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EFFECT OF HUMAN CORD BLOOD PLASMA EXOSOMES ON FIBROBLAST AGING

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ABSTRACT

EFFECT OF HUMAN CORD BLOOD PLASMA EXOSOMES ON FIBROBLAST AGING

Skin aging is a complex process accelerated by various factors, such as ultraviolet radiation (UV), genetics, environmental pollutants, smoking, and alcohol consumption. This aging process involves the deterioration of essential skin components like hyaluronic acid (HA), subcutaneous fat, collagen, and elastic fibers. Fibroblasts, the predominant cell type in the dermis, play a central role in skin aging, either directly or through interactions with other cells. Recent research has highlighted the potential of exosomes in addressing skin aging. Exosomes, small extracellular vesicles ranging from 30 to 120 nanometres, are secreted by all cell types and serve as crucial mediators of intercellular communication, genetic material transfer, and immune regulation. Human cord blood plasma is a promising source for skin regeneration and wound healing. This thesis aimed to investigate the impact of human cord blood plasma exosomes (CBE) on aging parameters in human fibroblast cells. Exosomes isolated from cord blood plasma were characterized and used to treat senior dermal fibroblasts to assess the effect of these exosomes on the cell adhesion, expansion, and proliferation of senior human fibroblast cells. Through various analyses, including flow cytometry and Nanoparticle Tracking Analysis key proteins on exosome surfaces and size and concentrations of exosomes were identified. Our results demonstrated that exosomes at an optimal concentration of 100 μg/ml enhances cell proliferation, increases telomerase activity, and improves wound closure rates. Flow cytometry results indicated a higher percentage of cells in the S and G2-M phases in the exosome-treated group, signifying increased cell cycle progression. Telomerase activity significantly increased from 22.24 IU/ml in normal conditions to 38.10 IU/ml with exosome treatment. Gene expression analysis showed downregulation of stress-related genes (p21 and p16) and upregulation of the proliferation marker Ki-67, while immunocytochemistry indicated higher expression of extracellular matrix proteins Collagen Type I and elastin in treated cells. These findings highlight the promise of cord blood plasma exosomes in medical applications, suggesting they could play a crucial role in modulating cellular environments to support tissue repair and combat cellular aging.

ÖZET

İNSAN KORDON KANI PLAZMA EKSOZOMLARININ FİBROBLAST YAŞLANMASINA ETKİSİ

Cilt yaşlanması, ultraviyole radyasyon, genetik, çevresel kirleticiler, sigara ve alkol tüketimi gibi çeşitli faktörlerin hızlandırdığı karmaşık bir süreçtir. Bu yaşlanma süreci, hyaluronik asit (HA), deri altı yağ, kollajen ve elastik lifler gibi temel cilt bileşenlerinin bozulmasını içerir. Dermisteki baskın hücre türü olan fibroblastlar, doğrudan veya diğer hücrelerle etkileşimler yoluyla cilt yaşlanmasında merkezi bir rol oynar. Son araştırmalar, eksozomların cilt yaşlanmasına çözüm bulma potansiyelini vurgulamıştır. 30 ila 120 nanometre arasında değişen küçük hücre dışı kesecikler olan eksozomlar, tüm hücre tipleri tarafından salgılanır ve hücreler arası iletişim, genetik materyal transferi ve bağışıklık düzenlemesinde önemli aracılar olarak görev yapar. İnsan kordon kanı plazması, cilt yenilenmesi ve yara iyileşmesi için umut verici bir kaynaktır. Bu tez, insan kordon kanı plazma eksozomlarının (CBE) insan fibroblast hücrelerindeki yaşlanma parametreleri üzerindeki etkisini araştırmayı amaçlamıştır. CBE karakterize edilmiş ve bu eksozomların yaşlanmış insan fibroblast hücrelerinin hücre çoğalması üzerindeki etkisini değerlendirmek için kıdemli dermal fibroblastlar tedavi edilmiştir. Akış sitometrisi ve Nanopartikül İzleme Analizi dahil olmak üzere çeşitli analizler yoluyla, eksozom yüzeylerindeki anahtar proteinler, eksozomların boyutu ve konsantrasyonları belirlenmiştir. Sonuçlarımız, 100 µg/ml'lik optimal konsantrasyondaki eksozomların hücre proliferasyonunu artırdığını, telomeraz aktivitesini artırdığını ve yara kapanma oranlarını iyileştirdiğini göstermiştir. Akış sitometri sonuçları, eksozomla tedavi edilen grupta S ve G2-M fazlarında daha yüksek hücre yüzdesine işaret etmiş; bu, hücre döngüsü ilerlemesinin arttığını göstermektedir. Telomeraz aktivitesi normal koşullarda 22,24 IU/ml'den eksozom tedavisiyle 38,10 IU/ml'ye önemli ölçüde artmıştır. Gen ekspresyonu analizi, stresle ilişkili genlerin (p21 ve p16) aşağı regüle edildiğini ve çoğalma işaretçisi Ki-67'nin yukarı regüle edildiğini gösterirken, immünositokimya, tedavi edilen hücrelerde hücre dışı matriks proteinleri Kollajen Tip I ve elastin'in daha yüksek ekspresyonunu göstermiştir. Bu bulgular, tıbbi uygulamalarda CBE vaadini vurgulamakta ve doku onarımını desteklemek, hücresel yaşlanmayla mücadele etmek için hücresel ortamların modüle edilmesinde önemli bir rol oynayabileceklerini öne sürmektedir.

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LIST OF ABBREVIATIONS

ANOVA	One-way analysis of variance
BOTOX	Botulinum toxin
CAHA	Calcium Hydroxyapatite
CBE	Cord blood plasma exosomes
DDR	DNA damage response
DEX	Dextran
ESCRT	Endosomal sorting complex required for transport
EVs	Extracellular vesicles
FBS	Fetal bovine serum
HA	Hyaluronic acid
HSCs	Hematopoietic stem cells
IPL	Intense pulsed light
iPSCs	Induced pluripotent stem cells
ILVs	Intraluminal vesicles
MVBs	Multivesicular bodies
MSCs	Mesenchymal stem cells
miRNAs	MicroRNAs
NER	Nucleotide excision repair
NTA	Nanoparticle tracking analysis
PLLA	Poly-L-lactic acid filler
PMMA	Polymethylmethacrylate filler
PRP	Platelet rich plasma
PDGF	Platelet-derived growth factor
PB	Peripheral blood
PEG	Polyethylene glycol
PSA	Penicillin, streptomycin, and amphotericin B
SD	Standard deviation
SEM	Standard error of the mean
SASP	Senescence-associated secretory phenotype
TGF-β	Transforming growth factor-beta
CBE	Human umbilical cord blood exosome

UV	Ultraviolet
VEGF	Vascular endothelial growth factor

1. INTRODUCTION

Aging is an unavoidable and intricate process that impacts all living creatures, including humans. While aging encompasses a multitude of physiological changes at various levels of organization within the body, the outward manifestation of aging on the skin is particularly visible and readily perceivable. The aging of the skin is a complex phenomenon affected by a combination of internal and external factors, with cellular aging and cellular senescence playing pivotal roles in its progression [1,2].

Cell aging, often referred to as replicative or intrinsic aging, is an inherent and genetically programmed process that occurs over time as cells divide and replicate. This type of aging is primarily driven by telomere shortening, oxidative stress, and cellular damage accumulation, leading to a gradual decline in cellular function and regenerative capacity [3]. The skin, as the body's largest organ, is significantly impacted by cell aging, resulting in changes such as reduced epidermal turnover, decreased collagen and elastin production, and impaired wound healing. These alterations manifest as wrinkles, sagging, and thinning of the skin, contributing to the overall appearance of aged skin [4].

The skin, positioned prominently at the forefront of the body, serves as an ideal platform for the observation and analysis of the impact of both external and internal factors contributing to aging. In the last five years, significant technological advancements have transformed our capacity to explore the processes of aging and senescence, particularly at the level of individual skin cells within their tissue context [5,6]. Innovations such as lineage tracing of cells in model organisms, intra-vital microscopy, sequencing, and mass spectrometry have empowered researchers to investigate the aging processes, signalling mechanisms, and communication dynamics within these cells. These state-of-the-art tools have also generated new hypotheses for in vitro experimental studies.

1.1. COMPARTMENTS OF SKIN

The skin, a remarkably intricate organ, is composed of two primary layers: the outermost epidermal layer and the inner dermal layer, intricately interconnected by the basement membrane. These layers harmoniously collaborate to give rise to the multifaceted structure we recognize as skin. In this complex context, various skin structures such as hair follicles, sebaceous glands, sweat glands, and nails form through a collaborative process involving both epithelial and mesenchymal cells located in the dermis. (Figure 1.1)

Directly beneath the dermis, we find the hypodermis, a layer comprising white adipose tissue housing adipocytes. These adipocytes mature by accumulating lipids over time. Together, these layers and structures combine to create the skin, a versatile organ that serves numerous essential functions in the human body [7,8].

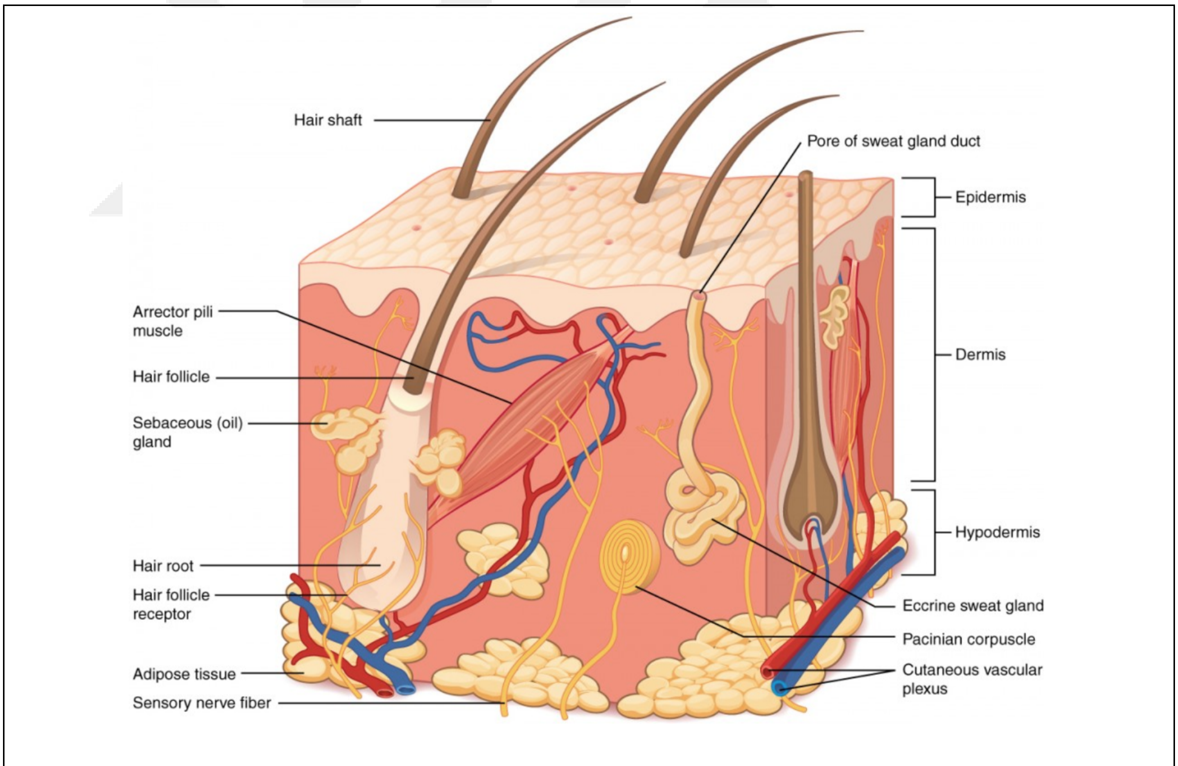

Figure 1.1. Skin tissue layers [8]

The epidermis, which constitutes the outermost layer of the skin, primarily consists of keratinocytes and presents itself as a complex, layered epithelial structure. These keratinocytes play a crucial role in maintaining the dynamic balance of the skin tissue. They undergo significant stages in their life cycle, commencing in the basal layer where they are firmly attached to the basement membrane (Figure 1.2). However, upon separating from this fundamental anchor, they cease proliferation and initiate a highly specialized process known as terminal differentiation. This involves reinforcing the cytoskeleton, forming an intercellular barrier, and ultimately culminating in a specific form of programmed cell death referred to as cornification. Cornified keratinocytes are vital for preserving skin function because they continuously produce a protective barrier that shields the skin from various environmental stresses [9].

Within the basal layer of the skin, stem cells are strategically positioned in two primary areas: the protrusion zone of hair follicles and the follicular epidermis. These stem cells play a critical role in ensuring the ongoing replenishment and regeneration of keratinocytes, which is essential for the epidermis to fulfil its crucial function as the protective barrier of the skin [10].

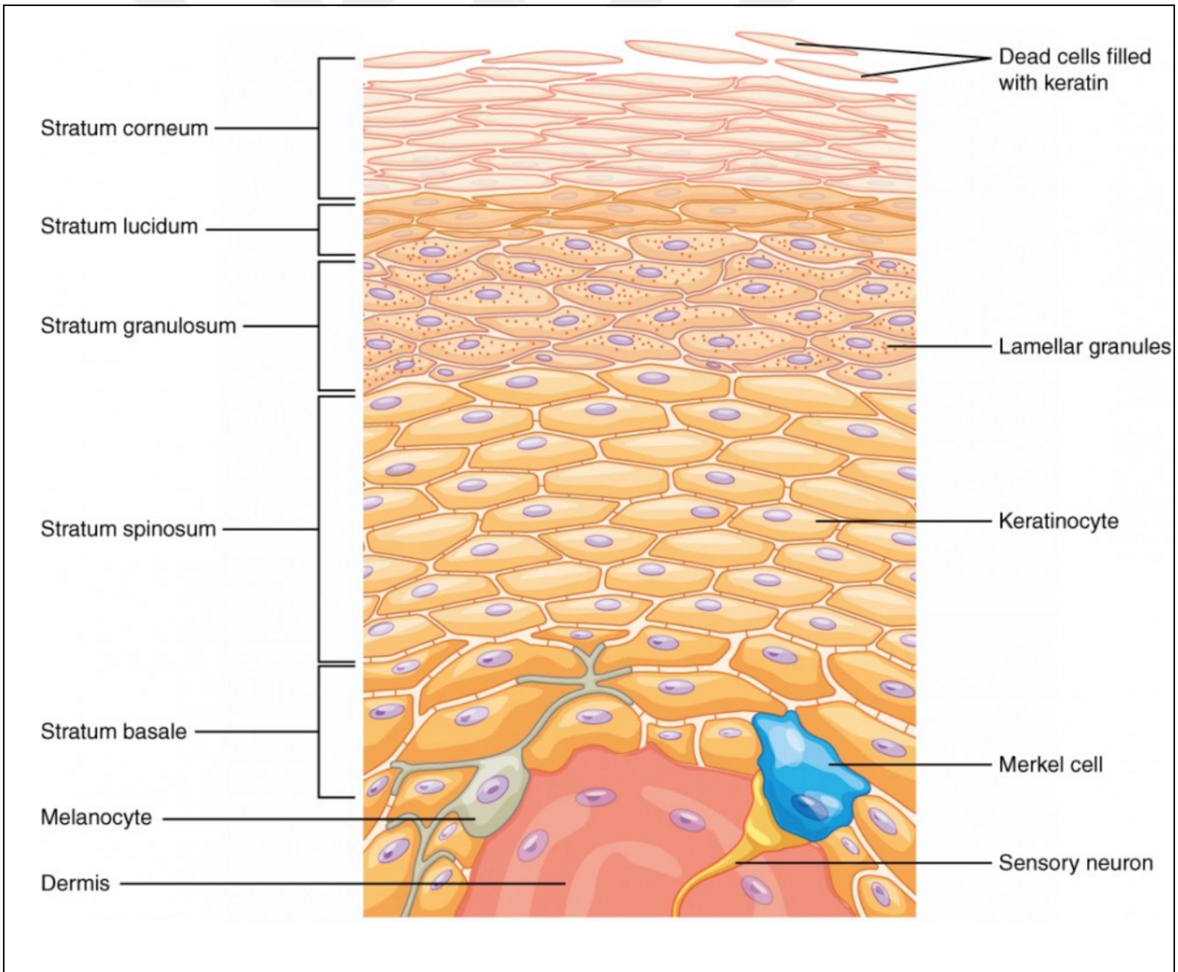

Figure 1.2. Layers of epidermis [10]

1.2. SKIN AGING PROCESS

The aging process has a significant impact on the epidermis, leading to the shrinkage of the epidermal layer and a decreased ability of the skin's barrier to heal after damage. These observations suggest a decline in the efficiency of the stem cell replenishment chain. Recent research underscores the critical role played by the stem cell microenvironment, also known as the epidermal niche, in maintaining epidermal homeostasis. This epidermal niche consists of both cell-intrinsic factors like epigenetics and cell-extrinsic factors, including growth factors, matrix composition, and cell-cell interactions. These factors influence both amplifying cells and stem cells, adding complexity to the cellular dynamics of the skin [11]. Importantly, these parameters can change due to external influences and the aging process [12].

