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# Delayed animal aging through the recovery of stem cell senescence by platelet rich plasma

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ABSTRACT

Aging is related to loss of functional stem cell accompanying loss of tissue and organ regeneration potentials. Previously, we demonstrated that the life span of ovariectomy-senescence accelerated mice (OVX-SAMP8) was significantly prolonged and similar to that of the congenic senescence-resistant strain of mice after platelet rich plasma (PRP)/embryonic fibroblast transplantation. The aim of this study is to investigate the potential of PRP for recovering cellular potential from senescence and then delaying animal aging. We first examined whether stem cells would be senescent in aged mice compared to young mice. Primary adipose derived stem cells (ADSCs) and bone marrow derived stem cells (BMSCs) were harvested from young and aged mice, and found that cell senescence was strongly correlated to animal aging. Subsequently, we demonstrated that PRP could recover cell potential from senescence, such as promote cell growth (cell proliferation and colony formation), increase osteogenesis, decrease adipogenesis, restore cell senescence related markers and resist the oxidative stress in stem cells from aged mice. The results also showed that PRP treatment in aged mice could delay mice aging as indicated by survival, body weight and aging phenotypes (behavior and gross morphology) in term of recovering the cellular potential of their stem cells compared to the results on aged control mice. In conclusion these findings showed that PRP has potential to delay aging through the recovery of stem cell senescence and could be used as an alternative medicine for tissue regeneration and future rejuvenation.



## 1. Introduction

Many diseases are originated from aging, including cardiovascular, Alzheimer's and Parkinson's disease along with osteoporosis and osteoarthritis due to both age-dependent senescence in tissue-specific stem cells and changes in their microenvironment [1,2]. Aging is related to the accumulation of degenerative factors emitted by senescent cells, such as free radicals, proteases, cytokines, and reactive oxygen species (ROS), which lead to increased rates of apoptosis and degeneration [3]. In addition, loss of functional stem cells in aging process would accompany loss of tissue and organ regeneration potential in bone [4], skin [5], brain [6] and hair [7]. Local microenvironments and niche cell interactions are indispensable for maintaining stem cell function. An age-related decrease in environmental factors is as important as an age-related decline in endogenous stem cell activity. Thus, increases in local and systemic factors have the potential to restore regenerative capacity of aged cells and maintain tissue viability [8].

During aging, the systemic connection between fat and bone maintains an important risk for vertebral and hip fractures, as well as aging-related diseases such as osteoporosis and diabetes. The increase at fat tissue mass is age-dependent, which accumulates in

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bone marrow, muscle, liver, and other ectopic sites [9]. Additionally, preadipocytes in fat tissue will release multiple adipokines and inflammation factors (eg. Leptin, PPARγ, TNF-a, and IL-6) to induce cell senescence and apoptosis by activating cellular stress response pathways [10,11]. The release of these growth factors and activation of transcription factors further affects mesenchymal stem cell differentiation to adipocyte or osteoblast in fat distribution, increasing susceptibility to lipotoxicity in bone marrow [12,13]. Interestingly, studies from Keller JN et al. found that oxidative stress accumulates in aged adipose tissue [14]. Oxidative stress is known to increase with aging and also alters the gene expression of important regulators of the cell cycle, such as p53, p16 and p19 and further affect the other molecular markers expression of aging (sirt1, c-fos and IGF-1). Alterations in these genes result in reduced stem cell proliferation and increased amount of cell hypotrophy and apoptosis [15]. Therefore, molecular mechanisms that affect adipogenesis and oxidative stress of stem cells can potentially regulate cell senescence and thereby the progression of aging.

