2 which induces carbohydrates reservation (glycogen and trehalose), thickening of the cell wall and several other changes as preparation of the cells for extended survival [163]. *R. rosea* has been suggested to induce translocation of Msn2/Msn4 into the nucleus thus inducing synthesis of protective proteins. Besides that, increased resistance of yeast may also be attributed to the range of biologically active substances such as polyphenols, present in *R. rosea* [164].

High concentration of *R. rosea* extract may result in death of yeast through increased production of ROS that exceeds the rate of their elimination by cellular defence mechanisms, leading to intensification of oxidation of biomolecules [165,166]. Bayliak, et al. reported that heat stress increased SOD activity in cells pre-treated with *R. rosea* aqueous extract but decreased catalase activity in cells under similar conditions. Interestingly, low concentrations of the extract activated defence mechanism and stimulated resistance to stress. Subsequently, low concentrations of *R. rosea* was suggested to act as a mild stressor to induce adaptive responses such as cellular resistance that result in improved maintenance and survival of yeast. Besides that, the extract was proposed to stabilize DNA and interact with mediators of signalling pathways to upregulate defences [167]. In line with these suggestions, an earlier study also noted that the effect of *R. rosea* on the lifespan and stress resistance of *S. cerevisiae* was mediated by hormetic mechanisms [168].

Chronological Lifespan

Chronological lifespan of yeast is often measured to indicate survival capacity. Chronological lifespan indicates the duration of cell survival in a non-dividing, quiescence-like state. It is commonly measured by culturing cells in liquid media, where cells enter a non-dividing state due to limited sources of carbon. When glucose becomes limited as a result of glycolysis, yeast enters the diauxic phase where growth begins to decrease, and aerobic utilization of ethanol takes place. Meanwhile, the viability of yeast is measured as the ability to resume mitotic growth when fresh medium is given. The ability of yeast to form new colonies on rich media is quantified as colony-forming units [169]. Replicative lifespan is used to measure the lifespan of mitotically active cells before they reach senescence. In this procedure, the total number of cell divisions of a single cell is scored and statistically validate by repetition (normally 40) to determine the average replicative lifespan of a certain genotype or condition.

The mean and the maximum survival time of yeast depend on the medium used and the strain tested. Limited nutrients or presence of external stresses that disturb cellular functions cause cells to switch to a nondividing stationary state and activate defence mechanisms for longer lifespan [170]. Incubation of yeast in water is another approach used to investigate chronological yeast lifespan. Yeasts incubated in water have low respiration rate and enter a non-dividing hypometabolic stationary phase where cells are highly resistant to stress and survive up to 3 months.

Research Limitations

S. cerevisiae proved to be a valuable model in which to study the relationship between healthspan and lifespan. As discussed earlier, the restoration of the oxidative status did improve the lifespan. However, like other unicellular organisms, the yeast model cannot be used to assess behavioural modifications such as those involving musculoskeletal and neuronal systems. Future studies can include measuring the mating rate between the two haploids of *S. cerevisiae*, as a type of 'behavioural response' observation.

Conclusion

Aging and degenerative diseases involves progressive loss of physiological function, which leads to unfavourable consequences, including morbidity and mortality. Animal models are used to elucidate the mechanisms involved in aging and diseases due to their shorter lifespan and ease of monitoring and manipulations. The lifespan of *C. elegans* and yeast are often measured to a certain efficacy of

interventions and treatments. Recent studies have embarked on determination of health measures such as oxidative stress resistance and movement capacity to further establish the potential of interventions in health improvements in these models besides using rodents and Drosophila. Only a handful of studies have compared health measures between different ages in aging models. Elucidation on the stages of deterioration is necessary in future studies to ease invention of targeted therapy. Apart from that, determination of health markers during the extended period of life as a result of intervention would determine whether the treatment or supplement involved actually prevented dysfunction and diseases at advanced age rather than prolongation of frailty. Not all animal models have parameters that cover all aspects of health. Measures of healthspan in Drosophila and yeast are limited because the assessment of behaviour is complicated while *C. elegans* is limited in terms of cardiac function and disease because it does not have a heart and circulatory system. However, *C. elegans* may be used in studies regarding human heart muscle as its body muscle contains sarcomeres similar to that of the heart. Hence, careful selection of an animal model to suit the measured health aspect is necessary to ensure achievement of research objective.

Conflicts of Interest

The authors declare no conflict of interest.

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