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褪黑激素與維他命 C 對於乳牙幹細胞幹性的影響

Effect of Melatonin and Vitamin C on the Stemness of Human Exfoliated Deciduous teeth Stem Cells

黃彥青

Yen-Ching Huang

指導教授：周涵怡 博士

Advisor: Han-Yi Elizabeth Chou, Ph.D.

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中文摘要

乳牙幹細胞（SHEDs）是間質幹細胞（MSCs）的一種，乳牙幹細胞有自我更新的能力及分化成多種細胞的潛力，包括分化成成骨細胞、軟骨細胞和脂肪細胞等等。由於分化的潛力，乳牙幹細胞能促進組織再生，加上乳牙幹細胞易於取得及分離，他被認為是再生醫學和幹細胞療法的良好細胞來源。相較於其他間質幹細胞，例如牙髓幹細胞（DPSCs）、骨髓幹細胞（BMSCs）和臍帶幹細胞（UC-MSCs）等等，乳牙幹細胞可以在試管內實驗中存活較久的時間，且乳牙幹細胞比其他間質幹細胞能表現出更高的增值能力及分化的潛力。在幹細胞療法或幹細胞的研究當中，繼代培養是取得一定數量幹細胞的主要方法，然而，連續的繼代培養會導致幹細胞退化，進而使幹細胞的幹性下降，而幹性包括了幹細胞的增殖能力和分化潛力。這種幹性的退化將影響幹細胞的品質和有效性，並最終阻礙其在幹細胞治療中的實際應用。根據先前研究顯示，褪黑激素及維生素 C 可以在培養細胞時調節細胞的活性。因此，為了研究褪黑激素及維生素 C 在幹細胞的退化和幹性之間的有效性，我在乳牙幹細胞的培養中分別加入褪黑激素及維生素 C 並培養至第二代及第五代，接著利用 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) 技術檢測乳牙幹細胞的細胞存活率，進而比較在培養中有無加入褪黑激素及維生素 C，對於幹細胞因為繼代培養而退化的情

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形。除了檢測細胞存活率之外，我也利用 Alizarin Red S 染色技術研究了乳牙幹細胞分化成成骨細胞的狀況，並分別比較了乳牙幹細胞繼代培養到第三代及第五代，在有無褪黑激素及維生素 C 的狀況下的分化情形。然而，根據先前的幹細胞研究顯示，乳牙幹細胞在培養時對環境很敏感，任何高濃度的外來因子都會影響他的狀態，而這會導致單獨加入褪黑激素或維生素 C 時受到濃度的限制。因此，我會進一步利用不同濃度比例將褪黑激素及維生素 C 合併來培養乳牙幹細胞，並研究乳牙幹細胞在合併培養後的細胞生長情形。而此研究結果顯示，在培養乳牙幹細胞時分別單獨加入褪黑激素及維生素 C，可以延緩幹細胞的退化並提高幹細胞的增殖能力及成骨分化潛力，而在加入褪黑激素與加入維生素 C 之間的組別則無顯著差異。至於將褪黑激素及維生素 C 以不同濃度比例合併後，此研究結果顯示可提高乳牙幹細胞的的增殖能力。這些結果可能對進一步的幹細胞研究和未來治療的應用有用。

關鍵字：乳牙幹細胞，幹性維持，褪黑激素，維他命 C，幹細胞分化

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Abstract

Stem cells from human exfoliated deciduous teeth (SHEDs) possess self-renewal capabilities and multi-lineage differentiation potential. Due to their differentiation potential, SHEDs promote tissue regeneration and are considered a valuable cell source in regenerative medicine and stem cell-based therapies. Compared to other mesenchymal stem cells (MSCs), SHEDs exhibit prolonged in vitro survival, a higher growth rate, and stronger differentiation potential. In stem cell research and therapies, in vitro serial subculture is the primary method for obtaining a sufficient quantity of stem cells. However, stem cells tend to deteriorate during serial subculture, resulting in diminished stemness, which includes their proliferative capacity and differentiation potential. This deterioration adversely affects the quality and efficacy of SHEDs, ultimately hindering their practical application in stem cell therapy. Previous studies have demonstrated that melatonin and vitamin C can modulate the functional activity of cells during culture. To assess the effects of melatonin and vitamin C on the stemness of SHEDs during deterioration, cell proliferation rates at passages 2 (P2) and 5 (P5) were compared using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Additionally, the osteogenic differentiation abilities at passages 3 (P3) and 5 (P5) were compared among the control, melatonin, and vitamin C groups. Studies have shown that SHEDs are sensitive to their culture environment, and high concentrations of exogenous factors can adversely affect their state. Consequently, the individual treatments of melatonin and vitamin C are limited in concentration. This study also investigates the combined treatment of melatonin and vitamin C at different concentration ratios. The

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combination treatment aims to address the limitations of individual treatments and enhance the stemness of SHEDs. The results indicated that separate treatments with melatonin and vitamin C significantly enhanced the proliferation rate and osteogenic differentiation ability of SHEDs compared to the control group, with no significant difference between the effects of melatonin and vitamin C. However, the combined treatment of melatonin and vitamin C significantly augmented the proliferation rate of SHEDs compared to the control group. These findings may be valuable for further stem cell research and future therapeutic applications.

Key words: Stem cells from human exfoliated deciduous teeth, Stemness, Melatonin, Vitamin C, Stem cell differentiation

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Chapter 1

Introduction

1.1 Stem cells and applications

Stem cells are unique cells with the capabilities of self-renewal and multilineage differentiation. These cells can divide to form more stem cells or undergo a process called stem cell differentiation to become specialized cells, which can range from immune cells to neurons [1]. No other cell type in the body possesses the innate ability to generate new cell types. Consequently, researchers believe that stem cell-based therapies hold significant promise for repairing damaged tissues and treating serious illnesses such as paralysis and Alzheimer's disease [2].

Stem cell therapy is a form of regenerative medicine that utilizes stem cells to promote the repair of dysfunctional or injured tissue [3]. Researchers grow stem cells in a lab and under the right condition, these stem cells can be expanded or differentiate into specific types of cells, such as blood cells or liver cells. The specialized cells can then be implanted into a person which has a damaged tissue [4]. For example, if the person has liver dysfunction, the cells could be injected into the liver. The healthy transplanted liver cells could then repair the dysfunctional liver. In addition to tissue regeneration and tissue repair of stem cells, applications of stem cells can be categorized into the five following areas: First, drug discovery and testing. When new drugs were discovered, investigating the interaction between the new drugs and human cells is important. Thus, researchers can utilize stem cells to create an in vitro model of human tissues, enabling laboratories

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to test the safety and efficacy of new drugs. This approach reduces the need for animal testing and provides more accurate perceptions into new drugs and the potential of clinical applications. Second, gene therapy. Inherited diseases such as albinism, are a serious problem in all ages. To investigate this problem, researchers can utilize stem cells to be genetically modified and then be implanted into the patient's body to fix mutations. Third, immunotherapy. Stem cells have been discovered in modulating the immune system, making them valuable in treating autoimmune diseases, such as multiple sclerosis, rheumatoid arthritis and preventing transplant rejection. Forth, disease modeling. New diseases happened every year, even previous diseases can mutate into a new cell line. To investigate more insights of the diseases, researchers can utilize stem cells to generate disease-specific cell lines, enabling laboratories to study disease progression and point out the therapeutic targets. Finally, personalized medicine. Stem cells can be used to progress the patient-specific therapies, developing treatments to a personalized genetic makeup. Stem cells can be used broadly and more research is ongoing.

1.2 The classification of stem cells

Stem cells can be classified into two main forms. The first form is embryonic stem cells, which are derived from the inner cell mass of an unused embryo that is three to five days old. The unused embryo, called a blastocyst, is a group of cells that forms when ovum is fertilized with sperm in a laboratory at an in vitro fertilization procedure. Embryonic stem cells are pluripotent stem cells, pluripotent stem cells can divide into more stem cells or can be differentiate into almost any cell type in the body. This variability of embryonic stem cells allows them to be used to regenerate or repair

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damaged tissue or organs [5]. However, the obstacle of embryonic stem cells research is that its source must be obtained from human embryos, which must be destroyed to obtain the stem cells and leads to several controversial questions and issues about the ethics.

The second form of stem cells is adult stem cells. Adult stem cells come from fully developed tissues such as the bone marrow, epithelial tissue and dental pulp. Adult stem cells have the ability to renew themselves or differentiate into new cells that can repair damaged tissue, yet this form of stem cells is more likely to differentiate only certain types of cells and there are typically a small number of stem cells that can be isolated from developed tissues [6]. Despite that adult stem cells have a more limited ability to give rise to various cells of the body, they are easier and have a higher successful rate to access when comparing with embryonic stem cells [7].

The adult stem cells can be further divided into four types, which are the hematopoietic stem cells, neural stem cells, skin stem cells and mesenchymal stem cells. Hematopoietic stem cells (HSCs), also known as blood stem cells, are cells present in blood and bone marrow. They can form mature blood cells, including the red blood cells that interact with oxygen, platelets that contribute to hemostasis and white blood cells that prevent infections. HSCs are used in the treatment of many diseases such as leukemia, lymphoma and sickle cell disease to repair a patient's hematopoietic system [8].

Neural stem cells (NSCs) are largely cells originating in the central nervous system. The brain and spinal cord are the central nervous system, other part of the body is the peripheral nervous system, which consists of nerves that connect the brain and spinal cord to the rest of the body. Central nervous system is composed of three major cell types. The first type is the neurons, they send information and messages through neurotransmitters

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to other neurons, such as the muscle cells or gland cells, and making them do actions. The second and the third type is astrocytes and oligodendrocytes, the two of them collectively called glial cells. Glial cells are non-neuronal cells that can reinforce the speed at which neurons send signals. NSCs have the potential to grow and differentiate into neurons and glial cells that are important to transmission and actions [9].

Skin stem cells (SSCs) are found in diverse niches within the skin, among these niches, hair follicle is the most studied. Skin is an important physical barrier against the environment and pathogens, thus, skin regeneration is essential for survival. Skin regeneration depends on multiple stem cell, among these stem cells, despite their different transcriptional, as well as different location, fall under the general term of SSCs. Skin wounds can heal normally; however, when it comes to some diseases or immense damage, they might impact the healing progress. Non-healing wounds represent a serious and life-threatening situation that may require advanced therapeutic strategies. As a result, SSCs have become an increasing focus into their role in wound healing and skin regeneration [10].

Mesenchymal stem cells (MSCs) are adult stem cells that isolated from different sources and they can differentiate into other types of cells, such as osteoblasts, chondrocytes and adipocytes [11]. Sources of MSCs include dental pulp tissue, bone marrow and umbilical cord tissue. The term "mesenchymal" refers to the embryonic origin of the cells, specifically cells that arise from the mesoderm germ layer. A germ layer is a primary layer of cells that forms during the early stages of embryonic development. There are three germ layers in animals, including the ectoderm (outer layer), the mesoderm (middle layer) and the endoderm (inner layer). Among the three germ

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layers, the mesoderm gives rise to various types of connective tissues, such as muscle, bone, cartilage, and fat.

1.3 Mesenchymal stem cells

Among these five types of adult stem cells, mesenchymal stem cells (MSCs) are the most widely studied stem cells. As the rapidly growing field of stem cell therapies and tissue engineering, MSCs have become a promising source due to their immense clinical potential and they can be easily obtained from various sources and expanded in vitro [12]. In addition, MSCs have a lower risk of immune rejection, as they are less immunogenic than other stem cells [13]. One of the important benefits of using MSCs for stem cell therapy is that MSCs can promote tissue repair by releasing growth factors and cytokines, which help recruit other cells to the injury site. These growth factors and cytokines can also promote the formation of new blood vessels essential for tissue repair [14].