Apart from keratinocytes, the epidermis also contains well-organized melanocyte networks that produce pigment, which is important for protecting the skin from UV radiation [13]. Additionally, the epidermis contains immune system dendritic antigen-presenting cells called Langerhans cells. These diverse cellular networks within the epidermis depend on the proper functioning of keratinocytes to remain in good condition; any malfunction in this coordination can result in age-related declines in pigment regulation and epidermal immune function [14, 15, 16]. The dermis, which has a lower cellular density than the epidermis, is abundant in an extracellular matrix and can be divided into two main layers: the papillary Fibroblasts are the primary cells in the dermis, originating from at least two different cell lineages during dermal development [17]. These cells undergo significant changes in their identity as aging occurs, a topic we will explore further below. The dermis also contains various other cell types, such as nerve endings, Schwann cells, endothelial cells that form blood vessels, pericytes, mast cells, tissue macrophages, and a variety of immune cells [18]. When it comes to the renewal of the outer skin barrier, the epidermis is made up of constantly proliferating keratinocytes, whereas the adult dermis is thought to be a post-mitotic tissue made up of specialized cells that divide seldom. The epidermis serving as the "first line of defence" against external stressors, especially those related to aging, is anticipated to be more important for these dermal cells as they rely more on mechanisms for healing functional damage and responding to stress. [19].

Skin undergoes significant changes as it ages, whether due to the passage of time or exposure to sunlight. Chronological aging often results in thinner skin with fine wrinkles and increased dryness, while photo-aged skin tends to develop a leathery appearance with coarse wrinkles. Wrinkles and skin sagging primarily occur due to alterations in dermal components during the aging process. This includes reduced the synthesis of dermal matrix components and changes in remodelling processes. In chronologically aging skin, the regular structure of dermal elastin deteriorates, and type I collagen, the most abundant protein in the dermal extracellular matrix, undergoes degradation, structural deterioration, and decreased production [20-21].

Changes brought about by external influences can be attributed to a variety of mechanisms in the dermal matrix. UVB rays cause keratinocytes to secrete matrix metalloproteinases, which break down the dermal matrix even when they do not reach the dermis. This causes "solar elastosis," or an accumulation of broken-up and crosslinked elastin in the upper dermis, which reduces elasticity. Additional factors that damage the dermal matrix include elastin modifications by UVA-induced reactive carbonyls and the accumulation of senescent cells due to UV exposure or aging. These senescent cells produce enzymes that break down the matrix and secrete lipids that can chemically alter proteins [22-28].

Skin aging, like aging in other organs, involves a gradual decline in functionality and regenerative potential. What distinguishes skin aging is its susceptibility to extrinsic factors influenced by lifestyle [22-26].

The most significant extrinsic factor contributing to skin aging is ultraviolet radiation, causing DNA damage and oxidative stress, accelerating cellular aging and senescence. The specific effects of ultraviolet radiation on cell types and skin compartments, as well as the types of aging-related damage induced, depend on the wavelength of the ultraviolet light [29].

In general, the well-established hallmarks of aging observed in various organs also apply to different compartments and cell types within the skin [30]. However, skin cells that remain in the body for extended periods suffer more significantly from the deterioration of cellular maintenance and repair mechanisms compared to highly proliferative cells that are frequently regenerated. [31].

1.3. CELL AGING AND FIBROBLAST CELLS

Cell senescence and the aging of fibroblasts play a pivotal role in the process of skin aging. Within the dermis, fibroblasts represent the most cell type and significantly influence various aspects of skin aging, whether through direct effects or interactions with other cells.

Laboratory-based aging experiments conducted on dermal fibroblasts provide valuable insights into the profound changes that occur as these cells undergo the aging process. These changes encompass a range of critical phenomena, including the accumulation of double-stranded DNA breaks, chromosomal and epigenetic irregularities, shortening or oxidative damage to telomeres (the protective caps on the ends of chromosomes), and a decline in DNA repair mechanisms. Aged fibroblasts, particularly those that have become senescent, face challenges in maintaining proteostasis, which involves the precise regulation of the cellular proteome in terms of both quantity and quality. This deterioration stems from abnormalities in processes such as protein synthesis, protein folding, protein degradation, and post-translational modifications, including protein oxidation and cross-linking [32].

In summary, the aging of fibroblasts within the skin’s dermis is a pivotal factor contributing to overall skin aging. These cells undergo molecular and cellular changes that significantly impact the skin’s structural integrity and functional capabilities over time. Damage to fibroblasts or the continuous signalling of such damage can trigger the aging process in these cells. This aging process, marked by a permanent stop in the cell cycle, entails significant changes in the synthesis, secretion, or vesicular release of certain biological components. In a biological context, in vivo senescence plays a crucial role in preventing damaged cells from potentially becoming cancerous and also contributes to the wound healing process. However, when senescent cells persist in tissues for extended periods and release their secreted factors, known as secretomes, it can contribute to age-related tissue deterioration and, in some cases, even promote tumour formation [33].

It is consistently possible to induce senescence in cultured fibroblasts by either reducing their ability to proliferate or subjecting them to UVB irradiation. It's crucial to remember that these techniques are somewhat artificial, and results from these kinds of studies must be confirmed in an in vivo setting [34]. Wei and colleagues conducted a noteworthy work wherein they utilized advanced bioinformatics approaches and RNA sequencing to uncover parameters associated with aging in a range of in vitro models. Using senescent fibroblasts from old human donors, their findings were verified. They identified the diabetes-associated p53-dependent Ras-related gene as a novel marker linked to aging. Non-senescent fibroblasts were more vulnerable to hydrogen peroxide-induced senescence when this diabetes-related gene was deleted, offering insights into possible aging mechanisms and indicators, including cellular senescence and its connection to conditions [35].

Another study by Cavinato and colleagues demonstrated that UVB-induced senescence in dermal fibroblasts led to impaired proteasome function. This impairment, seen as a potential compensatory mechanism for degrading oxidized proteins, induced autophagy. When autophagy was further blocked, the stressed cells underwent apoptosis [36].

Aging-related reductions in autophagic capacity were emphasized in a study comparing skin fibroblasts from aged mice. This research revealed that the expression of motor proteins responsible for moving autophagosomes was impaired in aged dermal fibroblasts, but not in senescent ones. This disruption resulted in reduced fusion between autophagosomes and lysosomes, leading to impaired removal of cellular cargo from the perinuclear space [37].

In summary, skin aging involves several interconnected factors that impact different cell types and contexts within the skin. One crucial aspect is the decline in autophagic activity, which leads to the inefficient removal of dysfunctional mitochondria, known as mitophagy. This deficiency amplifies oxidative stress in aging cells, resulting in the accumulation of malfunctioning mitochondria and increased production of harmful reactive oxygen species, exacerbating oxidative damage [38]. This concept sheds light on how skin aging is influenced by the specific and context-dependent effects of autophagy [39].

Additionally, recent research has highlighted the role of the immunoproteasome, a variant of the 20S proteasome, in regulating oxidatively modified proteins in cells. Studies in non-human primate dermal fibroblasts have shown a connection between species longevity and immunoproteasome activity. Drugs like rapamycin, known to extend lifespan in mice, increase immunoproteasome activity in primate cells, which not only aids in degrading oxidized proteins but also correlates with the expression of genes related to antigen presentation and immune signalling [40].

Surprisingly, continuous activation of the redox regulator Nrf2, a key player in cellular stress response, has been found to induce fibroblast senescence in both in vitro and in vivo settings. These senescent fibroblasts produce an abnormal extracellular matrix that enhances wound healing but also has pro-tumorigenic effects. This discovery highlights the context-dependent nature of senescent cell effects and their potential implications for tissue health and longevity [41,42]. In essence, understanding the complex dynamics of skin aging involves considering various factors, including autophagy, immunoproteasome activity, and redox regulation, while recognizing the context-dependent effects of senescent cells on tissue health and longevity.

1.4. PLASTICITY IDENTITY OF FIBROBLAST CELLS

The study of fibroblasts in aging skin has provided intriguing insights into the complex mechanisms underlying skin aging. Several noteworthy discoveries have been made in this area. Senescent cells communicate with their surroundings through physical, paracrine, and systemic means. A critical component of this communication is the senescence-associated secretory phenotype (SASP), which represents the secretome of senescent cells. The SASP is a complex mixture of immunomodulatory and proteolytic mediators, including lipids. It is considered the primary driver of both beneficial and detrimental effects associated with senescent cells in skin and other tissues (Figure 1.3.) [43].

Computational modelling has been employed to identify transcriptional regulators of the SASP across different cellular contexts. For instance, Meyer and colleagues identified NEMO as a predicted transcriptional regulator of the SASP and confirmed its role in regulating the SASP in response to DNA damage in murine dermal fibroblasts [44]. The persistence of senescent cells and their continued secretion of the SASP contribute to age-related tissue deterioration. Therefore, removing senescent cells has emerged as a promising strategy to reduce age-related damage and diseases [45]. The SASP plays a crucial role in recruiting cells from the innate and adaptive immune systems to target and clear senescent cells [46]. Understanding how senescent cells communicate and the role of the SASP in aging-related processes has opened new avenues for potential interventions to combat age-related skin damage and diseases.

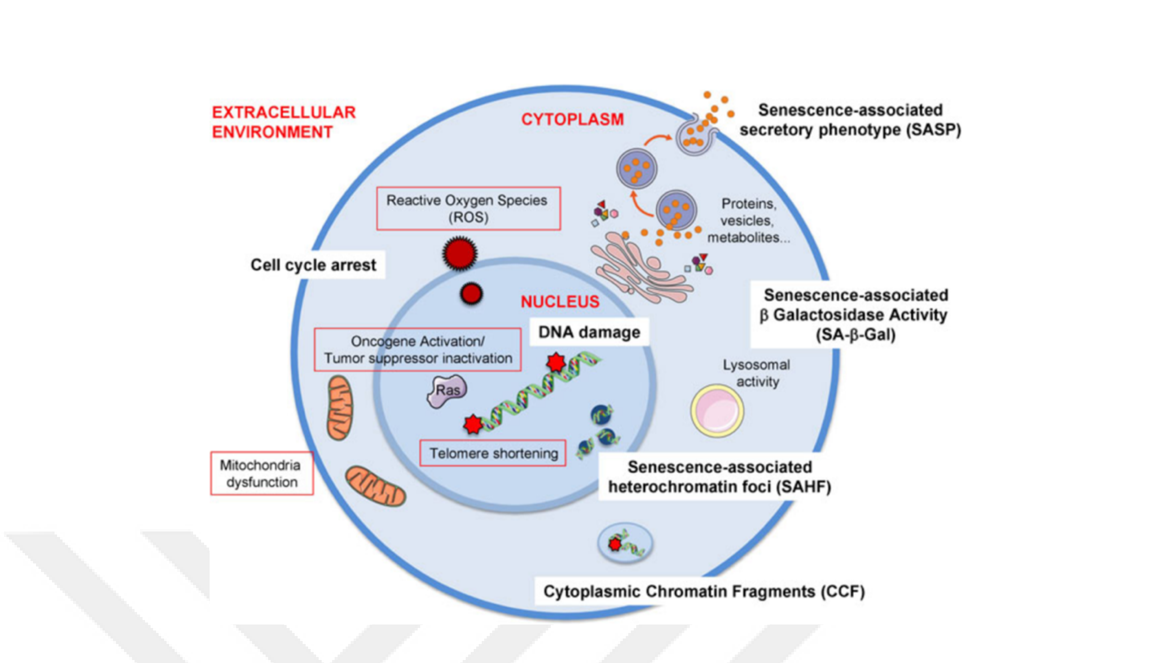

Figure 1.3. Triggers and biomarkers of cellular senescence [50]

Despite efforts to clear senescent cells, aged tissues continue to accumulate them, contributing to further tissue deterioration [47]. Recent research by *Pereira* and colleagues highlighted an interesting mechanism by which senescent dermal fibroblasts evade elimination [48].

Another intriguing concept is "bystander senescence," where senescent cells influence neighbouring cells in spatial proximity. This phenomenon has been observed in vivo for dermal fibroblasts [49].

Additionally, senescent fibroblasts have been discovered to communicate with neighbouring cells by transferring extracellular vesicles containing bioactive microRNAs. These vesicles have been shown to affect keratinocytes in organotypic models, inducing phenotypes resembling those of geriatric skin cells [50]. These findings underscore the complexity and significance of cell communication and plasticity in the context of skin aging. Understanding these mechanisms can provide valuable insights for developing strategies to mitigate the effects of skin aging and promote healthier skin in the aging population. Recent research into the aging of dermal fibroblasts has not only provided detailed insights into the canonical hallmarks of aging but has also revealed the remarkable functional plasticity displayed by these cells during the aging process. The application of advanced techniques such as deep sequencing, single-cell sequencing, and lineage tracing has greatly contributed to the progress in this field.

A study conducted by *Salzer,* and colleagues revealed significant transcriptomic differences between young and aged Pdgfra/CD34 double-positive mouse fibroblasts. While the expression of genes related to the extracellular matrix synthesis and secretion decreased in older fibroblasts as expected, there was a substantial increase in the expression of genes associated with inflammation, stress fiber formation, adipogenesis, and lipid metabolism in the aging group [51]. In this study, advanced techniques like single-cell RNA sequencing and lineage tracing were used to explore the diversity and adaptability of fibroblast populations as organisms age. Through transcriptomic analysis of fibroblasts from newborn mice, researchers identified distinct clusters representing papillary and reticular fibroblasts, with subclusters showing varying levels of cell activity among papillary cells. Lineage tracing experiments demonstrated that progeny of Lrig+ cells, which define newborn papillary fibroblasts, had already dispersed throughout the entire dermis by the age of two months. Additionally, some fibroblasts transformed into mature adipocytes, making it challenging to categorize fibroblasts solely based on their transcriptomic signatures as they age [52,53].

An intriguing finding was the modulation of cellular identity during aging. Caloric restriction in mice prevented the loss of papillary fibroblast identity and the acquisition of adipocytic features during the aging process, demonstrating that cellular identity is not fixed and can be influenced by interventions that slow down age-related deterioration [54]. Moreover, the reduction in cellular density within the murine dermis during aging was revealed to be a non-random process. It resulted from the cumulative loss of localized cell clusters and was not compensated for by the proliferation of activated cells. Instead, neighbouring cells with positionally stable characteristics extended their membranes to compensate for the lost density. This phenomenon was demonstrated through in vivo imaging by Marsh and colleagues [54]. Their findings offer valuable insights into the dynamic nature of fibroblast populations, cellular identity, and tissue density changes during aging.

In summary, recent findings highlight the remarkable adaptability and plasticity of dermal fibroblasts during the aging process. Advanced techniques have enabled researchers to unravel the complexity of fibroblast populations and their responses to aging, shedding light on potential interventions to modulate cellular identity and mitigate age-related changes in the skin.

1.5. KERATINOCYTES AND SKIN AGING

In a laboratory setting using monolayer culture, epidermal keratinocytes exhibit a senescent phenotype without displaying markers associated with terminal differentiation. This senescent state typically occurs after a considerably lower number of population doublings compared to fibroblasts. It's important to note that the persistent DNA damage signalling that reinforces senescence differs between epithelial keratinocytes and fibroblasts [55].

The process of senescence in epithelial keratinocytes can be divided into two phases. Initially, there is a growth arrest that is not influenced by factors such as telomere shortening, DNA damage response, or ATM/ATR signalling. Subsequently, a second phase follows, which was characterized by the replication of cell clones that manage to escape the initial halt in proliferation. While in vitro studies provide valuable insights into cell type-specific damage and repair mechanisms, examining the aging-related characteristics of keratinocytes within the epidermis is a complex challenge because, unlike melanocyte stem cells in the skin, which significantly decline with age, the number of keratinocyte stem cells in hair follicles remains relatively stable, except in areas affected by age-related hair loss [56]. The senescent state appears somewhat reversible during this phase [57]. It's worth noting that attempts to restore the proliferative capacity of keratinocytes through heterochronic transplantation have not yielded significant results, which suggests the involvement of niche factors in the aging process of the epidermis. Recent research has shed light on the damage, dysfunction, and repair mechanisms involved in aging epidermal keratinocytes. While proteostasis in aged keratinocytes has not been as extensively studied as in fibroblasts, it is of growing interest. One study identified an age-related decline in the proteasomal subunit PSMD8, and knockdown of this gene led to an aged epidermal phenotype in a three-dimensional model. Autophagy, while not crucial for epidermal barrier function, plays a role in preventing keratinocyte senescence and DNA damage. However, the interplay between autophagy and epidermal metabolism in epidermal stem cells requires further investigation [58,59]. Accumulating evidence suggests that the aging process in keratinocytes is influenced by various factors. While low NAD+ causes early differentiation and an increase in senescence markers, high NAD+ levels encourage keratinocyte proliferation and an undifferentiated state. Premature aging can be caused by mutations in the CSA gene, which codes for nucleotide excision repair (NER) of DNA. However, keratinocyte senescence can be delayed by correcting the CSA gene. Human age causes a decline in NER capacity, which may indicate that NER plays a role in both intrinsic and extrinsic keratinocyte aging. Collagen 17A1 is lost as a result of quiescent keratinocyte stem cells sustaining DNA damage response (DDR) signaling in elderly mouse hair follicles. The elimination of senescent cells by terminal differentiation and shedding is triggered by this loss, suggesting that collagen 17A1 is a key factor in the in vivo aging of epidermal stem cells.

