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Effects of Creatine and Nicotinamide on Experimentally Induced Senescence in Dermal Fibroblasts

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EFFECTS OF CREATINE AND NICOTINAMIDE ON EXPERIMENTALLY INDUCED SENESCENCE IN DERMAL FIBROBLASTS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

by

AVINASH SATYANARAYAN MAHAJAN
B.Pharm., SRM Institute of Science and Technology, India, 2018

2020
Wright State University
I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPER-VISION BY Avinash Satyanarayan mahajan ENTITLED Effects of Creatine and Nicotinamide in Experimentally Induced Senescence in Dermal Fibroblasts BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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Mahajan, Avinash Satyanarayan. M.S., Department of Pharmacology and Toxicology, Wright State University, 2020. Effects of Creatine and Nicotinamide in Experimentally Induced Senescence in Dermal Fibroblasts

Dermal fibroblasts provide structural support by producing collagen and other structural/support proteins beneath the epidermis. Fibroblasts also produce Insulin-like growth factor-1 (IGF-1), which binds to the IGF-1 receptors (IGF-1Rs) on keratinocytes to activate signaling pathways that regulate cell proliferation and cellular responses to genotoxic stressors like ultraviolet B radiation found in sunlight. Our group has determined that the lack of IGF-1 expression due to fibroblast senescence in the dermis of geriatric individuals is correlated with an increased incidence of skin cancer in geriatric patients. The present studies were designed to test the hypothesis that pro-energetics like creatine monohydrate and nicotinamide can protect fibroblasts against senescence. To that end, we used an experimental model of senescence in which primary human fibroblasts are treated with hydrogen peroxide (H₂O₂) in vitro, with senescence measured by staining for beta-galactosidase activity (+beta-gal), p21 protein expression and senescence associated secretory phenotype (SASP) cytokine mRNA levels. We also determined the effect of H₂O₂ on IGF-1 mRNA and protein expression. Our studies indicate that pretreatment with creatine monohydrate or nicotinamide protects human fibroblasts from the H₂O₂-induced cell senescence. These studies suggest a potential strategy for protecting fibroblasts in geriatric skin from undergoing stress-induced
senescence, which may maintain IGF-1 levels and therefore limit carcinogenesis in epidermal keratinocytes.
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Introduction

1.1 Statement of problem

Dermal fibroblasts synthesize IGF-1 which binds to its transmembrane IGF-1 receptor (IGF-1R) on the keratinocytes. Human keratinocytes cannot synthesize IGF-1 but when the IGF-1R gets activated it initiates several pathways which regulate cell survival, metabolisms, and apoptosis. When dermal fibroblasts undergo senescence, their ability to produce IGF-1 is impaired. Hence IGF-1R is not activated. This results in abnormal behavior of the keratinocytes which is exacerbated in stressful conditions like UV radiation or reactive oxygen species. Also, accumulation of senescent fibroblasts may pose a threat to the microtissue environment by inducing senescence in neighboring cells/tissues. Finding a solution to this problem may reveal novel therapies to combat senescence and senescence related diseases.

1.2 Significance

If dermal fibroblasts are protected against senescence, it may provide important insights into combating age-related skin diseases while ensuring appropriate response of keratinocytes to stress conditions.
1.3 Statement of purpose

The purpose of this project is to study the ability of creatine monohydrate and nicotinamide to protect dermal fibroblasts against senescence. We intend to know if treatment with nicotinamide or creatine can promote IGF-1 production while attenuating certain senescent phenotypes in fibroblast cells.

1.4 Hypothesis

Our main hypothesis is that pro-energetics like creatine or nicotinamide can protect human dermal fibroblasts from undergoing experimentally induced senescence.

1.5 Research objectives

- To demonstrate the effects of creatine monohydrate, nicotinamide and H₂O₂ on dermal fibroblasts.
- To demonstrate the effects of creatine monohydrate and nicotinamide on IGF-1 production and attenuation of the senescent phenotypes on stress-induced senescent fibroblast
1.6 Definitions

**Beta-Galactosidase:** It is a glycoside hydrolase enzyme which cleaves complex sugars to monosaccharides. This is an adaptation of the senescent cells to fill energy gaps.

**Creatine:** It is a pro-energetics used mostly in the bodybuilding industry. It boosts ATP production which leads to hypertrophy. It is also involved in several pathways in the mitochondria. It is non-essential and about 1g/day is produced in young adults.

**Dermal fibroblasts:** Cells present in the dermal layer of the skin which generate connective tissue and participate in wound healing.

**DMSO:** It is known as the universal solvent. They increase the permeability of compounds in the cells at low concentrations.

**Dulbecco’s Modified Eagle Medium (DMEM):** Growth media used to cultivate mammalian cells in vitro.

**IGF-1:** It is a growth hormone protein which is encoded by IGF-1 gene. It is primarily produced in the liver but other cells like muscle cells and dermal fibroblasts can synthesize it for regulating certain biological metabolic pathways.

**IGF-1R:** Transmembrane receptor present on many cell types (including the keratinocyte) to which IGF-1 binds.

**Nicotinamide:** It is an amide form of vitamin B3. It is an essential nutrient whose uses are well established in the field of cancer.

**p21:** It is a cyclin dependent kinase inhibitor which is a mediator of the p53 dependent cell cycle arrest due to DNA damage.
**PBS:** Phosphate Buffered Saline is a buffer solution that lacks calcium and magnesium. Its pH is suitable for various cell culture applications such as washing cells.

**Reactive Oxygen Species (ROS):** Reactive molecules containing oxygen molecules likesuch as superoxide, peroxides and hydroxyl radicals.

**Senescence Associated Secretory Phenotype (SASP):** These are inflammatory cytokines, immune modulators, growth factors, and proteases which are released by senescent cells.

**Senescence:** An irreversible growth arrest of the cells.

**X-gal:** It is a stain used to detect Beta galactosidase in cells.
Literature review

2.1 Aging

Micheal Rose defined aging in his thesis as *decline or loss of adaptation with increasing age, caused by a time progressive decline of Hamilton’s forces of natural selection* (1). Aging is inevitable and is characteristic of steady decline in physical, mental, and physiological activities (2). Evolutionary biologists suggested that it is in fact necessary for a race to survive and is a part of the natural selection to make resources available for younger generations. Recent attempts have defined aging as the impairment of cellular function along with attenuation of its ability to respond appropriately to stress and survival (3,4,5). This decline in certain abilities is seen in every organism that ages, ranging from a single cell to the most complex creatures. The age-related phenotype is well observed in mammals. These phenotypes can be visual signs of aging and/or age-related pathologies like macular degeneration, renal failure, osteoporosis, atherosclerosis, cardiac infarction, neurodegeneration, and many others (6,7). Evidence suggests that age related pathologies start to rise at the midpoint of a species lifespan and degeneration of a few tissues is quite common (8). In 1965 Hayflick found that human diploid cells in vitro have a finite lifetime and are indirectly related to the number of sub-cultivations at a ratio of splitting. Cloned cells of the diploid strains had the same doubling potential (9).

Contrary to the present developments, somatic mutations were believed to be the cause of aging (10,11). In 1958, Gioacchino Failla, an Italian American physicist, proposed that somatic mutations cause aging (12). This hypothesis was advocated by Leo Szilard in
1959. He mentions that a cell faces the most functional decline when in a chromosomal pair, either or both suffer aging, or if just one possibly hits aging and the other has an inherited defect (13). Harman D, in 1962, supported Failla’s hypothesis and bolstered it with the concept of the role of free radicals in aging (14). Alexander was the pioneer to hypothesize that DNA damage induces mutations and may cause aging. He implied that somatic mutations do not initiate the process of aging, based on certain experiments. However, he proposed that post mitotic cells tend to die as DNA damage gets accumulated in them throughout their lifespan. This hinders protein synthesis until it is lethal. Bernstein and Gensler provided arguments in the support of Alexander’s hypothesis in 1981. Tice and Setlow (1985), analysed various evidence and concluded in the support of the hypothesis that aging is caused due to damage in the DNA. This was also reviewed by several others, including Hart (1979), Gensler (1987), Ames (1985), Rattan (1989) and Hanawalt (1987). (1)

2.1.1 Theories of aging

Several theories were suggested to understand the causes of aging. Evolutionary theories of aging believe that aging has been a result of evolution to limit life span to benefit the younger generations (15,16). Some theories believe that it is random and that it is not of any significant value. Mutational accumulation is one of the proposed mechanisms which suggests that the decline in natural selection is due to reduction of adverse events after the peak of reproduction phase (17,15). Yet another theory throws light on aging between different species. According to this theory organisms with low reproduction rates and high survival use more resources to maintain somatic functions and the contrast happens
with organisms with high reproduction rates and low survival. This theory is called as the disposable soma theory (18,19).

