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METHODS ARTICLE

Characterization of Induction and Targeting of Senescent Mesenchymal Stromal Cells

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Mesenchymal stromal cells (MSCs) from older donors have limited potential for bone tissue formation compared with cells from younger donors, and cellular senescence has been postulated as an underlying cause. There is a critical need for methods to induce premature senescence to study this phenomenon efficiently and reproducibly. However, the field lacks consensus on the appropriate method to induce and characterize senescence. Moreover, we have a limited understanding of the effects of commonly used induction methods on senescent phenotype. To address this significant challenge, we assessed the effect of replicative, hydrogen peroxide, etoposide, and irradiation-induced senescence on human MSCs using a battery of senescent cell characteristics. All methods arrested proliferation and resulted in increased cell spreading compared with low passage controls. Etoposide and irradiation increased expression of senescence-related genes in MSCs at early time points, proinflammatory cytokine secretion, DNA damage, and production of senescence-associated β-galactosidase. We then evaluated the effect of fisetin, a flavonoid and candidate senolytic agent, to clear senescent cells and promote osteogenic differentiation of MSCs entrapped in gelatin methacryloyl (GelMA) hydrogels *in vitro*. When studying a mixture of nonsenescent and senescent MSCs, we did not observe decreases in senescent markers or increases in osteogenesis with fisetin treatment. However, the application of the same treatment toward a heterogeneous population of human bone marrow-derived cells entrapped in GelMA decreased senescent markers and increased osteogenesis after 14 days in culture. These results identify best practices for inducing prematurely senescent MSCs and motivate the need for further study of fisetin as a senolytic agent.

Keywords: senescence, MSCs, hydrogel, osteogenesis, senolytic agent

Impact Statement

The accumulation of senescent cells within the body has detrimental effects on tissue homeostasis. To study the role of senescent cells on tissue repair and regeneration, there is a need for effective means to induce premature cell senescence. Herein, we characterized the influence of common stressors to induce premature senescence in human mesenchymal stromal cells (MSCs). Irradiation of MSCs resulted in a phenotype most similar to quiescent, high-passage cells. These studies establish key biomarkers for evaluation when studying senescent cells *in vitro*.

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Introduction

Increased age is a comorbidity with musculoskeletal disease, marked by an increase in fracture incidence and rate of nonunion.[1–3] The average age of the U.S. population is steadily increasing, and 20% of the population is projected to be over age 65 by 2030.[4] Older populations suffer from lower bone mineral density, increased osteoporosis, decreased multipotent cell populations, lower expression of osteogenic genes, and corresponding impaired bone healing.[1,5] Failure of timely fracture healing can lead to fixation failure, resulting in morbid surgical repair. Revision surgeries commonly utilize autologous bone grafts from the iliac crest to improve healing potential, but this requires an additional procedure and is limited by tissue availability.[6]

Moreover, elderly patients may have reduced available bone volumes for such interventions, motivating the need for alternative strategies to treat these patients. Tissue-engineered approaches using autologous stem and progenitor cells are under widespread investigation, and mesenchymal stromal cells (MSCs) are a promising cell source for bone tissue engineering.[7,8] However, MSCs from older populations are less prevalent, less proliferative, and have reduced differentiation capacity.[9] To leverage the therapeutic potential of autologous cells, it is essential to develop effective methods that target and instruct MSCs for improved bone formation in the elderly.

Increased age is associated with an increase in cellular senescence, a quiescent state reached through proliferative exhaustion or by accumulation of cellular stress.[10,11] Senescent cells can be detrimental to tissue homeostasis due to the local inflammatory environment created by their secretome. Senescent cells are commonly identified by their broadened morphology, diminished proliferative capacity, upregulation of apoptotic inhibitors, telomere attrition, presentation of senescence-associated β-galactosidase, and production of a proinflammatory senescence-associated secretory phenotype (SASP).[12–14] However, the identification of senescent populations has been inconsistent, relying on a broad battery of assessments. The challenge of identifying senescent cells is further compounded by the variability of cells isolated from biological donors.

Replicative senescence is commonly employed to generate senescent populations *in vitro* but is time consuming and resource intensive. Stress-induced premature senescence, which relies upon DNA damage to induce the senescent phenotype, is an alternative approach to quickly generate a homogeneous senescent population for study. Common strategies to induce MSC senescence include acute treatment with hydrogen peroxide (H_2O_2),[15,16] chemotherapeutic treatments (i.e., etoposide),[17,18] or irradiation.[19,20] However, there is a need to comprehensively assess each method's efficiency to induce senescent markers and improve the consistency of future studies.

The importance of senescent cells has been established in a variety of disease states, including osteoarthritis,[21] atherosclerosis,[22] and insulin resistance in adipose tissue.[23] The selective clearance of senescent cells using senolytic agents has drawn increased study, including multiple clinical trials.[24–26] Senolytic agents reduce senescent populations through reduction of apoptotic antagonists.[24] Fisetin, a flavonoid with antioxidant properties, has demonstrated efficacy as a senolytic agent in multiple cell types *in vitro* as well as improved functional outcomes in an established senescent mouse model.[27,28] While the use of senolytics *in vivo* has resulted in improved bone health markers,[29] the application of senolytics on senescent populations of MSCs *in vitro* and the resultant senescent and osteogenic phenotype has not been studied.

