EVALUATION OF THE ANTI-AGING EFFECTS OF *Polyalthia longifolia* LEAF EXTRACT IN D-GALACTOSE-INDUCED AGING RAT MODEL

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by

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LIST OF SYMBOLS

%	Percentage
^{0}C	Degree Centigrade
ΔA	Change in Absorbance
ϵ	Epsilon
β	Beta
<	Less than
α	Alpha

LIST OF ABBREVIATIONS

AGEs	Advanced Glycation End Products
ACh	Acetylcholine
AChE	Acetylcholinesterase
b. wt./day	Body weight per day
BHT	Butylatedhydroxyltoluene
CAT	Catalase
DPPH	2,2-Diphenyl-2-Picrylhydrazyl
D-gal.	D-galactose
DNA	Deoxyribonucleic Acid
DTNT	5,5'-Dithiobis (2-Nitrobenzoic Acid
EDTA	Ethylenediaminetetraacetic Acid
FCR	Folin-Ciocalteu's Reagent
GAE	Gallic Acid Equivalents
GSH	Reduced Glutathione
IC_{50}	Half (50%) Maximum Inhibitory Concentration
NBT	Nitro blue Tetrazolium
PBS	Phosphate Buffered Saline
PD	Parkinsons Disease
PLME	*Polyalthia longifolia* Leaf Methanol Extract
QCE	Quercetin Equivalent
RONS	Reactive Oxygen and Nitrogen Species
ROS	Reactive Oxygen Species
SAAP	Senescent cells Antiapoptotic Pathways
SASP	Senescence Associated Secretory Phenotype
SOD	Superoxide Dismutase
TERT	Telomerase Reverse Transcriptase
TERC	Telomerase RNA Component
TNB	5-Thio-2-Nitrobenzoic Acid
VC	Vitamin C (Ascorbic Acid)
VE	Vitamin E (Alpha-tocopherol)

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PENILAIAN KESAN ANTI-PENUAAN EKSTRAK DAUN *Polyalthia longifolia* DALAM MODEL TIKUS PENUAAN YANG DIINDUKSI OLEH D-GALAKTOSA

ABSTRAK

Penuaan dikaitkan dengan tekanan oksidatif, pemendekan telomer, dan penuaan selular, yang menyumbang kepada neurodegenerasi dan kemerosotan kognitif. *Polyalthia longifolia*, tumbuhan yang kaya dengan polifenol dan mempunyai sifat antioksidan yang kuat, telah digunakan secara tradisional untuk pelbagai tujuan terapeutik. Kajian ini menilai potensi anti-penuaan dan neuroprotektif ekstrak metanol daun *Polyalthia longifolia* (PLME) pada dos 500 mg/kg/hari selama empat minggu dalam model tikus penuaan yang diinduksi oleh D-galaktosa. Analisis fitokimia menunjukkan kandungan fenolik (88.23 ± 0.001 mg GAE/g) dan flavonoid (66.97 ± 0.002 mg QE/g) yang tinggi, manakala spektrum FTIR mengenal pasti kumpulan fungsi utama (-OH, -CH, C=O, dan N-H), yang menunjukkan kehadiran sebatian bioaktif. PLME menunjukkan aktiviti antioksidan *in vitro* yang kuat dengan keberkesanan meneutralkan radikal DPPH, H_2O_2, dan O_2•–, masing-masing dengan nilai IC_{50} sebanyak 74.0 ± 6.0, 54.0 ± 3.0, dan 32.8 ± 1.0 µg/mL. Secara *in vivo,* pentadbiran D-galaktosa mencetuskan tekanan oksidatif berkaitan penuaan, menyebabkan peningkatan ketara ($P < 0.05$) dalam tahap malondialdehid serum (MDA: 9.66 ± 1.22 nmol/mL) serta penurunan kandungan glutation (GSH: 4.26 ± 1.61 µmol/mL) dan aktiviti enzim superoksida dismutase (SOD: 82.16 ± 3.01 U/mL) serta katalase (CAT: 11.71 ± 4.02 U/mL). Sebaliknya, rawatan dengan PLME secara signifikan mengurangkan tahap MDA (5.32 ± 0.64 nmol/mL) dan meningkatkan kandungan GSH (7.22 ± 2.87 µmol/mL) serta aktiviti SOD (106.09 ± 4.13 U/mL) dan CAT (15.22 ± 3.12 U/mL). Selain itu, PLME juga menunjukkan kesan anti-penuaan

dengan mengawal peningkatan berlebihan dalam penuaan selular, yang dibuktikan melalui pengurangan aktiviti β-galaktosidase serum (79.95 ± 4.33 pKat/mL) dan peningkatan tahap telomerase reverse transcriptase (TERT) (1,347.51 ± 103.34 pg/mL) berbanding kumpulan kawalan penuaan (β-galaktosidase: 125.63 ± 7.12 pKat/mL; TERT: 1,071.25 ± 125.18 pg/mL), menunjukkan peningkatan jangka hayat selular. PLME juga memperbaiki fungsi kognitif dan aktiviti kolinergik dengan mengurangkan aktiviti asetilkolinesterase (AChE) otak (0.59 ± 0.05 U/mg protein) dan meningkatkan tahap asetilkolin (ACh) (45.31 ± 4.20 µg/mg protein) berbanding kumpulan kawalan penuaan (AChE: 0.98 ± 0.07 U/mg protein; ACh: 26.11 ± 3.87 µg/mg protein). Analisis histologi mengesahkan perlindungan neuron hippocampal, sejajar dengan peningkatan dalam kesilapan ingatan kerja (WME: 8.22 ± 1.01) dan kesilapan ingatan rujukan (RME: 12.01 ± 1.22) dalam ujian maze radial, berbanding kumpulan kawalan penuaan (WME: 12.67 ± 1.40; RME: 19.93 ± 1.82). Kesimpulannya, PLME menunjukkan kesan anti-penuaan dan neuroprotektif yang ketara, berkemungkinan melalui aktiviti antioksidan yang kuat, pengawalan penuaan selular, dan peningkatan fungsi kolinergik. Penemuan ini menyokong potensi aplikasi PLME dalam pembangunan formulasi anti-penuaan semula jadi untuk menangani penuaan dan kemerosotan kognitif yang berkaitan dengan tekanan oksidatif.