The cellular theory of aging is based on the belief that immortal cell lines share many characteristics related to malignant cells (9). In 1930s, during cell culture technique advancements, it was established that a cell strain, irrespective of its origin, when cultivated in vitro can maintain a state of multiplication actively, for varied but a finite time period (20). Changing the composition of culture media and incubation conditions did not affect the aforementioned events. However, a contradiction to this was claimed by Carrel, stating that embryonic chick heart cells were kept in serial culture for 34 years (9). Despite this claim, no modern cell culture techniques have been able to sustain embryonic chick heart cells for more than a year, which invalidates Carrel’s claim. Since then, several cell lines have been established from mammalian tissues that multiply indefinitely. These cell lines were concluded to share certain properties with cancer cell lines. They are heteroploidy like tumor cells, which under suitable conditions they transform into tumor masses and other experimental methods have proved the similar characteristics of the cells to the description of tumor cells. But diploid cells do not exhibit these features. Since heteroploid cells are similar to tumor cells, only diploid cells are related to the cellular theory of aging, as they are mortal (9).

The DNA damage and repair theory suggests that decline in repair capacity or defect in the mechanism of repair causes gradual decline of cellular functions and a few alterations, leading to aging (21). Repair pathways like mismatch repair, homologous recombination and non-homologous end joining are reduced in non-dividing cells (22). Lack of repair causes accumulation of DNA damage forming adducts and lesions in the
genome and hence causing aging and tumor formation. Embryonic stem cells and germline cells don’t build up DNA damage as they repair mechanism is very efficient or the repair mechanism defective cells are immediately replaced with healthy ones, thereby decreasing the chances of inheriting mutations by daughter cells (23).

The free radical theory, as first suggested by D Harman states that aging is a consequence of formation of free radicals (14,24). Internal reactive oxygen species (ROS) forms up due to leaky respiration of mitochondria. A study on the mononuclear cells of humans reported the associations of fatigue and reduced vitality with DNA damage and ROS (25). However, it is argued that oxidative stress cannot cause aging, or its intervention delay the process. This is supported by studies which report antioxidants to be ineffective in increasing survival rates (26). The mitochondrial theory of aging is in lieu with the free radical theory. It states that mitochondrial DNA (mtDNA) damage causes functional decline of the cell. The mitochondrial membrane becomes susceptible to ROS leakage gradually over time. This supports the report that the mutations in mtDNA happens at faster rate than nuclear DNA (27).

### 2.2 Cellular senescence

The word senescence is derived from a Latin word *senex* which means old age. Biology defines senescence as a chain of deteriorative events that are followed by maturation and is linked with aging (28). When cells ceased to replicate in vitro, it was speculated that this behavior is repeated in the process of aging. These cells are at a risk of transforming
into tumorigenic cells. Before undergoing senescence, a cell is termed as pre-senescent or young. The cause for a pre-senescent cell to transform into a senescent one includes various stimuli (29). In fact, dysfunctional processes like DNA damage, oxidative stress, telomere shortening, organizational trepidation of the chromatin and other stressors are major causes (30,31,32).

Hayflick’s findings established that cells gradually cease division after definite replications. This paved way for two hypotheses. The first one stated that since it is known that cancerous cells proliferate indefinitely in culture, senescence may be a solution and act as a tumor suppressive mechanism (33). The second hypothesis stated that the behavior of a senescent cell is that which is seen in aging and hence it may be deleterious to the regenerative capacity of a cell (34). On pursuing these hypotheses new revelations were made in the field on cancer and aging. Senescent cells gradually develop in an environment which is filled with stressors. This affects the lifespan of an organism. A senescent cell suppresses tumorigenic effects as well the regenerative capacity of a cell. Therefore, the tumor suppressing activity was needed to be utilized for a short interval so that it does not affect the young functioning cells, hence minimizing the deleterious effects. This may prove to be beneficial to the young but may be otherwise for the old (29).

Mitotic cells proliferate and can also attain a reversible arrested state called quiescence (35,36, 37). In response to certain signals, quiescent cells resume proliferation. Such example would be tissue repair/ regeneration. Whereas, post-mitotic cells cease division permanently due to differentiation (37). Senescence is limited to mitotic cells. When these cells encounter various stressors, they irreversibly enter the growth arrest phase.
Not only do they stop proliferating but also obtain resistance to apoptotic signals. Their altered gene expression expresses characteristic senescent phenotypes. (29)

![Image of senescent phenotype](image)

**Figure 2.1** Various stressors turning presenescence cell into a senescent one (31).

### 2.2.1 Growth Arrest

A distinctive feature of cellular senescence is that cell is arrested in G1 phase of the cell cycle. They are not responsive towards growth factors and cease DNA replication. These features may vary depending upon the genetic make-up of the cell. Senescent WT mice fibroblasts showed G1 DNA content whereas a defect in the MKK7 signaling kinase caused a G2-M arrest (38). Even though it is well established that tumor cells proliferate indefinitely, treatment with certain anti-cancer agents has caused tumor cells to undergo
senescence (39,40). Findings so far suggest that growth arrest indeed happens during senescence, but the mechanism may differ based on the type and origin of the cell.

2.2.2 Resistance towards apoptosis

Apoptosis is a normal event that occurs as a tissue or organism develops. It is similar to the tumor suppressive effect caused by senescence (41,42). This resistance is adopted by many cells but not all and the mechanism may also differ. Human fibroblasts resist apoptosis when it is induced by ceramide. Endothelial cells lack this resistance (43). Although human fibroblasts are impervious to many types of apoptotic signals, they lack resistance to Fas receptor involved apoptosis (44,45). Therefore, whether a cell undergoes senescence or apoptosis can be manipulated using different stimulants.

2.2.3 Gene expression alteration

Senescence alters gene expression in a cell. Certain tumor suppressive genes are highly expressed which up regulates tumor suppressor pathways. Cell cycle inhibitors like p21 and p53 initiate a cascade of events which induces a senescence like growth arrest (46). Not only these senescent cells encode certain proteins which when released can change the surrounding tissue environment (47). On the other hand, senescent cells repress the encoding of proteins which promote cell cycle progression (48,49,50,51). Hence, senescence stage affects the regulation of certain genes, alters the micro tissue
environment, and possibly increases age related defects as senescence cells increase with age.

2.3 Causes of senescence

2.3.1 DNA damage

Benjamin Lewin defined DNA damage as- “Damage to DNA consists of any change that introduces a deviation from the usual double-helical structure”. A better adaptation of this would be- "Any modification in the physical and/or chemical structure of DNA resulting in an altered DNA molecule which is different from the original DNA molecule with regard to its physical, chemical and/or structural properties" (52,53).

Various factors affect the integrity of a genome. DNA damage is caused due to oncogenic stress, oxidative stress, defects in DNA repair mechanisms and by replicative exhaustion. Wearing off telomeres triggers cellular senescence upon replicative exhaustion (54,55,56,57). Telomeres prevent DNA damage response (DDR) to initiate on the chromosomes (58,59). DDR initiates when these telomeres shorten. Depletion of DDR factors impairs telomere maintenance (60,61). Telomere caps are formed when double stranded DNA along with telomere forms a T-loop. These caps are worn off due to stress. Telomere replication is carried out by telomerase and their somatic expression is incapable of recovering from telomere loss. Similar consequences are seen in when telomere attrition happens due to stress. This stress may be due radiation, reactive oxygen species (ROS), or carcinogens. These stressors cause single and double stranded breaks and due to simultaneous defects in the repair mechanism, keeps the damage unrepaired.
This induces DDR signaling persistently. This initiates a cascade of pathways which lead to senescence. (62,63)

![Figure 2.2 DNA damage in replicative and stress induced senescence (62).](image)

**Figure 2.2** DNA damage in replicative and stress induced senescence (62).

### 2.3.2 Reactive Oxygen Species

ROS are the consequences of aerobic metabolism (64). They include free radicals like hydroxyl radicals (‘OH), superoxide anions (O$_2^-$), and non-radical oxygen singlet and hydrogen peroxide (H$_2$O$_2$) (65,66,67). ROS is produced upon reduction of molecular oxygen by radiation or electron transfer reactions (68). When ROS levels increase in the cells, it induces a state known as oxidative stress. This state damages lipids, proteins, nucleic acids and can result in the inhibition of multiple types of enzymes (69,70). This intracellular oxidation is associated with senescence. Hydrogen peroxide, which is a major cause of oxidative stress, drives primary cells towards senescence (71,72). The
concentration of hydrogen peroxide impacts the levels of ROS accumulated in the cells, which in turn regulates the fate of the cells. Lower ROS levels causes senescence, whereas high concentration induces apoptosis. Lower levels of ROS triggers the p53/p21 pathway, triggering growth arrest (73).

Figure 2.3 Pathway causing ROS and leading to senescence (71).

2.3.3 UV (ultraviolet) radiation

UV radiation is comprised of three main components, namely UVA, UVB and UVC. UVC (200-280 nm) does not pass through the ozone layer, therefore exposure to it is difficult through natural means. On the other hand, UVA (315-400 nm) and UVB (280-315 nm) penetrate the skin and in the case of UVA can reach up to the dermis layer (74). Due to higher wavelength, UVA penetrates deeper through the skin (see Figure 2.4). They generate ROS by interacting with chromophores and photosensitizers, leading to damaged DNA (75,76). DNA generates dipyrimidine photoproducts upon interacting with UVB (77). This triggers the initiation of several pathways related to senescence,
growth, and tissue degeneration. Sub-lethal doses of UVB caused premature senescence in dermal fibroblasts (78,79,80). This was correlated with increased expression of p53, p21 and p16, involved in arrest of the cell cycle. Intracellular peroxide also was noted to increase in UVB exposed cells. Similar behavior was seen in human keratinocytes, with altered gene expression and increased ROS levels (81).