We hypothesized that the treatment of senescent MSC-laden constructs with fisetin would induce apoptosis, resulting in improved osteogenesis in the remaining MSCs. Herein, we characterized the response of human MSCs to common stressors to induce premature senescence. Furthermore, we assessed the response of stress-induced senescent MSCs and bone marrow-derived cells (BMDCs) from an older donor to fisetin treatment in a clinically relevant hydrogel.

Materials and Methods

Induction of senescence

Human bone marrow-derived MSCs (RoosterBio, Frederick, MD) were cultured in alpha minimum essential media (α-MEM) supplemented with 10% fetal bovine serum (Biotechne, Minneapolis, MN) and 1% penicillin–streptomycin (P/S; Gemini Bio Products, West Sacramento, CA) in standard cell culture conditions. MSCs were passaged at 80% confluency and used at passage 4–5 for all conditions, including low passage (LP) controls. Replicative induced senescence was accomplished with MSCs at high passage (HP) used at passage 13–15. Twenty-four hours before stress induction, MSCs were serum starved with α-MEM supplemented with 1% P/S to synchronize cell cycles between treatment groups.

MSCs at 80% confluency on tissue culture plastic (TCP) were treated with 200 μM H_2O_2 (Thermo Fisher, Fair Lawn, NJ) or 20 μM etoposide (Cayman Chemicals, Ann Arbor, MI) in complete media for 2 and 24 h, respectively. Replication-induced senescent MSCs were generated by seeding MSCs at 5000 cells/cm^2 on TCP and passaged at 80% confluency. Cells were repeatedly cultured on TCP using the same cellular density until proliferation was halted. To irradiate, MSCs were suspended in complete culture media at 2×10^5 MSCs/cm^3 and exposed to 10 sievert (Sv) using a MultiRad225 X-ray irradiator (Precision X-Ray, North Branford, CT). Following exposure to each stressor, MSCs were seeded on TCP at 6×10^3 MSCs/cm^2 in complete media until collection.

Fabrication of Gelatin methylcryloyl hydrogels and entrapment of MSCs

Gelatin methylcryloyl (GelMA; 300 bloom, MilliporeSigma, St. Louis, MO) was dissolved at 10% (w/v) in complete media with 0.6% (w/v) 2-Hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiophenone (Irgacure 2959) with agitation at 80°C for 30 min. MSCs (2.4×10^6 cells/cm^3) were suspended in complete media and mixed with an equal volume of the GelMA to yield a 5% GelMA/0.3% Irgacure solution containing 1.2×10^6 cells/cm^3. The final solution (0.025 cm^3) was dispensed into 4-mm-diameter × 1-mm-high molds and exposed to 20 mW/cm^2 ultraviolet (UV) light for 5 min before culture in low-retention culture plates. Hydrogels were loaded with MSCs as described in Table 1.

Table 1. Gelatin Methacryloyl Hydrogel Content by Cell Population for Fisetin Treatment Study

Group (± fisetin)	*LP*	*Irradiated*	*HP*
LP MSC content (%)	100	0	0
Irradiated MSC content (%)	85	15	0
HP MSC content (%)	85	0	15

HP, high passage; LP, low passage; MSC, mesenchymal stromal cells.

Isolation end entrapment of human BMDCs in GelMA

Bone reamings from the distal femur of a 55-year-old female undergoing traumatic long bone fracture repair were collected with consent following an Institutional Review Board protocol approved by the University of California Davis Health. Tissue was collected in buffered solution with heparin, centrifuged at 300 *g* for 5 min, and the pellet was digested with 0.5% (w/v) collagenase II (Worthington Biochemicals, Lakewood, NJ) in complete media for 30 min under agitation at 37°C. The digested sample was cleared of erythrocytes using an ammonium chloride potassium buffer (Thermo Fisher) for 5 min, leaving only mononuclear bone marrow derived cells (BMDCs). BMDCs were washed with phosphate-buffered saline (PBS), and cell number was quantified using a Countess II cell counter (Thermo Fisher).

BMDCs at 2.5×10^6 cells/cm^3 were mixed with 5% GelMA and 0.3% Irgacure, deposited in 8-mm-diameter × 1-mm-high molds, and exposed to 20 mW/cm^2 UV light for 5 min before transfer to suspension culture well plates. This resulted in gels containing 1.75×10^5 BMDCs.

Fisetin treatment of hydrogels

Hydrogels containing MSCs or BMDCs were incubated in complete media for 24 h after gelation. Hydrogels were then treated with 15 μM of fisetin (Selleck Chemicals, Houston, TX) dissolved in dimethyl sulfoxide (DMSO) in complete media or complete media with an equal volume of DMSO. After 48 h, hydrogels were washed with PBS and refreshed with complete media supplemented with 0.01 μM dexamethasone, 50 μg/cm^3 *L*-ascorbic acid 2-phosphatase, and 10 mM sodium β-glycerophosphate (osteogenic media, all from Sigma), with media changes every 48 h. Samples were collected for analysis after 14 days.