EVALUATION OF THE ANTI-AGING EFFECTS OF *Polyalthia longifolia* LEAF EXTRACT IN D-GALACTOSE-INDUCED AGING RAT MODEL

ABSTRACT

Aging is closely associated with oxidative stress, telomere shortening, and cellular senescence, contributing to neurodegeneration and cognitive decline. *Polyalthia longifolia*, a polyphenol-rich plant with strong antioxidant properties, has been traditionally used for various therapeutic purposes. This study investigated the anti-aging and neuroprotective potential of *Polyalthia longifolia* leaf methanol extract (PLME) at 500 mg/kg/day for four weeks against D-galactose-induced aging rat model. Phytochemical analysis of PLME revealed high phenolics (88.23 ± 0.001 mg GAE/g) and flavonoids (66.97 ± 0.002 mg QE/g) contents, while FTIR spectra identified key functional groups (-OH, -CH, C=O, and N-H), indicative of bioactive compounds. PLME demonstrated strong *in vitro* antioxidant activity, effectively scavenging DPPH, H_2O_2, and $O_2^{\bullet-}$ radicals, with IC_{50} values of (74.0 ± 6.0; 54.0 ± 3.0; and 32.8 ± 1.0) µg/mL, respectively. *In vivo,* D-galactose administration effectively induced aging-related oxidative stress, significantly ($P < 0.05$) elevating serum malondialdehyde (MDA: 9.66 ± 1.22 nmol/mL) while decreasing glutathione (GSH: 4.26 ± 1.61 µmol/mL) levels, and reducing superoxide dismutase (SOD: 82.16 ± 3.01 U/mL) and catalase (CAT: 11.71 ± 4.02 U/mL) activities. In contrast, PLME treatment significantly mitigated these effects, lowering MDA levels (5.32 ± 0.64 nmol/mL) while increasing GSH (7.22 ± 2.87 nmol/mL) concentration, as well as enhancing SOD (106.09 ± 4.13 U/mL) and CAT (15.22 ± 3.12 U/mL) activities. Additionally, PLME also exerted key anti-aging effects via modulating the excessive increase in cellular senescence by reducing serum β-galactosidase activity (79.95 ± 4.33 pKat/mL) and

enhancing telomerase reverse transcriptase (TERT) levels (1,347.51 ± 103.34 pg/mL) compared to the aging control group (β-galactosidase: 125.63 ± 7.12 pKat/mL; TERT: 1,071.25 ± 125.18 pg/mL), suggesting improved cellular longevity. Furthermore, PLME significantly ($P < 0.05$) improved cognitive function and cholinergic activity by decreasing brain acetylcholinesterase (AChE) activity (0.59 ± 0.05 U/mg protein) and increasing acetylcholine (ACh) levels (45.31 ± 4.20 µg/mg protein) relative to the aging control group (AChE: .0.98 ± 0.07 U/mg protein; ACh: 26.11 ± 3.87 µg/mg protein). Histological analysis confirmed that PLME preserved hippocampal neuroprotection, correlating with improved working memory errors (WME: 8.22 ± 1.01) and reference memory errors (RME: 12.01 ± 1.22) as observed in the radial maze test, compared to the aging control group (WME: 12.67 ± 1.40 and RME: 19.93 ± 1.82). In conclusion, PLME demonstrated significant anti-aging and neuroprotective effects, likely through its potent antioxidant activity, modulation of cellular senescence, and enhancement of cholinergic function. These findings support its potential application in the development of green anti-aging formulations to combat oxidative stress-related aging and cognitive decline.

CHAPTER 1

INTRODUCTION

Life expectancies are now on the increase. This could be due to the advancement in medical and community healthcare systems (Walsh et al., 2022). People are now living longer than the previous generations when infectious and communicable diseases are the primary causes of death (Foreman et al., 2018). While lifespan has increased over the years, the period of lifetime spent in good health (health span) has not increased proportionally. Therefore, many people spent a larger portion of their lifetime suffering from pain of age-related illnesses, such as cardiovascular and neurodegenerative disorders, and certain cancers. Thus, posing significant economic and healthcare challenges (Filfan et al., 2020). This demographic shift brings attention to the challenges connected to age-associated diseases and healthcare costs in elderly individuals (Giacalone et al., 2016). The endeavor of humans in striving to avoid aging has begun since ancient dynasties (Veith, 1950; Ong, 2005). Humans have been attempting to look for anti-aging solutions to combat aging and possess the power of immortality for prolonging life indefinitely (Dien, 1987). But, due to the impossibility of stopping aging and maintaining eternal youthfulness, scientists are now trying to shift the attention of the medical community from optimizing life span to optimizing health span (Wickramasinghe et al., 2020; Couteur & Barzilai, 2022). (Figure 1.1). To do this, it is essential to comprehend the fundamental mechanisms of aging and to create interventions to attack the root causes of all bodily defects of aging so as to hinder the aging process and prevent the onset of age-associated diseases in order to extend not only the life span but also the health span (The duration of time in which an individual maintains good health, reducing the burden of age-related diseases and disabilities (Martemucci et al., 2022; Bhatti et al., 2023) (Figure. 1.1).