**Figure 2.4** UVB radiation on human skin (82)

**2.4 Pathways of senescence**

Growth arrest due to senescence is mostly regulated by tumor suppressor pathways p53 and p16-RB (83). These pathways can interact together as well as function independently. These are initiated on stress stimuli. The tendency of cells to engage with these pathways is regulated by cell type and differences among species.
2.4.1 p53 pathway

Telomere dysfunctions and ionizing radiation which generate DNA damage response (DDR) cause senescence by the p53 pathway (84,85). HDM2 protein (in human) and MDM2 protein (in mice) regulate the p53 points at multiple points (86,87,88). They promote p53 degradation and HDM2 inhibition by alternate reading frame protein (ARF) (89,90). p21 is important in regulating senescence in a p53-dependent manner. p21 also causes growth arrest by mediating transient DNA damage. The exact mechanism which determines whether p21 causes senescence or transient growth arrest is not as yet known. Reduction of p21, p53 and DDR proteins has shown to attenuate DNA/telomere damage induced senescence and reverse cellular senescence in few cells (91).

2.4.2 p16-RB pathway

DDR causing stimulus may initiate p16-RB pathway that often occurs after the initiation of the p53 pathway (92). Epithelial cells are prone to initiate this pathway more than the dermal fibroblasts. Moreover, telomere disruption initiates p53 pathway in mouse cells. However, both p16 and p53 are engaged in human cells. ETS transcription factors are activated by RAS which induces the expression of p16. Mechanisms on how p16 regulation is stimulated is not well understood. It is postulated that attenuation of INK4a repressors like CBX7 and BMI1 induces p16 involved senescence (93,94,95). Overexpression of these repressors improved the replicative lifespan of human and mice fibroblasts (96,97,98)
Cells resume growth and replication upon inactivation of p53, which undergo senescence solely through the p53 pathway. RAS induced p16 expression can temporarily arrest growth, but limited proliferation can be obtained upon inactivation (99,100,101). However, cells that follow the p16-pRB pathway do not acquire lost proliferation ability despite inactivation of p53 and p16 pathways. p16 pathway generates senescence associated heterochromatin foci (SAHF) which silences genes responsible for proliferation of a cell (102,103). Simultaneously, while the SAHF develops, chromatin altering proteins undergo transient interactions. Once SAHF has been established it does not require p16 for its maintenance. This enables repressive chromatin, which makes the cellular senescence irreversible. While SAHF are absent in senescent cells, p16 pathway may induce their expression (104).

**Figure 2.5** Diagram depicts the p53 and p16 pathway of senescence (105)
2.5 Senescence Associated Secretory Phenotype (SASP)

Phenotype of a cell in an area and the physiochemical properties of factors around the cells in a tissue defines the tissue microenvironment. The factors that influence tissue microenvironment can be physical features like temperature, oxygen levels and certain chemical molecules like growth factors and cytokines. This microenvironment regulates behavior of a cell in the vicinity of the tissue. Microenvironment that is permissive, aggravates tumor growth. Accompanied by gene alterations, tumor cells evade inhibitive mechanisms and turn malignant. Cellular senescence, which is marked by cell growth arrest, promotes tumor suppression. However, studies show that certain secretory alterations change the microenvironment and promote oncogenic activities. This altered secretome is termed as SASP. Studies suggest the possibility of the existence of SASP secreted from fibroblasts of varying age group donors (106,107,108,109).

These factors are broadly classified into three groups based on their molecular mechanisms.

1) **Receptor binding factors:** These include cytokines (including TNF-α), chemokines and growth factors. The soluble signaling molecules bind to their respective surface receptors and initiate various cellular mechanisms. (110,111)

   **Interleukins:** IL-6, IL-8, IL-1α

   **Chemokines:** CCL-2, CCL-5, CCL-16, CCL-26, CCL-20, GRO-α, GRO-β.

   **Growth factors:** HGF, TGF, FGF, TGF-β and GM-CSF.
2) **Direct factors:** These factors cause the breakdown of membrane bound proteins and interfere in signaling mechanisms. This enables modification of microenvironment by the senescent cells. This group includes:

**Metalloproteases:** MMP-1, MMP-3, and MMP-10.

**Serine proteases:** urokinase plasminogen activator (uPA) and tissue plasminogen activator (tPA) (112, 113)

3) **Regulatory factors:** These components lack enzymatic activity and regulate the functions of direct factors and receptor binding factors by binding to them. For example, MMPs activity is inhibited by TIMP (114) and tPA and uPA activities are inhibited by PAI-1 (115).

### 2.5.1 Mechanism of SASP regulation

Cellular senescence is a time-consuming process and a gradual phenomenon. SASP is released in different phases. The preliminary onset begins when DNA damage occurs and may express such phenotype for about 36 hours. The following phase lasts for about 4-10 days and several SASP factors start to appear and intensify over time (116). Cells can participate in transcriptional and post transcriptional activities of SASP. SASP proteins like IL-8 and IL-6 are regulated by nuclear factor kappa light chain enhancer of active B cells (NF-kB) (117, 118, 119). Identification of DDR is necessary for post transcriptional regulation of SASP (120). Silencing DDR components has shown to reduce IL-6 and IL-8 expressions (121, 122, 123). Even though such evidence exists, the mechanism through which DDR is customary to SASP regulation is still to be understood. Recent studies have found components which trigger the DDR and eventually regulating SASP. ATM-
NEMO complex, GATA4 have shown to regulate DDR (124,125). There are some SASP regulatory mechanisms which are independent of DDR and stress-kinase p38 plays an important role in such mechanisms. Stress-kinase p38 activates the p16/Rb pathway and drives cells into senescence. Studies show that prevention of SASP release is associated with suppressed p38 expression (126). Due to the immense diversity of SASP components, and their varied regulations, it is difficult to give credit to a specific protein or mechanism. Many pathways are directly or indirectly associated with SASP release. This field is yet to be explored to substantially claim the molecular mechanisms of SASP components.

2.5.2 Function of SASP

When senescent cells release SASP components, they attract immune cells towards the specific tissue location and help in getting rid of senescent cells. This mechanism properly functions in healthy young organisms. However, this may not be seen in geriatric organisms or organisms with certain health complications. Since this regulation of immune cells signaling is impaired, it leads to the accumulation of senescent cells in the tissue microenvironment. Therefore, the positive side of SASP release is to eliminate temporary senescent cells, whereas the negative aspect is that it can cause chronic inflammation and other senescence related diseases upon persistent accumulation of senescent cells (127).

SASP components affect adjacent cells via the auto/paracrine pathway. This causes cell cycle arrest of the neighboring cells as well. It is followed by growth arrest and
development of senescence. When conditioned media, obtained from aged fibroblasts, was exposed to normal cells, the high levels of IL-6 and IL-1 factors increased ROS levels, DNA damage and eventually senescence (128). Studies show that inhibiting receptor binding of these compounds can prevent the onset of senescence in fibroblasts (129). Not only do SASP initiate cell cycle arrest but its deleterious effects have been associated with cancer progression. Increased amounts of IL-6 and IL-8 have made breast cancer cells resistant to therapy (130). The dual role of SASP is an interesting aspect to study about. While its effects may turn out to be beneficial in young tissues by promoting growth, proliferation and elimination of senescent cells, its effects may be otherwise in geriatric or functionally disabled cells.

2.6 IGF-1 receptor and senescence

Dermal fibroblasts occupy the dermal space beneath the epidermis. They are responsible to synthesize connective tissue (131). They also secrete IGF-1. Transmembrane IGF-1 receptors are activated when IGF-1 secreted by the dermal fibroblasts are bound to them. This initiates several pathways responsible for cell survival, proliferation, and apoptosis. However, in geriatric skin, dermal fibroblasts undergo senescence. This impairs the ability of the cells to secrete IGF-1(132) and causes human keratinocytes to behave inappropriately. DNA damage checkpoint signaling, and nucleotide excision repair are found to be damaged in keratinocytes with inactive IGF-1 receptors (133). The keratinocytes accumulate DNA damage when stressors like UVB or oxidative stress are exposed from time to time. Persistent DNA damage can cause malignancy (134).
2.7 Creatine

Primarily found in food via meat sources, Creatine is a nitrogenous organic acid. While mostly found in muscles; trace amounts of creatine have been found in brain and testes. Creatine can be endogenously synthesized from amino acids in the kidney and liver, producing about 1-3 g/day in the body. It is a proenergetic, as its major function is to phosphorylate ADP into ATP which provides energy. It has also shown to elevate IGF-1 mRNA expressions in adults consuming creatine. Its anticatabolic effect on the cell serves as antioxidant and reduces ROS levels. Its benefits have widely been established in muscle cells and is being applied in the field of sports and nutrition. It has also shown to play protective effects against mitochondria degradation, and age-related neurological disorders. Its effects in senescence have not been understood clearly(135).