Quantification of cell proliferation, metabolic activity, and caspase 3/7 activity

Samples were collected in passive lysis buffer (Promega, Madison, WI) and sonicated for 15 s for cellular dissociation. Total dsDNA content was determined by the Quant-iT DNA Assay Kit (Thermo Fisher) following the manufacturer's instructions. Relative metabolic activity was determined using an alamarBlue reagent (Thermo Fisher) diluted 1:10 in complete media, with fluorescence read as per the manufacturer's instructions. Metabolic values were normalized to total DNA content. Caspase 3/7 activity was quantified using the Caspase-Glo 3/7 assay (Promega) as per the manufacturer's instructions.

Quantitative polymerase chain reaction

RNA was isolated following cellular disassociation in TRIzol (Invitrogen, Carlsbad, CA) reagent as per the manufacturer's instructions. Hydrogels required sonication for 15 s in TRIzol to fully dissolve. Four hundred nanograms of RNA was reverse transcribed to complimentary DNA using the QuantiTect Reverse Transcription Kit (Qiagen, Germantown, MD) and normalized to a final concentration of 10 ng/μL.

Quantitative polymerase chain reaction was performed using Taq PCR Master Mix (Qiagen) in a QuantStudio 5 real-time PCR system (Thermo Fisher). Human specific primers for *GAPDH* (Hs02786624_g1), *CDNK1A* (Hs00355782_m1), and *CDNK2A* (Rn01470656_m1) were purchased from Thermo Fisher. Results were normalized to the endogenous control, *GAPDH*, to yield a ΔCt value for each time point and further normalized with the LP *GAPDH* ΔCt to determine the ΔΔCt.

Characterization of MSC senescence

MSC senescence was characterized using several commonly reported techniques as described below.

Senescence-associated cell staining

Senescence-associated β-galactosidase staining was performed using the Senescence β-Galactosidase Staining Kit (Cell Signaling Technology, Danvers, MA). Briefly, MSCs were washed with PBS and fixed in 10% buffered formalin at room temperature for 15 min. Cells were then treated with an X-gal staining solution at pH = 6 at 37°C overnight. MSCs were then counterstained with Hematoxylin and washed extensively before imaging (Nikon Eclipse, TE2000U). Positive cells were counted manually from three separate regions of each sample.

MSC morphology was quantified using β-galactosidase and Hematoxylin stained on images measuring 2592 × 1944 pixels using ImageJ (National Institutes of Health). Total cell area was calculated using the Huang thresholding function, omitting particles less than 100 μm^2 and normalized to cell number for each field of view. Image analysis was accomplished in triplicate using three random fields of view per sample.

γ-H2AX staining was performed using the γ-H2AX Staining Kit (Abcam, Cambridge, MA) following the manufacturer's instructions. Before the final wash, cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) to identify the cell nucleus. Positive-stained cells were identified manually by colocalization of γ-H2AX in DAPI-stained nuclei and presented as percentage of total cells present.

Quantification of the SASP

Cells in monolayer culture were refreshed with complete media 24 h before secretome collection, while hydrogels were refreshed with osteogenic media 24 h before secretome collection. Media were collected and frozen at −20°C until analysis. Secretion of IFN-γ, IL-6, TNFα, and IL-1β was quantified using the Proinflammatory 1 Kit (Mesoscale Diagnostics, Rockville, MD). Samples were diluted 1:1 in a diluent provided by the manufacturer and analyzed as per the manufacturer's instructions.

Osteogenic characterization

Intracellular alkaline phosphatase (ALP) activity was measured by incubating lysed cellular supernatant with 50 mM *p*-nitrophenyl-phosphate (pNPP).[30] Gels were dissolved in 1 M HCl for 72 h at 60°C with agitation and total calcium was measured using the Stanbio Calcium Liquid Reagent for Diagnostic Kit (Thermo Fisher).[31]