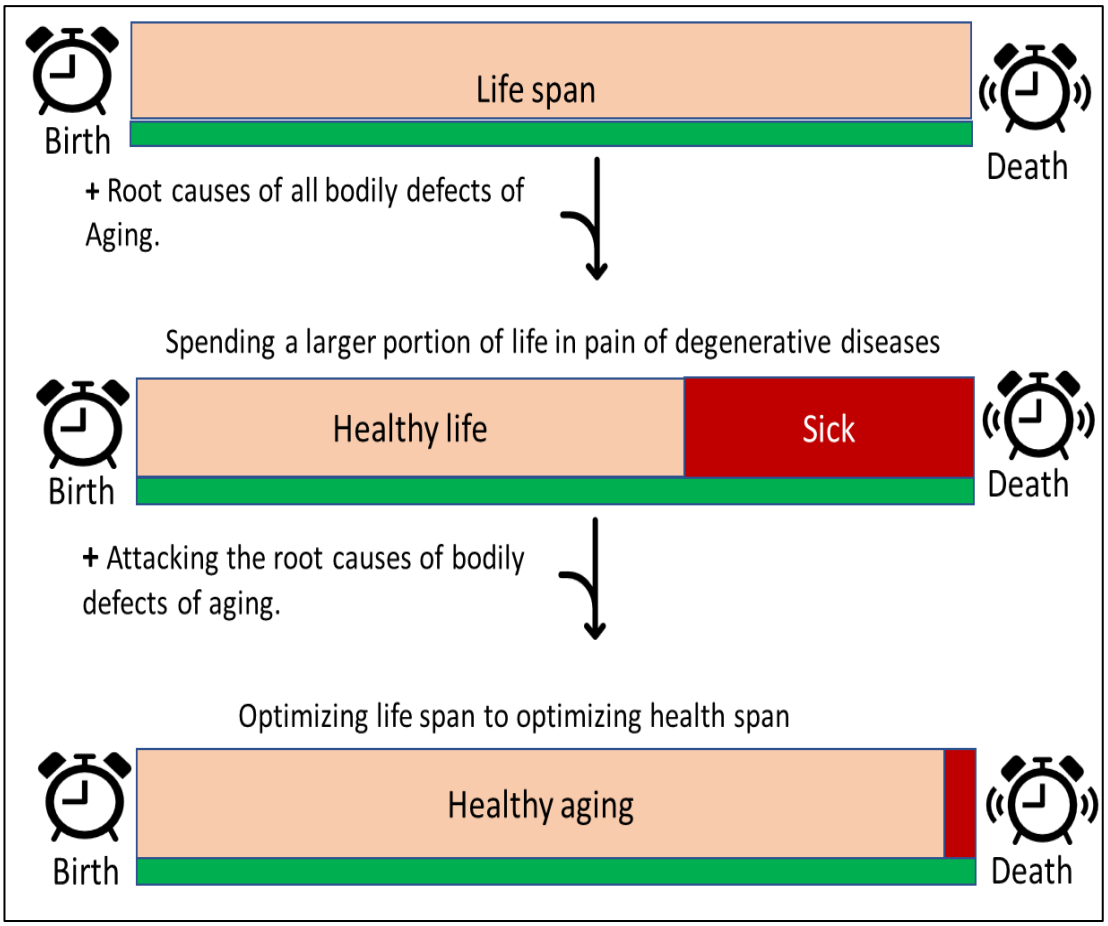

Figure 1.1 The concept of longevity: Promoting healthy aging by addressing the fundamental causes of bodily deterioration associated with aging.

The search for a novel antiaging formulation could be considered as the search for the cure for many diseases at the same time (Jothy et al., 2016). Aging can be defined as a natural and inevitable progression of living organisms from youth to old age, characterized by cellular division over time. This process is associated with a gradual decline in the capacity to withstand stress and diseases that could lead to reduced quality of life (Martel et al., 2020). It is a primary risk factor for numerous chronic non-communicable age-associated diseases including cardiovascular, neurodegenerative, immune, muscular, and metabolic diseases that are often disabling, costly to manage, and could eventually lead to death (Trubitsyn, 2020).

The normal functioning of cells involves their ability to divide, differentiate and regenerate appropriately. Such processes are governed by the genomic stability of the cells. The key factors that contribute to this stability are the telomeric ends (Telomeres) that protect the end of the chromosomes (Zhang et al., 2016). During the process of replicative cell division, telomeres gradually shorten in length with each round of replicative cell division. The shortening of telomere length with each cell division can ultimately trigger stress-related premature senescence resulting in permanent cell growth arrest (Yousefzadeh et al., 2018; Zhang et al., 2016).

Cellular senescence is a key factor in the aging process and the development of age-related diseases. A range of stimuli such as telomere shortening, U.V radiation, oncogene activation, chronic inflammation, abnormal mechanical or cellular stress (including oxidative and genotoxic stress, as well as exposure to chemokines and cytokines) can induce cellular senescence, which in turn can activate senescence-associated secretory phenotype (SASP) which is deleterious to the neighbouring cells (Ohtani, 2022). Once cells enter senescence, they usually become resistance to apoptosis due to the upregulation of the antiapoptotic pathways in senescent cells (Kirkland et al., 2017; Pignolo et al., 2020; Gasek et al., 2021; Chaib et al., 2022;). Senescent cells adversely affect their surrounding cells, tissues, and organs which occurs when the cells lose their specific functions and begin to secrete a mix of pro-tumorigenic and pro-inflammatory factors, termed the senescence-associated secretory phenotype (SASP) thereby inducing paracrine and endocrine damages to the neighbouring cells, tissues, and organs (Lämmermann et al., 2018; Chaib et al., 2022). Increasing evidence suggests that senescent cells contribute significantly to various age-related diseases, and their targeted removal has been shown to extend the lifespan and health-span of mice (Pignolo et al., 2020; Gasek et al., 2021). Although, studies

have shown that the elimination of senescent cells, along with the upregulation of telomerase activity, can enhance proliferation of young cells and slowdown the aging process. This can ultimately replenish telomere length in rejuvenating cells there by delaying the onset of aging and its complications (Borghesan et al., 2020).

Conducting research on anti-aging using medicinal plants involves investigating the potentials of the natural compounds present in plants to alleviate the impacts of aging and enhance overall health and longevity (Raina et al., 2023). For centuries, medicinal plants have been utilised in traditional medicine practices across the globe, and modern research seeks to validate and understand the scientific basis for their anti-aging properties (Zhang & Tsao, 2016; Li & Weng, 2017; Jamshidi-Kia et al., 2018). Ethnopharmacologically, *Polyalthia longifolia* is a traditionally known ayurvedic medicinal plant with diverse array of pharmacological and biological activities which has been extensively utilized in traditional folk medicine as general tonic for rejuvenation and for addressing many ailments such as gastrointestinal ulcers, rheumatic fever, and overall body pain (Sampath & Vasanthi, 2013; Yao LuiJin et al., 2019). Various *in vitro* and *in vivo* studies on *Polyalthia longifolia* have further validated its considerable potential for the management of numerous age-related diseases including cardiovascular, neurodegenerative, immune, muscular, inflammatory, and metabolic diseases (Dattatray et al., 2021). Recently, Hemagirri & Sasidharan, (2022a, 2022b) reported that the methanolic leaf extract of *Polyalthia longifolia* exhibits strong antioxidant and anti-aging properties. Specifically, the extract was shown to possess promising anti-aging effects by activating the SOD and SIRT1 genes and decreasing oxidative stress in *S. cerevisiae* BY611 yeast cells. Based on these findings from the cellular model, further studies are recommended to

investigate these effects using murine models and other experimental systems (Hemagirri & Sasidharan, 2022a, 2022b).

Repeated administration of D-galactose can lead to aging-associated symptoms in animals, including changes in biochemical markers, decreased reproductive capacity, degenerative alterations in neural cells, and memory deficit (Zhao et al., 2018; Liu et al., 2020a; Wang et al., 2024). The D-galactose-induced aging model in rats is considered as one of the most reliable model for generating outcomes that are similar to those observed in clinical research (Wang et al., 2018; Li et al., 2019; Liu et al., 2020b). At higher concentrations, D-galactose undergoes conversion into aldose and hydroperoxide through the catalytic action of galactose oxidase. This process results in the production of superoxide anions and oxygen-derived free radicals. These changes closely resemble the natural aging process (senescence model) manifesting as neurological decline, decrease antioxidant enzyme activity, and weakened immune responses (Zhao et al., 2018; Liu et al., 2020b). Considering the well-established antioxidant and anti-inflammatory properties of *Polyalthia longifolia* and the underlying mechanism of aging, this study aimed to investigate the effects of *Polyalthia longifolia* leaf methanolic extract (PLME) on D-galactose-induced aging in rats.