Figure 2.6 Flowchart of endogenous creatine synthesis (135)
2.8 Nicotinamide

The water-soluble form of niacin is termed as nicotinamide. It cannot be endogenously synthesized, hence is obtained from exogenous sources, mostly from diet. Nicotinamide is the precursor of NAD⁺ which is a coenzyme that participates in the salvage pathway. Nicotinamide is known to increase the synthesis of NAD⁺. It has shown positive effects on cell survival in many cell types. It is also known to promote fetal cell maturation, differentiation of embryonic stem cells to cells producing insulin. In the case of ischemic infarction, it protects brain cells from oxidative stress. It also plays a protective role on islet cells when free radical exposure is aggravated. Human primary fibroblasts had increased cellular lifespan when treated with nicotinamide. It has known protective effects against ROS levels as well. Its role in combating cellular senescence needs to be understood more clearly. (136)

![Nicotinamide as a precursor of the salvage pathway](image)

**Figure 2.7** Nicotinamide as a precursor of the salvage pathway (137).
Materials and methods

3.1 Introduction

This chapter discusses the materials, procedures and analyses performed to generate results corresponding to the aims of this study. All studies have been employed using in vitro model of senescence in dermal fibroblasts.

3.2 Cell culture

Neonatal dermal fibroblasts were used in this study. These were obtained as frozen cells and cultivated in a 100mm plate primarily. These cells are flattened, elongated and spindle shaped. These primary fibroblasts are capable of responding to various treatments given in vitro. These cells were cultured in plates of different sizes suitable for the experimental procedures in this study.

3.2.1 Cell growth and media storage condition

Neonatal dermal fibroblasts were grown in DMEM media consisting of low glucose, 100 U penicillin/ 0.1 mg/mL streptomycin (5mL), 2 mM glutamine (5 mL), and 50 mL 10% FBS FetalClone III. The cells were incubated at 37°C in a incubator containing 5% CO₂ and 20% O₂.
3.2.2 Cell passage

Media was removed from cells and plates were washed twice with PBS 1x. 2 ml of 0.05% trypsin-EDTA 1x was added to each plate and placed in the incubator for 3-5 minutes. Then upon dissociation of the cells from the plate, 8 ml of DMEM low glucose media was added to each plate to bring the volume to 10 ml and the cells were triturated. About 2 ml of the media containing cells were added to new plates and 8 ml media was added to bring up the volume to 10 ml. Before treating the cells, they were counted before adding to new plates to maintain equal number of cells in each plate. For consistency, cells were counted, grown and trypsinised at the same time intervals.

3.2.3 Changing media

To change media, old media was removed from the culture plate and washed twice with PBS 1X. 10 ml of fresh media was added. Media is changed every two days between passaging.

3.2.4 Cell counts

Cells were washed with 10 ml PBS 1X twice. 2 ml of 0.05% trypsin-EDTA was added and returned to the incubator for 3-5 minutes. As soon as the cells were detached, 8 ml of media was added. 10 µl of this suspended cell mixture was added to 10 µl of Trypan blue stain. 10 µl of the resulting mixture was pipetted onto a slide and the cell count was obtained on the Countess machine. For accurate values, average of two reading counts
were taken before plating. 200,000 cells were plated in each 100 mm plate. 100,000 cells were plated in each 60 mm plate. 20,000 cells were plated in each 35 mm plate.

3.2.5 Thawing cells stored in -80°C freezer

Vials containing cells were taken out from -80 °C freezer and allowed to thaw at room temperature. Cells were immediately transferred in a new 100 mm plate and 10 ml of fresh media was added. Cells were fed at regular intervals and passed upon reaching 90% confluency.

3.2.6 Treatments

The control plate did not receive any treatment apart from DMEM low glucose media. The vehicle treatment group was treated with sterilized ultrapure water.

3.2.6.1 Hydrogen peroxide treatment

H₂O₂ treated groups received varying concentrations H₂O₂ treatment (200, 400, 600, 800, 1000 µMs). Out of which, 600 µM was constantly utilized for later experiments. H₂O₂ was directly added to media to achieve desired concentration levels.

3.2.6.2 Creatine monohydrate treatment

Stock solution of 100 mM Creatine monohydrate (149.15 g/mol) was made in 10 ml of warm ultra-pure water by shaking vigorously for complete dissolution. Creatine monohydrate is more soluble in warm water than that at room temperature. This solution
was filter sterilized using a 0.2 µm pore size filter and saved at 4 °C (?) as the stock salutation.

3.2.6.3 Nicotinamide treatment

Similarly, 100 mM of Nicotinamide (122.12 g/mol) stock solution was made in 10 ml sterilized ultrapure water, filter sterilized using a 0.2 µm pore size filter, and saved in refrigerator before use.

3.3 Experimental model of senescence

Cells underwent experimentally-induced senescence with H₂O₂. 200,000 cells/100 mm plate, 100,000 cells/60 mm plate, 30,000 cells/35 mm plate were plated using fresh media. A volume of 10 ml, 5 ml, 2 ml were constantly maintained in 100 mm, 60 mm and 35 mm plates, respectively. These cells were incubated for 24 hrs in optimum conditions. Then, cells were pretreated with water vehicle or creatine monohydrate or nicotinamide in fresh media and incubated for 24 hrs in optimum conditions. The cells were treated with H₂O₂ in fresh media along with the vehicle or creatine monohydrate or nicotinamide treatment for 2 hrs. Later, cells were washed twice with PBS 1X and fresh media along with vehicle or creatine monohydrate or nicotinamide treatment was added. Cells were incubated for 72 hrs post treatments and cells were utilized for further experiments. A total of 8 treatment groups were used in this study.
3.3.1 Treatment groups

- **No treatment**: Cells were treated only with DMEM low glucose complete media.
- **Vehicle**: Cells were treated with vehicle of water.
- **H₂O₂ treatment**: Cells were treated with H₂O₂ at the concentrations of 200, 400, 600, 800, and 1000 µM.
- **Creatine monohydrate**: Cells were treated with varying concentrations of creatine monohydrate; 1, 5, 10, 20 mM.
- **Nicotinamide treatment**: Cells were treated with varying concentrations of nicotinamide; 1, 2.5, 5, 10, 20 mM.
- **Creatine monohydrate + nicotinamide treatment**: Cells were co-treated with 5 mM of each creatine monohydrate and nicotinamide.

3.4 MTT assay for cell proliferation

Media was removed post 24/48/72 hrs of treatment to obtain a time response. Varying doses of treatments were given to obtain a dose response. 1.25 ml of 5 mg/ml MTT solution was added to 23.75 ml of Epilife medium (for 0.25 mg/ml MTT final concentration MTT final concentration). 2 ml of this MTT-containing medium was added to each plate and incubated for 45 min at 37 °C. Medium was then removed and replaced with 1 ml of DMSO to solubilize MTT dye and was well mixed. 100 µl of the resulting solution was transferred to a 96-well plate, 3 wells per sample. Using the Microtek R plate reader, absorbance was read at 570 nm. Cell survival was calculated by an average of 3 identical wells and normalized to the no treatment group.
3.5 β-galactosidase staining

For this assay experimental model of senescence was incorporated in 35 mm plates. Post 72 hrs incubation cells were stained for β-galactosidase using abcam senescence detection kit.

3.5.1 Reagent preparation.

20 mg of X-gal was dissolved in 1ml DMSO to prepare a 20X stock solution. Excess X-gal was stored at -20°C (protected from light). The remaining reagents were provided with the kit.

3.5.2 Sample preparation

Media was removed and cells were washed twice with PBS 1X. Cells were fixed with 0.5ml0.5 ml of fixative solution for 15-20 minutes at room temperature. Meanwhile, staining solution is prepared using the reagents provided with the kit. For each plate, 940 µl of staining solution, 10 µl of staining supplement, 50 µl of 20X X-gal in DMSO were added and mixed thoroughly. This is termed as the staining solution mix. After 15-20 minutes of fixation, the cells were washed thrice with PBS 1X. 1ml of the staining solution mix was added to each plate. The plates were sealed with paraffin paper and incubated overnight in CO2 free incubator at 37°C. The next day, pictures of cells were taken under a microscope using the Biotek Cytation 5 machine.
3.6 Cell lysate preparation

Media was removed from the cells. The cells were washed with PBS 1X. Later, 5ml of 1X PBS was added and cell were scraped using a cell scraper and collected in 15 ml conical tubes. The cells were centrifuged for 5-10 minutes at 3000 rpm. Supernatant was discarded and the cell pellet was added with 200 µl of RIPA lysis buffer with Triton X-100. The cells were vortexed for 30 seconds thrice at an interval of 5 minutes. Later, the mixture was transferred to 1.5 ml centrifuge tubes and centrifuged at 13,000 rpm for 20 minutes. The lysate was transferred to another tube and the pellet was discarded.