Statistical analysis

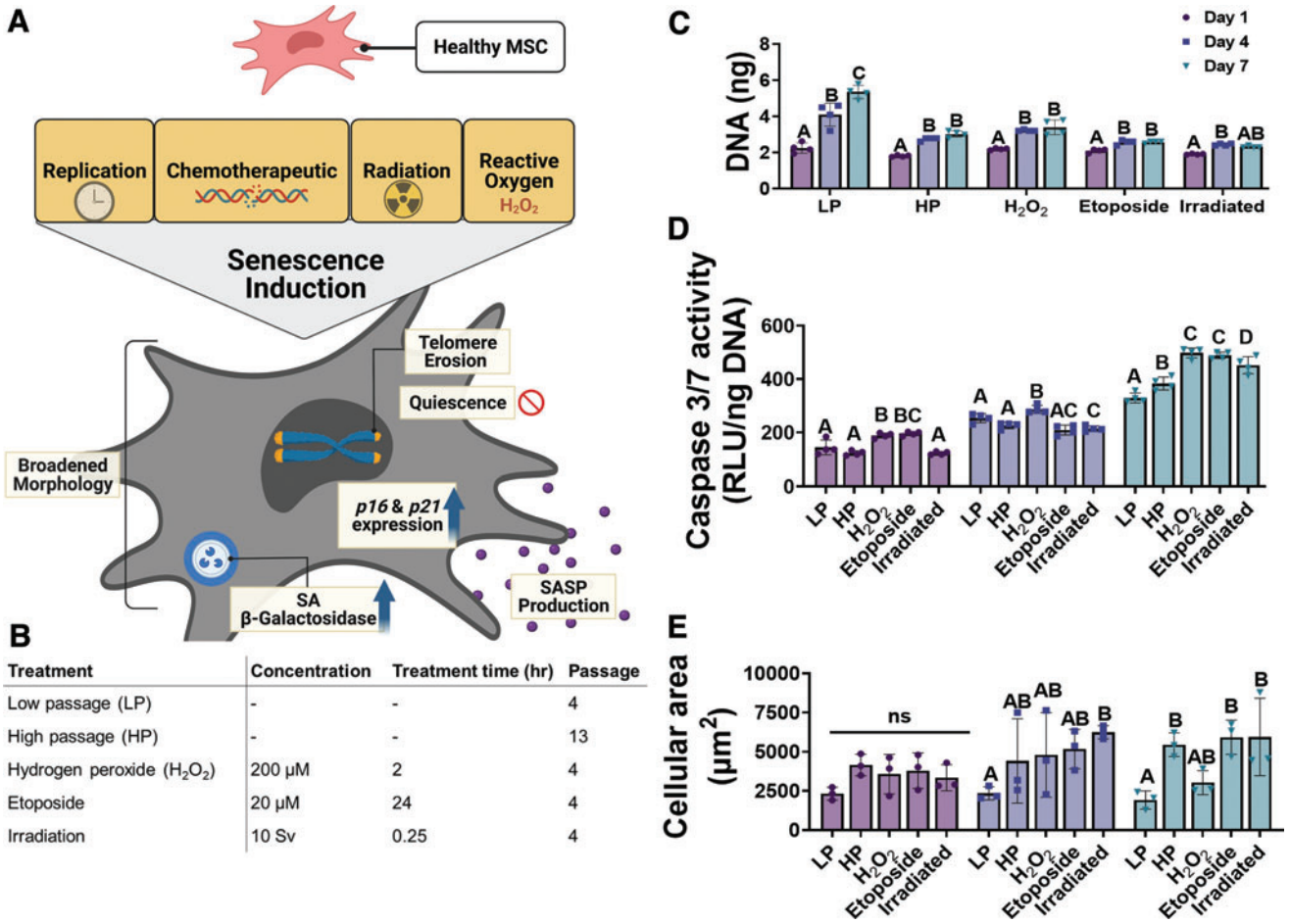
Data are presented as mean ± standard deviation. Statistical analysis was performed using Prism 9.3.1 (GraphPad, San Diego, CA) software utilizing one-way analysis of variance (ANOVA) with *post hoc* Tukey's test, two-way ANOVA with *post hoc* Šidaks multiple comparison test, or an unpaired Student's *t*-test depending on the number of groups tested. Letter designation denoting significance between groups was used to facilitate clarity. Groups with different letters indicate significance ($p < 0.05$) while groups with similar letters are not statistically significant.

Experiment

MSC quiescence is dependent on the type and magnitude of stress induction

Reactive oxygen species, chemotherapeutics, and irradiative stress are common stressors to induce premature senescence, yet there is no consensus on necessary dosage, application time, or senescent marker outputs (Fig. 1A). This represents a significant hurdle for studies in the field and to effectively compare results among groups. Replicative exhaustion is generally accepted as a mechanism to induce senescence, and stress-induced methods should aim to yield a similar phenotype. After an extensive literature review, we identified several common induction stimuli to prematurely induce senescence in MSCs as follows: acute exposure to H_2O_2 (200 µM for 2 h), etoposide (20 µM for 24 h), or irradiation (10 Sv; Fig. 1B). After acute exposure to these stimuli, we measured the response of MSCs over 7 days *via* a battery of common assays to discern the occurrence of senescence.

DNA content is an indicator of MSC proliferation, and we observed increases in DNA content for MSCs in the LP



B

Treatment	Concentration	Treatment time (hr)	Passage
Low passage (LP)	-	-	4
High passage (HP)	-	-	13
Hydrogen peroxide (H_2O_2)	200 µM	2	4
Etoposide	20 µM	24	4
Irradiation	10 Sv	0.25	4

FIG. 1. Stress-induced premature senescence methods induce similar outcomes on proliferation and apoptosis. **(A)** Schematic illustrating different methods to generate stress-induced premature senescence and typical markers to identify the senescent population. **(B)** Table describing the different treatment groups assessed for senescent markers in this study. **(C)** Quantification of DNA content; **(D)** caspase 3/7 activity; and **(E)** MSC spreading as determined by ImageJ analysis of cellular area in monolayer culture at day 1, 4, and 7. Data are mean ± standard deviation ($n = 3$ or 4). MSC, mesenchymal stromal cell. Color images are available online.

group over 7 days as expected (Fig. 1C). However, we detected arrest of cell proliferation by all stress-induced methods after day 4, as occurs during senescence. Furthermore, the lack of MSC proliferation was not due to contact inhibition as confirmed by bright field microscopy. We detected increases in caspase 3/7 activity, an indicator of apoptosis, across each stress-induced mechanism but in a temporal manner. The greatest caspase 3/7 activity was observed at day 7 in H_2O_2- and etoposide-treated cells (Fig. 1D). Indeed, increases in caspase activity were greater than that observed in HP MSCs. These data suggest that stress-based approaches to induce premature senescence can be overly cytotoxic, leading to cell death, and not necessarily inducing cellular quiescence.