1.1 Problem Statement and Rationale of the Study

Aging is accompanied by chronic, non-communicable disorders including Parkinson's and Alzheimer's, arthritis and cardiovascular diseases which are often debilitating and expensive to manage (Kuppen et al., 2023). These will undoubtedly place more burden on the economy and further place additional pressure on public health care systems, which are already overwhelmed by the burden of communicable

diseases (Sorrenti et al., 2022). The World Health Organisation (WHO) has projected that due to rising life expectancy, the global population of older adults aged 60 and above will exceed 1.2 billion by the year 2025. Moreover, the occurrence of aging and age-associated ailments may double every five years in individuals over the age of 65 (Ghilain et al., 2021). While certain medications including Galantamine, Donepezil, Huperzine and Rivastigmine have been recommended for treating various age-associated conditions, they are expensive and the outcomes are often unsatisfactory (Sahoo et al., 2018; Majolo et al., 2024). Also, they are not free from potential side effects, such as drowsiness, diarrhoea, vomiting, and headache. Consequently, there is a growing preference for natural compounds over synthetic drugs or chemicals to mitigate the risk of adverse drug reactions and reduce the financial burden on the modern healthcare system. As a result, natural products are often seen as more trustworthy (Wilkinson et al., 2012; Menghani et al., 2021). Given these concerns, there is a strong need to search for and develop safer and more effective options from natural sources to address aging and its related health challenges. This has prompted the evaluation of medicinal plants as potential sources of anti-aging agents, based on their traditional use as therapeutic remedies as supported by numerous scientific findings (Espinosa-Leal & Garcia-Lara, 2019; Menghani et al., 2021). It has been postulated that *Polyalthia longifolia* leaf methanolic extract could address this need, as it has been reported to exhibit significant antioxidant and anti-aging effects in the *S. cerevisiae* BY611 yeast cell model (Hemagirri & Sasidharan, 2022a, 2022b). To the best of our knowledge, the anti-aging effects of *Polyalthia longifolia* on murine models have not yet been investigated. Therefore, there is a need for preclinical studies using murine models to further explore the anti-aging potential of this promising medicinal plant for the future development of green anti-aging formulations.

1.2 Research Hypothesis

1. PLME may contain bioactive phytochemicals and may exhibit significant *in vitro* antioxidant activity by scavenging free radicals.

2. PLME may mitigate oxidative stress in D-galactose-induced aging rats by enhancing antioxidant enzymes (SOD, CAT, and GSH) and reducing lipid peroxidation (MDA) levels.

3. PLME may delay aging process in D-galactose-induced aging rats by enhancing telomerase activation (TERT levels) and reducing β-galactosidase activity.

4. PLME may enhance cognitive function and memory performance in D-galactose-induced aging rats, leading to improved learning ability and fewer errors in the eight-arm radial maze test.

5. PLME may exhibit neuroprotective effects in aging rats by increasing brain acetylcholine levels, inhibiting acetylcholinesterase activity, and preserving hippocampal neuronal integrity.

1.3 General Objective (Aim) of the Research Work

This study aims to explore and evaluate the anti-aging effects of *Polyalthia longifolia* leaf methanol extract (PLME) in a D-galactose-induced aging rat model.

1.3.1 Specific Objectives

The specific objectives listed below were investigated to accomplish the purpose of this research.

1. To analyse the phytochemical composition of PLME and evaluate its *in vitro* free radical scavenging activity using various chemical assays.
2. To investigate the effects of PLME administration on body weight, organ indices, and antioxidant biomarkers (SOD, CAT, GSH, and MDA) in D-galactose-induced aging rats.
3. To assess the effects of PLME on telomerase activation and its senotherapeutic potential by measuring Telomerase Reverse Transcriptase (TERT) levels and β-galactosidase activity in the serum and organ homogenates of the rats.
4. To evaluate the effects of PLME administration on cognitive function and memory abilities using the eight-arm radial maze test.
5. To determine the neuroprotective effects of PLME against D-galactose-induced brain damage by measuring acetylcholine (ACh) levels, evaluating acetylcholinesterase (AChE) activity, and conducting histopathological analysis of the hippocampus.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Aging can be explained as an inevitable biological progression of living organisms from youth to old age through the gradual process of cell division over time (Xue et al., 2021). It is a highly complicated and multifaceted process marked by the gradual and persistent loss of body functions and a continuous decline in the capacity to resist stress and diseases, ultimately impacting the overall physiology of living organisms (Leyane et al., 2022). This multifactorial and complex process is a key risk factor for a range of chronic non-communicable age-associated disorders, including cardiovascular, neurodegenerative, immune, muscular, and metabolic disorders, which are not only disabling but also costly to manage and can eventually lead to mortality (Trubitsyn, 2020). Although aging is an inherent and inescapable biological process, it can however be delayed, and age-related diseases can also be prevented, as demonstrated by numerous studies (Frank et al., 2021; Amorim et al., 2022; Cheng et al., 2022; Xenos et al., 2022). Gerontology, the scientific study of aging, explores the complex molecular processes underlying the aging phenomenon and contributes valuable insights that extend beyond the realm of basic science (Ok, 2022; Marino et al., 2023). Understanding aging at the molecular level is crucial for developing targeted interventions to promote healthy aging and improve quality of life. Therefore, studying aging biology and mechanisms can inform strategies to slow aging and reduce the risk of age-related disorders (Marino et al., 2023).

2.2 Historical Perspective on the Search for Anti-aging Solutions

The pursuit of anti-aging solutions dates back to ancient dynasties (Ong, 2005; Veith, 1950). Throughout history, humans have sought ways to combat aging or achieve immortality (Dien, 1987). For instance, the ancient China emperor Qin Shi Huang (Qin dynasty) was fascinated by antiaging pills because he was afraid of death or being killed by his enemies (Ong, 2005). He sent a ship with hundreds of young men and women in search of magical anti-aging plants or an elixir of life, but they never returned (Dien, 1987; Kuwayama, 1991; Sanft, 2008). Not only the ancient Chinese emperors were enthusiastic about immortality, but people of all ages were interested in eternal youth. Yet, no one had succeeded in achieving the goal of living an immortal life (Kuwayama, 1991). Despite this, the quest continues. Since aging and death are inevitable, efforts now focus on slowing aging and preventing age-related diseases to promote a longer, healthier lifespan (Franklin & Tate, 2009).