Senescence-accelerated prone mouse (SAMP8) is associated with age-dependent functional decline in cell survival and function, as well as age-associated brain dysfunction. Additionally, SAMP8 mice were found to have higher levels of oxidative stress due to generation of large amounts of reactive oxygen species [16]. Not only do these mice display high oxidative stress status, but they also have high adipogenesis, indicating a good model to determine senescence and aging in cellular and animal level, respectively. Especially, stem cells derived from bone marrow (BMSCs) or adipose tissue (ADSCs) could be predisposed to age-related changes which reduce their ability to contribute to endogenous repair processes [17]. It has recently been proposed that aging BMSCs could shift from pro-osteoblastogenesis to pro-adipogenesis due to an accumulation of age-related bone marrow fat [18]. Even from these observations it remains unclear whether senescence of stem cells is responsible for the pathogenesis of aging in animal.

Previously, we demonstrated that platelet rich plasma (PRP) extended life span, improved survival and decreased adipogenesis in SAMP8 mice [19,20]. In this study, we hypothesized that PRP was able to delay aging through examining the characteristics of cells including cell growth (cell proliferation and colony formation), cell differentiation (osteogenesis and adipogenesis) and cellular senescence (gene, SA-β-gal activity, and oxidative stress); as well as animal morphology including life span, weight change and phenotype examination such as morphology (skin glossiness, collagen density, cataracts and lordokyphosis of the spine), bone formation and behavior (passivity and reactivity).

## 2. Materials and methods

### 2.1. PRP preparation

Human whole blood was purchased from Taipei Blood Center and fractionated using the platelet rich plasma (PRP) with MCS blood cell separation system (Haemonetics Corp., USA), and then bovine thrombin (100 IU bovine thrombin/150 ml PRP) was used to remove aggregated fibrin and the product was centrifuged for 6 min (3000 rpm at room temperature). To ensure the most appropriate concentration and consistency of the purified PRP used in this study, transforming growth factor beta 1 (TGF-β1) was quantitatively analyzed using a Quantikine enzyme-linked immunosorbent assay (ELISA) kit (#DB100, R&D Diagnostics, Wiesbaden, Germany) to calibrate the purified PRP concentration. Previously, results showed that a 750 pg/ml concentration of TGF-β1 in PRP was optimal for cell proliferation and differentiation [20]. Purified PRP was dissolved with alpha-minimum essential medium (alpha-MEM) media (containing 1% fetal bovine serum and 1% prostate-specific antigen) to prepare the PRP solution for use in this study.

### 2.2. Cell characterization

First, we isolated the bone marrow stem cells (BMSCs) and Adipose-derived stem cells (ADSCs) from young (1-month old) and aged (10-month old) SAMP8 mice. All femurs and tibias (to isolate BMSCs) and adipose tissues (to isolate ADSCs) were collected from SAMP8 mice before and after PRP treatment. The adherent BMSCs were harvested from bone fragments with alpha-MEM media after being cultured for 1 week. ADSCs required tissue digestion by alpha-MEM media containing 10% Fetal bovine serum (FBS), 0.1% collagenase type IV solution and 1% prostate-specific antigen (PSA) at 37 °C for 1 h and removal of the supernatant (centrifuged at 1500 rpm, 10 min), then, after culturing for 3 days, harvest the adherent cells. These cells were cultured in the alpha-MEM media containing 10% FBS and 1% PSA. The culture media was changed every two days, and cells were passaged after 80–90% confluence. Subsequently, the stem (CD) markers profiles were determined using flow cytometry (FACSCalibur, Taipei Medical University) and the positive markers were CD44 and CD105, while the negative markers were CD34 and CD45.