Senescent MSCs are frequently identified by gross differences in morphology while in culture, commonly described as exhibiting increased spreading reminiscent of a fried egg shape. When evaluating MSCs on TCP, we quantified cells with larger area (Fig. 1E) and observed increasingly spread and broadened morphology in stress-induced groups when assessing morphology in β-galactosidase-stained MSCs (Fig. 2A, ii-v) compared with LP MSCs (Fig. 2A, i). Collectively, these data demonstrate that the selected methods to induce stress-induced senescence effectively halt MSC proliferation and induce increases in apoptosis similar to replicative induced cell senescence.

β-galactosidase and DNA damage markers are dependent upon induction method

Senescence-associated β-galactosidase was first identified as a marker for senescent diploid fibroblast populations by Hayflick and Moorehead nearly 60 years ago[10] and remains a common metric to identify senescent cell populations in culture. We did not observe differences in β-galactosidase staining at early time points (Fig. 2A). However, there was a distinct increase in the frequency of β-galactosidase-positive cells by day 7 in HP, etoposide, and irradiation-treated groups (Fig. 2C). This finding suggests that β-galactosidase

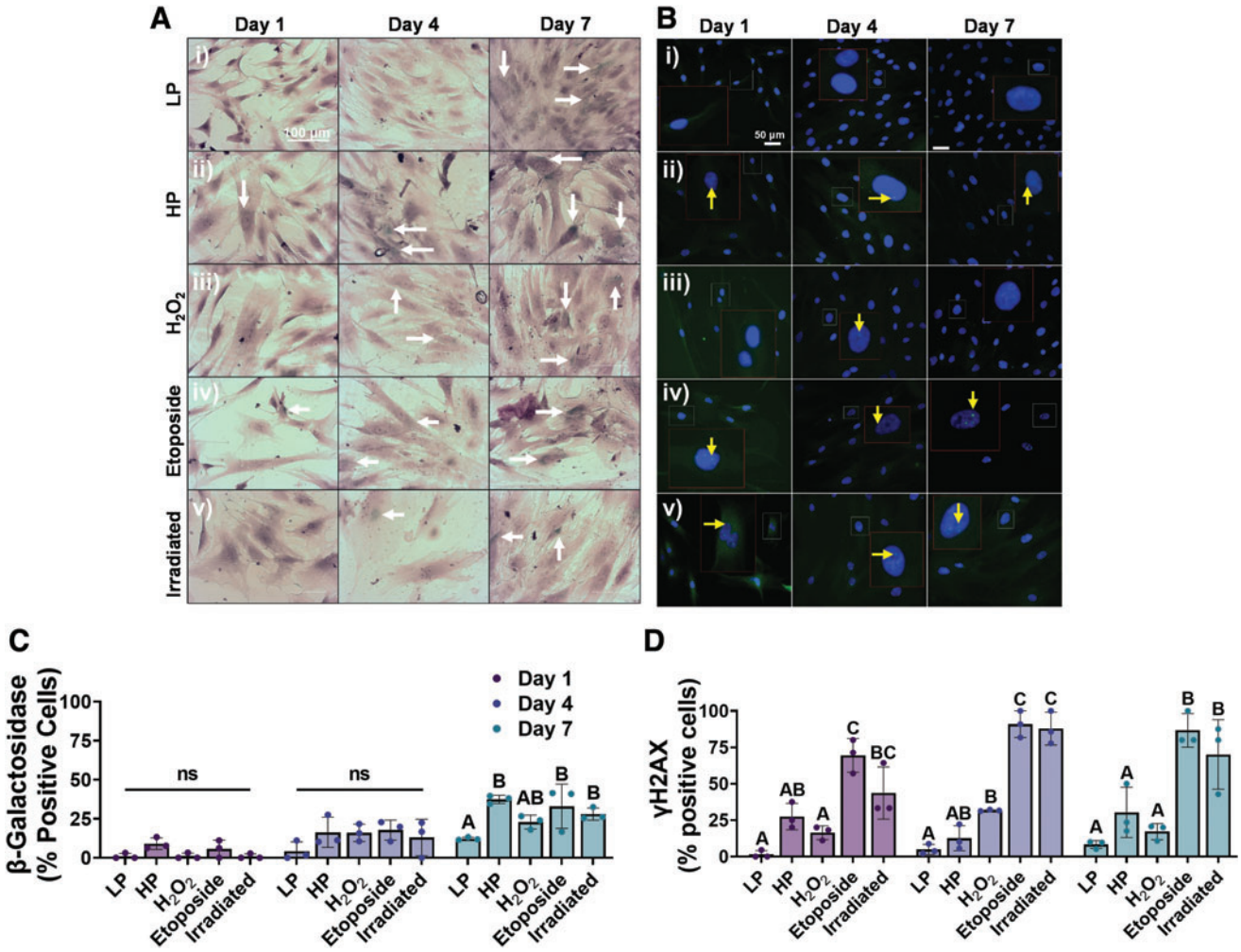


FIG. 2. β-galactosidase and γ-H2AX expression is increased in stress-induced premature senescent MSCs. **(A)** Stress-induced methods result in increases in β-galactosidase staining over time (positive *blue* staining is identified by *white arrows*, scale bar = 100 µm, 20× magnification). MSCs are counterstained with Hematoxylin (*purple*). **(B)** Stress-induced methods increase the frequency of γ-H2AX (positive *green* staining is identified by *yellow arrows*, scale bar = 50 µm, 40× magnification) colocalized in the cell nucleus (*blue*) of MSCs over time quantification of the frequency of MSCs presenting **(C)** β-galactosidase or **(D)** γ-H2AX in the nucleus. Data are mean ± standard deviation ($n = 3$). Letter designation indicates statistical differences between groups. Color images are available online.

expression is time dependent for various induction methods. Additionally, the frequency of β-galactosidase staining was increased in the LP group, indicating some dependence on either culture time or contact that may be a confounding factor when utilizing this method to identify senescent cells in culture.