Furthermore, studies have looked into the flexibility and communication of keratinocytes during aging. As long as basal epidermal keratinocytes express Col17A1, PDGFRα+ fibroblasts form junctional structures that hold the keratinocytes in place. As we age, these structures deteriorate and may no longer be as effective in protecting epidermal stem cells because fibroblasts and keratinocytes do not physically connect as much. Due of UVB-induced photodamage, melanocytes connected to Col17A1-positive stem cells may delaminate with keratinocytes, causing dyspigmentation. This interaction also affects melanocytes associated to these stem cells [60-63]. These findings highlight the complex mechanisms involved in the aging of keratinocytes and its impact on skin health.

In aged epidermis, keratinocytes exhibit a reduced activation state of IGF-1R, leading to defective DNA damage responses and an increased vulnerability to mutation accumulation. This phenomenon is not exclusive to keratinocytes but stems from the decreased IGF-1 support provided by aging dermal fibroblasts [64].

Understanding of signals originating from aged or senescent keratinocytes has primarily been derived from in vitro studies, with limited in vivo observations. In a mouse model of oncogene-induced senescence, senescent keratinocytes release SASP, which initially enhances the local plasticity and stemness of neighbouring epithelial cells, facilitating senescent cell clearance. However, prolonged SASP exposure results in the accumulation of stem cells lacking regenerative capabilities and induces senescence in nearby cells.

The regulation of keratinocyte SASP involves factors like NFkB and the RNA-binding protein YBX1, which decreases in aging cells and promotes SASP cytokine translation. Another mouse model with induced mitochondrial dysfunction exhibited an atypical SASP in keratinocytes, lacking the major inflammatory component but accelerating keratinocyte differentiation, suggesting that senescent keratinocytes can be eliminated through the differentiation. Additionally, the epidermis of aged mice was found to contribute to increased cutaneous and serum inflammatory cytokine levels, particularly in response to barrier disruption, indicating adaptability of the keratinocyte SASP to stress [65-69]. These findings provide insights into the complex role of keratinocytes in skin aging and inflammation.

1.6. SKIN AGING TREATMENTS

Skin aging treatments encompass a wide range of interventions aimed at mitigating or reversing the visible signs of aging on the skin. These treatments are designed to address the cellular and structural changes that occur as the skin ages, restoring a more youthful appearance and improving skin function.

1.6.1. Topical Retinoids

Topical retinoids, derived from vitamin A, have long been recognized as a gold standard in the treatment of skin aging. They offer a multifaceted approach to rejuvenating the skin by addressing various cellular-level changes associated with aging [70].

Topical retinoids are renowned for their multifaceted skin benefits, primarily stimulating collagen production to enhance skin elasticity and structure. They accelerate cell turnover, facilitating the rapid shedding of dead and damaged cells for smoother, more even-toned skin. This action not only unclogs pores and reduces blemishes but also effectively addresses aging and acne-prone skin [70]. Moreover, retinoids can diminish hyperpigmentation by inhibiting melanin production, leading to an improved complexion and skin tone. Certain formulations, particularly those combined with moisturizers, bolster skin hydration by reinforcing the skin's moisture barrier, thus alleviating dryness and peeling. Additionally, they play a crucial role in preventing photoaging by repairing UV-induced damage and fortifying the skin against future harm, making them integral to anti-aging skincare routines. Available in various types and strengths, from over-the-counter options like retinol to prescription-strength variants, retinoids offer a spectrum of potency and efficacy. However, it's vital to use them as prescribed, typically at night, followed by a moisturizer to mitigate dryness, and to incorporate broad-spectrum sunscreen into daily routines to protect against heightened UV sensitivity [70, 71].

1.6.2. Dermal Fillers

Dermal fillers, such as hyaluronic acid-based injectables, are popular for restoring volume and reducing the appearance of wrinkles and folds. These minimally invasive treatments plump up sagging skin, add contour, and provide immediate results. Dermal fillers are commonly used for enhancing lips, filling nasolabial folds, and rejuvenating the under-eye area [72].

Dermal fillers are an array of substances designed to combat aging and enhance facial features, with options including hyaluronic acid (HA), calcium hydroxyapatite (CaHA), poly-L-lactic acid (PLLA), and polymethylmethacrylate (PMMA) fillers, each possessing distinct characteristics and applications. HA fillers, celebrated for their versatility, leverage the moisture-retaining properties of hyaluronic acid, a substance naturally found in the body, to immediately address wrinkles, folds, and enhance lip volume, with the added benefit of reversibility. CaHA fillers, on the other hand, offer a longer-term solution by stimulating collagen production, ideal for deep lines and restoring volume in areas like cheeks and temples. PLLA fillers distinguish themselves by gradually bolstering collagen synthesis, targeting sagging skin and volume loss with effects that unfold over months for a subtly rejuvenated appearance. Lastly, PMMA fillers provide durability and semi-permanent volume enhancement, suited for deep wrinkles and facial augmentation, featuring microspheres suspended in a gel for both immediate and enduring results [73].

1.6.3. Botulinum Toxin (Botox)

Botulinum Toxin, commonly known as Botox, is a highly popular injectable treatment for both cosmetic and medical purposes. Its mechanism of action involves temporarily blocking nerve signals to muscles, leading to muscle relaxation. In the cosmetic realm, Botox is widely used to reduce the appearance of dynamic wrinkles caused by repeated facial muscle contractions, with common treatment areas including the forehead, glabella, and crow's feet. Beyond its aesthetic applications, Botox has medical uses, including treating conditions like chronic migraines, hyperhidrosis, muscle spasms, and various neurological disorders. The procedure is relatively quick, minimally invasive, and administered by trained professionals. Results become noticeable within days to a week and typically last around three to six months, necessitating periodic maintenance. Botox's safety profile, customizable nature, and temporary effects make it a sought-after option for individuals looking to rejuvenate their appearance or address specific medical conditions with minimal downtime [74].

1.6.4. Chemical Peels

Chemical peels are a popular and effective cosmetic procedure used to rejuvenate the skin's appearance. By applying specially formulated chemical solutions to the skin's surface, chemical peels exfoliate and remove the damaged outer layers, stimulating the growth of new, healthier skin cells. They are suitable for addressing a range of skin concerns, including fine lines, sun damage, age spots, acne scars, and uneven pigmentation. The depth of the peel varies, from superficial to deep, with different recovery times associated with each. Superficial peels have minimal downtime, while deeper peels may require more extended recovery periods. To achieve the best results and minimize potential risks, individuals should consult with a qualified skincare professional who can assess their skin's specific needs and recommend the most suitable type and depth of chemical peel. Overall, chemical peels offer a transformative solution for achieving smoother, brighter, and more youthful-looking skin [75].

1.6.5. Laser and Light Therapies

Laser and light therapies represent cutting-edge solutions for enhancing skin health and appearance. These treatments, ranging from fractional laser therapy to intense pulsed light (IPL) therapy, leverage targeted wavelengths of light to address a diverse array of skin concerns. By stimulating collagen production, reducing pigmentation irregularities, and smoothing out wrinkles, laser and light therapies offer remarkable rejuvenation benefits. Their versatility extends to treating medical conditions such as rosacea and spider veins. Recovery time varies depending on the treatment intensity, with some therapies requiring minimal downtime while others may necessitate several days of recovery. With customized treatment plans tailored to individual needs and the guidance of skilled dermatologists or skincare professionals, laser and light therapies offer an effective and transformative approach to achieving smoother, more youthful, and healthier skin. [76].

1.6.6. Cultured Fibroblast Therapy and Exosome Treatments

Fibroblast therapy and exosome treatments represent cutting-edge regenerative medicine approaches with promising applications in skincare and anti-aging. Cultured fibroblast therapy utilizes fibroblast cells, possessing a distinctive capacity to synthesis collagen to promote tissue repair and rejuvenation. Exosomes are tiny vesicles secreted by stem cells that contain bioactive molecules, including growth factors and signalling proteins, which can modulate cellular processes. [77,78].

1.6.6.1 Cultured Fibroblast in Anti-Aging

Cultured fibroblast treatments represent a fascinating intersection of cell biology, regenerative medicine, and cosmetic science, utilizing fibroblasts pivotal in wound healing and tissue repair to harness the body's intrinsic repair mechanisms, promoting skin regeneration and improving tissue function. Fibroblasts, a type of cell making up the connective tissue, play a critical role in wound healing by producing the extracellular matrix (ECM) and collagen, essential for providing structural support to tissues. These cells, prevalent not only in the skin but also in organs and tendons, highlight their importance across various bodily functions. The journey of cultured fibroblast treatments began in earnest in the 1980s with early research focusing on their potential for healing and tissue regeneration. This period saw the exploration of cultured fibroblast injections to promote healing, with promising results in experimental studies and clinical trials. As the field evolved, techniques for isolating fibroblasts from a patient's own tissues, expanding them in culture, and utilizing them for various therapeutic purposes also developed. This approach has several advantages, including a reduced risk of immune rejection and the ability to tailor treatments to individual patients. One of the primary applications of cultured fibroblast treatments has been in tissue engineering and the treatment of chronic wounds, such as diabetic and venous ulcers. Additionally, the ability to genetically modify fibroblasts has opened new avenues for delivering therapeutic genes directly to tissues, expanding the potential uses of these cells in regenerative medicine [79].

In the 2000s, the use of cultured fibroblasts expanded into the cosmetic industry with the goal of improving skin quality and reducing the appearance of wrinkles. This application leverages the fibroblasts' ability to produce collagen and other components of the ECM, crucial for maintaining skin elasticity and appearance. Today, cultured fibroblast therapy involves isolating, expanding, and potentially modifying fibroblasts to enhance their regenerative capabilities. These cells can then be transplanted back into damaged skin areas, contributing to tissue regeneration, reducing scar tissue formation, and enhancing overall skin quality and appearance. This therapy has shown effectiveness in treating a variety of skin conditions and injuries, underscoring the critical role fibroblasts play in skin regeneration [79].

1.6.6.2 Extracellular Vesicles

Exosome treatments harness the regenerative capability of exosomes released by stem cells. These tiny vesicles contain a potent cocktail of growth factors and signalling molecules that can be applied topically or injected into the skin. Exosomes can stimulate cellular regeneration, reduce inflammation, and improve skin texture and tone [80].

1.7. EXOSOMES

The concept of exosomes was initially formulated as small nanoparticles responsible for cellular waste disposal, as elucidated in the study by *Trams* et al. in 1981. These intriguing extracellular vesicles, classified as lipid membraned particles, typically measuring between 30 to 100 nano meters in size, have garnered significant attention in recent years, as outlined by *Doyle* et al in 2019 [80,81]. Research has confirmed their presence in a wide range of cell types, including dendritic cells, tumor cells, stem cells, as well as in salivary and blood cells. Furthermore, a fascinating discovery has expanded the horizons of exosome research, revealing the existence of exosome-like nanoparticles derived from plant cells [82].

The content of exosomes can vary depending on the type of originating cell, encompassing nucleic acid molecules such as miRNA and mRNA, proteins, and lipids. The lipid membrane enveloping exosomes is adorned with surface receptors like CD9, CD81, and CD63, along with heat shock proteins like HSP70 and HSP90. These surface markers not only facilitate the transportation of substances to recipient cells but also play a pivotal role in the recognition of exosomes [83]. In the realm of plant biology, analogous extracellular vesicles are referred to as "exosome-like" due to their striking similarity in terms of morphology, content, and release mechanism [84]. However, the study of exosome-like nanoparticles derived from plants remains a relatively novel and burgeoning area of investigation, holding immense potential for uncovering fascinating insights into intercellular communication and molecular transport mechanisms in the plant kingdom.

1.7.1. Biogenesis of Exosomes

The formation and release of exosomes, crucial players in intercellular communication, involve a complex and finely orchestrated cellular process. Multivesicular bodies (MVBs) are central to exosome production. MVBs are generated through a process of budding into the endosomal lumen, wherein they encapsulate endosomes destined for degradation in lysosomes. However, MVBs also serve a significant role in transporting materials beyond the cell membrane through fusion with the plasma membrane, facilitating cargo delivery not only to lysosomes but also to the extracellular space, as elucidated by Gruenberg *et al.* in 2004 [85-87]. Exosomes, contained within MVBs, are secreted into the extracellular milieu in mammalian cells through membrane fusion events. These exosomes are subsequently transmitted to recipient cells via three known mechanisms, as described by Trams et al. (1981): (i) by direct release into the cytosol following plasma membrane fusion, (ii) by binding to receptor-ligand interactions, and (iii) through endocytosis pathways [88]. While some extracellular vesicles (EVs) are formed directly on the cell's surface, exosome biogenesis traditionally commences within the cell. The process starts with an endocytosis mechanism, wherein portions of the cell membrane are internalized, forming intracellular compartments known as endosomes [89]. Within these endosomes, the membrane budding process recurs, eventually resulting in the accumulation of small vesicles, referred to as intraluminal vesicles (ILVs). The entire structure, filled with ILVs, is termed as MVB. During ILV formation, these vesicles are loaded with various cargo, including lipids, proteins, and RNAs, although the precise details of this process are still under investigation [90]. One pathway involved in cargo loading is mediated by the endosomal sorting complex required for transport (ESCRT), although other mechanisms, such as lipid rafts, are also implicated [91].

Notably, even in the absence of functional ESCRT components, cells can still produce exosomes, albeit with specific disruptions in the generation of particular ILV types. Some of these ESCRT-independent mechanisms may involve transiently formed lipid rafts enriched in specific lipids and protein receptors, which facilitate the inclusion of select molecules into ILVs. Once the MVB's maturation is complete, it can migrate to and fuse with the cell's membrane, releasing its vesicular contents into the extracellular space in the process. This fusion event marks the birth of exosomes. However, it's important to note that MVBs can also merge with lysosomes, leading to the breakdown of the MVB and the recycling of its contents [92]. While the biochemical intricacies of exosome biogenesis are still being unravelled, recent findings have shed light on some of the regulatory factors. For instance, the protein NDRG1, implicated in endosomal trafficking to lysosomes, was found to enhance the release of functional exosomes when blocked [93]. Additionally, an increase in the protein Rab27a, responsible for docking MVBs with the cell membrane, was observed to accelerate exosome discharge [94].

Exosomes, upon their release into the extracellular milieu, interact with other circulating EVs in the body, facilitating intercellular communication. However, distinguishing whether an exosome-like EV has been produced through the MVB pathway or via direct budding from the cell surface remains challenging, as they exhibit strikingly similar properties (Figure 1.4.) [95]. Moreover, there is supporting evidence indicating that plant cells also produce nanovesicles with exosome-like characteristics. These vesicles originating from plants are thought to utilize a mechanism similar to MVB-mediated exosome release for their secretion into the extracellular environment. Research has unveiled that the vesicles found in the extracellular surroundings of plants exhibit a morphology that closely resembles mammalian exosomes [96]. This interesting similarity between exosome-like vesicles in mammals and plants presents exciting opportunities to investigate the various functions of these nanovesicles in facilitating the intercellular communication across different biological realms [97].

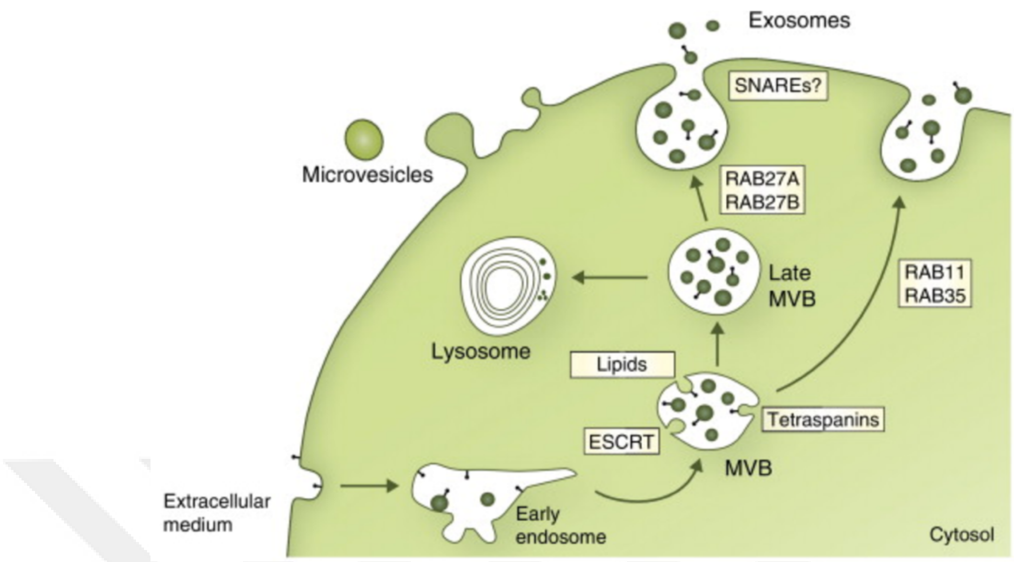

Figure 1.4. Biogenesis of exosomes [97]

1.7.2. Molecular Composition of Exosomes

Exosomes, well-known for their role in intercellular communication, exhibit a diverse molecular composition that can vary depending on their cell of origin. These tiny vesicles typically contain lipids, proteins, and nucleic acids [98]. Exosomes contain nucleic acids, specifically mRNA and miRNA, both in mammalian and plant-derived exosomes, although the quantities can differ significantly. Mammalian exosomes typically carry around 100-300 miRNAs, whereas plant-derived exosomes tend to have fewer than 100 [99]. Mammalian exosomes are rich in cholesterol but have a relatively low number of phospholipids. In contrast, plant-derived nanovesicles are abundant in certain plant lipids, such as phospholipids and galactolipids, which can constitute up to 98 % of their composition. Despite extensive research, some proteins in plant exosomes remain unidentified due to limited information in this area.