In addition, MSCs are also being assessed in clinical studies to treat diseases such as osteogenesis imperfecta, atopic dermatitis, Parkinson's, and many others [15]. MSCs can self-renew and also exhibit multi-lineage differentiation. The differentiation process of MSCs is a complex interplay of genetic and epigenetic factors. The genetic factors include the expression of specific transcription factors and signaling molecules, such as bone morphogenetic proteins (BMPs) or Wingless-related integration sites (Wnts), while the epigenetic factors include changes in DNA methylation, histone modification, and non-coding RNA expression [16]. The process of MSCs differentiation can be divided into three stages. The first stage is the activation of specific genes, which leads to the expression of transcription factors. The second stage is the initiation of cell proliferation

and the formation of specific cell types. The final stage is the maturation of the cells, which leads to the formation of functional cells [17]. It is important to investigate the differentiation ability of MSCs, because the source of the stem cells, the conditions and the microenvironment of the cell cultivation may impact their differentiation ability, and influence their clinical application ultimately.

The International Society for Cellular Therapy's Mesenchymal and Tissue Stem Cell Committee (ISCT) has suggested three main standards for identifying MSCs. First, a mesenchymal stem cell must be plastic-adherent when maintained in standard culture conditions. Second, a mesenchymal stem cell must express CD73, CD90 surface marker, and lack expression of CD34 surface marker. Third, a mesenchymal stem cell must have the ability to differentiate into osteoblasts in vitro. According to the three standards, there are several stem cells isolated from different tissues have been identified as a type of MSC, the most widely known is bone marrow–derived MSCs (BMSCs). However, different sources for obtaining stem cells were discovered, for example, dental tissues. Stem cells that isolated from dental tissues, such as dental pulp-derived stem cells (DPSCs), dental follicle progenitor cells (DFPCs), stem cells from apical papilla (SCAP), periodontal ligament stem cells (PDLSCs), stem cells that isolated from human exfoliated deciduous teeth (SHEDs), etc. also have the characteristics of MSCs like self-renewal ability, multi-lineage differentiation capacity and immunomodulatory features. Among these dental tissue-derived MSCs, SHEDs are the most widely studied and were considered a promising source for stem cell therapies due to their great proliferation and differentiation capacity [18]. SHEDs are firstly identified by Miura et al. in 2003 [19]. According to recent studies, SHEDs may be beneficial to several physical defects, such

as cartilage tissue defects [20], tracheal fistula [21] and calvarial bone defects [22], in addition, SHEDs have been reported to have the ability to relieve the symptoms of Alzheimer [23] and atopic dermatitis [24].

1.4 Stem cells from human exfoliated deciduous teeth (SHEDs)

SHEDs are derived from the dental pulp tissue of human deciduous teeth. Dental pulp is a highly vascularized connective tissue located within the pulp chamber of both deciduous and permanent teeth, surrounded by the hard tissue known as dentin. The dental pulp is composed of an extracellular matrix and various cell types, including fibroblasts, endothelial cells, immune cells, and stem cells. Stem cells within the dental pulp can differentiate into odontoblasts, which form the outer surface of the dental pulp. The primary function of odontoblasts is dentinogenesis, the formation of dentin, which is crucial for maintaining tooth vitality [25]. Dental pulp serves as a source of different stem cell populations, such as dental pulp stem cells (DPSCs) in permanent teeth and stem cells from exfoliated deciduous teeth (SHEDs). The resorption of the roots of deciduous teeth plays a significant role in the development of permanent teeth. Due to differences in their development and biological characteristics, deciduous and permanent teeth are markedly distinct [26]. In addition, deciduous teeth are considered to have fewer environmental and genetic influences than permanent teeth. Deciduous teeth are believed to experience fewer environmental and genetic influences compared to permanent teeth. Consequently, SHEDs are expected to exhibit higher proliferation rates, greater cell viability, and enhanced differentiation potential compared to DPSCs [27].

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1.5 Current problems of SHEDs

Numerous studies have reported that SHEDs possess significant potential in regenerative treatments and tissue engineering. This potential is attributed to their abilities for self-renewal and multi-lineage differentiation, enabling them to differentiate into osteoblasts, adipocytes, and endothelial cells [28]. SHEDs have been proposed as a promising source of stem cells for regenerative therapies. However, obtaining a sufficient quantity of stem cells for clinical applications necessitates in vitro expansion. Several studies have reported potential challenges associated with the in vitro expansion of SHEDs. Notably, repeated expansion is linked to an accelerated rate of cellular deterioration, resulting in a decrease in stemness, including reduced proliferation ability and differentiation potential [29].

1.6 Factors that can regulate the stemness of stem cells

The deterioration affects the quality and efficacy of SHEDs and ultimately impact their clinical application. Based on the results of previous studies, researchers are increasingly inclined to select the factors [30], including metformin, lysine demethylase 3A (KDM3A), melatonin and vitamin C, that can regulate the deterioration, enhance the proliferative rate and differentiation ability of SHEDs when cell culturing. Metformin is a first-line medication for type II diabetes, there are studies reported that metformin and stem cells play an important role in physiological activities and pathological processes [31], thus researchers have studied the effect of metformin on stem cells in recent years. KDM3A is a type of histone demethylase, studies have reported that KDM3A participates in metabolic modulation and regulates the deterioration of stem cells by its

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overexpression, which blunts DNA damage response and thus reducing cellular deterioration [32].

Melatonin is one of the growth hormones, which is mainly produced in a circadian manner by the pineal gland, numerous studies have shown that melatonin has an effectiveness on inflammation and wound healing in several organs including the liver [33], intestine [34], and salivary glands, etc. In addition to wound healing, researchers have found that melatonin can enhance the proliferation, differentiation and delay the deterioration of SHEDs by regulating several signaling pathways, such as cyclooxygenase-2 (COX-2) and mitogen-activated protein kinase (MAPK) pathways [35].

Vitamin C, also known as ascorbic acid, has been reported as a strong antioxidant and has several physiological functions, such as removing reactive oxygen species and facilitating the mitosis which can suppress the effects of aging during stem cell culturing [36], vitamin C also downregulates various pro-inflammatory factors, such as interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α through Toll-like receptor 4 (TLR4) signaling pathway [37].

Among these factors, melatonin and vitamin C are the most widely used for stem cell culturing, due to their chemical stability, which allows them to act mildly with stem cells and other factors, in addition, they have stronger anti-inflammatory and antioxidant effects on cells than the other two factors because of their direct reaction to cellular signaling pathways [38]. As a result, melatonin and vitamin C will be the factors that I used in this study.

1.7 The significance of our study

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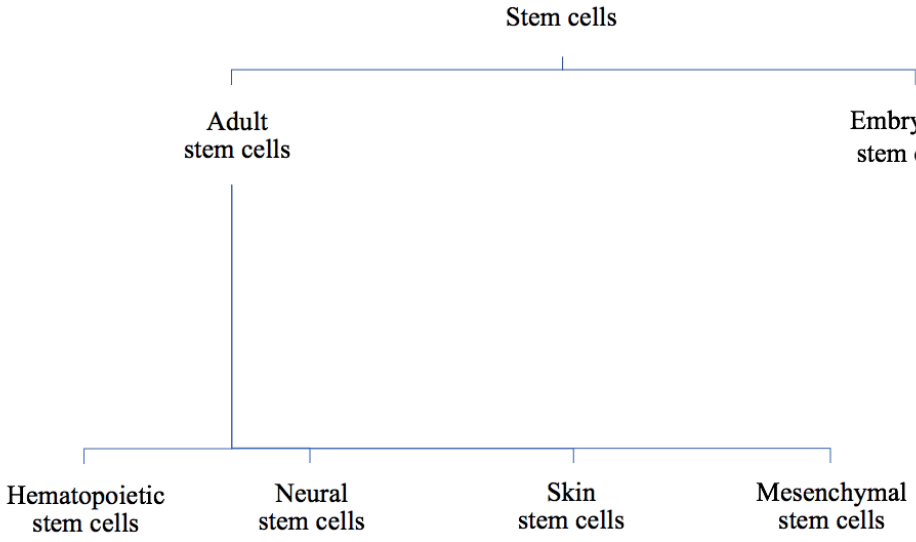
There are studies investigating stem cells treated with melatonin or vitamin C, for example, dental pulp mesenchymal stem cells treated with melatonin [35]; corneal epithelial stem cells treated with vitamin C [63]; the regulation of adipose-derived stem cell by melatonin [64]; effect of vitamin C on bone marrow-derived mesenchymal stem cell [65], but there are few studies investigating stem cells from human exfoliated deciduous teeth treated with melatonin or vitamin C. However, despite the observed applications and advantages of melatonin and vitamin C presented previously, the individual treatment of melatonin and vitamin C were often bounded by the upper limit of the concentration when applying to stem cells [41] [58]. Stem cells are extremely sensitive to environments, any foreign factors or materials will affect the state of stem cells, if the concentration of the foreign factor got too high, it could lead to a decreasing of cell viability, thus the individual treatment of melatonin and vitamin C on stem cells will be limited by the concentration. As a result, the difference between our study and current studies is that we combine melatonin and vitamin C to treat stem cells from human exfoliated deciduous teeth.

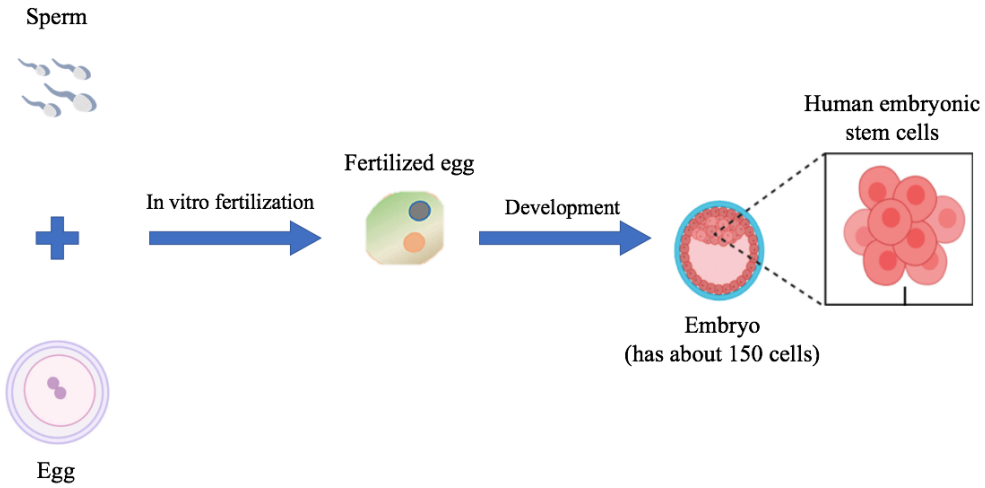
1.8 Aim and approach

SHEDs are considered a promising source for regenerative therapies. However, their clinical application is hindered by deterioration, leading to decreased cell proliferation and differentiation potential. Previous studies have indicated that melatonin and vitamin C can regulate stem cell deterioration. The specific aim of this research project is to investigate the effect of melatonin and vitamin C on the stemness of SHEDs. We will examine the roles of melatonin and vitamin C in maintaining SHED stemness by

comparing cell viability and differentiation capacity. Additionally, we will investigate the effect of combined melatonin and vitamin C treatment on SHED cell viability, assessing whether this combination offers superior protection against deterioration compared to individual treatments.



The classification of stem cells, which can be broadly classified to Adult or Embryonic stem cells. Adult stem cells can be further sub-classified into four types: hematopoietic, neural, epithelial stem cells, skin, and mesenchymal stem cells.