2.3 Pathogenesis and Pathophysiology of Aging

Aging is a natural and inevitable dynamic biological process, affecting all living organisms as a result of the cumulative effects of cell replication over time (Trubitsyn, 2020). The aging process is closely connected with the accumulation of biochemical toxins, such as reactive oxygen and nitrogen species, which ultimately contributes to age-related diseases (Liguori et al., 2018). Aging manifests through skin wrinkles, muscle degeneration, cataracts, and hair graying, resulting from an increased number of aged cells (Wang & Bennett, 2012; Keshavarz et al., 2023). Cell division, differentiation, and regeneration depend on genomic stability, primarily maintained by telomeres, which protect chromosome ends (Kim et al., 2016). During replicative cell division, telomeres shorten with each replication, eventually leading to stress-induced premature

senescence and permanent growth arrest to prevent damaged DNA replication (Yousefzadeh et al., 2018). Cellular senescence, a key factor in aging and age-related diseases, can be triggered by telomere shortening, oncogene activation, UV radiation, chronic inflammation, and oxidative stress (Ohtani, 2022). Research has revealed that, once cells enter a state of senescence, they usually become resistance to apoptosis due to the activation of the antiapoptotic pathways (Kirkland et al., 2017). they affect their surrounding cells, tissues, and organs by losing their specific cellular functions and secrete some pro-inflammatory and pro-tumorigenic factors, collectively known as SASP, causing damage to surrounding tissues and organs (Lämmermann et al., 2018). Evidence suggests that senescent cells drive many age-related diseases, and their targeted removal can extend lifespan and healthspan in mice (Pignolo et al., 2020). Studies indicate that eliminating senescent cells and activating telomerase can replenish telomere length, slow aging, and mitigate age-associated disease.

2.4 Cellular Senescence: The Basic Halmark of Aging

Many of the aging markers such as telomere attrition, mitochondrial dysfunction, oncogene activation, UV radiation, and oxidative stress are major triggers of cellular senescence (Campisi & d'Adda di Fagagna, 2007; Franco et al., 2022). This state of cellular senescence is often marked by the upregulation of key cell cycle inhibitors, such as p16INK4a, p21CIP1, p15INK4b, p14ARF, and the p53-dependent DNA Damage Response (DDR), resulting in irreversible cell cycle arrest, typically in either the G1 or G2 phase of the cell cycle (Campisi & d'Adda di Fagagna, 2007; Borghesan et al., 2020). Senescent cells resist apoptosis due to the activation of anti-apoptotic pathways (PI3K/AKT and BCL-2) and induce senescence in surrounding cells through the persistent secretion of senescence-associated secretory phenotype (SASP)

factors (Dörr et al., 2013; Chaib et al., 2022). The accumulation of senescent cells, worsened by declining immune function, contributes to tissue dysfunction and age-related diseases (Dörr et al., 2013). However, eliminating senescent cells and neutralizing SASP through senotherapeutic approaches may help extend health span in aging individuals (Rodier et al., 2009; Kang et al., 2015; Gasek et al., 2021).

2.5 Theories of Aging

Theories form the foundation of science, structuring and interpreting facts. Aging is a complex, multifactorial process driven by oxidative stress, inflammation, DNA damage, telomere attrition, and epigenetic changes, ultimately leading to cellular senescence (Ok, 2022; Marino et al., 2023). Understanding aging at the molecular level is essential for uncovering its mechanisms and developing interventions (Giorgi et al., 2018; Horvath & Raj, 2018). While aging is inevitable, studying its processes can promote healthier aging (Aunan et al., 2017; Zis et al., 2017). Despite recent advancements in molecular biology and genetics, the mechanisms that determines human lifespan remain unravelled (Jin, 2010; Marques et al., 2010; Cordeiro & Wood, 2023). Previously, aging was thought to be a fixed, genetically driven process, with Alexis Carrel's early 1900s experiments mistakenly suggesting that human cells might possess immortality (Reggiani, 2006; Park, 2016). However, in the 1960s, Leonard Hayflick refuted this by establishing the "Hayflick limit," setting a maximum cell division threshold that implies a human lifespan of approximately 115 years (Hayflick, 1965). With modern scientific advancements, numerous aging theories have emerged, considering both intrinsic and extrinsic factors. These theories propose that controlling certain conditions may slow aging and extend lifespan (Finkel & Holbrook, 2000). Biological theories of aging are categorized into two main groups: *Programmed*

Theories and Error (Damage) Theories (Borras et al., 2020). Programmed theories suggest aging follows a biological timetable influenced by gene expression changes that regulate defense, repair, and maintenance mechanisms. In contrast, error theories attribute aging to accumulated damage from environmental stressors (Borras et al., 2020). Some of the prominent biological theories of aging are explained hereunder.

2.5.1 Cellular Senescence Theory of Aging

The cellular senescence theory of aging suggests that DNA damage, oxidative stress, and telomere shortening drive cells into senescence, leading to their gradual dysfunction and impaired division (Bengtson & Settersten Jr, 2016; Guo et al., 2022). Over time, the accumulation of senescent cells accelerates aging. These cells secrete pro-inflammatory molecules, known as the senescence-associated secretory phenotype (SASP), which promotes chronic inflammation, tissue dysfunction, and age-related diseases (Guo et al., 2022).

2.5.2 Telomere Shortening Theory of Aging

The telomere shortening theory of aging states that repeated cell divisions progressively shorten telomeres, ultimately impairing cellular repair and replication, leading to aging (Deka et al., 2022; Goldsmith, 2022). Telomeres are repetitive DNA sequences (TTAGGG) that protect chromosome ends from deterioration and fusion during replication (Cohen & Bryan, 2023). Due to the "end-replication problem," telomeres gradually shorten with each cell division. When critically short, cells enter replicative senescence to prevent damaged DNA replication. Telomerase, an enzyme that extends telomeres, is largely inactive in most adult somatic cells, contributing to gradual telomere attrition and aging (Deka et al., 2022; Cohen & Bryan, 2023).

2.5.3 Mitochondrial Theory of Aging

he mitochondrial theory of aging proposes that mitochondrial function declines over time due to accumulated damage from reactive oxygen species (ROS), by-products of energy production. This damage reduces energy production, increases oxidative stress, and impairs cellular function, accelerating aging (Guo et al., 2022). Mitochondria, essential for cellular energy production through oxidative phosphorylation, generate ROS, which can damage mitochondrial DNA and proteins. Over time, this damage impairs mitochondrial efficiency, leading to decreased energy output and heightened oxidative stress, ultimately compromising cellular function and contributing to aging (Goldsmith, 2022; Umare et al., 2022).

2.5.4 Free Radical Theory of Aging

The free radical theory of aging suggests that accumulated oxidative damage from free radicals disrupts the balance between free radical production and antioxidant defense, leading to cellular dysfunction, inflammation, and age-related diseases (Polidori & Mecocci, 2022). Free radicals are highly reactive molecules with unpaired electrons, produced during normal cellular processes, that can damage lipids, proteins, and DNA. Although cells have antioxidant defense systems to counteract these effects, their efficiency declines with age, allowing oxidative damage to accumulate and accelerate aging (Polidori & Mecocci, 2022).