3.7 Bradford Assay

To prepare BSA standards, 800 µl of PBS was added to 6 microcentrifuge tubes. Then, 0, 1, 2, 3, 4 and 5 µl of 2 mg/ml BSA were added to the tubes, respectively. To prepare samples, 795 µl of PBS was added to microcentrifuge tubes. To this 5 µl of samples were added. Finally, 200 µl of Bradford reagent was added to both standard and sample tubes. 150 µl was pipetted from the tubes and added to 96 well plate. The standard and samples were loaded in triplicates. Absorbance was measured at 595 nm. This was used to measure the protein amount in each sample.
3.8 Western blot

For this technique, the experimental model of senescence was incorporated in 100 mm plates.

3.8.1 Reagent preparation

3.8.1.1 1.5M Tris HCl pH 8.8 (4X) (500ml)

300 ml water was added to a beaker with stirring rod. To this 91 g Tris base (for 1.5 M Tris-HCl final) was added. pH was adjusted to 8.8 with concentrated HCl. Water was added to bring up the volume to 500 ml. Later, this was autoclaved and stored at room temperature.

3.8.1.2 0.5 M Tris-HCl pH 6.8 (4X) (for 500 ml)

300 ml water was added to beaker with stirring rod. 30.3 g Tris base (for 0.5 M Tris-HCl final) was added. pH was adjusted to 6.8 with concentrated HCl. Later, this was autoclaved and stored at room temperature.

3.8.1.3 30% acrylamide (29:1 ratio of acrylamide: bisacrylamide) (for 200 ml)

58 acrylamide and 2g bisacrylamide were added to 100 ml water in a beaker with a stir bar. Mixture was stirred until complete dissolution was achieved. The volume was brought up to 200 ml with water and stored in a glass bottle wrapped in an aluminum foil at 4°C
3.8.1.4 10% SDS

15 g SDS was added to 100 ml of water in 200 ml beaker with stir bar. This was stirred until complete dissolution was achieved. The volume was brought up to 150 ml with water. Later, this mixture was filter sterilized and stored at room temperature.

3.8.1.5 10% Ammonium persulfate (APS)

0.5 g of ammonium persulfate was added to a 15 ml conical tube. 5 ml of deionized water was added to the conical tube. The mixture was stored at 4°C.

3.8.1.6 6X SDS-PAGE Sample Buffer (loading dye/buffer)

A mixture of 6 ml of 100% glycerol, 12 ml 0.5 M Tris-HCl pH 6.8, 1.8 g DTT, 1.2 g SDS and 600 μl 1% Bromophenol blue was prepared. The mixture was aliquoted in 1.5 ml centrifuge tubes and stored at -20°C.

3.8.1.7 10X SDS-PAGE Running Buffer (4 L)

3 L of distilled water was added to a 4L beaker with a stir bar. Then, 121.2 g Tris base, 576 g Glycine and 40 g SDS were added and stirred until dissolved. The volume was brought upto 4L with distilled water.

3.8.1.8 1X SDS-PAGE Running Buffer (4 L)

300 ml of 10X SDS-PAGE Running Buffer was added to a 4L bottle and the volume was brought upto 3L with distilled water.
3.8.1.9 Semi-dry Transfer Buffer (4 L)

2 L of distilled water was added to a 4L beaker with stir bar. Then, 23.2 g Tris base, 11.72 g glycine, and 1.48 g SDS were added. To this 800 ml methanol was added after SDS dissolved. The volume was brought up to 4L with distilled water. This solution was transferred to a 4L bottle with a spigot.

3.8.1.10 10X TBS (Tris-buffered saline) – (for 1 L)

To prepare 1 L of 10X TBST, 60.55 g Tris base and 78.9 g NaCl was added to 700 ml water in beaker with stirring rod. The pH was adjusted to 7.5 with concentrated HCl. To this 10 ml Tween-20 was added and the volume was brought up to 1L with water.

3.8.1.11 10X TBS-T (Tris-buffered saline + Tween) – (for 4 L)

3 L of distilled water was added to a 4 L beaker with a stir bar. Then 242.2 g Tris base and 315.6 g NaCl were added. The pH was adjusted to 7.4-7.5 with concentrated HCl. Later, 40 ml Tween-20 was added, and the volume was brought up to 4L with water.

3.8.1.12 1X TBST (1X Tris-buffered saline + Tween-20) – (for 1 L)

1X TBS-T was prepared from 10X TBS by adding 100 ml of 10X TBS to 900 ml of water and then adding 1 ml of Tween-20.
3.8.1.13 0.5% Ponceau S stain (100 ml)

0.5 g of Ponceau S was added to a 100 ml bottle. Then, 100 ml of 5% acetic acid (diluted in water) was added to the bottle and dissolved.

3.8.1.14 5% milk in 1X TBST (50 ml)

2.5 g non-fat dry milk was added per 50 ml 1X TBST in a 50 ml conical tube. This was shaken until the milk powder completely dissolved in 1X TBST. This was stored at 4°C.

3.8.2 Preparing SDS-PAGE gels

3.8.2.1 Resolving Gel 12% (for one 1.5 mm thick mini gel)

In a conical tube 3.4 ml water, 2.5ml 1.5M Tris-HCl pH 8.8, 4 ml 30% acrylamide, 100µl 10% SDS, 50 µl 10% APS and 5 µl Tetramethylethylenediamine (TEMED) was added. 8 ml of the solution was added between the glass plates (until the level of the green clamp behind the glass was reached. After pouring resolving gel, the gel was gently covered with 70% ethanol to flatten gel, to remove bubbles and allow polymerization for about 45 minutes. Later, ethanol was removed, and the gel was rinsed thrice with distilled water.

3.8.2.2 Stacking Gel (4%) (for one gel)

In a conical tube, 1.5 ml water, 625 µl 0,5 M Tris-HCl pH 6.8, 325 µl 30% acrylamide, 25 µl 10% SDS, 12.5 µl 10% APS, 1.5 µl TEMED and 25 µl bromophenol blue were
added. The upper portion of the glass was filled with the stacking gel solution. A 15-well comb was inserted into the stacking gel and left to polymerize for 30 minutes.

These gels were wrapped in a wet paper towel and stored at 4°C to avoid drying up.

3.8.3 Procedure

3.8.3.1 Gel Electrophoresis

10 µg of protein was obtained from each treatment group and added to a 1.5 ml centrifuge tube. To this 4µl of 6X SDS was added. The volume was brought up to 25µl with RIPA buffer. The mixture was boiled at 95°C for 5 minutes and centrifuged at max speed for 30 seconds. 25 µl of this mixture was added to each well and gel electrophoresis was conducted for 45 minutes at 200V.

3.8.3.2 Membrane Transfer

The gel was removed from the glass plates and soaked in semi-dry transfer buffer. Alongside, nitrocellulose membrane and thick blot paper were soaked in transfer buffer. A sandwich was prepared in the transfer unit in the order of blot paper, membrane, gel, and blot paper. The transfer was set at 25 V/2.5 Amps for 15 minutes.

3.8.3.3 Blocking

After the membrane transfer, the membrane was briefly washed thrice with 1X TBST. 0.5% ponceau stain was added and rocked on the shaker for 2 minutes to observe the
protein bands on the membrane. A brief wash of about 3 times was followed. 10 ml of 5% milk in TBST was added and rocked on the shaker for 30 minutes to allow blocking.

3.8.3.4 Primary Antibody

After blocking, 4 brief washes with 1X TBST were followed. Varying dilutions of primary antibody were used based on the protein of interest.


The membrane was rocked with primary antibody overnight at 4°C.

3.8.3.5 Secondary antibody

Primary antibody was removed and four 5-minute washes with 1X TBST were given. Then the membrane was rocked for 1 hour with secondary antibody (1:5000). Five, brief, 5-minute washes were followed, and the membrane was exposed to ECL substrate. The membrane was then viewed under the Chemidoc system. Chemiluminescence was observed at high resolution and appropriate settings (1st exposure after 5 sec, then 60 images over the next 600 sec)

3.9 RT-QPCR

For this technique, the experimental model of senescence was incorporated in 60 mm plates.
3.9.1 RNA isolation

Media was removed and the cells were washed with PBS 1X. Cells were trypsinised with 1 ml 0.05% trypsin-EDTA for 5 minutes at 37°C in the incubator. 4 ml of fresh media was added. The cells were transferred in a conical tube and centrifuged at 3000 rpm for 5-10 minutes. Cell pellets were collected after discarding the supernatant. Cells were disrupted by adding 350 µl of RLT buffer. The lysate was pipetted into a QIAshredder spin column placed in a 2 ml collection tube and centrifuged for 2 minutes at full speed. 350 µl of 70% ethanol was added to the homogenized lysate and mixed well. This mixture was transferred to a RNeasy spin column placed in a 2ml collection tube and centrifuged at maximum speed for 15 seconds. The flow through was discarded. Then, 700 µl of RW1 buffer was added to the RNeasy spin column and centrifuged at maximum speed for 15 seconds. The flow through was discarded. 500 µl of RPE buffer was added to the RNeasy spin column and centrifuged at maximum speed for 15 seconds to wash the spin column membrane. 500 µl of RPE buffer was added again and centrifuged at maximum speed for 2 minutes. RNeasy spin column was placed in a new 2ml collection tube and centrifuged at full speed for 1 minute to eliminate any possible carryover of RPE buffer. The RNeasy spin column was placed in a new 1.5ml collection tube and 50 µl of RNase free water was directly added and centrifuged at full speed for 1 minute. The flow through obtained was the eluted RNA.