γ-H2AX is a fluorophore that labels epitopes of nucleotides after double-strand breaks and is used to identify senescent cells.[11] The frequency of γ-H2AX-positive nuclei was immediately higher in the etoposide and irradiative induced groups and remained higher than other groups across all collection points (Fig. 2B). This result was expected, as the mechanism of senescent induction for both treatments is driven by DNA double-strand breaks, while the mechanism for replicative induced senescence is purported to be due to telomere attrition.[32] Thus, cytochemical staining for β-galactosidase and γ-H2AX are effective markers for detecting senescent cells, but the intensity and frequency of staining is dependent upon the method of induction.

Transcriptomic and secretion profiles of stress-induced mechanisms are temporally expressed

The expression of genes, such as *p16* (*CDKN2A*) and *p21* (*CDKN1A*), are upregulated in response to genetic stressors that damage DNA, resulting in cessation of proliferation.[13] We observed increased expression of *p16* in replicative induced senescent groups at the earliest time point compared with all other groups (Fig. 3A). However, this difference was lost at later time points. All stress treatments induced increases in *p21* expression at day 1 (Fig. 3B), and etoposide treatment induced significantly greater *p21* expression than that observed in the replicative induced group. These differences were not retained at later time points but a trend of increased expression over the LP control was observed through day 4. These data confirm that *p16* and *p21* expression, common markers of cell senescence, are not linear and are temporally dependent in MSCs.

Beyond quiescence, senescent cells can be detrimental to local tissues. Senescent cells secrete multiple proinflammatory cytokines, and this SASP is another method to identify senescent populations.[12,33] Senescent cells disrupt tissue homeostasis through the autocrine and paracrine effects of inflammatory factors such as IFN-γ, IL-6, and IL-1β. In these studies, production of IFN-γ was not significant until day 7, with irradiated MSCs secreting more than all other groups. IL-6 in the conditioned media of MSCs undergoing stress-induced methods was significantly increased compared with the LP group at day 7 (Fig. 3D). Interestingly,