1.7.3. Sources of Exosomes

Numerous studies have highlighted the regenerative potential of exosomes derived from progenitor and stem cells. Mesenchymal stem cell (MSC)-derived exosomes, in particular, have been extensively studied and tentatively endorsed in regenerative medicine for treating various diseases. These exosomes, which are cell-free alternatives to cell-based therapies, have been used to facilitate tissue regeneration and wound healing [100]. However, the exact contents of exosomes responsible for their regenerative effects remain incompletely understood. Exosomes isolated from MSCs originating from different tissues have shown promise in tissue recovery and wound healing. Challenges such as targeted treatment and the complexity of exosomal contents remain, but efforts have been made to mitigate these challenges [101].

Recent studies have unveiled the presence of exosome-like nanovesicles derived from edible plants, shedding light on their role in promoting organ and bodily homeostasis through food absorption. These exosome-like nanoparticles, released by various edible plants such as grapes, grapefruits, ginger, and carrots, exhibit anti-inflammatory properties, particularly beneficial in the context of inflammatory bowel diseases. This interspecies interaction demonstrates the potential impact of plant-derived exosome-like nanoparticles [102,103].

Platelet rich plasma (PRP), an autologous whole blood rich in platelets, has gained prominence in treating various medical conditions, including wound healing, cell proliferation enhancement, ligament repair, and apoptosis induction, especially in the field of osteonecrosis over the past five years. Although initially employed for cosmetic and anti-aging purposes, PRP is increasingly used in musculoskeletal disorders. Platelets are known to secrete a high quantity of growth factors and cytokines, which stimulate cell proliferation [104]. However, despite the accelerated research in PRP-based experiments, the effects of PRP-derived exosomes on tissue regeneration, anti-aging, and wound healing remain relatively unexplored in clinical treatments. Little research has been conducted to investigate the potential of PRP exosomes in regenerative medicine and plastic surgery applications [105]. In various studies, platelet-derived exosomes and exosome-like nanoparticles from PRP have been investigated for their potential therapeutic applications, particularly in conditions like osteoarthritis, chronic skin wounds, and osteonecrosis induced by glucocorticoids. These exosomes are rich in growth factors such as platelet-derived growth factor (PDGF), transforming growth factor-beta (TGF-β), and vascular endothelial growth factor (VEGF), which are known to support tissue healing and cartilage regeneration. However, the precise mechanisms through which PRP-derived exosomes promote tissue regeneration and wound healing remain to be fully understood [106-109]. Further research is needed to uncover the underlying processes and mechanisms involved in these potential therapeutic applications.

1.7.4. Extracellular Vesicles from Cord Blood Plasma

Extracellular vesicles derived from cord blood plasma have garnered significant attention in the context of skin aging. These vesicles, often referred to as exosomes, play a pivotal role in intercellular communication and have been investigated for their potential impact on the aging process of the skin [110, 111]. Research has demonstrated that exosomes, including those derived from cord blood plasma, can circulate in biological fluids and participate in cell-to-cell communication. In the context of skin aging, these exosomes are of particular interest due to their potential to influence biological activities in specific cell types involved in skin health and rejuvenation. EVs originating from human umbilical cord blood (UCB) [112] have come into focus as a compelling subject for both experimental and clinical investigation due to their intricate composition and the therapeutic potential they harbour. UCB, denoting the residual blood residing within the umbilical cord and placenta postpartum, boasts a rich repository of hematopoietic stem cells (HSCs), mesenchymal stem cells, and a diverse array of stem or progenitor cell types [113]. These UCB-derived cellular entities offer distinct advantages over their adult stem cell counterparts, predominantly characterized by their relative immaturity, donor safety, reduced incidence of graft-versus-host disease, and diminished ethical considerations. Consequently, UCB has emerged as an invaluable reservoir of transplantable cells poised to address a broad spectrum of human afflictions [114,115].

Parallel to the abundance of stem and progenitor cells, UCB exhibits a wealth of exosomes, minute extracellular vesicles secreted by most nucleated cells and ubiquitous in biological fluids [116]. While the precise biological functions of UCB-derived exosomes remain enigmatic and necessitate further elucidation, contemporary investigations offer intriguing insights. Recent inquiries suggest that these exosomes possess the capacity to bolster angiogenesis and expedite the healing of cutaneous wounds by augmenting the proliferation and migration of endothelial cells and fibroblasts [117]. Additionally, investigations have unveiled differences in protein expression profiles within exosomes derived from human UCB during normal pregnancies and those afflicted by pre-eclampsia. These findings underscore the therapeutic and biomarker potential inherent in UCB-derived exosomes, paving the way for subsequent in-depth exploration of the underlying molecular mechanisms [118,119]. In the realm of epigenetic gene regulation, microRNAs (miRNAs) have garnered significant attention due to their ability to negatively modulate mRNA translation and stability. Recently, exosomal miRNAs, carried within exosomes, have emerged as key players in this field. These exosomal miRNAs are believed to influence the biological activities of specific cell types through umbilical cord blood (UCB) exosomes. There is still a knowledge vacuum about the miRNA expression profile within UCB exosomes, despite prior studies concentrating on profiling miRNAs in UCB, UCB-derived cells, and cord blood buffy coat layers. Conversely, adult peripheral blood (PB) and unified cord blood (UCB) are now important sources of hematopoietic stem cells (HSCs) for transplantation in a range of medical disorders. Clinical evidence suggests that UCB transplantation, as opposed to HSC transplantation from adult donors, is linked to a noticeably decreased risk of leukemic recurrence. The specific causes of this occurrence are still unknown, though. The varied clinical results observed in UCB and PB transplantation may be explained by differences in immunological characteristics, physiological settings, and miRNA profiles found in exosomes derived from various sources or under different physiological situations [120–122]. Further research in this area could shed light on the underlying mechanisms.

2. THE AIM OF THE STUDY

Exosomes play a fundamental role in cellular communication, serving as vital carriers of information between cells. It is well-established that diverse cell types secrete exosomes, and these vesicles exhibit notable variability in terms of their cellular cargo. While the medicinal applications of cord blood plasma have a long history, the realm of cord blood plasma exosomes represents a recently discovered area that warrants in-depth exploration and research.

To this end, the primary objectives of this study are delineated as follows: (a) the isolation and comprehensive characterization of exosomes derived from cord blood plasma; (b) a systematic evaluation of the in vitro effects of these exosomes on primary fibroblast cell lines, with a particular focus on their influence on cellular proliferation and migration capabilities; (c) an examination of telomerase enzyme activity within fibroblast cells.

3. MATERIAL AND METHODS

3.1. MATERIALS

3.1.1. Chemicals

DMEM high glucose (Gibco 41966, Thermo Fisher Scientific, USA), DMEM low glucose (Gibco 31885, Thermo Fisher Scientific, USA), Heat inactivated Fetal Bovine Serum, NB4 (Serve, Collagenase, USA) (FBS) (Gibco 10500-056, Thermo Fisher Scientific, USA), Penicillin-Streptomycin (PS) (Gibco 15140-122, Thermo Fisher Scientific, USA), Dimethyl Sulphoxide (DMSO) (Santa Cruz Biotechnology sc-202581, USA), Phosphate Buffered Saline (PBS) (Lonza BE17-517Q, Switzerland), Trypsin-EDTA (Gibco 25200056, Thermo Fisher Scientific, USA), Polyethylene glycol (#81310, Sigma-Aldrich, USA), Dextran (#9004-54-0, Sigma-Aldrich, USA), , Anti-CD9 antibody (ab2215, Abcam, USA), Anti-CD63 antibody (ab8219, Abcam, USA), Anti-Hsp70 antibody (ab61907, Abcam, USA), Aldehyde/Sulfate Latex Beads,4 % w/v, 4 µm (A37304, Thermo Fisher Scientific, USA), MTS assay (3-(-4,5-dimethyl-thiazol-2-yl) – 5 - (3-carboxymethoxy- phenyl)-2-(4-sulfo-phenyl)-2H-tetrazolium, Sigma), Human Telomerase ELISA Kit (# EEL019, Thermo Fisher Scientific, USA).

3.1.2. Instruments

Laminar Flow Cabin (Heal Force Class II Biosafety Cabinet A2, China), CO2 incubator (In-Vitro Cell ES NU-5800, NuAire, USA), Inverted Microscope (Nikon Eclipse, TS100, Netherlands), Low-Speed Centrifuge (Gyrozen 416, Korea), -80°C freezer (New Brunswick, U410, Germany), Microplate reader (Bio-tek ELx800, USA), Mini Trans-Blot Cell Blotting System (Bio-Rad, USA), High-Speed Centrifuge (Sigma Aldrich 3-18KS, Germany), Shaking Water Bath (Stuart SBS40, UK), Nano-Drop 2000 (Thermo Fisher Scientific, USA),Confocal Laser Scanning Microscope (Zeiss LSM 700, Germany), Guava easyCyte Flow Cytometers (Merck Milipore, Germany), Muse® Cell Analyzer (Merck Millipore, USA), ELISA plate reader (Biotek, USA), FACS Calibur (BD Biosciences, USA), NTA (NS300).

3.1.3. Cell Lines

Senior-aged dermal fibroblasts isolated from an aged donor were purchased from Onkim Stem Cell Technologies.

3.2. METHODS

3.2.1. Exosome Isolation from Cord Blood Plasma

Exosome isolation was performed on cord blood plasma samples obtained from Onkim Stem Cell Technology. To remove any remaining cellular debris, an initial centrifugation step was conducted, and the resulting supernatant was carefully transferred to a new tube. Further clarification was achieved by centrifuging the supernatant at 16,000×g for 30 minutes. Exosomes were then isolated using an aqueous two-phase system (ATPS) as described in the literature. This involved preparing an ATPS isolation solution by mixing Dextran (DEX) and Poly (ethylene glycol) (PEG) in distilled water. The cell lysate was combined with the ATPS isolation solution and centrifuged at 1000×g for 10 minutes to separate the phases. After discarding about 80% of the volume, an equal amount of ATPS isolation solution was added, followed by another round of centrifugation at the same speed. Finally, the upper phase was discarded, and the exosome-rich bottom phase was filtered through 0.22 µm filters. This thorough process successfully yielded isolated cord blood plasma exosomes, which are promising for various research applications.

3.2.2. Exosome Characterization and Qualification

Exosome characterization and quantification were performed using various methods. For quantification and size distribution determination, nanoparticle tracking analysis (NTA) was used. This involved employing a 488 nm laser and following instrument manual recommendations for dilutions (1:40 with distilled water). NTA software version 3.3.301 was used to capture and analyze nanoparticles, with 60-second intervals and at least five recordings.

Flow cytometry was utilized to analyze exosome surface antigens. After calculating the exosome particle number per milliliter via NTA, 5 µg of exosomes were coupled with 5 µl of aldehyde/sulfate-coated latex beads (4 % w/V, 4 µm) and incubated at room temperature for 15 minutes. Subsequently, the exosomes were incubated with 2 % BSA in PBS for 2 hours to minimize nonspecific antibody binding. Glycine was then added to reach a final concentration of 100 mM, and the mixture was further incubated on a shaker for 30 minutes. Following incubation, cold PBS was added, and the mixture underwent centrifugation at 2700 g for 3 minutes. The exosomes were then resuspended in PBS and divided into separate tubes. Each tube contained specific plasma antibodies (CD9 and CD63) conjugated to Alexa Fluor 488, and they were incubated overnight. The next day, exosomes were pelleted through centrifugation at 2700 g for 3 minutes, resuspended, and analysed using Flow Cytometry.

Exosome quantification was conducted using the Lowry protein assay kit, a well-established method for measuring protein content in extracellular vesicles. In this assay, 4 µl of exosomes were mixed with 16 µl of distilled water, and 5 µl of this mixture was added to three distinct wells within a 96-well plate. Subsequently, 25 µl of Reagent A solution and 220 µl of Lowry B solution were introduced to each well, followed by a 30-minute incubation period. The absorbance of the plate was measured at 750 nanometers using a Multiscan Spectrophotometer, and the protein concentrations of unknown samples were determined based on the known absorbance values of BSA standards.

For SEM analysis, exosomes were isolated, fixed, dehydrated, dried, and coated with a conductive material. In the SEM chamber, an electron beam scanned the exosome sample, and detectors captured emitted secondary and backscattered electrons, generating high-resolution 3D-like images. SEM enabled researchers to observe exosome size, shape, and surface characteristics, providing insights into their physical properties and potential roles in biological processes.

3.2.3. Primary Fibroblast Cell Culture and Characterization

In the process of cell culture, senior-aged dermal fibroblasts purchased from Onkim Stem Cell Technologies were seeded into culture dishes with a DMEM High media, both supplemented with 100 units/mL Penicillin and 100 µg/mL Streptomycin (1% PS) and 10% (v/v) FBS (Fetal Bovine Serum), and cell counting was performed using trypan blue staining. For passaging, cells reach to 75-80% confluency were transferred to larger culture dishes. Cells not immediately used were prepared for freezing in a freezing solution (10% (v/v) Dimethyl sulfoxide (DMSO) and 90% (v/v) FBS). Cryovials containing the cells are labelled and gradually frozen to -150°C using a programmable freezer.

Flow cytometric analyses were conducted utilizing the FACS Calibur flow cytometry device. Following determination of the number of cells removed by trypsinization at the end of the 3rd passage. Subsequently, a portion of the cells was set aside for flow cytometric analysis. These cells were incubated with phycoerythrin (PE) monoclonal antibodies targeting cell surface markers, along with appropriate isotype controls, for 45 minutes at room temperature, shielded from light. For fibroblasts, CD11b/34/45/73/90/105/44/29 monoclonal antibodies were utilized. Following incubation, the cells were washed and resuspended in 400µl washing solution before being analyzed on the FACS Calibur flow cytometry device.

3.2.4. Cell Viability

The investigation aimed to evaluate the viability of primary cultured fibroblast cells using the MTS assay, which utilizes 3-(4,5-dimethyl-thiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS). For this assay, cord blood exosomes were prepared at four different concentrations, ranging from 50 µg/ml to 500 µg/ml. These exosome solutions were prepared in DMEM with 10% FBS and 1% penicillin, streptomycin, and amphotericin B (PSA). Approximately $5x10^3$ cells were seeded into individual wells of 96-well plates and allowed to attach for 24 hours under the conditions described in Section 3.2.3. After the initial incubation, the cell culture medium was replaced with DMEM containing exosomes at various concentrations (50 µg/ml to 500 µg/ml). Cell viability was assessed at three different time intervals: 24-, 48-, and 72-hours following treatment with CBE. At each time point, 10 µl of a PBS-glucose solution with a final concentration of 10% MTS was added to each well and incubated for 1 hour at 37°C in a humidified atmosphere with 5% CO2. The absorbance of the wells was then measured at 490 nm using an ELISA plate reader (Biotek, USA) to determine the extent of cell viability.

3.2.5. Scratch Assay

A scratch assay was conducted to assess the effect of cord blood plasma exosomes on cell migration and wound healing. In this experiment, $2x10^5$ cells were initially seeded into 6-well plates and incubated under the conditions described in Section 3.2.3. Once the cells adhered to the substratum, a controlled scratch was made across the cell monolayer using a sterile 1000 µl micropipette tip. The cells were then promptly rinsed twice with PBS. The cell culture medium was replaced with either a treatment medium containing exosomes or a control medium without exosomes. The cells were incubated again at 37°C under the conditions previously mentioned in Section 3.2.3.