3.9.2 Reverse Transcription

RNA concentration was determined using the Nanodrop spectrophotometer. Equal amounts of RNA were pipetted for reverse transcription. 12 µl of samples were pipetted in an 8 tubes strip and 2 µl of 7x genomic DNA wipeout buffer was added. This was
incubated in the thermocycler at 42°C for 5 minutes. The samples were kept on ice and a master mix of the reverse transcription was prepared. For one reaction the master mix requires 4 µl of RT buffer, 1 µl of primer and 1 µl of RT enzyme. Each reaction was added to each tube of the 8 tubes strip. This was then incubated for 15 minutes at 42°C followed by a 5-minute incubation at 95°C. The RT reaction was stored in -20°C.

### 3.9.3 Q-PCR

A QPCR requires 10 µl reactions in the form of triplicates. This is for reference gene and gene of interest each. For 1 reaction 5µl of 2X Taqman fast universal PCR master mix, 0.5 µl of 20X Taqman primer, 0.5 µl of RT reaction and 3 µl of molecular biology grade water were added to each well of a 96-well plate. In this study, the reference gene used is Beta-2-microglobulin and varying gene of interests. The 96 well plate was placed in the Biorad Q-PCR machine for 40 cycles. Ct values were obtained from the software and then the Livak method or the ΔΔCt method was used to calculate the expression fold change compared to no treatment group.
Results

4.1 Effect of agents on dermal fibroblasts

These studies were designed to observe the effects of various agents used in this study on dermal fibroblasts.

4.1.1 Hydrogen peroxide decreases cell survival

Hydrogen peroxide causes oxidative stress in cells (138). To observe its effects on dermal fibroblasts, we treated cells with varying doses of H₂O₂. As per figure 4.1, decrease in cell survival was observed two days after H₂O₂ treatment. Significant decrease in cell survival was seen in H₂O₂ on days 2 and 3, relative to control. The circled part in figure 4.1 represents the significance.

4.1.2 Creatine monohydrate does not affect cell survival

To observe the effects of creatine monohydrate alone on dermal fibroblasts, cells were treated with varying doses of creatine monohydrate and MTT assay was performed on different time points. There was no significance decrease in cell survival observed in cells which were treated with creatine monohydrate. Ranging from low dose to high dose of creatine, none affected cell survival, as shown in figure 4.2.
4.1.3 Nicotinamide decreases cell survival at a high dose

To observe the effects of nicotinamide on dermal fibroblasts, cells were treated with varying doses of nicotinamide. Figure 4.3 explains that, there was no significant change in cell survival in cells treated with up to 10 mM of nicotinamide. However, 20 mM of nicotinamide treated cells decreased the cell survival, starting Day 2 and on Day 3. Indicating cytotoxicity of nicotinamide at higher doses.

Figure 4.1 Effects of varying doses of H₂O₂ on fibroblast cell survival. Cells either received no treatment (NT), 200 µM, 400 µM, 600 µM, 800 µM, 200µM, 400µM, 600µM, 800µM or 1 mM of H₂O₂. The circled area in the graph indicates significance between the decreased cell survival found on day 2 and 3 on 800µM and 1mM H₂O₂ treatment, relative to day 0 and 1 of the same treatments.
Figure 4.2 Dose response of fibroblast cell to increasing concentrations of creatine monohydrate (1, 5, 10, 20 mM) on fibroblast cell survival.
Figure 4.3 The effect of varying doses of nicotinamide (NAM) on fibroblast cell survival. The circled area in the graph indicates significance between the decreased cell survival found on day 2 and 3 on 20mM NAM treatment, relative to day 1 of the same treatment.
Figure 4.4: Effects of creatine monohydrate, nicotinamide and H\textsubscript{2}O\textsubscript{2} on β-galactosidase activity. Cells received no treatment (NT), H\textsubscript{2}O\textsubscript{2} (600µM, 800µM), creatine monohydrate (Cr 5mM, Cr 10mM) or nicotinamide (NAM 5mM, NAM 10mM) were subjected to X-gal stain 72 hours post treatment. A significant increase in the number of cells positive for β-galactosidase was seen in both the H\textsubscript{2}O\textsubscript{2} treated groups. Whereas, creatine
monohydrate and nicotinamide treated groups did not differ from no treatment. The data depicted are mean SE percentage of β-galactosidase positive cells (mean of n=3). Groups were compared using one-way ANOVA and Tukey’s post-hoc test. Differences in samples were considered significant if P value is less than 0.05. P<0.0001 (####) compared to NT.

4.1.4 H₂O₂ increases the percentage of beta-galactosidase positive cells.

To determine the β-galactosidase activity due to treatment, different doses of H₂O₂, creatine monohydrate and nicotinamide were used on human dermal fibroblasts. They were stained with X-gal 72 hours post treatment. Dermal fibroblasts responded with an increase in the number of cells stained for β-galactosidase in the H₂O₂ treated groups significantly when compared to control NT (Figure 4.4). Creatine and nicotinamide did not increase the number of β galactosidase stained cells.

4.2 Effect of Creatine monohydrate on IGF-1 production and senescence markers in oxidative stress induced senescent fibroblasts.

To determine this effect, cells received no treatment (NT), vehicle, H₂O₂ (600µM), creatine monohydrate (Cr 5mM, Cr 10mM) + H₂O₂ (600µM).
4.2.1 Creatine monohydrate decreases the percentage of beta-galactosidase positive H$_2$O$_2$-treated cells.

Cells received creatine monohydrate pretreatment 24 hours post plating cells and received H$_2$O$_2$ treatment 48 hours post plating alongside creatine monohydrate treatment. 72 hours post H$_2$O$_2$ treatment, creatine monohydrate significantly decreased the percentage of β galactosidase-positive cells following H$_2$O$_2$ treatment (Fig 4.5).
**Figure 4.5:** Effect of creatine monohydrate on β-galactosidase activity. Cells receive no treatment (NT), vehicle, H2O2 (600µM), creatine monohydrate (Cr 5mM, Cr 10mM) + H2O2 (600µM). The cells were subjected to X-gal stain 72 hours post treatment. A significant increase in the number of cells positive for β-galactosidase was seen in the H2O2 treated cells. Whereas, creatine monohydrate treated cells significantly reduced β galactosidase %. The data depicted are the percentage of β-galactosidase positive cells (mean and SD of n=3). Groups were compared using one-way ANOVA and Tukey’s post-hoc test. Differences in samples were considered significant if P value was less than 0.05. P<0.0001 (****), P<0.001 (**), both compared to H2O2 and P<0.0001 (####) compared to NT.
4.2.2 Creatine monohydrate increased IGF-1 production in stress induced dermal fibroblasts

Cells received creatine monohydrate pretreatment 24 hours post plating cells and received $\text{H}_2\text{O}_2$ treatment 48 hours post plating alongside creatine monohydrate treatment. 72 hours post $\text{H}_2\text{O}_2$ treatment, as shown in Fig 4.6 (A), creatine monohydrate increased IGF-1 protein expression, and mRNA expression, as shown in Fig 4.6 (B), in stress induced cells, significantly, when compared to the $\text{H}_2\text{O}_2$-treated group.
A

<table>
<thead>
<tr>
<th></th>
<th>NT</th>
<th>Veh</th>
<th>NT</th>
<th>Cr5</th>
<th>Cr10</th>
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<td>H₂O₂</td>
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IGF-1
Actin

B

H₂O₂ - 600µM

Normalized expression IGF-1/actin

IGF-1 Expression fold change

H₂O₂ - 600µM
**Figure 4.6:** Effect of creatine monohydrate on IGF-1 production. Cells receive no treatment (NT), vehicle, H₂O₂ (600µM), creatine monohydrate (Cr 5mM, Cr 10mM) + H₂O₂ (600µM). Creatine monohydrate pretreatment was given 24 hours post plating and was constantly maintained until cells were harvested. H₂O₂ treatment was given 48 hours post plating cells. Western blot and RT-QPCR performed revealed that creatine monohydrate increased IGF-1 protein expression(A) and IGF-1 mRNA expression (B) in stress induced cells, respectively. The data depicted are mean SD expression IGF-1 protein (A) and mRNA (B) (mean of n=3). Groups were compared using one-way ANOVA and Tukey’s post-hoc test. Differences in samples were considered significant if P value is less than 0.05. P<0.0001 (****), compared to H₂O₂ and P<0.0001 (####) compared to NT.