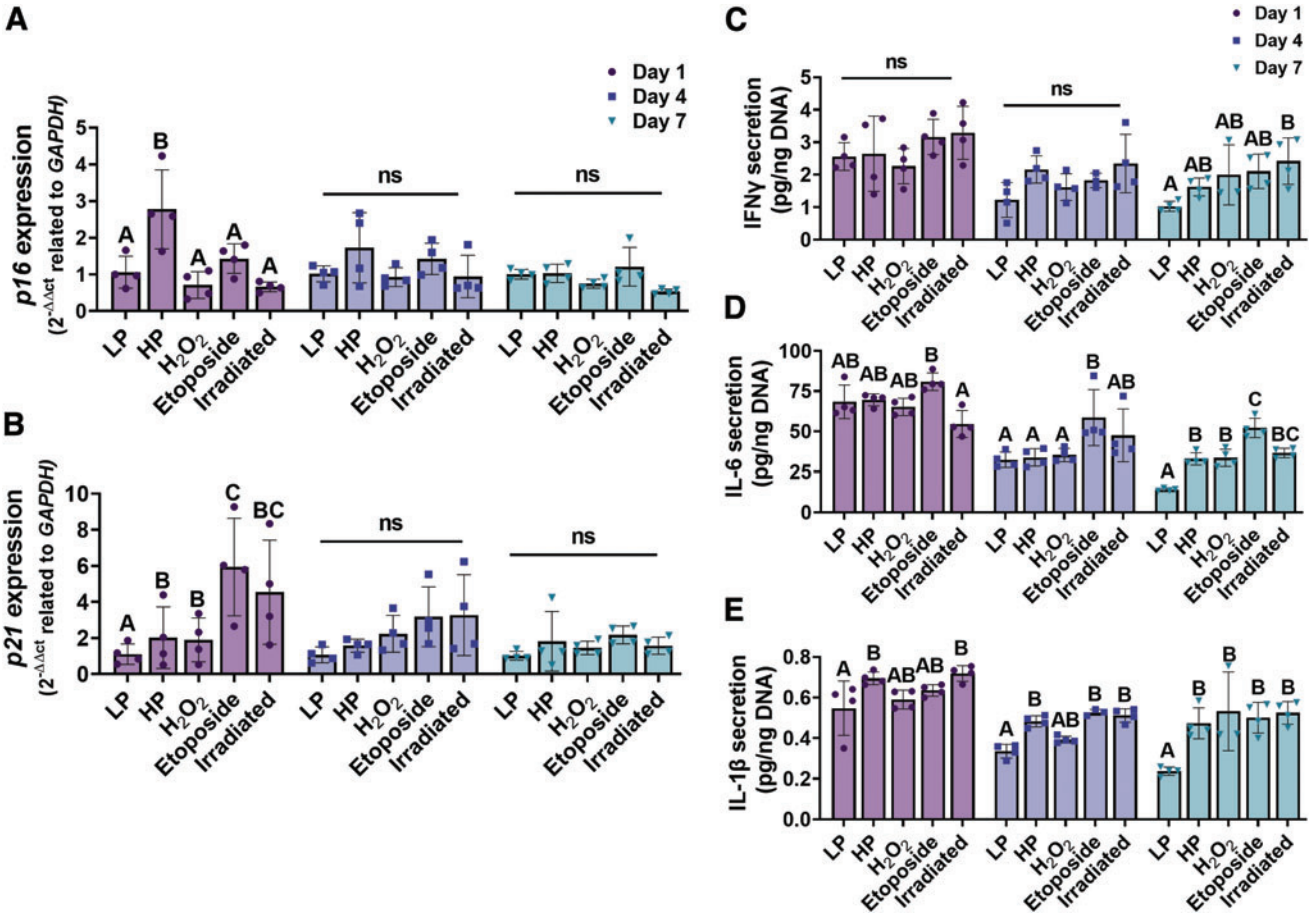


FIG. 3. Senescence-related genes are expressed early and then dampened while inflammatory factors are consistently elevated compared with low passage controls. **(A)** *p16* and **(B)** *p21* expression revealed differences among treatment groups only at day 1. Secretion of **(C)** IFN-γ; **(D)** IL-6; and **(E)** IL-1β is increased in stress-induced senescent populations compared with LP MSCs, with differences more evident at later time points. Data are mean ± standard deviation ($n = 4$). Letter designation indicates statistical differences between groups. LP, low passage. Color images are available online.

Table 2. Outcome of Stress-Induced Senescent Methods on Mesenchymal Stromal Cells Compared with Replicative Induced Senescence

Assessment	*H_2O_2*	*Etoposide*	*Irradiation*
Caspase 3/7 activity	+	+	+
Cellular spreading	=	=	=
β-galactosidase expression	−	=	=
γ-H2AX expression	=	+	+
p16 expression	−	−	−
p21 expression	=	+	=
IFN-γ secretion	=	=	=
IL-6 secretion	=	+	=
IL-1β secretion	=	=	=

+ indicates increased presentation, = indicates comparable presentation, and − indicates decreased presentation compared with replicative induced senescence.

MSCs treated with etoposide secreted the most IL-6 at all collection points. IL-1β production increased with the HP and irradiative treatments at day 1 collection (Fig. 3E), with all stress induction methods generating more IL-1β than the LP group by day 7.

These data demonstrate that irradiative and etoposide treatment generate a phenotype that is most similar to replicative induced senescence marked by proliferation arrest, spread morphology, senescence-associated β-galactosidase, and SASP profile (Table 2). However, etoposide treatment was more toxic to cell populations as measured by apoptosis and increased *p21* expression when compared with the replicative induced group. Based on these findings, we selected irradiation to prematurely induce MSC senescence in our remaining studies. However, due to the availability of gamma irradiation equipment, etoposide could be a useful alternative for inducing senescence in MSCs.

Fisetin treatment of MSCs in GelMA does not affect cell phenotype

Having established irradiation as a consistent and effective means to induce senescence in MSCs, we interrogated the potential of fisetin as a senolytic agent to clear senescent cells from a three-dimensional (3D) culture. We entrapped a mixture of prematurely induced senescent MSCs with LP MSCs in GelMA (Table 1) and quantified the senescent and osteogenic response to an acute treatment of fisetin. We selected an 85:15 ratio of healthy-to-senescent cells based on the reported occurrence of senescent cell populations in various tissue compartments *in vivo*.[34,35] After 14 days in culture following acute treatment with fisetin, we did not detect any differences in DNA content in any group (Fig. 4A). Similarly, we did not observe differences in metabolic activity (Fig. 4D).