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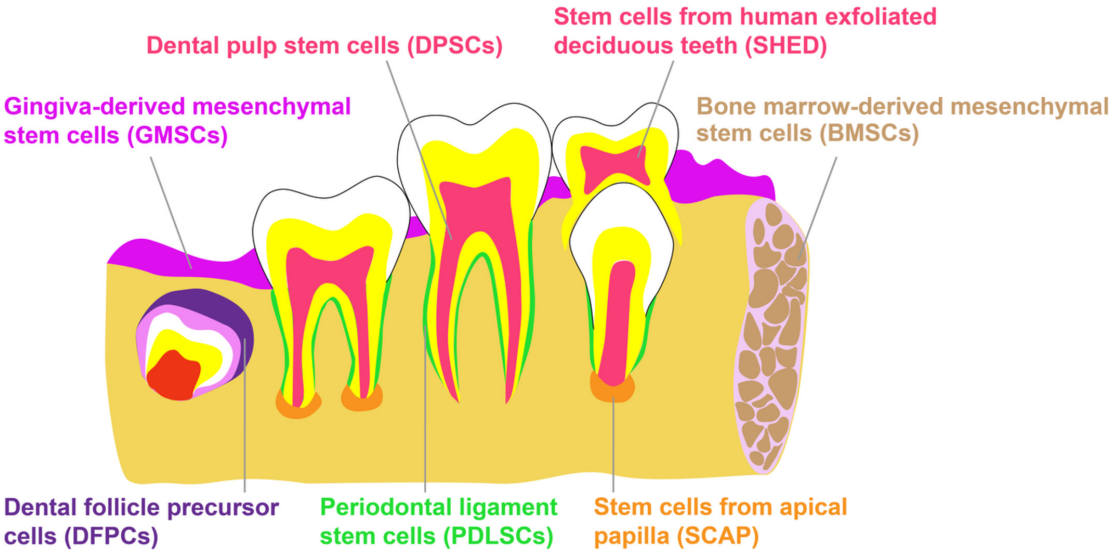
The origin of the human embryonic stem cells. Eggs are fertilized with sperm in the laboratory at an in vitro fertilization procedure, forming a fertilized egg. After three to five days of development, the human embryonic stem cells can be derived from the inner cell mass of the embryo.



Dental tissues are promising sources for isolating stem cells. Stem cells that isolated from dental tissues, such as dental pulp-derived stem cells (DPSCs), periodontal ligament stem cells (PDLSCs), stem cells from apical papilla (SCAP), dental follicle progenitor cells (DFPCs), stem cells that isolated from human exfoliated deciduous teeth (SHEDs), etc. they all have the characteristics of MSCs.

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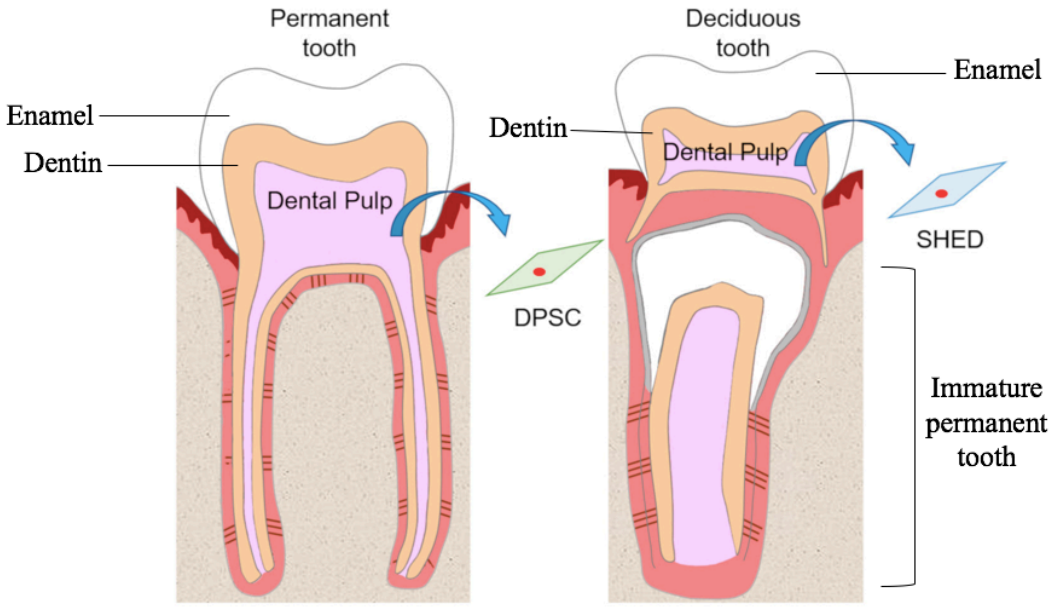

DPSCs and SHEDs. DPSCs are derived from the dental pulp of human permanent tooth, while SHEDs are derived from the dental pulp of human exfoliated deciduous tooth.

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Chapter 2

Methodology and Experiment Design

2.1. Isolating stem cells from human exfoliated deciduous teeth (SHEDs)

Isolation of stem cells from the human exfoliated deciduous teeth (SHEDs) can be achieved by two methods [39]. The first method is to separate the crown and root surface at the cementum-enamel junction using bone forceps. This separation exposes the dental pulp, which is rich in stem cells and thus can be scraped to isolate SHEDs. Since this method performs a wide access opening, it can unfortunately damage the stem cells and thus is not recommended by the protocols for the isolation of stem cells. The second method is to scrape the dental pulp cavity of the deciduous tooth crown with a tweezer. Compared to the first method, the second method takes a much less intrusive way to access the opening and likely keeps the deciduous tooth complete and damage-free [40]. Since the success of the experiment requires that the deciduous teeth remain in excellent condition, the second method was chosen for isolating SHEDs and the scraping on the crown of a complete and damage-free deciduous tooth was performed as detailed next.

A healthy exfoliated deciduous tooth was provided by National Taiwan University Children's Hospital. Following the aseptic techniques, the freshly extracted tooth was first wrapped in sterilized gauze and then placed into the cell culture hood for experiments, as shown in Figure 1A. Once SHEDs were exposed using the second method mentioned above, the stem cells were extracted by pipetting techniques. Specifically, a solution of

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10 μL of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin solution (PS) was used. For convenience, this solution is called the controlled medium hereafter. The controlled medium was pipetted down to the crown to suspend the stem cells in the medium. The stem cells were further mixed with the medium by pipetting up. Through several times of pipetting up and down, the stem cells and the medium became well mixed. The resulting turbid medium that contained SHEDs was then aspirated and placed into a 6-cm cell culture dish plate that contained 4 mL of DMEM supplemented with FBS and PS, which is referred to as the culture medium hereafter. SHEDs were cultured at 37^0 C in a humidified atmosphere with 5% CO_2 with the culture medium replaced every two days, and SHEDs were observed every 24 hours using an inverted phase-contrast microscope. The results of the observation after 24 hours of cell culturing showed that the isolation of SHEDs wasn't satisfactory, the cell viability and proliferation were too low, as shown in Figure 1B. As a result, SHEDs isolated from the deciduous tooth that provided by Dr. Yu-Zhi Jiang would not use for the research. The SHEDs that used for subsequent experiment were from the previous stock from lab.

2.2. Subculture procedure

When the stem cells grow to a high density (e.g., 80% confluence was achieved), the cells must be collected and placed into a new culture dish plate to allow more space and nutrition for cells to grow. This procedure is called cell subculture. In general, cells can be classified into two groups: adherent cells and suspension cells. For adherent cells such as SHEDs, they have secreted extracellular matrix comprising macromolecules and

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minerals such as collagen, enzymes, hydroxyapatite, and glycoproteins. When this type of the cells is placed in the dish plate, the cell does not adhere to the bottom of the dish directly but through the connection built by these proteins. Thus, when performing cell subculture of SHEDs, the protein connection between the cells and the dish must break. Since trypsin, which is an enzyme in the first section of the small intestine that digests protein molecules, can break the protein connection by cutting long chains of amino acids into smaller pieces, we use trypsin for cell subculture in this study.

The subculture of SHEDs involves several steps. First, the old culture medium was removed, and 1 mL of normal saline was used to clean the stem cells and to wash out any wastes produced during the cell growth. The washout step was repeated three times. After the washout, 1 mL of 0.05% trypsin (Gibco; Thermo Fisher Scientific, Inc.) was added to the dish plate to break the protein connection. To ensure that trypsin infiltrated the dish and acted completely, the dish was first swayed slowly and gently, and then placed into the incubator for one minute. Once one minute was up, the dish was taken out from the incubator and tapped gently around the edge to detach the stem cells from the side of the dish. Finally, the dish was placed under the inverted phase-contrast microscope for observation. If the protein connection was broken successfully and stem cells were suspended in the trypsin, we would see the stem cells floating slowly under the microscope.

Note that stem cells should not stay in trypsin for too long to avoid trypsin excessively damaging the proteins of the cells. Hence, after the observations, the dish was put back into the cell culture hood and 1 mL of DMEM medium supplemented with FBS and PS was added to terminate the acting of trypsin. To ensure that all the stem cells were

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suspended in the medium, the dish was swayed gently again. Then, pipetting techniques were applied to extract the stem cells. During the pipetting, the dish plate should be slightly tilted so that the pipetted medium can run through the cells thoroughly. After repeating pipetting several times, the turbid medium that contained stem cells was aspirated and placed into a new 6-cm dish plate. Before placing the dish back in the incubator for continuing cell culture, more swaying was performed to ensure that the stem cells could grow evenly in the dish. This entire procedure of subculture was performed four times; in other words, stem cells were sub-cultured to Passage 5 (P5) for the subsequent experiments.

2.3. Experiment design

While SHEDs have been considered a viable source for anti-aging treatment due to their ability to regenerate damaged tissues and improve overall cellular function, they will inevitably undergo functional deterioration. SHEDs lose stemness during aging and typically start to deteriorate at about Passage 5–7, limiting their applications in therapy. However, significant progress has been made in understanding the SHEDs phenotypic changes and possible mechanisms that drive their senescence. In particular, many in vitro studies have shown that melatonin and vitamin C can interact with stem cells in multiple ways, which delays the senescence and maintains the stemness of stem cells. Hence, the following experiments aim to investigate whether treatments with melatonin and vitamin C impact stemness, including proliferation, senescence and differentiation of SHEDs.

Table 1 lists the five groups of SHEDS examined in this study. The first group is the cells with a controlled medium without any treatment of melatonin and vitamin C.

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The second group is cells with melatonin treatment only, while the third group is cells with vitamin C only. Even though a few studies have suggested that melatonin and vitamin C treatment could enhance stem cell proliferation individually, their combined effects remain unclear. Thus, the 4th and 5th groups are specifically designed to understand how the combination of two solutions influences the proliferation of stem cells.

As shown in Table 1, the concentration of vitamin C in both the 4th and 5th groups has been kept at 25 μM. This is because a number of studies have suggested that the proper concentration of vitamin C for cell culture ranges from 25 to 50 μM, depends on which stem cells we are using. 50 μM should be the upper limit of the concentration applied to the cells; otherwise, the acid within vitamin C would induce cell lysis and damage the cells [41]. To investigate the suitable concentration of vitamin C on the culturing of SHEDs, we have tested the cell viability of SHEDs under the concentration of 50 μM and 25 μM of vitamin C. The results showed that the 50 μM concentration of vitamin C was too high, leading to cell death after 24 hours of cell culturing (Sup. 1). However, SHEDs under the concentration of 25 μM of vitamin C have proliferated successfully. As a result, the concentration of vitamin C that used for the research will be 25 μM. As for melatonin, several studies have indicated that the concentration ranges from 50 to 100 μM is suitable for stem cells when culturing. To investigate the suitable concentration of melatonin on SHEDs, we have compared the cell viability of SHEDs under the concentration of 50 μM and 100 μM of melatonin. The results showed that the treatment of 50 μM melatonin and 100 μM melatonin has a similar effect on the cell proliferation of SHEDs (Sup. 2). As a result, we will consider 50 μM of melatonin.

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Combining with 25 μM of vitamin C, we hypothesize that the optimal concentration of melatonin may not need to be as high as 50 μM. Therefore, we tested the performance of two combinations with Group 4 using a melatonin concentration of 25 μM and Group 5 using a melatonin concentration of 50 μM. Finally, it is important to note in this study, all groups have a duplicate to ensure the quality and replicability of the experiments.

2.4. Assessing the stemness of SHEDs

The impacts of melatonin and vitamin C treatments are assessed by examining the degree of the deterioration and osteogenesis of SHEDs, as detailed in the following subsections.