2.5.5 Inflammation Theory of Aging

The inflammation theory of aging suggests that chronic low-level inflammation, or "inflammaging," contributes to tissue damage, cellular dysfunction, and age-related diseases (Li et al., 2023). With age, the immune system becomes dysregulated, increasing the production of pro-inflammatory molecules like chemokines and cytokines. This persistent inflammation, triggered by factors such as cellular damage,

infection, and senescent cell accumulation, accelerates aging and promotes neurodegenerative and cardiovascular diseases (Polidori & Mecocci, 2022; Li et al., 2023).

2.5.6 Hormonal Theory of Aging

The hormonal theory of aging suggests that declining levels of key hormones, including growth hormone, sex hormones, and insulin-like growth factor 1 (IGF-1), contribute to aging (Bono et al., 2023). Hormones regulate essential processes like metabolism, growth, and reproduction. Age-related hormonal changes impact metabolism, tissue maintenance, and overall health, leading to physiological decline and age-related diseases (Giampapa et al., 2022).

2.5.7 Epigenetic Theory of Aging

The epigenetic theory of aging suggests that accumulated epigenetic changes alter gene expression, impair cellular function, and increase susceptibility to age-related diseases (Guo et al., 2022). Epigenetics involves modifications like DNA methylation and histone changes that regulate gene expression without altering the DNA sequence. Environmental factors, lifestyle, and genetics influence these modifications, which over time can disrupt cellular function and tissue maintenance, accelerating aging (Wang et al., 2022b).

2.5.8 The Glycation and Cross-Linking Theory of Aging

The glycation and cross-linking theory of aging suggests that the accumulation of advanced glycation end products (AGEs), formed by the non-enzymatic reaction between sugars and proteins, contributes to aging and age-related diseases by promoting inflammation and oxidative stress (Addi et al., 2022). AGEs form through a multi-step process, beginning with sugar molecules binding to proteins, lipids, or nucleic acids,

leading to stable compounds that undergo further modifications (Mengstie et al., 2022; Parwani & Mandal, 2023). Over time, AGEs cross-link proteins, reducing tissue flexibility and impairing function, particularly in long-lived proteins like collagen (Kim et al., 2017). Additionally, AGEs can also interact with receptors for AGEs (RAGEs), leading to triggering chronic inflammation and oxidative stress, which contribute to diseases such as diabetes, cardiovascular disorders, cataracts, and neurodegeneration (Kim et al., 2017; Rowan et al., 2018).

2.6 Oxidative Stress: A Unifying Factor in Most of the Theories of Aging

While various aging theories offer unique perspectives, none of the theory fully explain the complexity of the aging process alone (Jin, 2010; Hussain & Kayani, 2020). Also, no single theory fully accounts for the complexities of aging, as the process is too intricate to be explained by a single framework (Hussain & Kayani, 2020). Currently, no consensus exists on a definitive aging theory. Many theories are not mutually exclusive but rather overlap and interact, particularly through oxidative stress, which plays a central role in their interconnection (Johnson et al., 2019; Hajam et al., 2022). Despite its limitations, the free radical theory remains one of the most widely supported aging theories (Park et al., 2022). Growing evidence suggests that reactive oxygen species (ROS) are major contributors to aging, causing damage to mitochondria, proteins, and DNA while also accelerating telomere shortening and the formation of advanced glycation end products. This highlights the interconnectedness of the free radical, telomere, and mitochondrial theories of aging (Juszczyk et al., 2021; Hajam et al., 2022) (Figure 2.1). Further evidence supports the free radical theory, particularly in research on lifespan-extending agents. A key observation is the correlation between species' lifespans, metabolic rates, and antioxidant defense effectiveness (Campisi et al., 2019). Additionally, studies show that enhancing antioxidant enzyme expression in

animals significantly extends lifespan (Park et al., 2022). As a result, plant-derived compounds, especially natural phenols and flavonoids, have gained attention for their antioxidant properties and potential to counteract aging-related effects, such as mitochondrial dysfunction and ROS accumulation.

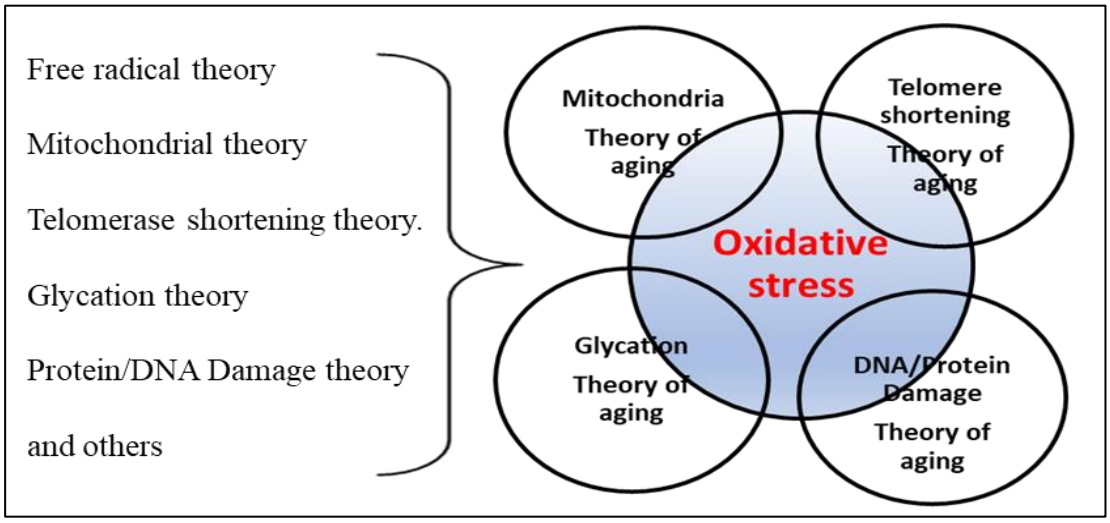

Figure 2.1 Oxidative stress: The common key factor connecting most aging theories

2.7 Oxidative Stress and Antioxidants

Oxidative stress is a complex biochemical phenomenon that occurs at the molecular level in living organisms. It results from an imbalance between the production of reactive oxygen and nitrogen species (RONS) and the ability of biological systems to detoxify these harmful molecules (Hussain & Kayani, 2020). While RONS are vital for cellular processes, their excessive production damages biomolecules like DNA, proteins, and lipids, contributing to aging and disease (Hussain & Kayani, 2020). Cells utilize enzymatic and non-enzymatic antioxidants to neutralize ROS, maintain redox balance, and protect against oxidative damage-related diseases (Papaccio et al., 2022; Sharma & Mehdi, 2023). It has been elucidated by Chakkittukandiyil et al. (2022) that antioxidant enzyme expression and activity are tightly regulated by signaling

pathways, particularly the Nrf2 pathway. Under oxidative stress, Nrf2 is activated, translocates to the nucleus, and triggers the expression of antioxidant response element (ARE) genes, encoding antioxidant enzymes and related proteins (Vomund et al., 2017; Saha et al., 2020)**.**