To monitor the scratch healing process, images of the scratch were taken at the start of the experiment and at the 16th hour using an inverted microscope (Carl Zeiss Microscopy, LLS, Thornwood, NY, USA). The wound closure rate was analyzed using the ImageJ software (NIH, Bethesda, MD) by calculating the initial gap size (Ai) minus the final distance (Af) between the wound edges. This result was divided by the initial gap size, and the final outcome was multiplied by 100 to determine the percentage of wound closure. The mathematical equation (3.1) used for this calculation is as follows:

$$\% \text{ wound closure} = [(Ai-Af)/Ai] \times 100 \tag{3.1}$$

3.2.6. Telomerase Enzyme Activity Assay

In the preparation process, a standard solution was created by reconstituting 120µl of the standard (160IU/ml) with an equal amount of standard diluent, resulting in an 80IU/ml standard stock solution. Serial dilutions were then made from the stock solution to produce standard points at concentrations of 40IU/ml, 20IU/ml, 10IU/ml, 5IU/ml, 2.5IU/ml, and 1.25IU/ml of telomerase standard with human telomerase antibody (160IU/ml), with the standard diluent serving as the zero standard. Reagents, standard solutions, and samples were brought to room temperature for the assay, which was conducted at room temperature as well. The assay involved adding specific volumes of standards and samples to appropriate wells, followed by incubation, washing, and substrate addition. The reaction was stopped with a stop solution, and the optical density (OD value) for each well was quickly assessed by employing a microplate reader configured to 450 nm within 10 minutes of the stop solution being added. This stepwise process ensures accurate and precise measurement of the target analyte Collect the 15 ml of CBE treated fibroblast cell supernatant in sterile tubes. Centrifuge the samples at 2000-3000 RPM for 20 minutes, then carefully transfer the supernatant to a new sterile tube, avoiding the sediment. For cellular components, dilute the cell suspension in PBS (pH 7.2-7.4) to achieve a concentration of approximately 1 million cells/ml. Subject the suspension to repeated freeze-thaw cycles to lyse the cells and release their contents, then centrifuge at 2000-3000 RPM for 20 minutes. Finally, collect the supernatant without the sediment.

3.2.7. Cell Cycle Analysis

To evaluate the effects of cord blood exosomes on senior-aged fibroblast cells, a cell cycle analysis was performed using a Flow Cytometer (BD Biosciences, USA). Briefly, 3.5×10^5 senior-aged fibroblast cells were seeded in a T-75 cell culture flask. After 24 hours, once the cells had adhered to the flask surface, they were treated with 100 µg/ml of CBE in DMEM low glucose medium for 24 hours. For the control, cells were treated with DMEM low glucose medium containing 20% FBS for 24 hours. For the serum-starved group (SS), cells were treated with DMEM containing 20% FBS for 12 hours, followed by 12 hours without serum to synchronize all cells in the G1 phase.

The cells were then detached by trypsinization, collected into a Falcon tube containing the treatment medium, and centrifuged at 300 × g for 5 minutes. After centrifugation, the cells were washed with PBS, pelleted again, and fixed with 4% paraformaldehyde. Subsequently, the cells were pelleted, incubated in 0.1% Triton X for 20 minutes, and centrifuged at 350 × g for 5 minutes at 4°C. Afterward, 0.05 mg/ml of RNase was added per sample and incubated for 30 minutes. Following incubation, the cells were centrifuged at 350 × g for 5 minutes at 4°C, resuspended in 300 µl of PBS, and 1.5 µl of PI was added, except for the gating group during flow cytometry.

After this preparation, 10,000 cells were analyzed using the Flow Cytometer. This entire procedure was repeated at least three times to ensure accuracy and reproducibility.

3.2.8. Cellular Reactive Oxygen Species Detection

To investigate the impact of cord blood exosomes on senior-aged fibroblast cells, the cells were cultured in standard cell culture media. After harvesting, 25,000 cells were seeded into a dark, 96-well microplate and allowed to attach overnight. The experimental group received treatment with 100 µg/ml of cord blood exosomes for 24 hours. The control group was treated with DMEM low glucose medium containing 20% FBS for the same duration. The serum-starved (SS) group was treated with DMEM containing 20% FBS for 12 hours, followed by 12 hours without serum.

Following treatment, the media was aspirated, and 100 µL/well of 1X Buffer was added. After removing the buffer, the cells were stained by adding 100 µL/well of diluted DCFDA Solution and incubated at 37°C in the dark for 45 minutes. Subsequently, the DCFDA

Solution was aspirated, and 100 µL/well of 1X Assay Buffer was added. Fluorescent intensity was immediately measured using a fluorescence plate reader (BioScience MicroPlate Reader, US).

3.2.9. RNA Isolation for Gene Analysis

Senior-aged fibroblasts at a density of 3.5 × 10^5 cells per well were seeded in a T-25 culture flask and incubated overnight at 37°C with 5% CO2. The experimental group received treatment with 100 µg/ml cord blood exosomes in DMEM low glucose medium. For the control group, cells were treated with DMEM low glucose medium containing 20% FBS for 24 hours. For the serum-starved (SS) group, cells were exposed to DMEM with 20% FBS for 12 hours, followed by 12 hours without serum. After 24 hours, cells were harvested, and total RNA was extracted from each cell line using TRIzol™ reagent (#15596026, Thermo-Fisher) according to the instruction for use. To extract RNA, TRIzol (400 µl) was added to cell pellets and incubated for 5 minutes. Chloroform (100 µl) was then added, samples were mixed and incubated for 3 minutes, followed by centrifugation at 12,000 × g for 15 minutes to separate the phases. The upper phase was transferred to a new tube, and isopropanol (200 µl) was added, followed by mixing and a 10-minute incubation. After centrifugation at 12,000 × g for 10 minutes, RNA collected as a white pellet at the tube bottom, and the supernatant was discarded. Pellets were then mixed with 75% ethanol (400 µl), vortexed, and centrifuged at 7500 × g for 5 minutes. Pellets were air-dried for about 5 minutes, solubilized in 30 µl of RNase/DNase-free water, and RNA concentrations were measured using a Nanodrop. Samples were stored at -80°C.

3.2.10. cDNA Synthesis by Reverse Transcription PCR

The cDNA was synthesized from isolated RNA samples using the cDNA Kit (High Capacity/ Cat no: 436814). To remove genomic DNA, a mixture was prepared with 2 µl of 7X gDNA Wipeout Buffer, 1500 ng of template RNA, and RNase-free water, making a total volume of 14 µl. This reaction mixture was incubated at 42ºC for 2 minutes and then placed on ice. Next, the reverse-transcription master mix was prepared. For the reaction, 1 µl of Quantiscript Reverse Transcriptase, 4 µl of 5X Quantiscript RT Buffer, 1 µl of RT Primer Mix, and 14 µl of the template RNA (after genomic DNA removal) were combined. The mixture was then processed in a thermal cycler at 42ºC for 15 minutes followed by 95ºC for 3 minutes. The resulting cDNA concentrations were measured using a Nanodrop.

3.2.11. Quantitative Real-Time Polymerase Chain Reaction (RT-PCR) Assay

Target primers were designed using Primer-BLAST, an online tool from the National Center for Biotechnology Information, and synthesized by SentBioLab (Ankara, Turkey). The sequences are provided in Table 3.1. To assess the gene expression levels of p21, p16, ki67, MCP1 cDNAs from samples were utilized for q-PCR analysis. The q-PCR was performed using Promega GoTaq® qPCR and RT-qPCR Systems. For the reaction, 500 ng of cDNA was used as the template, and the PCR master mix was prepared as follows: 1.75 µl of nuclease-free water, 1.25 µl of 1 µM primer, and 5 µl of SYBR Green (2x). The q-PCR was conducted according to the conditions specified in Table 3.2. The housekeeping gene 18S rRNA served as the normalization control.

Table 3.1. Primers used in RT-PCR assays

Gene	Species	Orientation	Sequence
p21	Human	F	5’- TGGAGACTCTCAGGGTCGAAA-3’
		R	5’- GGCGTTTGGAGTGGTAGAAATC-3’
p16	Human	F	5’- CCCAACGCACCGAATAGTTA-3’
		R	5’- ACCAGCGTGTCCAGGAAG-3’
Ki67	Human	F	5’- TCCTTTGGTGGGCACCTAAGACCTG-3’
		R	5’- GATGGTTGAGGTCGTTCCTTGATG-3’
MCP1	Human	F	5’- ACTGAAGCTCGTACTCTC-3’
		R	5’- CTTGGGTTGTGGAGTGAG-3’

Table 3.2. RT-PCR settings

Cycle	Repeat	Step	Duration	Temperature
Initial Denaturation	1	1	5 minutes	93 ^{o}C
Denaturation	40	1	60 seconds	93 ^{o}C
Annealing		2	60 seconds	55 ^{o}C
Extension		3	60 seconds	72 ^{o}C
Final Extension	1	1	10 minutes	72 ^{o}C
Melt Curve	110	1	12 seconds	-0.5 ^{o}C/cycle
Hold	1	1	-	4^{o}C

3.2.12. Immunocytochemistry Analysis

3000 Fibroblast cells from senior-aged donors were plated in an 8-well plate and incubated overnight. The control group was maintained in DMEM medium (complete), while the experimental group was treated with 100 µg/mL CBE for 24 hours. Following the treatment, the cells were fixed with 2% paraformaldehyde for 30 minutes at room temperature. After fixation, the cells were washed three times with PBS-T (PBS containing 0.1% Tween 20). Permeabilization was achieved by treating the cells with 0.1% Triton X-100 for 5 minutes, followed by an additional wash with PBS-T. For detecting collagen Type I, cells were incubated overnight at 4°C in a solution containing 2% goat serum and 0.2% primary antibody against collagen Type I. For elastin detection, cells underwent a similar treatment using a primary antibody against elastin (Thermofisher/MA5-41583). Following the incubation, cells were washed with PBS and then incubated with 0.4% goat anti-mouse AlexaFluor-647 antibody for 1 hour at 4°C, followed by a gentle wash. The nuclei were stained with a 0.1% DAPI solution for 20 minutes at 4°C, then washed with PBS. Observations were performed using a confocal laser scanning microscope, and ImageJ software was utilized to quantify the levels of Type I collagen and elastin.

3.2.13. Statistical Analysis

In Section 3 of the results, statistical analysis of the data was performed, with values presented as means ± standard deviation (SD) or means ± standard error of the mean (SEM). The standard deviation for each experiment was derived from data spanning three to ten independent experiments. Parametric statistics between two data sets were compared using a two-tailed multiple t-test. For comparisons involving more than two different conditions, a one-way analysis of variance (ANOVA) with Geisser-Greenhouse correction was employed. Statistical significance was set at $*P \leq 0.05$. Tube formation and tube length analysis were conducted using the Wimasis Image analysis tool. These analyses were carried out using the statistical software GraphPad Prism 6 (GraphPad Software, USA).

4. RESULTS

4.1. DETECTION OF EXOSOME SIZE AND PARTICLE NUMBER USING NTA

NTA was employed to assess the diverse sizes and concentrations of exosomes while monitoring their Brownian motion. Based on the data presented in Figure 4.2, the average exosome size was calculated to be 95±4.3 nm, with a mode at 100.1±3,5 nm. Additionally, the concentration of exosomes was determined to be 2.68e+013 +/- 3.80e+012 particles/ml particles per milliliter, and the number of particles observed per frame was 23.8 +/- 3.2 particles/frame.

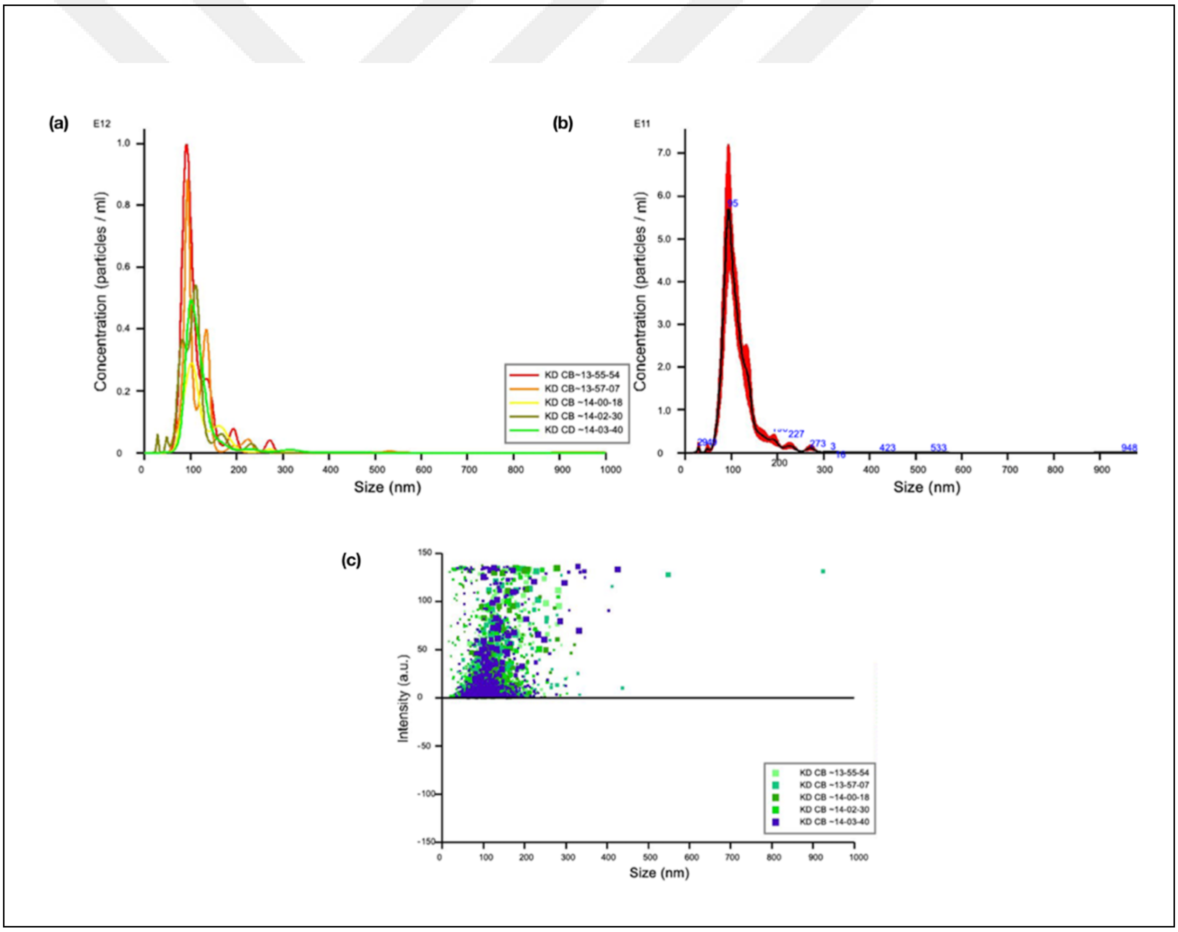

Figure 4.1. NTA of CBE showing particle size, concentration and their variety. (a) FTLA concentration / size, (b) FTLA concentration /size, (c) Intensity /size of exosome distribution. n =3 groups.

4.2. FLOW CYTOMETRY-BASED SURFACE MARKER AND MORPHOLOGIC CHARACTERIZATION OF EXOSOMES

Exosomes were analyzed by examining the surface proteins present on their membranes. The kit includes a component for capturing exosomes using magnetic beads of 6-micron size, coated with an anti-human CD63 antibody. These beads can be identified in a cytometer based on their physical properties, measured by FSC and SSC. Additionally, the kit contains a biotinylated anti-human CD9 antibody and streptavidin conjugated with phycoerythrin (PE), forming a detection system. This allows for the identification of exosomes on the bead surface via the cytometer's FL2 (PE) fluorescent channel. The beads can also be recognized by other fluorescent detectors in the cytometer, aiding in distinguishing bead populations and eliminating duplicates in the analysis. The primary detection antibody provided is the Anti-CD9 biotin, and the secondary detection reagent is Streptavidin-Phycoerythrin (PE), used for identifying biotinylated antibodies. These surface indicators include conjugated CD9 and CD63 (plasma membrane proteins), which are abundantly present on the exosome membrane. The presence of these markers was determined using magnetic beads coated with specific antibodies for these proteins (Figure 4.1). To ensure accuracy, NC beads with no exosomes were employed, and positive results were observed for CD9/CD63 at 1,67%, 89,54%, and 85,22% respectively.