### 4.2.3 Creatine monohydrate reduces the expression of markers associated with senescence.

#### 4.2.3.1 Creatine monohydrate reduced SASP expression.

Cells received creatine monohydrate pretreatment 24 hours post plating cells and received H₂O₂ treatment 48 hours post plating alongside creatine monohydrate treatment. 72 hours post H₂O₂ treatment, as shown in Fig 4.7, creatine monohydrate treated cells showed reduced expression fold change of SASP mRNA compared to H₂O₂ treated cells.
A

B

IL-6 Expression fold change

0 1 2 3 4 5 6

NT Vehicle H2O2 Cr 5mM +H2O2 Cr 10mM +H2O2

IL-8 expression fold change

0 1 2 3 4 5

NT Vehicle H2O2 Cr 5mM +H2O2 Cr 10mM +H2O2

H2O2 600µM

****

****

****

****
Figure 4.7 Effect of creatine monohydrate on SASP. Cells receive no treatment (NT), vehicle, H$_2$O$_2$ (600µM), creatine monohydrate (Cr 5mM, Cr 10mM) + H$_2$O$_2$ (600µM). Creatine monohydrate pretreatment was given 24 hours post plating and was constantly maintained until cells were harvested. H$_2$O$_2$ treatment was given 48 hours post plating cells. RT-Q PCR performed revealed that creatine monohydrate reduced SASP mRNA, that is, IL-6 mRNA expression (A), IL-8 mRNA expression (B) and TNF-α mRNA expression (C) in stress induced cells. The data depicted are mean SD expression IGF-1 protein (A) and mRNA (B) (mean of n=3). Groups were compared using one-way ANOVA and Tukey’s post-hoc test. Differences in samples were considered significant if P value is less than 0.05. P<0.0001 (****), compared to H$_2$O$_2$ and P<0.0001 (####) compared to NT.

H$_2$O$_2$ increased the IL-6 expression fold change about 4.5 times to that of no treatment in human dermal fibroblasts. Creatine monohydrate treatment reduced it to about two times to that of no treatment. While there was clear significant difference when creatine monohydrate was treated, there was no significant difference among the creatine monohydrate treated groups. Similar findings were seen in IL-8 and TNF-α expression.
fold changes (Fig 4.7). H$_2$O$_2$ treatment increased the IL-8 expression about 4 times and TNF-α expression about 3.5 times when compared to no treatment. Creatine monohydrate reduced it twice as much as no treatment in both cases as well.

**Figure 4.8:** Effect of creatine monohydrate on p21 expression. Cells receive no treatment (NT), vehicle, H$_2$O$_2$ (600µM), creatine monohydrate (Cr 5mM, Cr 10mM) + H$_2$O$_2$ (600µM). Creatine monohydrate pretreatment was given 24 hours post plating and was constantly maintained until cells were harvested. H$_2$O$_2$ treatment was given 48 hours post plating cells. Western blot performed revealed that creatine monohydrate reduced p21 protein expression significantly in stress induced cells. The data depicted are mean SD expression p21 protein (mean of n=3). Groups were compared using one-way ANOVA
and Tukey’s post-hoc test. Differences in samples were considered significant if P value is less than 0.05. P<0.0001 (** ** **), compared to H2O2.

### 4.2.3.2 Creatine monohydrate reduced H2O2-induced p21 expression

P21 protein expression was lowered by creatine monohydrate treatments (5,10 mM) significantly. Cell lysates were prepared 72 hours post stress inducing treatment and kept on ice to avoid protein degradation. These levels of Cr brought down P21 levels similar to those of the control (NT) treatment as shown in Fig (4.8)

### 4.3 Effect of nicotinamide on IGF-1 production and senescence markers in oxidative stress induced senescent fibroblasts

To determine this effect, cells received no treatment (NT), vehicle, H2O2 (600µM), nicotinamide (NAM 2.5mM, NAM 5mM) + H2O2 (600µM) and nicotinamide + creatine monohydrate cotreatment (NAM 5mM + Cr 5mM) + H2O2 (600µM)

#### 4.3.1 Nicotinamide decreased β galactosidase % in H2O2 treated cells

Cells received nicotinamide and nicotinamide + creatine monohydrate combination pretreatment 24 hours post plating cells and received H2O2 treatment 48 hours post
plating alongside pretreatment. 72 hours post H₂O₂ treatment, nicotinamide and combination of creatine and nicotinamide significantly decreased the percentage of β-galactosidase in stress induced cells compared to H₂O₂ treated cells (Fig 4.9). However, the combination treatment of creatine monohydrate and nicotinamide significantly increased β-galactosidase % when compared to the nicotinamide treated cells when stress is induced, but there was a significant reduction when compared to H₂O₂ treated cells.

### 4.3.2 Nicotinamide increased IGF-1 production in stress induced dermal fibroblasts

Cells received nicotinamide and nicotinamide + creatine monohydrate combination pretreatment 24 hours post plating cells and received H₂O₂ treatment 48 hours post plating alongside pretreatment. 72 hours post H₂O₂ treatment, as shown in Fig 4.10 (A), nicotinamide and nicotinamide + creatine monohydrate increased IGF-1 protein expression, and mRNA expression, as shown in Fig 4.10 (B), in stress induced cells, significantly, when compared to H₂O₂ treated group.
Figure 4.9: Effect of nicotinamide and nicotinamide + creatine monohydrate combination on \( \beta \)-galactosidase activity. Cells receive no treatment (NT), vehicle, \( \text{H}_2\text{O}_2 \) (600\( \mu \text{M} \)), nicotinamide (NAM 2.5mM, NAM 5mM) + \( \text{H}_2\text{O}_2 \) (600\( \mu \text{M} \)) and nicotinamide
and creatine monohydrate (NAM 5mM + Cr 5mM) + H2O2 (600µM). The cells were subjected to X-gal stain 72 hours post treatment. A significant increase in the number of cells positive for β-galactosidase was seen in the H2O2 treated cells. Whereas, nicotinamide and combination of creatine monohydrate and nicotinamide treated cells significantly reduced β galactosidase %. The data depicted are mean SD percentage of β-galactosidase positive cells (mean of n=3). Groups were compared using one-way ANOVA and Tukey’s post-hoc test. Differences in samples were considered significant if P value is less than 0.05. P<0.0001 (****), compared to H2O2, P<0.0001 (####) compared to NT, P<0.001 (@@@), compared to NAM 5mM + H2O2 600µM and P<0.01 ($$) compared to NAM 2.5mM + H2O2 600µM
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**H₂O₂**

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**IGF-1**

**Actin**

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**H₂O₂** - 600µM

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Figure 4.10: Effect of nicotinamide on IGF-1 production. Cells receive no treatment (NT), vehicle, H₂O₂ (600µM), nicotinamide (NAM 2.5mM, NAM 5mM) + H₂O₂ (600µM) and creatine monohydrate + nicotinamide (Cr 5mM + NAM 5mM) + H₂O₂ (600µM). Creatine monohydrate and nicotinamide pretreatment was given 24 hours post plating and was constantly maintained until cells were harvested. H₂O₂ treatment was given 48 hours post plating cells. Western blot and RT-QPCR performed revealed that pretreatment increased IGF-1 protein expression(A) and IGF-1 mRNA expression (B) in stress induced cells, respectively. The data depicted are mean SD expression IGF-1 protein (A) and mRNA (B) (mean of n=3). Groups were compared using one-way ANOVA and Tukey’s post-hoc test. Differences in samples were considered significant if P value is less than 0.05. P<0.01 (**), P<0.05 (*) both compared to H₂O₂ and P<0.0001 (####) compared to NT.

However, not much effect was seen due to the combination of creatine monohydrate and nicotinamide. Its response was similar to individual nicotinamide/creatine treatments.
4.3.3 Nicotinamide reduced senescent markers expression.

4.3.3.1 Nicotinamide reduced SASP expression.

Cells received nicotinamide or nicotinamide along with creatine monohydrate pretreatment 24 hours post plating cells and received H$_2$O$_2$ treatment 48 hours post plating alongside pretreatment. 72 hours post H$_2$O$_2$ treatment, as shown in Fig 4.11, pretreated cells showed reduced expression fold change of SASP mRNA compared to H$_2$O$_2$ treated cells.
Figure 4.11 Effect of nicotinamide on SASP. Cells receive no treatment (NT), vehicle, H$_2$O$_2$ (600µM), nicotinamide (NAM 2.5mM, NAM 5mM) + H$_2$O$_2$ (600µM) and creatine monohydrate + nicotinamide (Cr 5mM + NAM 5mM) + H$_2$O$_2$ (600µM). Creatine monohydrate and nicotinamide pretreatment was given 24 hours post plating and was constantly maintained until cells were harvested. H$_2$O$_2$ treatment was given 48 hours post plating cells. RT-Q PCR performed revealed that pretreatment reduced SASP mRNA, that is, IL-6 mRNA expression (A), IL-8 mRNA expression (B) and TNF-α mRNA expression (C) in stress induced cells. The data depicted are mean SD expression IGF-1
protein (A) and mRNA (B) (mean of n=3). Groups were compared using one-way ANOVA and Tukey’s post-hoc test. Differences in samples were considered significant if P value is less than 0.05. P<0.0001 (****), P<0.001 (***) , P<0.01 (**), all compared to H2O2 and P<0.0001 (####) compared to NT.