2.4.1. Examining the cell viability of SHEDs using MTT assay

Since SHEDs start deteriorating roughly at P5, the treatment effectiveness of melatonin and vitamin C will be quantified by comparing the cell viability between P2 and P5. The smaller the changes between P2 and P5 are, the more effective the treatment is.

SHEDs from groups listed in Table 1 were placed into a dish plate with 6 wells. The controlled medium and the treatment solution were replaced every two days, and SHEDs were subcultured as mentioned above. The viability at P2 and P5 was observed, and the cell growth curve was quantified using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The MTT assay is a colorimetric assay used to measure cellular metabolic activity as an indicator of cell viability and proliferation. This method has been widely used due to the simplicity of its protocol – only requires

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adding two reagents to the assay wells without the need for removing liquid or washing the cells. The measurement principle is based on the reduction of MTT, which is a yellow tetrazolium salt. When cells are viable with active metabolism, they will convert MTT into an insoluble, purple-colored formazan product by mitochondrial dehydrogenase. When cells die, they lose the ability to convert MTT into formazan, then the color of MTT remains yellow. Hence, the purple color formation serves as a useful and convenient marker of the viable cells.

To quantify the proliferation and viability of stem cells, we used a spectrophotometer that measures the amount of light absorbed by a suspension or a solution of an organic molecule at the wavelength of yellow light (i.e., 570 nm). The purple color formation from viable cells will correspond to a low absorbance at 570 nm. Hence, the higher the absorbance of a spectrophotometer reading is, the more viable cells are. The reading of the spectrophotometer, i.e., the optical density (OD) values, can then be used as a measure of cell viability.

To summarize, the assay was performed through the following steps. First, prepare a mixed solution with DMEM and MTT using a ratio of 1:9. Second, aspirate the old controlled medium, add 1.5 mL of the mixed solution in each well, and place the cells into the incubator for three hours. After the incubation, the formazan product of the MTT tetrazolium will deposit in the culture medium and near the cell surface, and accumulate as an insoluble precipitate inside cells. These will lead to the misreading of the spectrophotometer. Hence, the third step is to solubilize the formazan prior to recording absorbance readings, using agents such as isopropanol, dimethyl formamide, and dimethyl sulfoxide (DMSO). Among those agents, DMSO is the most preferred one

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because it is aprotic, relatively inert, nontoxic, and stable at high temperatures. Therefore, we added 1 mL DMSO in each well, swayed the dish slowly to ensure the formazan was solubilized completely, and then placed the dish into a spectrophotometer to quantify the cell viability.

2.4.2. Examining the deterioration degree of SHEDs

To compare the deterioration degree of SHEDs from P2 to P5 between the controlled, melatonin and vitamin C treatment groups, the percentage of deterioration was calculated in each group. The calculation of deterioration degree is an important indicator, the more deterioration indicates the less stemness is.

The calculation method is as follows: First, the value of P2 in each group minus the value of P5 in each group, then the value was divided by P2. The corresponding value of P2-P5/P2 in each group were then used to indicate a percentage of cell deterioration. The resulting percentage can be used to measure the degree of deterioration – the lower the percentage is, the lesser the deterioration is.

2.4.3. Examining the osteogenesis of SHEDs

In addition to the assessment mentioned above, the impact of melatonin and vitamin C treatment was also assessed based on the stemness of SHEDs by observing their osteogenesis ability. Osteogenesis is a process of the osteogenic differentiation of stem cells, which is critical for osteoblast formation. The osteogenesis process is regulated by several signaling pathways such as cell–cell interactions, paracrine factors, and mechanical signals, which activate a plethora of signaling molecules belonging to

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osteoblasts. These pathways are related to the condition of stem cells; for example, the stemness of stem cells will influence the abilities of differentiation. Therefore, osteogenesis can be used to indicate the stemness of SHEDs [42].

The detailed procedure for examining the osteogenesis of SHEDs includes the following steps. First, we use subcultured cells at P3 since this is the passage with the greatest potential to differentiate. For these cells, the controlled medium will be replaced with the osteogenic differentiation medium to provide a nutrient environment for inducing osteogenesis. The differentiation medium contains dexamethasone, ascorbic acid 2-phosphate, and sodium β-glycerophosphate (StemPro™ Osteogenesis Differentiation Kit; cat. No. A1007201; Gibco; Thermo Fisher Scientific, Inc.). SHEDs were cultured in the differentiation medium for 14 days with the medium replaced every two days. After 14 days of incubation, the alizarin red S staining technique was performed to observe whether SHEDs have been successfully differentiated into osteoblasts.

Alizarin red S solution is commonly used to detect calcium nodules that is formed by osteoblasts in the late stage of differentiation. The detection is based on a fact that the solution can react with calcium via its OH groups and thus stain the cell. To apply the alizarin red S staining technique, we first removed the former differentiation medium and then added 1 mL of 4% paraformaldehyde to fix the cells for 15 minutes at room temperature. Fixing cells in space immediately following removal from cell culture conditions is important, because it not only allows staining solutions to access the interior of the cells, but also limits autolysis and putrefaction. 4% paraformaldehyde is the preferred fixative for preventing the process of cellular decay. It maintains the cell morphology, integrity, and structure by building covalent cross-links between molecules.

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thereby effectively preserving cells and tissue components. After 15 minutes of fixation, we removed the 4% paraformaldehyde and washed out the excess fixative twice with phosphate buffered saline (PBS). Finally, 1 mL of 40 mM alizarin red S (Sigma-Aldrich; Merck; cat. No. TMS-008) was added to stain the cells for 20 minutes at room temperature, and then the excess dyes were washed out twice with PBS afterward. Red dyes will attach if SHEDs have successfully differentiated into osteoblasts, and thus the osteogenesis of stem cells can be observed under a microscope. To quantify the calcium nodules formed by osteogenesis, analysis of OD values from the spectrophotometer was performed.

Product	Company
Melatonin	Sigma-Aldrich
Vitamin C	Sigma-Aldrich
Osteogenesis differentiation kit	Thermo Fisher Scientific
Alizarin Red staining solution	Sigma-Aldrich

2.5. Statistics

The P values reported in the result section were calculated by the one way ANOVA test, assessing the statistical significance of the difference between the controlled group and the melatonin, vitamin C treatment groups. Three significance levels were chosen, including *P<0.05, **P<0.01 and ***P<0.001. The latter corresponds to a higher significance level than the others.

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Chapter 3

Results

3.1. The isolation of stem cells from human exfoliated deciduous teeth (SHEDs)

A deciduous tooth was provided by the National Taiwan University Children's Hospital. Immediately after extraction, the tooth was wrapped in sterilized gauze (Figure 1A). Due to the presence of bacterial stains, the tooth was wiped as clean as possible with the sterilized gauze. In a cell culture hood, the dental pulp cavity of the tooth crown was carefully scraped with tweezers to expose the stem cells. Given the small and delicate nature of the tooth, the crown was gently scraped multiple times to create a small groove containing dental pulp crumbs. Once the dental pulp cavity was adequately exposed, stem cells were isolated. The stem cells were then suspended in the culture medium. The mixture was pipetted several times until the medium became turbid, indicating the presence of stem cells. The turbid medium containing SHEDs was transferred to a 6-cm cell culture dish, which was swayed gently to ensure even distribution of the stem cells.

SHEDs were cultured at 37°C in a humidified atmosphere with 5% CO2, with the culture medium replaced every two days. The SHEDs were observed every 24 hours using an inverted phase-contrast microscope. After 24 hours of culturing, the results showed that the isolation of SHEDs was unsatisfactory, with low cell viability and proliferation (Figure 1B). Consequently, the SHEDs isolated from the tooth provided by the hospital

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were not used for the research. Instead, SHEDs from the previous lab stock were used for subsequent experiments.

3.2. The morphological, proliferative alteration and observation of stem cells from human exfoliated deciduous teeth (SHEDs)

Following isolation, most SHEDs without treatments exhibited a spindle shape, small size, and low granularity during continued culture to P2 (Figure 21A). However, long-term culture to P5 led to stem cell deterioration, characterized by decreased cell proliferation and significant morphological changes. At P5, SHEDs appeared notably larger with irregular and elongated shapes. These findings suggest that SHEDs undergo deterioration during serial subculture.

3.3. Impacts on the morphological alteration and cell viability of SHEDs with treatments

Figure 3 shows the morphological alteration and proliferation of SHEDs during subculture from P2 to P5. Visually, as shown in Figure 3A, the SHEDs with either the melatonin or vitamin C treatment show better proliferation compared to the controlled group for both P2 and P5, and the proliferation of SHEDs are decreased at P5 in all groups. Focusing on the changes from P2 to P5, we see that SHEDs with treatments have lower morphological alteration comparing with the controlled group, SHEDs with treatments at

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P5 still remain spindle shape, small size, and low granularity. In addition, the proliferations of SHEDs deteriorate in all three groups in terms of their numbers and morphology, and that the degree of the deterioration is smaller when SHEDs are treated with the melatonin and vitamin C.

Besides the observation of morphological alteration, the cell viability by MTT assay is important too. The measurement principle is based on the reduction of MTT, which is a yellow tetrazolium salt. When cells are viable with active metabolism, they will convert MTT into an insoluble, purple-colored formazan product. Hence, the purple color formation serves as a useful and convenient marker of the viable cells. Quantitatively, figure 3B shows that SHEDs with melatonin and vitamin C treatments exhibit a much higher cell viability at P2 and P5 than those without the treatments. Based on the statistical analysis, the difference in absorbance at 570 nm between the groups with and without the treatments is statistically significant with a P-value less than 0.001.

To assess the deterioration degree of SHEDs from P2 to P5 between the controlled, melatonin and vitamin C treatment groups, the percentage of deterioration was calculated. Figure 3C showed that SHEDs with melatonin or vitamin C treatment have a lower percentage of deterioration from P2 to P5. Regarding the treatment medium, the impacts of melatonin and vitamin C are similar and not significantly different. In sum, these results indicate that even though SHEDs lose their proliferative potential during deterioration, melatonin and vitamin C can delay the deterioration of SHEDs.

3.4. Impacts on the osteogenesis ability of SHEDs

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Figure 4 summarizes the potential impact of the treatment on the osteogenesis ability of the SHEDs between the controlled, melatonin and vitamin C treatment groups at P3 and P5, the cells were cultured in osteogenic induction medium for two weeks and each group was performed in two replicates. Alizarin Red S staining showed that after the osteogenic induction process was performed, calcified nodules was observed. The detection is based on the fact that the solution can react with calcium via its OH groups and thus stain the cells. To assess the formation of nodules, stained Alizarin Red was extracted using 10% cetylpyridinium chloride buffer solution and the OD value of the solution was measured at 570 nm.

As illustrated in Figures 4A and 4B, no matter whether the group is with or without the special retreatment with melatonin and vitamin C, both the size and number of Alizarin Red-positive condensed nodules of SHEDs are generally larger in the early passage (P3) than those in the later passage (P5), which indicates a deteriorated osteogenesis ability of SHEDs from P3 to P5. The deterioration is less severe for the groups treated with melatonin and vitamin C. This qualitative assessment is confirmed by the OD values in Figure 4C. The differences in OD values between groups with and without treatments are statistically significant, but the difference between the melatonin treatment and the vitamin C treatment is not. In short, both the melatonin treatment and the vitamin C treatment enhance the osteogenesis ability of the SHEDs and slow the deterioration of osteogenesis. The performances of the melatonin treatment and the vitamin C treatment on the osteogenesis enhancement are rather similar.

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3.5. Impacts on the morphological alteration and cell viability of SHEDs with the combination treatment

Among the previous results, melatonin and vitamin C treatment could enhance stem cell proliferation individually. To further investigate the relation of the combination of melatonin and vitamin C, the combination treatment was performed. The concentration ratio of melatonin and vitamin C will be 1:1 and 2:1, while the concentration of melatonin is 25 μM and 50 μM, and the concentration of vitamin C will be kept at 25 μM. The reason why the concentration of vitamin C had to keep at 25 μM is because I have tested the cell viability of SHEDs under the concentration of 50 μM and 25 μM of vitamin C, the results showed that the 50 μM concentration of vitamin C was too high, leading to cell death after 24 hours of cell culturing (Sup. 1), while SHEDs under the concentration of 25 μM of vitamin C have proliferated successfully. As a result, the concentration of vitamin C that used for the combination treatment will be kept at 25 μM.