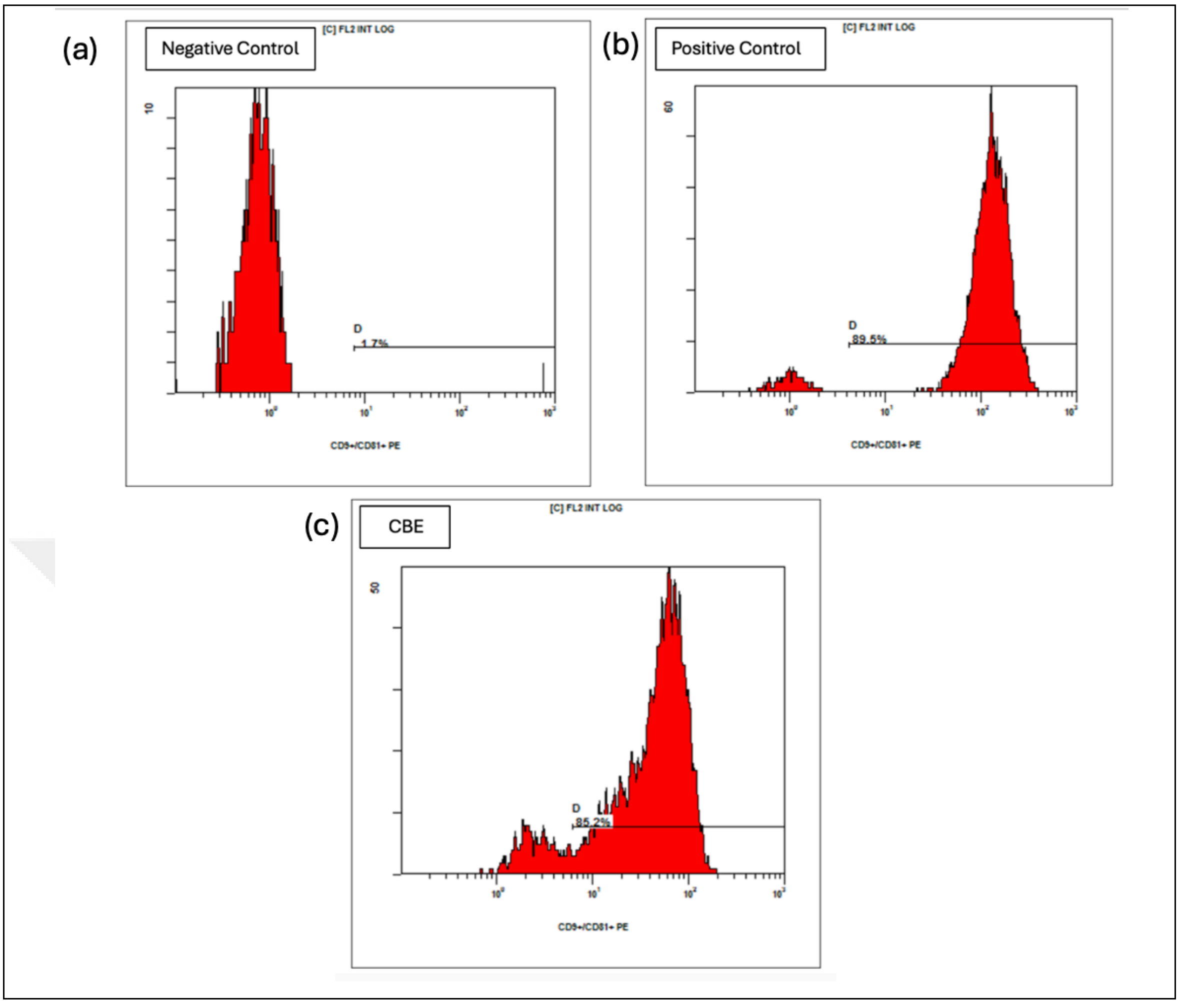

Figure 4.2. Flow cytometry analysis of CBE. (a) NC, (b) CD9/CD63 conjugated proteins, (c) cord blood exosomes probed with CD63/CD9 conjugated proteins.

The surface marker characterization of CBE was conducted using scanning electron microscopy. The results indicated that the exosomes ranged in size between 40 and 100 nm, as demonstrated by the scanning electron microscope analysis. This information suggests that exosomes derived from umbilical cord blood fall within a specific size range, which is important for understanding their properties and potential applications. (Figure 4.2.)

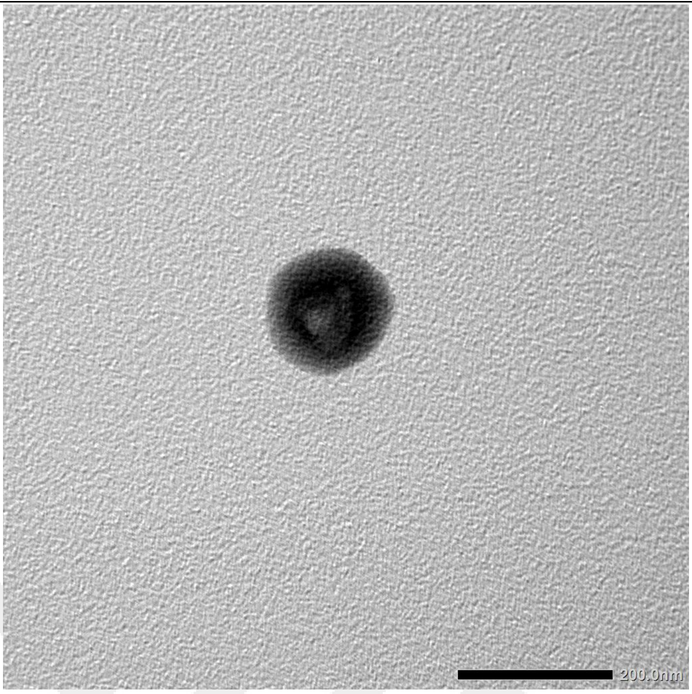

Figure 4.3. Characterization of CBE via Scanning Electron Microscopy Flow cytometry analysis of CBE.

4.3. FIBROBLAST SURFACE ANTIGEN CHARACTERIZATION VIA FLOW CYTOMETRY

The surface proteins on the membranes of the primary fibroblast cell suspension have been examined. These fibroblast specific surface markers include CD29(+), CD44(+), CD 73(+), CD90 (+) and CD 105 (+) which are abundantly present on the fibroblast membrane, while cells were expected to be negatively stained for CD34(-), CD45(-) and CD 146 (-) markers. Detection of these markers involved utilizing magnetic beads that were coated with antibodies specific to these proteins (see Figure 4.3). To ensure accuracy, NC beads with fibroblast were employed, and positive results were observed for markers as 100%, 0,23%, 0,02%, 100%, 99,62%, 100%, 100% and 0,03% respectively.

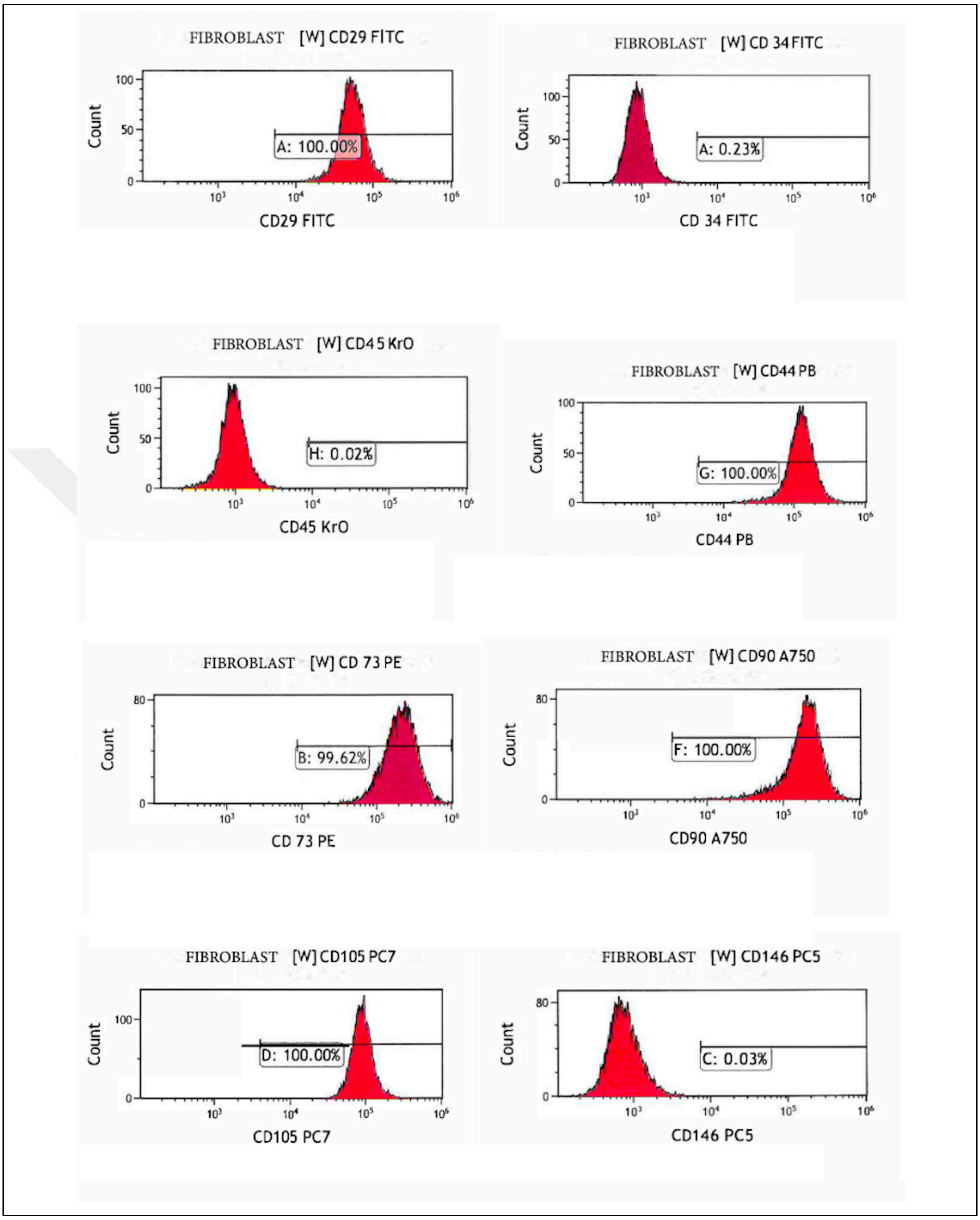

Figure 4.4. Characterization of senior-aged dermal fibroblasts cell culture via flow cytometry.

4.4. ASSESSMENT OF CELLULAR PROLIFERATION

After characterizing the exosomes, their effect on the senior-aged dermal fibroblasts was evaluated through the MTS assay, which depends on mitochondrial activity. Conversely, concentrations of 50µg/ml, 200 µg/ml, and 500 µg/ml resulted in diminished cellular viability by the third day of observation. Particularly noteworthy was the notable increase in cell activity observed at the 100 µg/ml dosage. (Figure 4.5.)

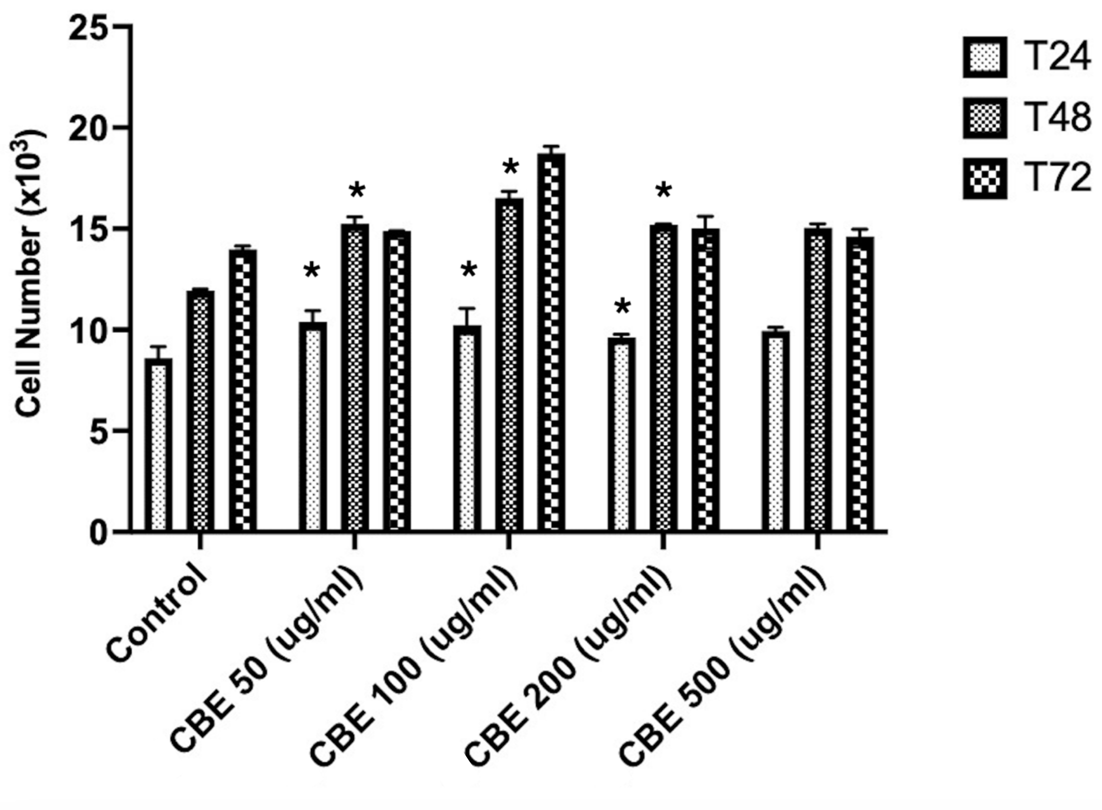

Figure 4.5. The effects of CBE on the proliferation of fibroblast cells. The control was conducted using a complete growth medium. n=3, *P<0.05.

Taking outcomes from three independent experiments into account, the 100ug/ml concentration was selected as the optimal dose. At the culmination of the third day of experimentation, it was observed that the population of cells subjected to a 100 µg dose of CBE had reached a count of 15,000 cells, while the control group, devoid of the CBE treatment, tallied 11,000 cells.

4.5. EFFECT OF CBE TREATMENT ON TELOMERASE ENZYME ACTIVITY OF SENIOR-AGED DERMAL FIBROBLASTS

The activity of telomerase is pivotal in preserving the length of telomeres and preventing the replicative senescence of fibroblast cells. What's noteworthy is that elevating telomerase activity not only impacts telomere length and hence cellular proliferation but also preserves overall cellular function and long-term immune function. In DMEM High media, supplemented with 10% (v/v) FBS, the telomerase enzyme activity measures at 22.24 IU/ml. However, it was observed that treatment with DMEM High media supplemented with 100µg/ml of cord blood plasma exosomes resulted in an increase in telomerase enzyme activity to 38.10 IU/ml in fibroblast cells.

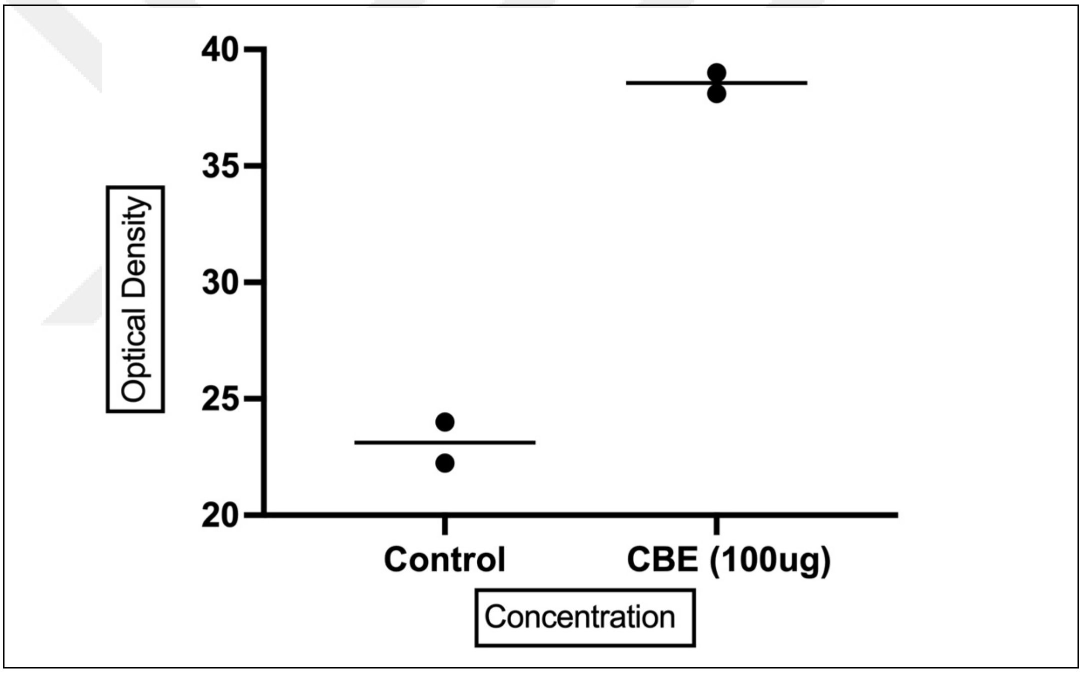

Figure 4.6. The effects of CBE on fibroblast cell telomerase enzyme activity. Control is conducted using complete growth medium. n=3

4.6. EFFECT OF CBE TREATMENT ON MIGRATION OF SENIOR-AGED DERMAL FIBROBLASTS

A scratch assay was conducted to assess the effect of cord blood plasma exosomes on cell migration and wound closure. To achieve this, 100µg/ml concentrations of exosomes were utilized. This concentration was chosen based on the MTS analysis results, which demonstrated enhanced the proliferation of senior-aged fibroblast cells.