2.7.1 Reactive Oxygen and Nitrogen Species

Reactive oxygen and nitrogen species (RNOS) are highly reactive molecules containing oxygen and nitrogen atoms, characterized by their unpaired electrons (Hajam et al., 2022). They play pivotal roles in cellular signaling, redox regulation, and various physiological process. However, an imbalance in their production and scavenging leads to oxidative and nitrosative stress, contributing to diseases like cancer, neurodegenerative and cardiovascular disorders, and inflammation (Curieses Andrés et al., 2023). Free radicals arise from intrinsic and extrinsic sources, as well as cellular metabolism, producing reactive oxygen species (ROS) that elevate oxidative stress across tissues and organs. When the body's antioxidant defense fails to counteract this stress, stress-sensitive intracellular signaling pathways are triggered, producing harmful gene products that contribute to aging-related complications (Pizzino et al., 2017). Reactive species include paramagnetic free radicals, such as (superoxide anion ($O_2^{\bullet-}$), hydroxyl radical ($HO^{\bullet}$), peroxyl radicals ($ROO^{\bullet}$), nitric oxide ($NO^{\bullet}$), and nitrogen dioxide ($NO_2^{\bullet}$)), and diamagnetic molecules like (hydrogen peroxide (H_2O_2), peroxynitrite (ONOO-), and nitrous oxide (N_2O)), which form through reactions involving these radicals. Previously regarded solely as toxic agents, some physiological free radicals, like superoxide and nitric oxide, are now recognized as mediators of enzyme- and gene-regulated processes in both normal and pathological conditions (Shields et al., 2021; Tretter et al., 2021). Growing evidence from experimental and

clinical studies suggests that ROS and RNS plays significant roles in the development of aging and age-associated conditions (de Almeida et al., 2020).

2.7.2 Sources of Reactive Oxygen and Nitrogen Species (RONS)

Reactive oxygen and nitrogen species (RONS) are generated within biological systems through both endogenous and exogenous processes (Aranda-Rivera et al., 2022). These species are typically formed via mechanism such as the incomplete reduction of molecular oxygen or electron transfer reactions. The primary sources of RONS include endogenous metabolic processes and external environmental factors (Tabassum et al., 2020; Aranda-Rivera et al., 2022; Berdiaki et al., 2023). Endogenous sources of RONS include mitochondria, where electron leakage during oxidative phosphorylation contributes to ROS production (Tabassum et al., 2020). Enzymatic reactions involving xanthine oxidase, cyclooxygenase, and nitric oxide synthase (NOS) also produce RONS as part of cellular signaling and catalytic cycles (Aranda-Rivera et al., 2022). Additionally, endoplasmic reticulum (ER) stress and immune cell activity during inflammation can trigger RONS generation (Tabassum et al., 2020; Aranda-Rivera et al., 2022). Exogenous sources of RONS include environmental factors such as UV radiation, ionizing radiation, pollutants, and toxins (Aranda-Rivera et al., 2022). The metabolism of xenobiotics, including certain drugs and chemicals, can also generate ROS (Chaudhary et al., 2023). Furthermore, diet and lifestyle factors, such as consumption of specific foods, smoking, and alcohol intake, have been shown to elevate RONS levels (Chaudhary et al., 2023). Together, these endogenous and exogenous sources contribute to the overall oxidative stress within biological systems, influencing cellular function and health.

2.7.3 The Antioxidant Enzyme System

The body's defense against oxidative stress and the maintenance of redox homeostasis relies on a network of antioxidant enzymes. These enzymes neutralize reactive oxygen species (ROS) and free radicals, preventing oxidative damage to cellular structures (Sachdev et al., 2023; Tretter et al., 2021).

Key enzymatic antioxidants include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GSH). SOD is further classified into three types: manganese-containing SOD, Iron-containing SOD, and Copper-Zinc-dependent SOD. The primary defense mechanism against the harmful effect of superoxide ($O_2^{\cdot-}$) involves the action of Superoxide dismutase (SOD), which catalyzes the conversion of superoxide ($O_2^{\cdot-}$) into hydrogen peroxide (H_2O_2) and oxygen (O_2) (Ighodaro & Akinloye, 2018). Catalase then detoxifies H_2O_2 into water and oxygen, preventing cellular damage. Similarly, glutathione peroxidase can also neutralize H_2O_2 using reduced glutathione (GSH), which is converted into oxidized glutathione (GSSG). Glutathione reductase regenerates GSH from GSSG by oxidizing NADPH to NAD^+ (Ighodaro & Akinloye, 2018; Satyanarayana, 2021; Tretter et al., 2021; Sachdev et al., 2023) (Figure 2.2). Glutathione, the major intracellular reducing agent, plays a crucial role in protecting cells from lipid peroxidation. A decline in GSH levels may indicate increased oxidative stress (Hajam et al., 2022).

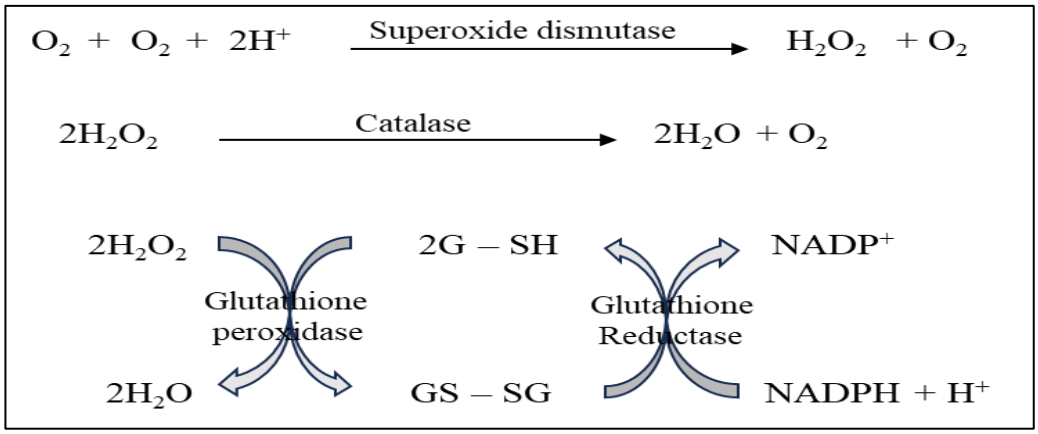

Figure 2.2 The antioxidant enzyme system.

GSH: reduced glutathione; GSSG: oxidized glutathione. Reduced GSH detoxifies H_2O_2; peroxidase catalyzes this reaction. NADPH is responsible for the regeneration of reduced glutathione from its oxidized form. (Adapted and modified from Satyanarayana, 2021).