The results shown in Figure 5A indicated that comparing with the controlled group, SHEDs with the combination treatment of melatonin and vitamin C have lower morphological alteration during serial subculture from P2 to P5, SHEDs with treatments at P5 still remain spindle shape, small size, and low granularity. Furthermore, SHEDs with combination treatments significantly enhances the cell proliferation of SHEDs at both P2 and P5.

In addition to the observation of morphological alteration, the cell viability by MTT assay is an important indicator of cell deterioration. The quantification of cell viability by MTT assay, as shown in Figure 5B, indicated that despite the decreasing of viability at P5, the combination treatment still enhances and maintains the proliferation of SHEDs.

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and the effect was better than the separate treatment of melatonin and vitamin C, while there is no significant difference between the different concentration ratio of the combination treatment. The percentage of deterioration was calculated to examine the deterioration degree of SHEDs from P2 to P5 in Figure 5C. The deterioration degree was compared between the controlled, separate treatment and the combination treatment group. The result indicates that SHEDs with the combination of melatonin and vitamin C treatment group have a lower percentage of deterioration from P2 to P5 comparing with other groups.

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Chapter 4

Discussion

4.1. The melatonin and vitamin C treatment on the deterioration of SHEDs

SHEDs offer significant advantages for tissue regeneration and clinical therapy due to their straightforward isolation process and multi-lineage differentiation potential. Previous studies have demonstrated that all mesenchymal stem cells, including SHEDs, eventually undergo cellular deterioration following in vitro expansion, exhibiting limited proliferative capacity and differentiation potential over time [43]. Melatonin and vitamin C have been reported to have an influence on stem cells culturing, which enhances the proliferation capacity and differentiation potential of stem cells. To figure out the relation between the deterioration of SHEDs, melatonin and vitamin C, the cell viability at P2 and P5 of the controlled group and the melatonin, vitamin C treatment groups were compared.

The results of cell proliferation and viability by microscope images and MTT assay have showed that SHEDs at early passage numbers exhibited differences in growth, SHEDs undergo considerable alterations during in vitro serial subculture, including spontaneous transformation, reduced capacity for proliferation and morphological alterations. However, a comparison of the cell viability and the deterioration degree at P2 and P5 of the controlled group and the melatonin, vitamin C treatment groups, has shown that the proliferation ability of SHEDs was highly increased by the treatment of melatonin and vitamin C at both P2 and P5, which delayed the deterioration of SHEDs. Meanwhile,

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there is no significant difference between melatonin and vitamin C treatment, they have the same effect on the proliferation and deterioration of SHEDs.

4.2. The melatonin and vitamin C treatment on the osteogenesis of SHEDs

Previous studies demonstrated that SHEDs underwent deterioration and exhibited reduced differentiation potential because of serial subculture in vitro [43]. To investigate the influence of melatonin and vitamin C on the osteogenic differentiation ability of SHEDs, the Alizarin Red S staining technique was performed and the formation of calcified nodules were compared between the controlled group and the treatment groups at P3 and P5.

The results of OD value showed that the formation of calcified nodules was decreased from P3 to P5 in the controlled, melatonin, and vitamin C treatment groups, this indicated that the osteogenic differentiation ability of SHEDs was reduced due to the deterioration. However, comparing the controlled group with the treatment groups, the formation of nodules was highly increased. Taken together, the treatment of melatonin and vitamin C can enhance the osteogenic differentiation potential at early passage, while maintaining the ability to differentiate from deterioration of SHEDs. As for the individual effect of melatonin and vitamin C, the results showed that there is no significant difference between them, they showed the same effect on the differentiation and deterioration of SHEDs.

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4.3. The combination treatment of melatonin and vitamin C on the proliferation of SHEDs

According to our previous results, the separate treatment of melatonin and vitamin C could enhance the proliferation capacity and differentiation potential of SHEDs. To further investigate the relation between melatonin and vitamin C, the combination treatment at different ratio of concentration was performed.

The results of cell viability at P2 and P5 by MTT assay within the controlled and the combination treatment group have demonstrated that the combination of melatonin and vitamin C can enhance the cell proliferation and viability of SHEDs. Furthermore, the comparison of deterioration degree indicated that SHEDs with the combination of melatonin and vitamin C treatment can reduce the deterioration from P2 to P5, and the effectiveness is better than the separate treatment of melatonin or vitamin C, while these is no significant difference between the different concentration ratio of the combination treatment.

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Chapter 5

Conclusion

According to the analyses above, we conclude the following:

1. SHEDs undergo deterioration during the serial subculture in vitro. Deterioration results in morphology transformation, arrested proliferation and reduced differentiation ability.
2. The individual treatment of melatonin and vitamin C augments the proliferation capacity and osteogenesis ability of SHEDs at both early and later passage, thus delaying the deterioration and maintaining the stemness of SHEDs.
3. The combination of melatonin and vitamin C treatment enhances the proliferation ability of SHEDs at both early and later passage.
4. Melatonin and vitamin C showed the same effect on the proliferation and osteogenesis of SHEDs. The combination of melatonin and vitamin C at the different concentration ratio of 1:1 and 2:1 has the same effect on the proliferation of SHEDs, there is no significant difference between the two-different concentration ratio.

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Subsequent studies suggested

Different concentration ratio of the combination of melatonin and Vitamin C

We investigated the effects of combining melatonin and vitamin C at concentration ratios of 1:1 and 2:1. Our results indicated no significant difference between these two ratios. Due to time constraints, we were unable to examine other concentration ratios. Therefore, to better understand the relationship between melatonin and vitamin C on stem cells, further studies exploring a wider range of concentration ratios are necessary.

Multiple lineage differentiation

SHEDs possess multifaceted lineage differentiation capabilities, encompassing osteogenesis and neurogenesis. While our investigation has primarily centered on SHEDs' osteogenic potential due to its relevance in treating bone defects, there exist significant applications for neurogenesis, such as neural repair and regeneration. Therefore, exploring SHEDs neurogenic capacity is crucial for identifying their secondary applications.

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Chapter 6

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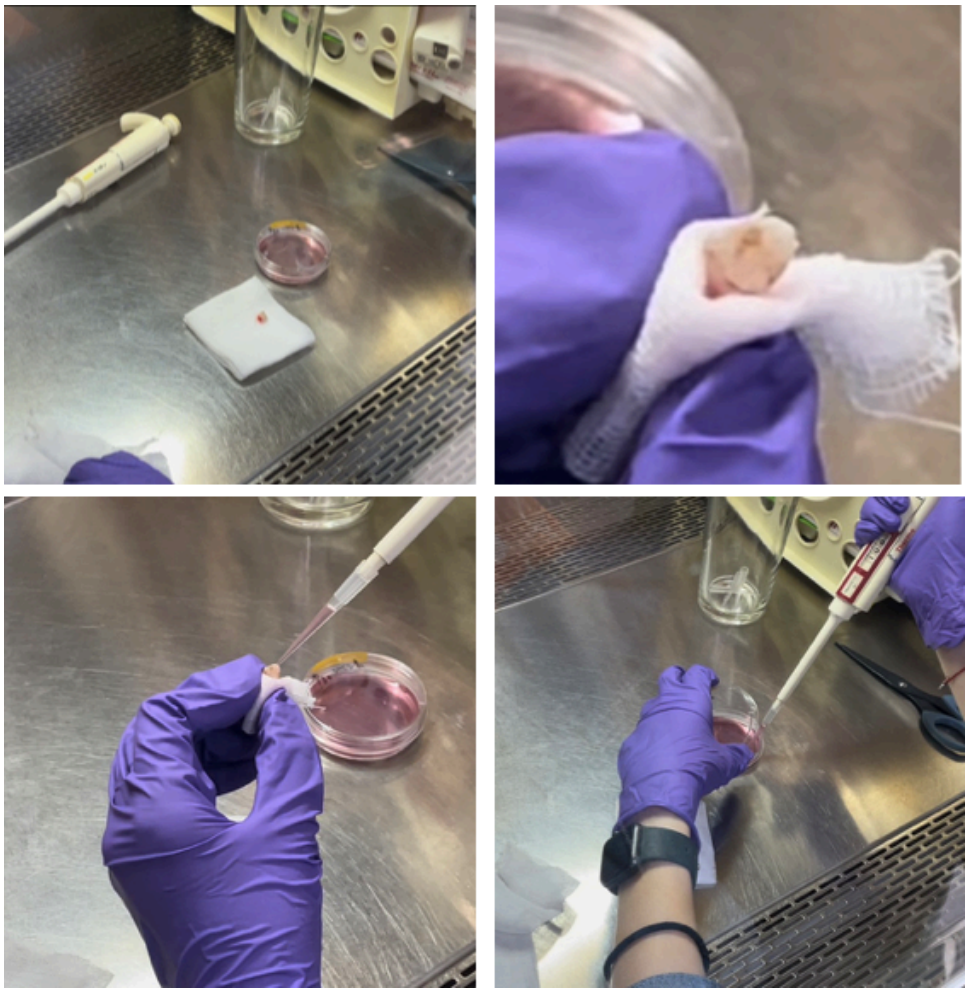
Chapter 7

Figure and Legend

(A)



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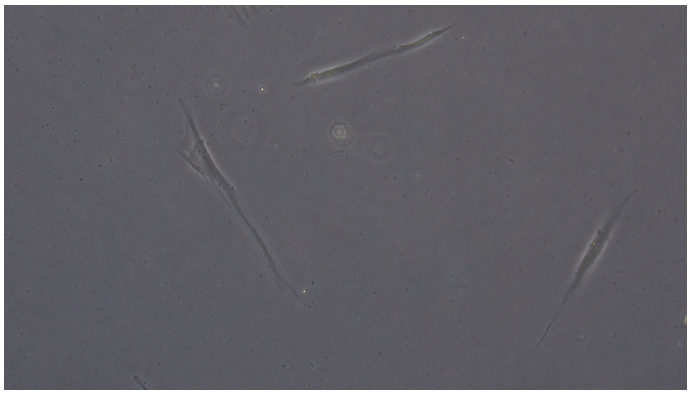

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Figure 1. (A) The exfoliated deciduous tooth without dental caries was provided by Dr. Yu-Zhi Jiang. SHEDs were isolated from the deciduous tooth by aseptic techniques. (B) The observation of SHEDs by microscope after 24 hours of cell culturing.