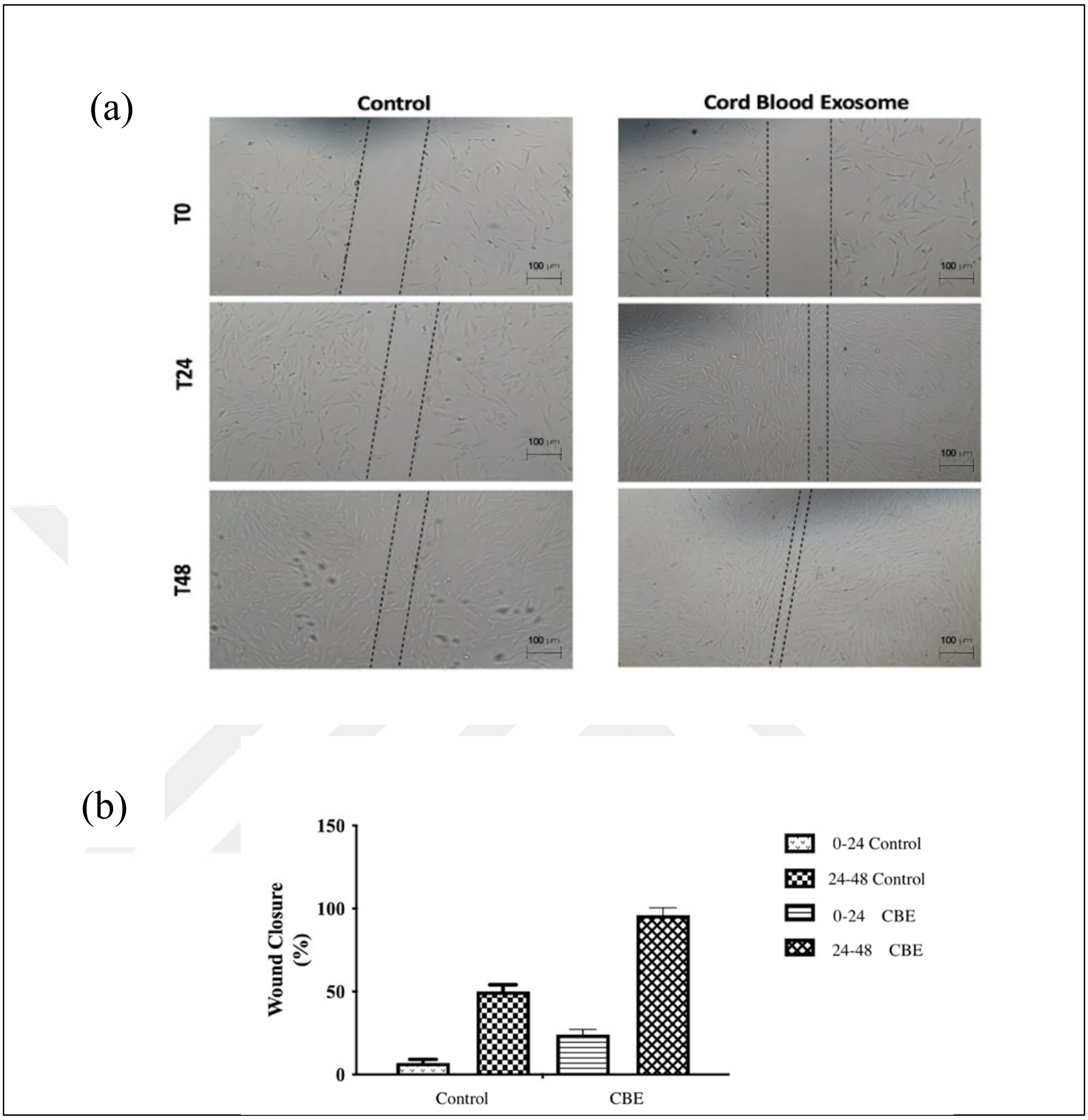

Figure 4.7. Scratch assay analysis of Fibroblast cells (a) Depicts representative images of Fibroblast cells treated with cord blood plasma exosomes at 0, 24, and 48 hours. (b) Shows the wound closure rates in Fibroblast cells treated with exosomes. Data are shown with ± SD, n=3, *P<0.05.

Results show that treatment with 100 µg/ml of exosomes resulted in fibroblast cells achieving wound closure rates of 49.78 ± 1.315% and 98.23 ± 1.045% within 24 and 48 hours, respectively. In contrast, cells treated with only the growth medium exhibited significantly lower wound closure rates of 12.07 ± 2.654% at 24 hours and 36.56 ± 1.212% at 48 hours. After 24 hours, wound closure in fibroblast monolayers, which were mechanically scratched with a pipette, significantly increased in groups treated with CBE compared to the control group, with an average closure rate of 33.3% (n=3). By 48 hours post-wound creation, it was observed that wounds in scratched fibroblasts incubated with CBE were completely closed, whereas in the control group, the closure rate remained below 50%. (Figure 4.7.)

4.7. CELL CYCLE ANALYSIS

The cell cycle analysis was conducted on senior aged fibroblast cells treated with CBE versus controls. The CBE group received exosome treatment for 24 hours, while the control group had no treatment, and the SS group was maintained in serum-free media after the initial 12 hours. The flow cytometry results are displayed in Figure 4.8. As expected when cells were serum starved (SS), cell cycle arrest was seen in the majority senior-aged fibroblasts with 80% in G0-G1, 10,32% S, and 14.3 in G2/M phases. The distribution in control group was detected as 63.1 % in G0-G1, 11.3 % S, and 24.1 in G2/M. When the G0-G1 phases are analyzed, the CBE group showed significantly lower population of G0-G1 cells with 49.9% compared to the SS and control group. In agreement with the wst-1 results, the CBE group exhibited a higher percentage of cells in the S and G2-M phases with 15.6 and 36.3%, respectively, indicating a much higher proliferation rate compared to the control group.

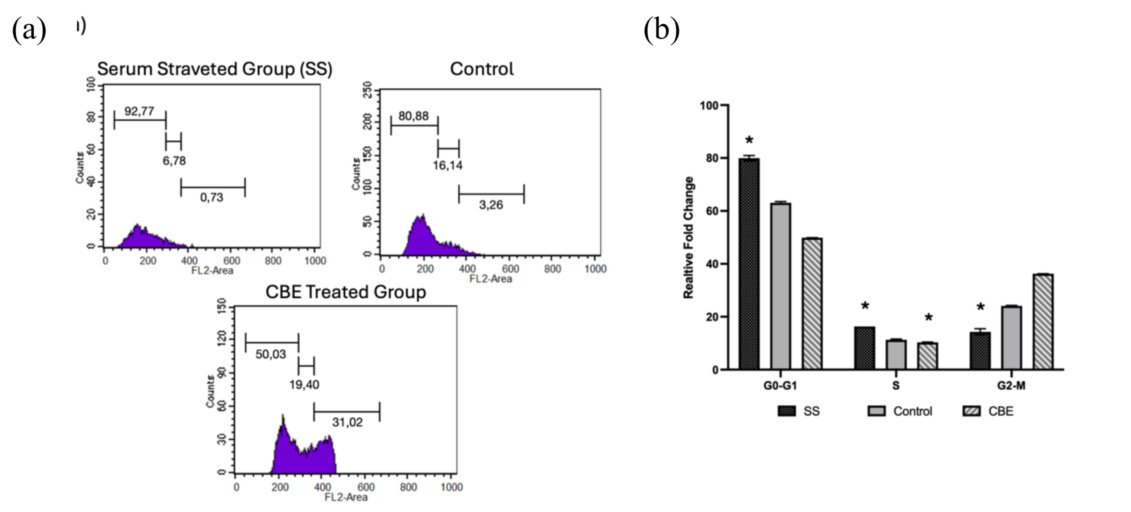

Figure 4.8. Cell cycle Analysis of senior aged fibroblast cells in different group (a) Cell cycle analysis results (b) quantification data of each group; n=3, *P<0.05.

4.8. CELLULAR REACTIVE OXYGEN SPECIES DETECTION

ROS detection analysis was conducted following the manufacturer's protocol. Senior aged fibroblasts were serum starved in order to induce ROS generation as suggested in the literature [123]. SS cells displayed significantly 1.3-fold higher ROS level when compared to Control group. Exosome treatment on the other hand led to a 1.5-fold decrease in ROS levels compared to control cells as DCFDA fluorescent intensity of 0.007 was recorded for CBE while the control group had an intensity of 0.01 (Figure 4.9). These results clearly indicate that the CBE group exhibited lower ROS activity compared to the control groups.

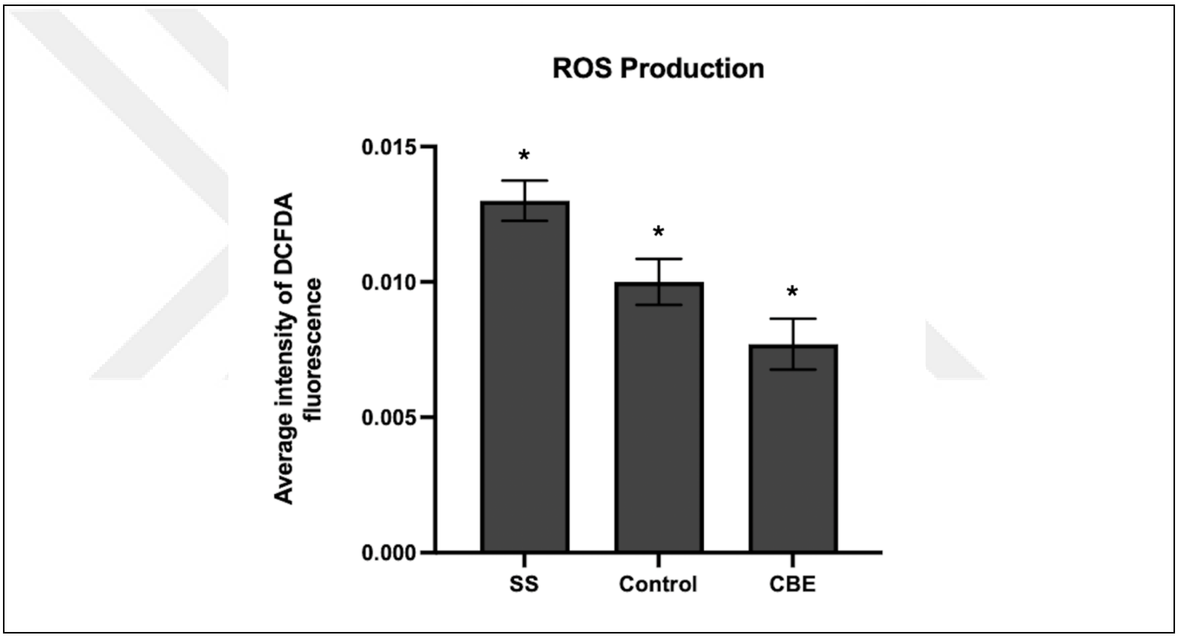

Figure 4.9. Detected ROS measured within senior aged fibroblast cells n=3, *P<0.05.

4.9. RNA ISOLATION FOR GENE ANALYSIS

As p21, p16, Ki67 and MCP1 genes are reported as the markers of cellular senescence, the expressions of these genes were measured using the RT-PCR in SS, control and CBE groups. The data indicate that p21 and p16 are significantly upregulated in SS conditions, with fold changes of 4.25 and 6.38, respectively, indicating increased cellular stress and senescence. In addition, Ki-67 shows a significant downregulation in SS (0.14), suggesting reduced cellular proliferation under these conditions. The expression levels of p21 and p16 was x fold lower in control group when compared to SS showing that senior fibroblasts were sensitive to serum starvation and respond with a sudden cell cycle arrest in Go-G1. Similarly, there was and x fold increase in Ki-67 expression level in control cells when compared to SS. The CBE treatment led to a respective 0.52 and 0.22 fold decrease in p21 and p16 expression in comparison to control group. As expected, Ki-67 was significantly upregulated in CBE (6.32 fold), suggesting enhanced cellular proliferation, which may promote tissue repair and regeneration. When compared to control, MCP-1 was downregulated in CBE by 0.28 fold, indicating reduced inflammation, while serum starvation resulted in a 3.9 fold increase in the expression of this gene. These findings suggest that CBE treatment may have potential anti-aging effects by reducing cellular stress and inflammation while promoting cellular proliferation.

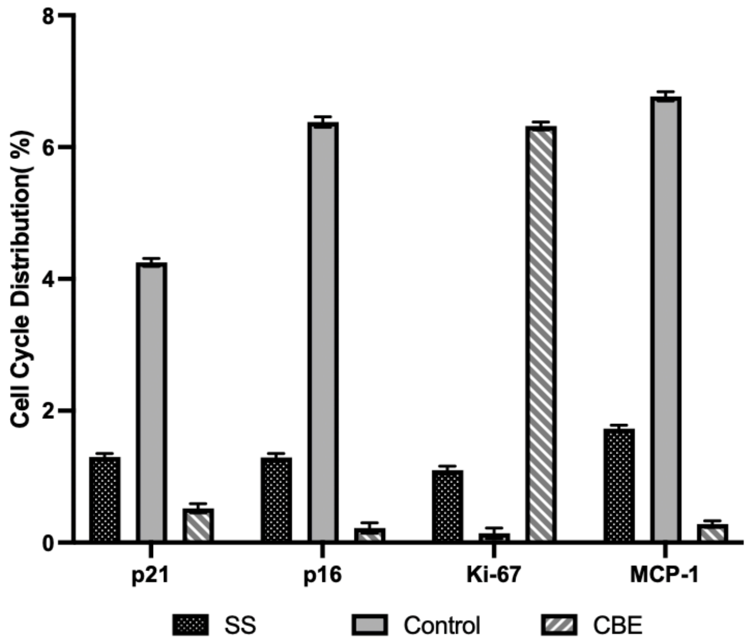

Figure 4.10. Relative Fold Changes in the Expression Levels of p21, p16, Ki-67, and MCP-1 genes n=3, *P<0.05.

4.10. IMMUNOCYTOCHEMISTRY ANALYSIS

The levels of Collagen Type I and elastin expression in untreated senior-aged fibroblasts (control) and those treated with CBE were analyzed using immunocytochemistry with confocal microscopy. Cells were labeled with antibodies specific to Collagen Type I and elastin. The results of the immunocytochemistry showed that the untreated control cells exhibited weaker staining compared to the CBE-treated group for both Collagen Type I and elastin. The graph representing the data demonstrates significant differences in fluorescence intensity between the control and CBE-treated groups for both Elastin and Collagen Type I.

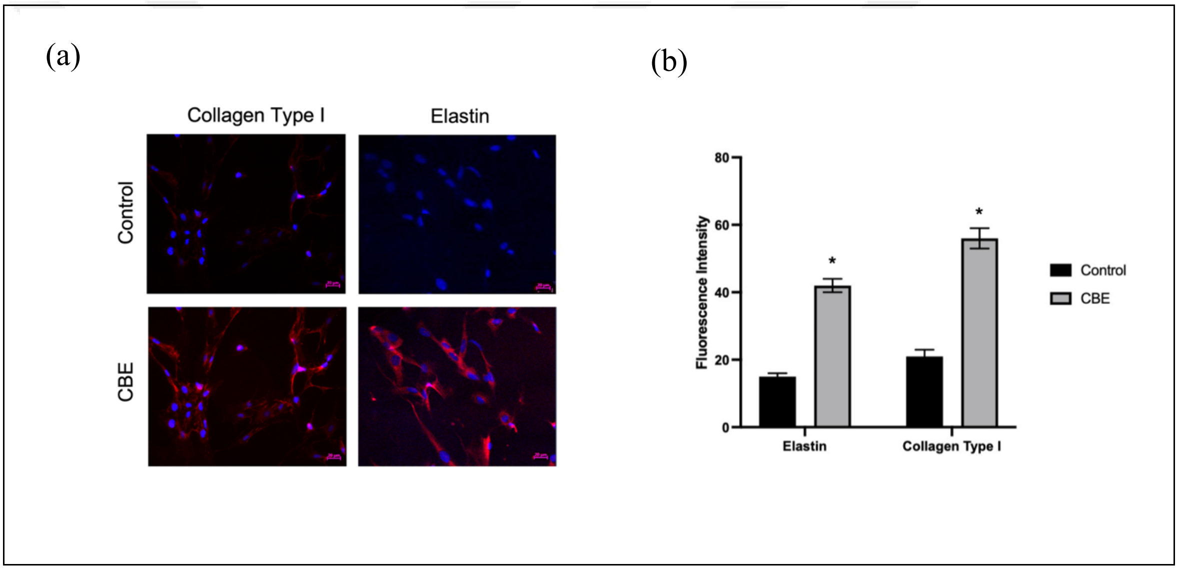

Figure 4.11. The location and quantification of elastin and collagen type I protein expression in senior-aged fibroblast cells. The data are presented with a standard deviation (± SD) and a sample size of n=3. Scale bar indicates 20 µm.