2.7.4 The Non-enzymatic Antioxidants

Notably, at times, certain reactive oxygen species can bypass the action of the antioxidant enzyme system. In such instances, the escaped free radicals can be neutralized using the none-enzymatic chain-breaking antioxidants such as water-soluble ascorbate (Vitamin C), lipid-soluble vitamin E, and ubiquinone (Kurutas, 2015; Shields et al., 2021). These antioxidants counteract oxidative stress by donating electrons or hydrogen atoms to stabilize ROS, preventing damage to biomolecules like proteins, lipids, and DNA (Mirończuk-Chodakowska et al., 2018). Key non-enzymatic antioxidants include vitamins [Vitamin A (β-carotene), Vitamin E (α-tocopherol), and Vitamin C (ascorbic acid)], which directly neutralize ROS (Kurutas, 2015; Shields et al., 2021). Additionally, various plant-derived compounds (e.g., flavonoids and polyphenols) are antioxidant compounds found in various plant-based foods, they possess antioxidant properties and can scavenge ROS (Sedighi et al., 2017; Zhang & Tsao, 2016). Furthermore, antioxidant minerals (e.g., Selenium, Manganese, Copper, Zinc, Chromium etc.) don't possess antioxidant activity on their own but are essential for the functioning of certain antioxidant enzymes (Abbas et al., 2018). Other non-

enzymatic antioxidants include Tripeptides (e.g., Glutathione. (GSH), a tripeptide presents in high concentrations in cells, which serves a central role in non-enzymatic antioxidant defense by participating in redox reactions and neutralizing ROS (Mirończuk-Chodakowska et al., 2018). Also, Metal-binding proteins (e.g., Some proteins, like ferritin, sequester iron, a metal that can catalyze harmful Fenton reactions, mitigating the generation of hydroxyl radicals (˙OH) (Valko et al., 2016).

2.8 Biochemical Mechanisms Connecting Aging, Oxidative stress, and Antioxidants

A key contributor to the aging process is oxidative stress, which occurs when the body produces more reactive oxygen species than its antioxidant defense can effectively neutralize (Das et al., 2023). ROS are highly reactive byproducts of cellular metabolism, particularly aerobic respiration. As aging progresses, antioxidant defense mechanisms become less efficient, increasing susceptibility to oxidative stress (Chaudhary et al., 2023). Over time, accumulated oxidative damage—triggered by environmental toxins, radiation, and metabolic processes—affects essential cellular components, including lipids, proteins, and DNA. This damage can impair DNA replication, induce cellular senescence, and contribute to tissue and organ dysfunction, leading to age-related disorders such as cardiovascular diseases, neurodegenerative conditions, and certain cancers (Chaudhary et al., 2023). The intricate relationship between aging, oxidative stress, and antioxidants underscores the balance between cellular damage and antioxidant defense. Research suggests that enhancing antioxidant systems can mitigate oxidative stress, reducing cellular damage (Pisoschi et al., 2021; Roychoudhury et al., 2021; Verhaegen et al., 2022). Therefore, understanding this interplay is crucial for developing strategies to promote healthy aging and extend lifespan (Santos et al., 2021; Chaudhary et al., 2023).

2.9 Biochemical Mechanisms Connecting Aging process, Telomere and Telomerase

Telomeres and telomerase activity are intricately linked to the aging process and have a significant role in this context (Ferrucci et al., 2020). Telomere shortening is a natural outcome of cell division and serves as a contributing factor to cellular senescence and the aging process (Fathi et al., 2019). Nevertheless, the enzyme telomerase has the potential to counteract telomere attrition, but its regulation is complex, and its use as an anti-aging intervention requires further investigation (Ferrucci et al., 2020).

2.9.1 Telomere and Telomerase

The study of telomere biology began centuries ago with the work of scientist Hermann Muller and led to various discoveries (Blackburn, 2000; Chakravarti et al., 2021). In 2009, Elizabeth Blackburn, Carol Widney Greider, and Jack William Szostak received the Nobel Prize in Physiology or Medicine as award for their pioneering findings on telomerase in model organisms like ciliate *Tetrahymena thermophilia* (Varela & Blasco, 2010). Among their various findings on telomeres and telomerase include the discovery of the mechanisms by which telomeres safeguard the chromosomes (Varela & Blasco, 2010). In addition, many studies have clearly demonstrated that telomere function and telomere replication are intricately connected to both the process of aging and cancer development in human cells (Harley & Villeponteau, 1995; Zakian, 2012).

2.9.2 Molecular Structure of Telomere and its Function

The term *telomere* originates from the Greek words *telos* (end) and *meres* (part), referring to the terminal regions of eukaryotic chromosomes. Telomeres consist of non-coding, tandemly repeated DNA sequences (5’-TTAGGGTTAGG-3’)n at the ends of

both chromosome arms (Jain & Cooper, 2010) Their linear structure in eukaryotic DNA creates an *end replication problem*—during cell division, the very ends of one DNA strand cannot be fully copied, leading to progressive chromosomal shortening (Kumari & Jat, 2021). Without telomeres, critical genes at chromosome ends would be lost with each replication cycle, threatening cell survival. Instead, telomeres form protective caps that prevent this loss (Stewart et al., 2012). In humans and other vertebrates, telomeres contain hexanucleotide (5'-TTAGGGTTAGGG-3')n tandem repeats (Moyzis et al., 1988). The structure of telomeres can trigger DNA damage responses if their ends are mistakenly identified as double-stranded DNA brakes by the DNA repair machinery. Therefore, to prevent this, telomeres are safeguarded by shelterin, a specialized protein complex (Ghilain et al., 2021) (Figure 2.3). For this reason, telomeres are referred to as protective end-caps that protect the end part of the DNA from unfolding. Shelterin not only protects telomeres from DNA damage responses—preventing fusion events that could lead to chromosomal abnormalities and cancer—but also stabilizes the *D-loop-T-loop* structure and regulates telomere elongation (Mir et al., 2020; Ghilain et al., 2021). The shelterin complex consists of six proteins: TRF1 and TRF2, which bind double-stranded telomeric DNA, and their associated partners RAP1 (linked to TRF2) and TIN2 (linked to TRF1). Additionally, the single-stranded overhang is bound by POT1, which connects to TPP1. TPP1 serves as a molecular fulcrum, linking POT1 and TIN2, facilitating telomerase recruitment and activation (Rajavel et al., 2014; Jafri et al., 2016; Wang et al., 2020) (Figure 2.3).

Each shelterin protein plays a distinct role—TRF2 shapes telomeric DNA to facilitate *t-loop* formation, RAP1 supports TRF2, and POT1-TPP1-TIN2 is critical for telomerase activation (Veverka et al., 2019; Wang et al., 2020). Telomeres are essential for chromosome stability, yet they gradually shorten with each cell division due to