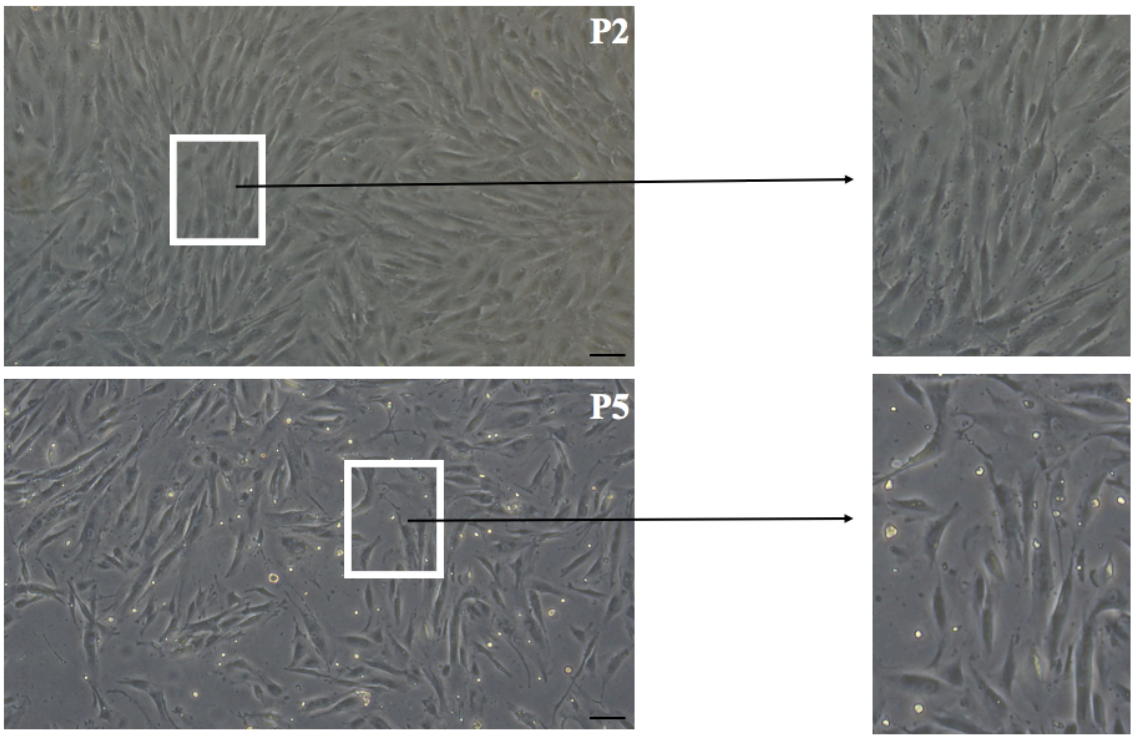

Figure 2. Images illustrating the morphology and proliferation of SHEDs at Passage 2 (P2; upper panel) and Passage 5 (P5; lower panel) with a scale bar of 100 μm. The images boxed by the white rectangles are zoomed in and shown at right.

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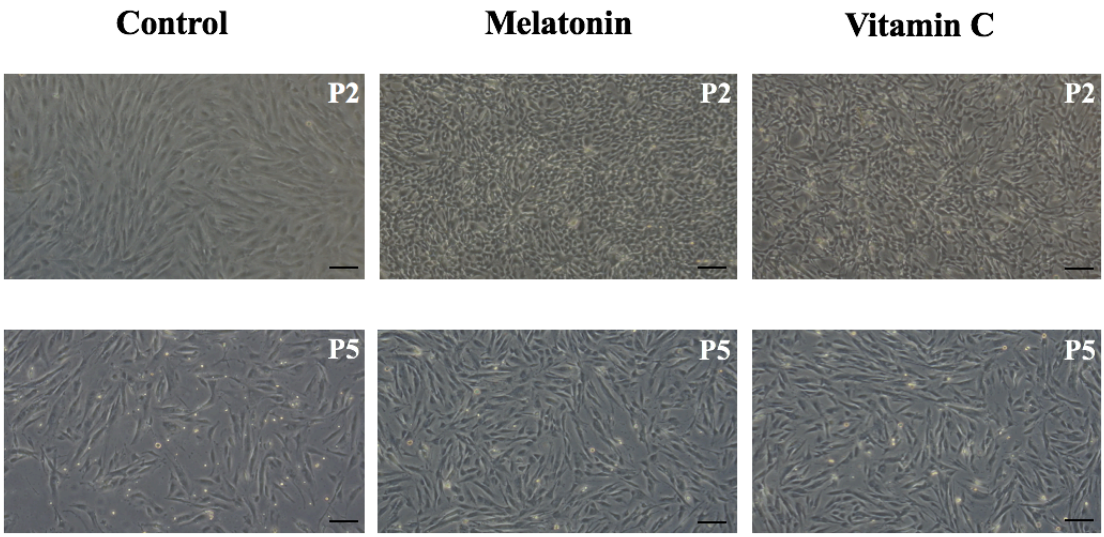
(A)



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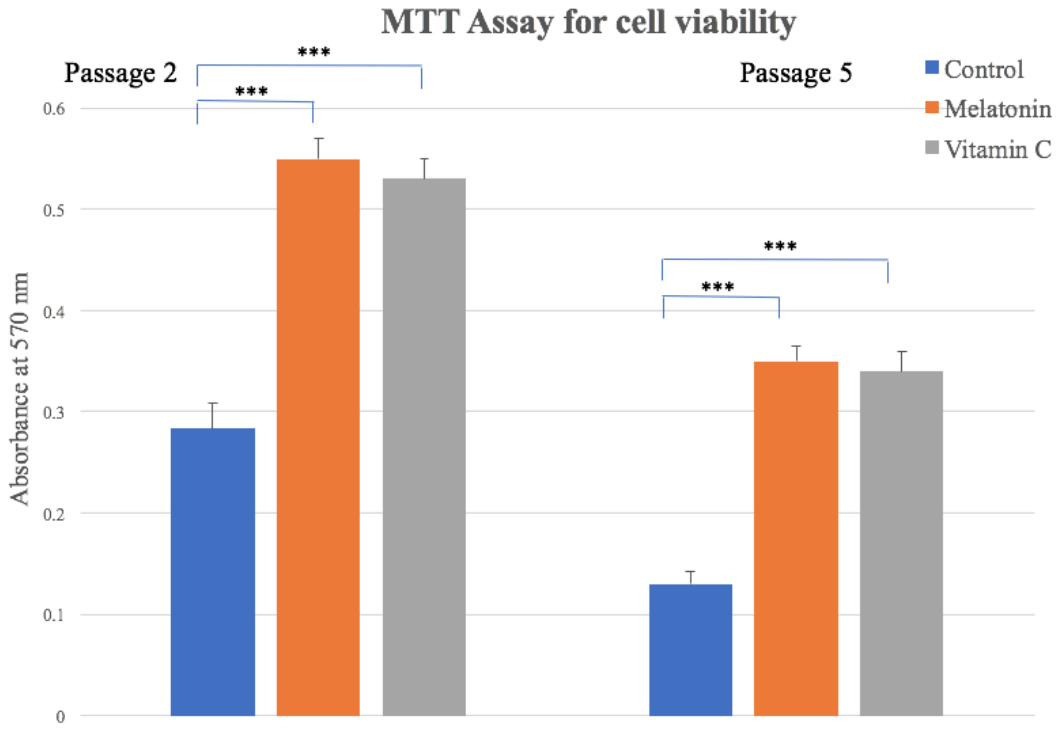

Melatonin	–	50μM	–	–	50μM	–
Vitamin C	–	–	25μM	–	–	25μM
Veh (PBS)	+	–	–	+	–	–

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(C)

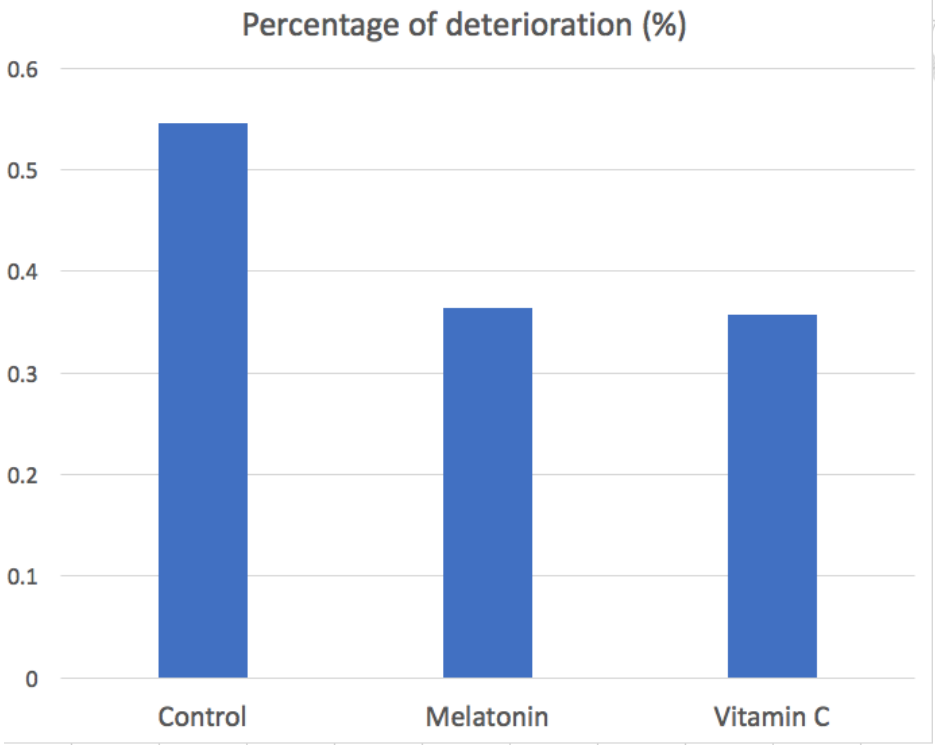

Figure 3. (A) Images illustrating the deterioration of the proliferation of SHEDs during serial subculture from P2 (upper panel) to P5 (lower panel) with a scale bar of 100 μm, using the controlled medium (left), melatonin (50 μM) treatment (middle), and vitamin C (25 μM) treatment (right). (B) A bar chart of light absorbance at 570 nm showing the cell viability of SHEDs at P2 (left) and P5 (right) for various treatment mediums. (C) The percentage of deterioration of SHEDs was compared between the controlled group, melatonin (50 μM) treatment group and vitamin C (25 μM) treatment group. Data are presented as the mean ± standard error of the mean. *P<0.05, **P<0.01 and ***P<0.001, as indicated. SHED, stem cells from human exfoliated deciduous teeth.

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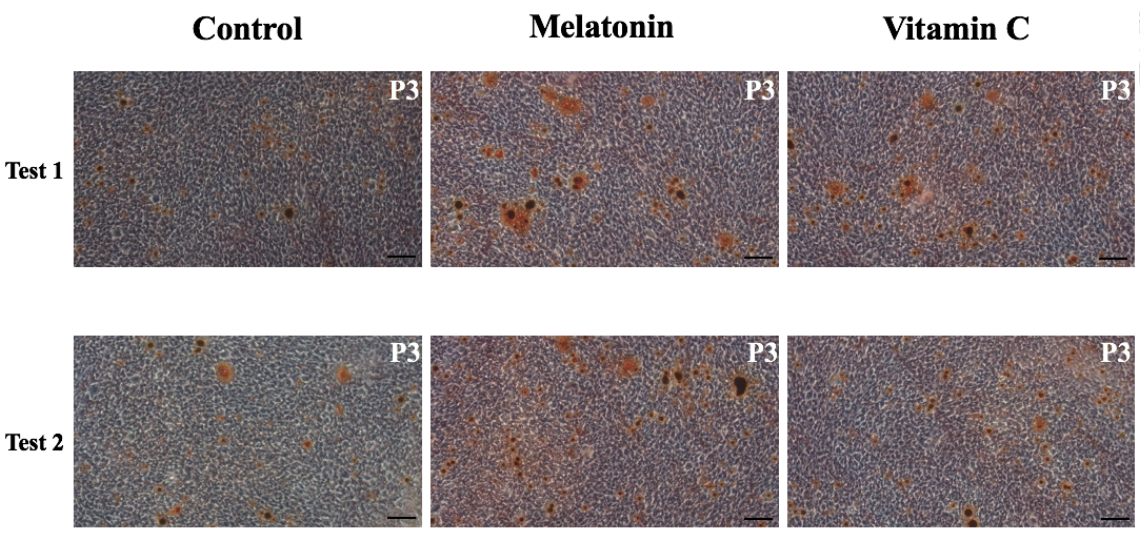