5. DISCUSSION

This study conducts an in-depth examination of the crucial role exosomes play in combating the skin aging, with a specific focus on their remarkable ability to revitalize aged skin. The phenomenon of skin aging is well-acknowledged within the scientific community, and it is notably accelerated by a multitude of factors, chief among them being the relentless assault of UV radiation. The aging progression is evident in the gradual degradation of vital skin elements, such as the depletion of HA, subcutaneous fatty tissue, collagen, and elastic fibers [110,111]. The pace at which this degeneration unfolds is influenced by an intricate interplay of diverse elements, ranging from genetic predisposition to pernicious environmental factors like exposure to the harmful effects of cigarette smoke and excessive alcohol consumption [111]. In this complex narrative of skin aging, fibroblasts emerge as the central actors. The aging process is significantly impacted by fibroblasts, which constitute the predominant cell type in the dermal layer of the skin. They orchestrate the aging symphony, either through direct actions or via intricate interactions with neighbouring cells, thereby steering the course of this intricate biological phenomenon. Fibroblasts, specialized cells located within the connective tissues of the body, play a pivotal role in the synthesis of collagen. This essential protein is foundational for providing structural support and strength to various bodily components, including the skin, bones, and several organs. The collagen fibers produced by fibroblasts are crucial elements of the extracellular matrix, contributing significantly to the tissue's firmness and elasticity, which are vital for maintaining the skin's youthful appearance and overall organ integrity [112, 113] Over time, or under the influence of environmental factors such as UV radiation or pollution, these collagen fibers can undergo degradation or fail to align correctly within the extracellular matrix. This disruption or collapse of the collagen network leads to a marked reduction in the skin's elasticity and firmness, a phenomenon most visibly manifested through the formation of wrinkles. Such changes not only signify the skin's aging process but also underscore the indispensable role of fibroblasts and collagen in preserving skin health and structure. The occurrence of these visual signs of aging is a direct testament to the compromised functionality of fibroblasts and the collagen they produce. As the natural capacity of fibroblasts to synthesize collagen diminishes with age or due to detrimental environmental exposures, the pursuit of interventions to counteract these effects becomes paramount [114]. Cultured fibroblast treatments emerge as a promising avenue in this regard.

By reintroducing cultured fibroblast cells back into the skin, there is a notable increase in collagen synthesis, which in turn, can lead to a reduction in the appearance of wrinkles and an improvement in skin texture and firmness. However, the application of such treatments is not without its challenges, particularly when fibroblast cells derived from older individuals are considered [115, 116]. These cells may exhibit reduced growth potential or viability when cultured, a consequence directly linked to the aging process. To address this obstacle, innovative approaches involve the use of cord blood exosomes. These are aimed at enhancing the proliferation rate and telomerase enzyme activities of senior aged fibroblast cells. Telomerase plays a critical role in maintaining the length of telomeres, protective caps on chromosome ends, thus potentially extending the lifespan and vitality of these cells within culture environments [117]. Through such advancements, it is possible to overcome the limitations posed by the aging of fibroblast cells, thereby offering a more effective means of restoring skin's youthful properties by enhancing collagen production and mitigating the signs of aging [118]. The research presented in this thesis encompasses an investigation into the effects of cord blood plasma exosomes on various aspects of cellular behaviour and function, with a particular focus on fibroblast cells. The discussed results and their implications provide insights into the possible therapeutic uses of exosomes within the realms of regenerative medicine and cell biology [119-122].

Exosomes, diminutive exosomes secreted by various cell types, have recently emerged as potent and versatile tools in the arsenal against skin aging. Characterized by their minuscule size, typically ranging from 30 to 120 nanometers, these exosomes are multifaceted agents that partake in a plethora of pivotal biological activities. Their repertoire includes acting as messengers for intercellular communication, facilitating the transmission of crucial signalling cues, mediating the transfer of vital genetic material, and intricately regulating immune responses. Exosomes exhibit an astonishing and distinctive capacity to not only repair and rejuvenate aging skin but also to mitigate inflammation. Their diminutive size equipped the exosomes with a remarkable ability to effortlessly traverse the formidable barrier presented by the skin's outermost layer [123,124].

A particularly intriguing source for the pursuit of regenerative and wound-healing endeavours is CBE. This biological fluid, enriched with a plethora of nutrients, encompasses a treasure trove of growth factors, hormones, interleukins, cytokines, and an array of signalling proteins. What sets cord blood plasma apart is the abundance of these biologically active molecules, a concentration that far surpasses those found in the peripheral blood of adults by a considerable margin. The unique composition of cord blood plasma renders it an enticing source for research into regenerative processes [125].

The primary objective of this study was to delve deeply into the multifaceted parameters underlying skin aging and, more importantly, to discern the impact of exosomes sourced from human cord blood plasma on senior-aged dermal fibroblasts. This endeavour involved the meticulous isolation of exosomes from human umbilical cord blood plasma. Subsequent cultivation of dermal fibroblast cells was carried out [126,127], with each set of cells being nurtured in a complete growth medium enriched with exosomes from the respective plasma sources. The effectiveness of these protocols was rigorously evaluated through a series of assessments, including the determination of the number of adherent cells, the extent of their proliferation, and precise size measurements of the exosomes. Special attention was given to the mechanisms governing skin rejuvenation and the activation of telomerase enzyme activity in fibroblast cells [128]. The initial phase of this study involved characterizing the cord blood plasma exosomes. Flow cytometry was employed to analyze the surface proteins present on exosome membranes, with specific attention given to CD9 and CD63. The successful detection of these surface markers using magnetic beads coated with specific antibodies confirmed the presence of exosomes. This characterization provides a solid foundation for subsequent experiments and ensures the integrity and identity of the exosomes used in the study. Focusing on the characterization of cord blood plasma exosomes and employing flow cytometry to analyse surface proteins, particularly CD9 and CD63, is well-grounded in current scientific methodologies. The use of magnetic beads coated with specific antibodies for the successful detection of these exosome surface markers is a validated approach in exosome research. This technique ensures the precise identification of exosomes, thereby laying a robust foundation for the integrity and identity verification of the exosomes used in subsequent experiments. Flow cytometry is indeed a widely used and effective method for the characterization of exosomal surface proteins. It enables the measurement of exosome size and structure and has been employed in various studies to determine the cellular origin of single extracellular vesicles (EVs), including exosomes. The detection of CD63, CD9, and other exosomal markers through flow cytometry, utilizing immuno-magnetic isolation and on-bead flow cytometry methods, confirms the presence and allows for the quantitative assessment of exosomes in biological samples [129, 130].

The size and concentration of exosomes were determined using NTA. The data revealed crucial physical properties of exosomes, including an average size of 95±4.3 nm and a mode at 100.1±3.5 nm. Additionally, the concentration of exosomes in the sample was quantified at 2.68e+013 particles/ml. These findings are fundamental in understanding the biological behaviour and interactions of exosomes with target cells. Studies have highlighted the utility of NTA in various contexts, emphasizing its role in accurately and reproducibly measuring extracellular vesicles, thereby facilitating a better understanding of their secretion dynamics, composition, and potential as biomarkers or therapeutic agents [131]. For example, NTA has been used to study the secretion of extracellular vesicles in cancer cells, revealing insights into their role in cell communication and tumor progression. Furthermore, the repeatability and accuracy of NTA measurements have been critically evaluated, underscoring the technique's reliability for exosome research [132].

Characterization of primary fibroblast cells is essential for assessing their response to exosome treatment. Flow cytometry analysis revealed the presence of specific surface markers on fibroblast membranes, including CD29, CD44, CD73, CD90, and CD105. This characterization ensures that the fibroblast cells used in the study exhibit the expected phenotype, laying the groundwork for subsequent experiments. Research has shown that markers like CD105, CD90, CD44, CD29, and CD73, which are traditionally used to define MSCs, are also expressed on human skin or lung fibroblasts, albeit with varying levels of expression that distinguish them from MSCs. This overlap in marker expression underscores the importance of comprehensive characterization to accurately identify and differentiate fibroblast cells in studies, particularly those investigating cellular responses to treatments [133].

The study investigated the impact of exosome treatment on fibroblast cell proliferation using the MTS assay. The results indicated that 100ug/ml exosome concentration significantly enhanced cell proliferation and viability. However, lower concentrations (50ug/ml, 200ug/ml, and 500ug/ml) led to reduced cell viability. The concentration-dependent effect observed suggests that cord blood plasma exosomes have a notable influence on fibroblast cell growth, which could have significant implications for regenerative medicine. The current understanding of the literature on the role of mesenchymal stem cell (MSC)-derived exosomes in enhancing cell proliferation and viability is supported by observations from various sources. This understanding specifically underscores the significant impact that exosome treatment can have on the growth of fibroblast cells, highlighting its potential for applications in regenerative medicine. The effect, which is dependent on concentration, emphasizes the importance of optimizing exosome dosages for therapeutic purposes [134].

The role of telomerase in maintaining telomere length and preventing replicative senescence is crucial for cellular function and longevity. The study demonstrated that treatment with 100ug/ml of cord blood plasma exosomes led to a substantial increase in telomerase enzyme activity, from 22.24 IU/ml to 38.10 IU/ml. This finding suggests that exosomes may play a pivotal role in preserving cellular function and immune function by preventing telomere shortening and senescence. The scratch assay examined how exosomes impact cell migration and the healing of wounds, essential processes in tissue regeneration and repair. Results revealed that 100ug/ml exosome treatment significantly improved wound closure rates compared to control cells. This discovery implies that exosomes possess the potential to enhance cell migration and accelerate wound healing, offering promising prospects for therapeutic interventions in tissue repair and regeneration. Research on exosomes from human umbilical cord blood plasma demonstrates their significant impact on wound healing processes. For example, a study found that these exosomes can accelerate cutaneous wound healing through mechanisms involving the promotion of angiogenesis and fibroblast function, suggesting a substantial role in enhancing cellular repair and regeneration [135]. Another study highlighted the use of exosomes from human cord blood plasma for cutaneous wound healing by promoting fibroblast function, angiogenesis, and differentiation of M2 macrophages, which are pivotal in transitioning from inflammation to proliferation during the wound healing process [136].

The cell cycle arrest experiment provided insightful data on the impact of CBE on senior aged fibroblast cells. Our flow cytometry results, depicted in Figure 4.8, reveal that the CBE group displayed a significantly higher percentage of cells in the S and G2-M phases compared to the SS group. Specifically, the CBE group exhibited a 22% increase in the G2-M phase over a 24-hour period relative to the SS group. This phase of the cell cycle is critical for the synthesis of cellular components necessary for mitosis, suggesting that exosomes can accelerate cellular preparation for division. Furthermore, the analysis of the G0-G1 phases showed a 20% increase in cell division in the CBE group compared to the SS. These findings are supported by previous studies that have shown exosomes can enhance cell cycle progression by delivering growth-promoting molecules such as microRNAs and proteins

[137, 138]. MicroRNAs are known to regulate gene expression post-transcriptionally, thereby influencing key cellular pathways involved in proliferation and differentiation. For example, miR-21, commonly found in exosomes, has been shown to promote cell proliferation and inhibit apoptosis by targeting PTEN and other tumor suppressor genes [137]. Additionally, exosomal proteins such as growth factors can activate signalling pathways that lead to cell cycle progression. The increase in the G2-M phase indicates that cord blood exosomes can effectively stimulate cell proliferation, potentially counteracting age-related cellular senescence and promoting tissue regeneration [138].

Reactive oxygen species (ROS) detection is pivotal in understanding cellular oxidative stress and damage. In our study, ROS levels were assessed following the manufacturer's protocol, with results illustrated in Figure 4.9. The CBE group, treated with exosomes for 24 hours, exhibited a DCFDA fluorescence that was significantly lower than the intensities of the SS and control groups. These results clearly demonstrate that CBE reduces ROS activity in senior aged fibroblast cells. The reduction in ROS levels suggests that exosomes may confer a protective effect against oxidative stress, which is known to contribute to cellular aging and damage [138]. This protective effect is likely due to the antioxidant enzymes and regulatory molecules present in exosomes, as supported by other studies indicating that exosomes can modulate oxidative stress pathways. For instance, exosomes from mesenchymal stem cells have been shown to carry antioxidant enzymes such as catalase and superoxide dismutase, which can reduce oxidative damage in recipient cells [139].

Gene expression level impact of cord blood exosome treatment on aging markers in fibroblasts, particularly focusing on the expression levels of p21, p16, Ki-67, and MCP-1. The significant upregulation of p21 and p16 in SS suggests an enhanced cellular response to cellular stress, characteristic of aging cells. Both p21 and p16 are critical regulators of the cell cycle, inducing senescence to prevent the proliferation of damaged cells. The marked increase in the expression of these cyclin-dependent kinase inhibitors in SS highlights the cells' attempts to respond cellular stress evoked by serum starvation. In contrast, the downregulation of p21 and p16 in CBE cells indicates lower levels of cellular stress and a reduced induction of senescence. This environment is likely more conducive to tissue maintenance and repair, suggesting that cord blood exosome treatment may mitigate some aspects of cellular aging by reducing the burden of senescent cells. Ki-67, a marker of cellular proliferation, shows significant upregulation in CBE, indicating a high rate of cell division. This heightened proliferation may be beneficial for tissue repair and regeneration, counteracting some of the degenerative aspects of aging. In contrast, the significant downregulation of Ki-67 in control suggests reduced cellular proliferation under conditions of high stress and damage, which can contribute to impaired tissue regeneration and functional decline typical of aging tissues.

MCP-1 is a key player in recruiting monocytes to sites of inflammation, and its upregulation in SS (6.79-fold) points to heightened inflammatory responses. Chronic inflammation, or "inflammaging," is a well-recognized contributor to the aging process and the development of age-related diseases. Elevated MCP-1 levels in control suggest that aging tissues are likely experiencing persistent inflammation, contributing to further cellular damage and dysfunction. The downregulation of MCP-1 in CBE indicates lower levels of inflammation in cord blood exosome treated cells, which is beneficial for maintaining healthy tissues [141].

The expression of Collagen Type I and elastin, key components of the extracellular matrix, was examined through immunocytochemistry analysis using confocal microscopy. As shown in Figure 4.11, untreated senior aged fibroblast cells displayed weaker staining for Collagen Type I and elastin compared to cells treated with cord blood exosomes. The enhanced staining intensity in the CBE group indicates higher expression levels of these proteins, suggesting that exosome treatment promotes the synthesis of structural proteins crucial for maintaining skin integrity and elasticity [142]. This upregulation of Collagen Type I and elastin could potentially mitigate the effects of aging on the skin by improving its structural properties. These findings are consistent with studies demonstrating that exosomes can enhance extracellular matrix remodelling and increase the production of collagen and elastin in fibroblast cells. For example, exosomes from adipose-derived stem cells have been shown to enhance collagen synthesis and deposition in fibroblasts, thereby improving skin elasticity and reducing wrinkles [143, 144].

The correlation between parameters indicates that CBE reduce oxidative stress and senescence markers (p21 and p16), thereby promoting cell cycle progression and increasing cellular proliferation (indicated by Ki-67 upregulation). This suggests that exosome treatment can effectively rejuvenate aged fibroblast cells by creating a cellular environment that favours healthy cell division and tissue regeneration, potentially counteracting age-related cellular senescence and promoting overall skin health.

6. CONCLUSIONS

This study has conducted a comprehensive analysis of the pivotal role of exosomes in addressing skin aging, particularly focusing on their extraordinary capability to rejuvenate aged skin. The aging of skin is a multifaceted process exacerbated by various factors, primarily UV radiation, leading to the degradation of essential skin components such as hyaluronic acid, collagen, and elastic fibers. Fibroblasts, the primary cell type in the dermal layer, play a crucial role in this process through their synthesis of collagen, which is vital for maintaining skin structure and elasticity.

Our research highlights the promising potential of cord blood plasma exosomes in enhancing the proliferation and functionality of aged fibroblast cells. By isolating and characterizing exosomes from human umbilical cord blood plasma, and subsequently cultivating senior aged dermal fibroblasts with these exosomes, we observed significant improvements in cell proliferation, telomerase activity, and wound healing capabilities. Moreover, the findings suggest that exosomes can mitigate oxidative stress, reduce senescence markers, and enhance collagen synthesis, thereby promoting skin rejuvenation.

The implications of this study extend to the potential therapeutic uses of exosomes in regenerative medicine. The ability of exosomes to cross the skin barrier and deliver bioactive molecules makes them a potent tool for skin repair and anti-aging treatments. This research underscores the importance of further exploration into exosome-based therapies, which could revolutionize approaches to skin aging and other degenerative conditions.

In future studies, an expansion of the investigation can be undertaken to further elucidate the mechanisms underlying the effects of cord blood exosomes on aging parameters in fibroblast cells. Specific emphasis can be placed on elucidating the gene expression levels involved in this process and dissecting the intricate molecular pathways through which cord blood exosomes exert their influence. This future research can utilize advanced molecular biology techniques to analyse gene expression profiles in fibroblast cells exposed to cord blood exosomes. Transcriptomic analyses, such as RNA sequencing, could be employed to comprehensively examine changes in gene expression patterns induced by cord blood exosome treatment. By comparing the transcriptomes of treated and untreated fibroblast cells, the aim would be to identify key genes and regulatory pathways modulated by cord blood exosomes that are relevant to aging processes.

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