As depicted in Figure 4.11, H2O2 treatment increased the IL-6 expression fold change about 4.5 times to that of no treatment in human dermal fibroblasts. Pretreatment reduced it to about two times to that of no treatment. While there were clear significant differences when pretreatment was given to the cells, there was no significant difference among the pretreatment groups. Similar findings were noted in IL-8 and TNF-α expression fold changes. H2O2 treatment increased the IL-8 expression about 4 times and TNF- α expression about 3.5 times when compared to no treatment. Pretreatment reduced it twice as much as no treatment in both cases as well.
**Figure 4.12:** Effect of nicotinamide on p21 expression. Cells receive no treatment (NT), vehicle, H$_2$O$_2$ (600µM), nicotinamide (NAM 2.5mM, NAM 5mM) + H$_2$O$_2$ (600µM) and creatine monohydrate + nicotinamide (Cr 5mM + NAM 5mM) + H$_2$O$_2$ (600µM). Creatine monohydrate and nicotinamide pretreatment was given 24 hours post plating and was constantly maintained until cells were harvested. H$_2$O$_2$ treatment was given 48 hours post plating cells. Western blot performed revealed that pretreatment reduced p21 protein expression significantly in stress induced cells. The data depicted are mean SD expression p21 protein (mean of n=3). Groups were compared using one-way ANOVA
and Tukey’s post-hoc test. Differences in samples were considered significant if P value is less than 0.05. P<0.0001 (****), compared to H₂O₂.

### 4.2.3.2 Nicotinamide reduced H₂O₂-mediated p21 expression

P21 protein expression was lowered by nicotinamide treatments (2.5, 5 mM) and combination of creatine monohydrate and nicotinamide (Cr 5mM + NAM 5mM) in stress induced cells significantly. Cell lysates were prepared 72 hours post stress inducing treatment and kept on ice to avoid protein degradation. These brought down levels were similar to that of the control (NT) as shown in Fig (4.12)
Discussion

Cellular senescence is a gradual transformation of the cell into a state of arrested growth. It is not a “one-time phenomenon” but takes a certain time period to develop. Multiple studies have suggested that cell cycle is arrested in the G1 phase, but evidence also suggests that G2 phase may also be arrested. Senescence can be useful due to its tumor suppressive effect as cancer cells proliferate indefinitely. On the other hand, this suppressive effect may be deleterious to tissue regenerative capacity, growth, and differentiation. In young organisms, where cellular metabolic pathways are well regulated, a short-term tumor suppressive exposure may be beneficial in combating cancers while not affecting the regenerative capacity of normal healthy cells. But this may not be the case in older organisms. Cells in old organisms, may not function at their peak due to replicative exhaustion or stress induced DNA damage. This results in altered gene expression and hence affecting the normal functioning of the cell. Such is seen in human dermal fibroblasts. Young fibroblasts secrete connective tissues and other metabolic signals, one of them being IGF-1. This insulin like growth factor binds to its transmembrane receptors present on the keratinocytes. This binding activated several pathways responsible for cell survival/metabolism/apoptosis. This initiates an appropriate response towards to stress signals. Either the cell initiates regeneration or causes apoptosis. But, in geriatric fibroblasts, the production of IGF-1 is impaired due to the onset of senescence is the microtissue environment. Inactivation of IGF-1 receptors ultimately leads to an inappropriate response when stress is induced. The cells enter
growth arrest phase and may cause mutations upon stress induction. This has been associated with malignancy.

Our study uses hydrogen peroxide as means to induce stress and cause senescence. H₂O₂ is known to increase superoxide ions and free radicals, which increase ROS levels in the cell. This is the primary mechanism which causes DNA damage. Cell proliferation is affected due to these changes. This was confirmed in our study by performing MTT assay. Our findings suggest that decline in cell proliferation was seen on Day 2 and Day 3 post H₂O₂ treatment, in 800µM and 1mM H₂O₂ exposure (Fig 4.1). There was no significant difference in cell proliferation on Day 0 and Day 1. No effect of creatine monohydrate and nicotinamide was seen on cell proliferation and we found that creatine monohydrate even at higher concentrations did not have a significant difference in cell proliferation on all the time points post treatment (Fig 4.2). However, nicotinamide treated cells experienced decreased cell proliferation at 10mM and 20mM concentration on Days 1 and 2 (Fig 4.3). Decreased growth marks the onset of cellular senescence and this was confirmed by performing β-galactosidase staining. β-galactosidase is an enzyme which cleaves complex sugars to monosaccharides. Cells usually follow Krebs cycle to produce energy, but the adaptation to cleaving sugar molecules is a way senescent utilize to meet energy requirements. Hence, it is a very important marker for confirming senescence. Based on our findings, dermal fibroblasts treated with 600 µM or 800 µM H₂O₂ showed a significance increase in the number of cells positive for β-galactosidase compared to no treatment. Whereas creatine and nicotinamide treatment did not increase the number of β-galactosidase positive cells relative to no treatment (Fig 4.4).
IGF-1 production is important to ensure normal cellular function. In an attempt to look at the effects of creatine monohydrate and nicotinamide on stress induced dermal fibroblasts, we pretreated the cells with the mentioned pro energetics before inducing senescence. Our findings suggest that \( \text{H}_2\text{O}_2 \) reduced IGF-1 protein expressions and mRNA expression, while creatine monohydrate (5, 10 mM) increased IGF-1 protein expression (Fig 4.6 A) and mRNA expression (Fig 4.6 B) in stress induced cells relative to \( \text{H}_2\text{O}_2 \). Similarly, nicotinamide and combination of creatine monohydrate and nicotinamide also increased IGF-1 protein expression (4.10 A) and mRNA expression (4.10 B) relative to \( \text{H}_2\text{O}_2 \) in stress induced cells. These findings suggest that creatine monohydrate and nicotinamide pretreatment increased IGF-1 expression in both transcriptional and post transcriptional levels. However, the combination treatment of both the pro energetics did not have any additive or synergistic effect. \( \beta \)-galactosidase expression was also reduced when pro energetics were pretreated in stress induced cells (Fig 4.5 and Fig 4.9, respectively).

SASP components are an important phenotype of senescent cells. While they can be beneficial in young cells by attracting immune cells and eliminating senescent cells, in geriatric cells immune signaling is compromised, hence leads to accumulation of senescent cells in the surrounding tissue environment. Our studies have examined IL-6, IL-8 and TNF-\( \alpha \), which are some of the most important SASP factors. These are known to cause chronic inflammation and drive pre-senescent cells to senescence (112). Our findings suggest that pretreatment with creatine monohydrate (5mM and 10mM) in stress induced cells decreased each of the SASP components expression fold change relative to \( \text{H}_2\text{O}_2 \) treated cells. \( \text{H}_2\text{O}_2 \) increased the expression fold change of IL-6, IL-8 and TNF-\( \alpha \)
for about 4.5 times, 4 times and 3.5 times relative to no treatment, respectively. Pretreatment with creatine monohydrate decreased the expression fold to 2 times relative to no treatment (Fig 4.7 A, B, C). In line with these findings, pretreatment with nicotinamide (2.5, 5 mM) and the combination of nicotinamide and creatine monohydrate (NAM 5mM+ Cr 5mM) reduced IL-6, IL-8, TNF-α expression fold change in stress induced cells, significantly, relative to H2O2 treated cells (Fig 4.11 A, B, C).

Senescence is induced by p21 due to its ability to cause transient DNA damage(139). It is regulated in a p53 dependent manner. Hence, relative to our study it is an important marker for the onset of senescence. Our findings suggest that pretreatment with creatine monohydrate (5mM and 10mM) in stress induced cells decreased p21 protein expression, relative to H2O2 treated cells while H2O2 treatment increased p21 expression (Fig 4.8). Similarly, pretreatment with nicotinamide (2.5, 5 mM) and the combination of nicotinamide and creatine monohydrate (NAM 5mM+ Cr 5mM) reduced p21 protein expression in stress induced cells, significantly, relative to H2O2 treated cells (Fig 4.12).

Our, study so far has shown the protective effects of creatine monohydrate and nicotinamide. Their ability to decrease senescence can be utilized to answer questions in age related diseases. Our group suggests that creatine monohydrate/ nicotinamide upon topical treatment may decrease senescence of fibroblasts. Creatine monohydrate and nicotinamide treatment decreased the number of cells positive for beta galactosidase. So, pretreatment with the proenergetics did not boost IGF-1 production or decrease the expression of senescent markers in each cell, they prevented certain cells from undergoing senescence resulting in an overall increase in IGF-1 and decrease in senescence markers. This could be an answer to various age-related diseases since
senescent fibroblasts have been associated with accelerated ill-effects of aging like non-melanoma skin cancers. Senescent dermal fibroblasts impair the IGF-1 receptor activation and cause an anomaly in the behavior of human keratinocytes. When exposure to UVB and other forms of stressors happens over a period of time, it leads to accumulation of DNA damage and may cause malignancy (140). Our findings serve as preliminary results and testing the effect of these proenergetics in other aging models is important to substantiate our findings.
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