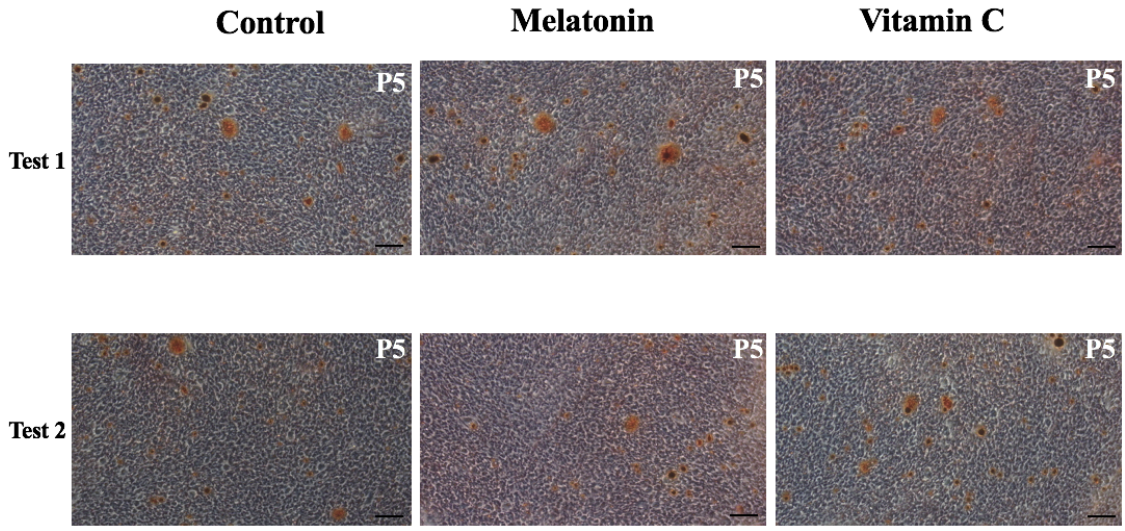

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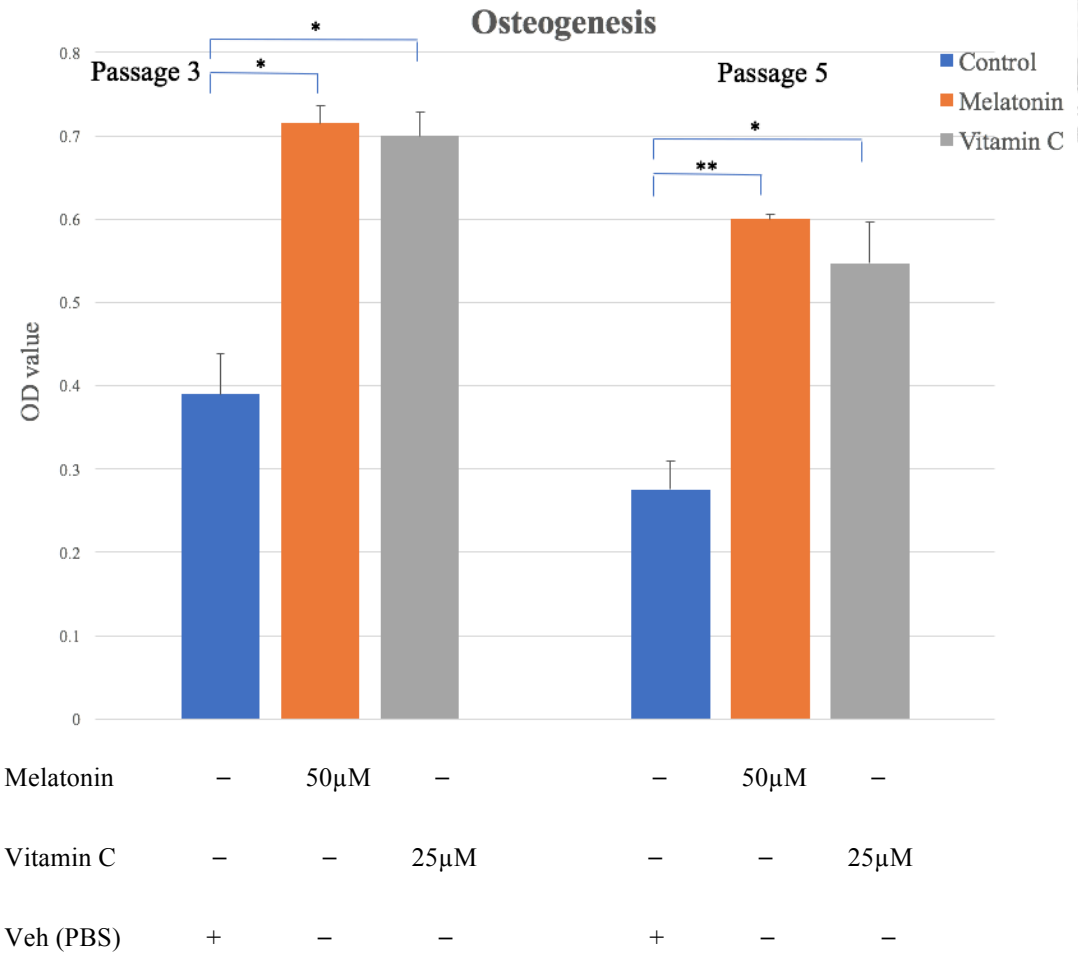

Figure 4. Images illustrating the osteogenesis of SHEDs at P3 (A) and P5 (B) observed in the controlled group (left), melatonin (50 μM) treatment group (middle), and vitamin C (25 μM) treatment group (right). Two tests for each group were performed and both shown in this figure. The scale bar corresponds to a length of 100 μm. (C) A bar chart of OD value at P3 (left) and P5 (right) for various treatment groups. Data are presented as the mean ± standard error of the mean. *P<0.05 and **P<0.01, as indicated. SHED, stem cells from human exfoliated deciduous teeth.

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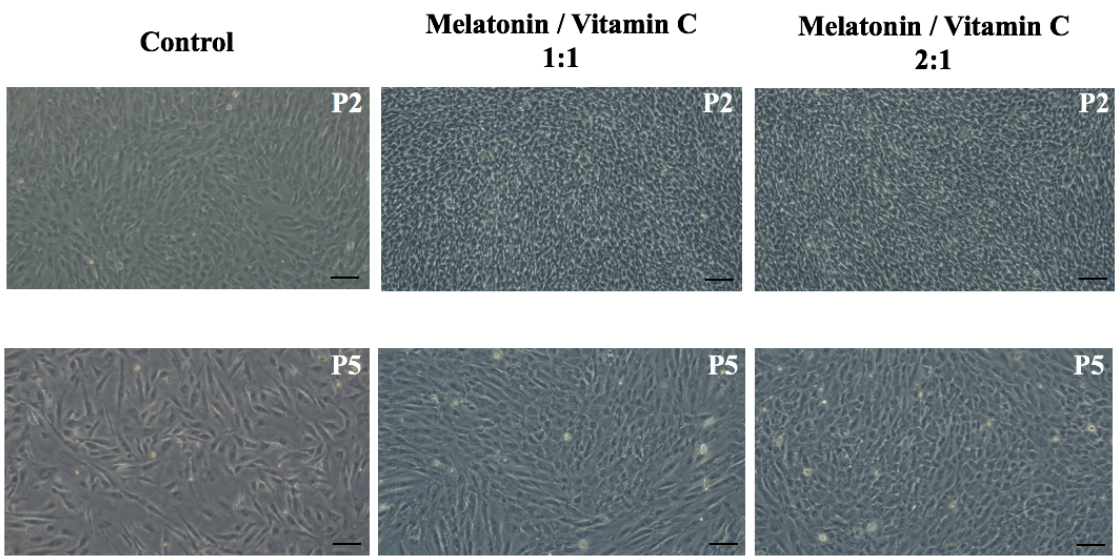
(A)



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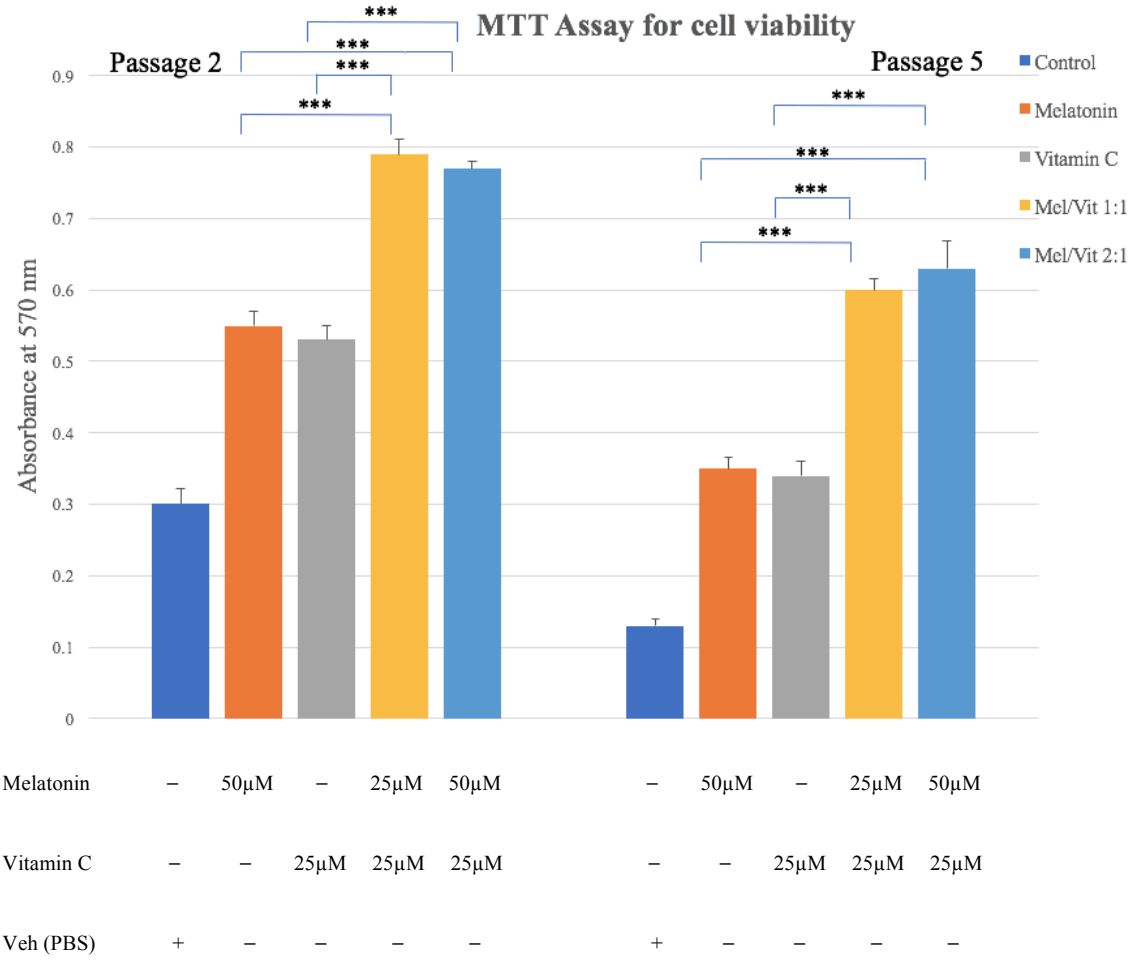

Melatonin	–	50μM	–	25μM	50μM		–	50μM	–	25μM	50μM
Vitamin C	–	–	25μM	25μM	25μM		–	–	25μM	25μM	25μM
Veh (PBS)	+	–	–	–	–		+	–	–	–	–

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(C)