### 2.3. Determination of osteogenic/adipogenic differentiation

To examine their osteogenic differentiation, isolated stem cells were cultured with or without PRP solution in alpha-MEM supplemented with 10% fetal bovine serum (FBS), 0.1 μM dexamethasone (Sigma–Aldrich), 10 mM β-glycerophosphate (Sigma–Aldrich), and 50 m M ascorbic acid (Sigma–Aldrich) for 21 days and then fixed in 10% formalin for 10 min, and analyzed by Alizarin Red S staining for 15 min at room temperature. After the bound staining was eluted with 10% cetylpyridinium chloride, the degree of osteogenesis was quantified by measuring absorbance of supernatants in spectrophotometer at 540 nm. For adipogenic differentiation, isolated stem cells were cultured with or without PRP solution in alph-MEM supplemented with 10% FBS, 1 μM Dexamethasone, 10 μg/ml insulin, and 0.5 mM 3-methyl-1-isobutylxanthine (sigma) for 2 days. Thereafter, the cells were changed to maintenance medium (alpha-MEM, 10% fetal calf serum, and 10 μg/ml insulin) every 2 days, and induced for adipogenesis over a 21-day period and then fixed as described above and observed by 0.5% Oil red O staining in 60% isopropyl alcohol for 15 min at room temperature. For quantification, the lipid droplets were extracted by 100% absolute isopropyl alcohol and absorbance was measured at 540 nm (Thermo Varioskan Flash, Taipei Medical University) according to the previously described protocol [19].

### 2.4. Cell proliferation, colony formation and senescence associated β-galactosidase (SA-β-gal) staining

The isolated stem cells were seeded in 6-cm dishes ($1 \times 10^5$ cells/per dish) in PRP solution (as described above) or basal medium (alpha-MEM supplemented with 10% FBS). After 48 h, these cells were collected and counted by trypsin and ethylenediaminetetraacetic acid treatment. For the colony formation assay, isolated stem cells ($1 \times 10^4$) were cultured in 6 cm dishes containing PRP solution or basal medium. At day 14, these cells were fixed in 4% formaldehyde and stained with 0.5% crystal violet (Fisher Scientific, Pittsburgh, PA) in methanol for 10 min, and formed colonies were then counted. The SA-β-gal assay is widely used as a biomarker of cellular senescence. The galactosidase activity was determined at pH6, the positive cells can lead to the formation of a blue precipitate. In this study, the isolated cells were fixed with 4% formaldehyde and then stained by SA-β-gal assay (SA-β-gal staining kit, CS0030, Sigma).

### 2.5. Experimental animals

The experiment protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Taipei Medical University and we monitored animal condition for signs of pain and suffering including anorexia, weight loss, ambulation difficulty, infection, etc. If distress was detected, treatment, analgesia, or humane euthanasia would have been utilized as appropriate. Young-senescence-accelerated prone mouse strain (SAMP8) mice were used by isolating their stem cells (ADSCs and BMSCs), and aged −SAMP8 mice were divided into 3 groups: isolated stem cells group, aged group (receiving PBS) and PRP group (receiving PRP/per month). Both PBS and PRP solution (10ul) were injected with a microsyringe into the bone marrow. Briefly, hair from the right hind limb was removed and a 26-gauge needle was inserted into the surface of the tibia through the patellar tendon, followed by injection of PRP solution (10ul) into the bone marrow cavity. Life span; body weight; and appearance of eyes, skin and hair after treatment period were examined in the aged and PRP group. Each group contain 8 animals.

### 2.6. Evaluation of animal aging

After 6 months of PRP treatment, aging phenotype and behavior were examined in the mice. We collected various mouse tissues (skin, brain and bone) and fixed them with 10% formalin prior to embedding the samples in paraffin. Skin sections (10 μm) were stained with Masson's trichome staining, while brain sections (10 μm) were incubated with primary antibody (beta-amyloid, Millpore) at 1:200 dilution for 1 days at 4 °C followed by use of biotinylated secondary antibodies with Avidin/Biotin Complex (ABC) Kits (Vector Labs #PK-6100) and diaminobenzidine (DAB) detection kits (Vector Labs # SK-4100) according to the standard protocol. For bone analysis, the extent of spine lordokyphosis was determined using X-ray (Taipei Medical University Hospital) analysis. Also, bone tissues were collected from the femurs and imaged using a SkyScan-1076 Micro-CT system (Skyscan, Beligum) in accordance with our previously described protocol [19]. Bone sections were then stained with hematoxylin eosin (H&E) staining in order to examine bone