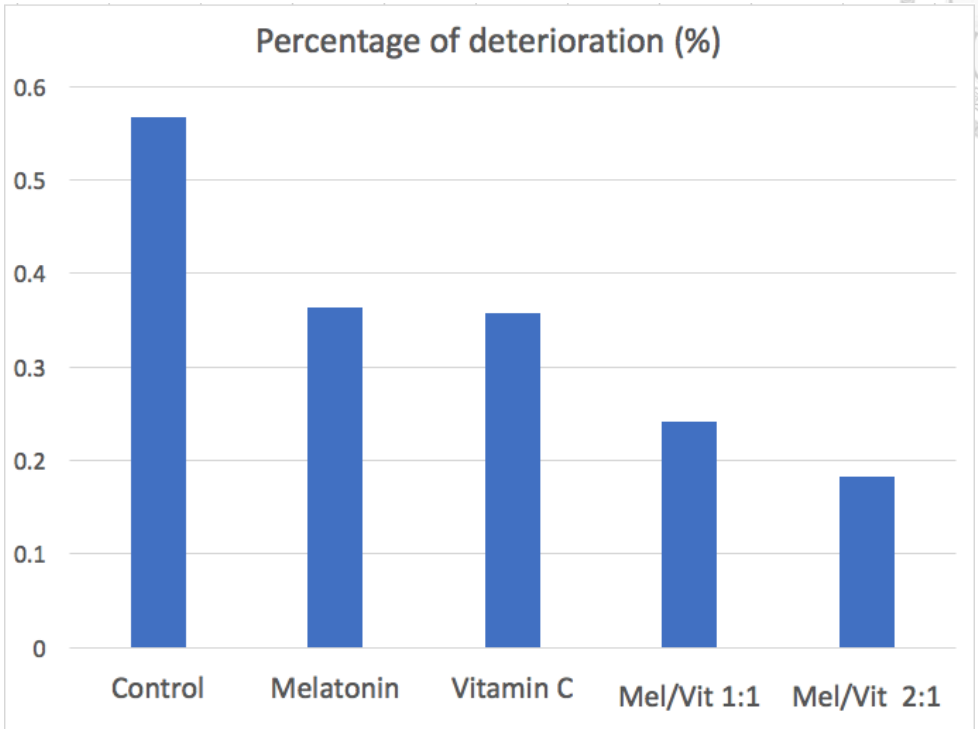

Figure 5. The cell proliferation and cell viability of SHEDs between the controlled group, separate treatment group and combination treatment of melatonin and vitamin C at P2 and P5. (A) The proliferation of SHEDs in the controlled group and the different ratio of concentration of combination treatment at P2 and P5. (B) The cell viability of SHEDs at P2 and P5 was compared between the controlled group, separate treatment group and combination treatment of melatonin and vitamin C by MTT assay. (C) The percentage of deterioration of SHEDs was compared between the controlled group, separate treatment and combination treatment of melatonin and vitamin C. Data are presented as the mean ± standard error of the mean. *P<0.05, **P<0.01 and ***P<0.001, as indicated. SHED, stem cells from human exfoliated deciduous teeth.

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Chapter 8

Tables

Group ID	Melatonin	Vitamin C
1	—	—
2	50 μM	—
3	—	25 μM
4	25 μM	25 μM
5	50 μM	25 μM

Table 1. A summary of cell groups examined in the study. All groups are mixed with the controlled medium but with various concentrations of melatonin and vitamin C. Group 1 is the controlled group, which is not treated with additional nutrition but only with the controlled medium that contains Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin solution. For Groups 2–5, the melatonin and the vitamin C powder (Sigma-Aldrich) are dissolved in distilled water to reach the specified concentrations.

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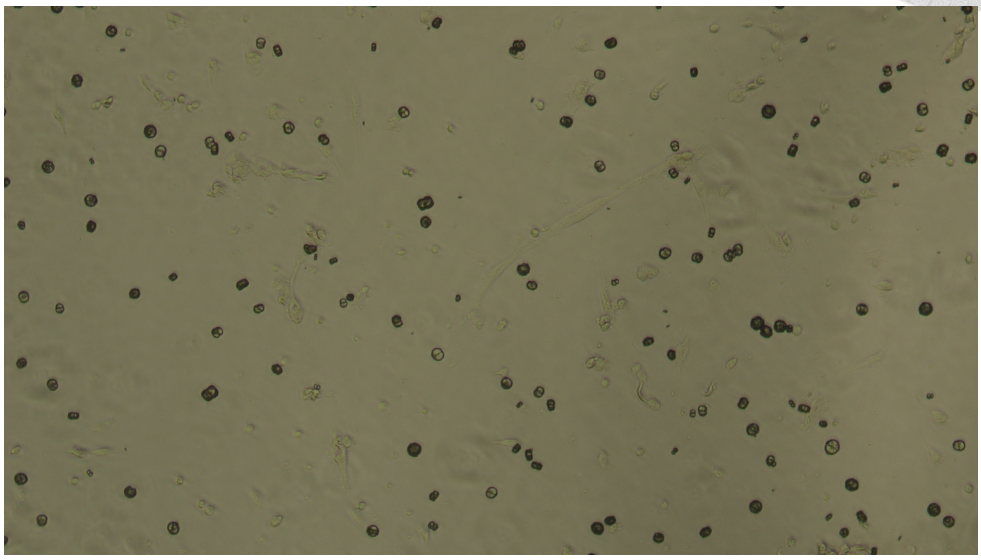
Supplementary Data



Supplementary 1 SHEDs were cultured in DMEM+FBS+PS with the treatment of vitamin C at the concentration of 50 μM

To determine the suitable concentration of vitamin C that used for the cultivation of SHEDs, 50 μM of vitamin C was first used. The result showed that the 50 μM concentration of vitamin C was too high, leading to cell death.

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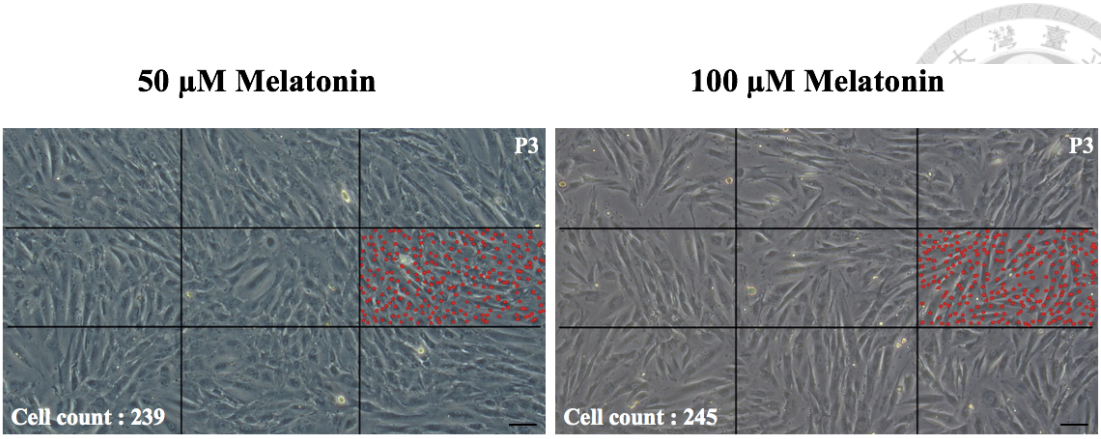

Supplementary 2 SHEDs were cultured in DMEM+FBS+PS with the treatment of melatonin at the concentration of 50 μM and 100 μM

To determine the suitable concentration of melatonin that used for the cultivation of SHEDs, the concentration of 50 μM and 100 μM of melatonin were used. The result showed that SHEDs with the treatment of 50 μM melatonin and 100 μM melatonin has a similar effect on cell proliferation. The cell proliferation was assessed by cell count.

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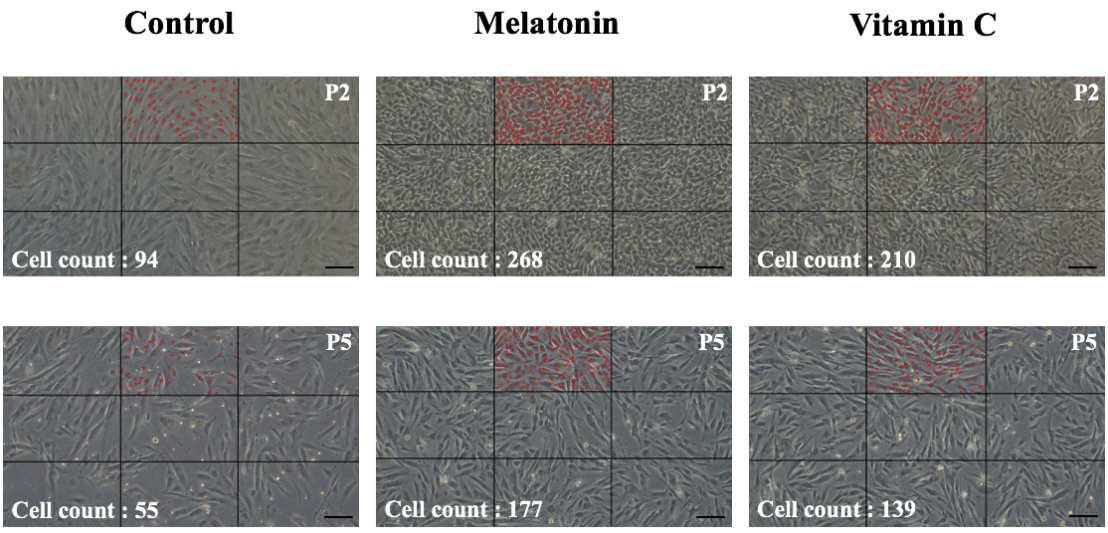

Supplementary 3 The cell count of SHEDs in the control, melatonin and vitamin C treatment groups at P2 and P5

For the experiment, cell count was performed to ensure the accuracy of cell viability between the controlled, melatonin and vitamin C group at P2 and P5.

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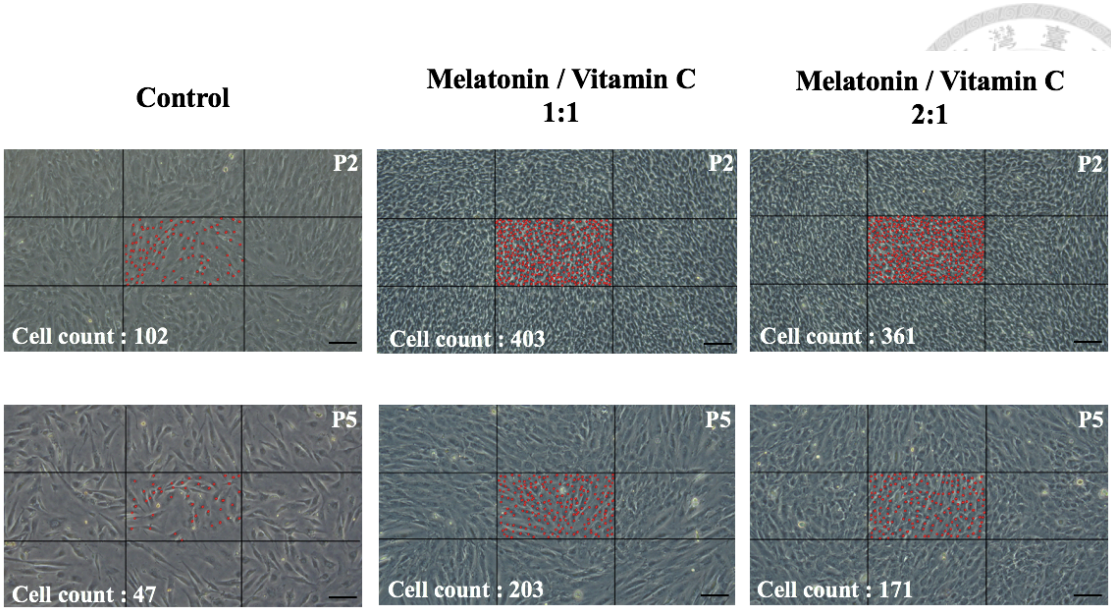

Supplementary 4 The cell count of SHEDs in the control, combination treatment of melatonin and vitamin C group at P2 and P5

For the experiment, cell count was performed to ensure the accuracy of cell viability between the control, combination treatment of melatonin and vitamin C group at P2 and P5.

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	P2-P5 / P2	% of deterioration
Control	0.546	54.6 %
Melatonin	0.364	36.4 %
Vitamin C	0.358	35.8 %

	P2-P5 / P2	% of deterioration
Control	0.567	56.7 %
Melatonin	0.364	36.4 %
Vitamin C	0.358	35.8 %
Mel / Vit 1:1	0.241	24.1 %
Mel / Vit 2:1	0.182	18.2 %

Supplementary 5 The calculation of cell deterioration of SHEDs

To access the deterioration degree of SHEDs from P2 to P5 between the controlled, melatonin and vitamin C treatment groups, the percentage of cell deterioration was calculated. The lower the percentage is, the fewer the deterioration is.

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