The expression of apoptotic antagonists *p16* and *p21* was also unaffected by the application of fisetin at 14 days (Fig. 4B, C), contrary to what has been reported in tissues from animals treated with fisetin.[28] Intracellular ALP activity, measured using the pNPP assay, (Fig. 4E) and calcium deposition (Fig. 4F) were similarly unaffected by fisetin treatment. These data suggest that the chosen fisetin dosing

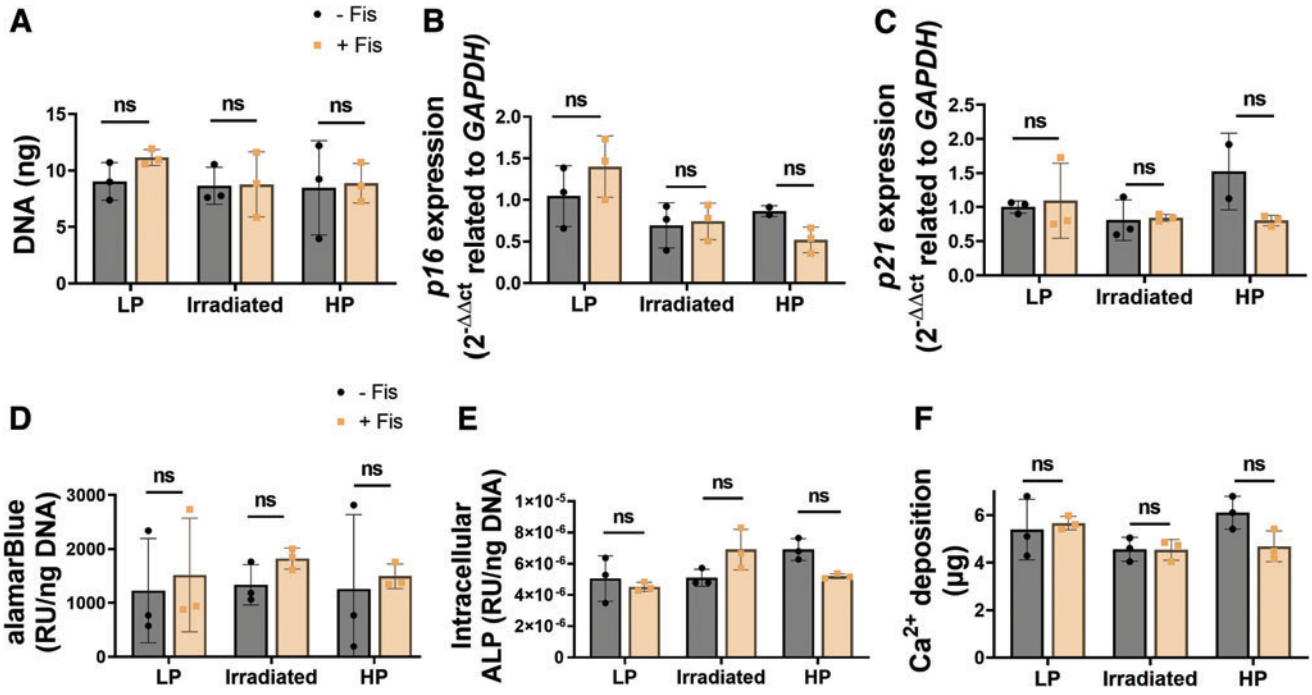


FIG. 4. Senescent and non-senescent MSCs encapsulated in GelMA hydrogels are not responsive to treatment with fisetin 14 days post-treatment. **(A)** DNA content was not altered by fisetin treatment. Expression of **(B)** *p16* and **(C)** *p21* were quantified by qPCR. **(D)** Metabolic activity was not altered by fisetin treatment. **(E)** Intracellular ALP activity and **(F)** calcium deposition were not affected by fisetin treatment. Data are mean ± standard deviation ($n = 3$). ALP, alkaline phosphatase; GelMA, gelatin methacryloyl; qPCR, quantitative polymerase chain reaction. Color images are available online.

regimen was insufficient to alter MSC senescent and osteogenic phenotype or perhaps earlier time points are necessary to observe significant differences among treatment groups.

Acute fisetin treatment reduces senescent markers and increases osteogenesis in BMDCs

BMDCs derived from cortical bone tissue reamings are under investigation as an alternative cell source for bone tissue engineering and contain a heterogeneous cell population of MSCs, hematopoietic progenitor cells, adipocytes, osteocytes, and endothelial cells.[36,37] To translate the use of fisetin to the more relevant clinical condition of heterogeneous cell samples, we collected and entrapped BMDCs in GelMA hydrogels, which ensures retention of the entire population for study without loss of mononuclear cells that do not easily adhere to TCP. Fisetin treatment reduced BMDC cellularity 14 days after treatment (Fig. 5A). Upon analysis, BMDCs had a lower expression of *p16* and *p21* 14 days post-treatment with fisetin (Fig. 5B, C). Furthermore, constructs exposed to fisetin exhibited reduced secretion of IL-6 (Fig. 5D) and increased metabolic activity (Fig. 5E). Compared with untreated gels, we detected increased expression of ALP activity (Fig. 5F) and calcium deposition (Fig. 5G) in constructs treated with fisetin. These findings indicate that the heterogeneous population of BMDCs was responsive to fisetin treatment as evidenced by reduction in senescent markers and increases in osteogenic markers 14 days after treatment.

Discussion

Senescent cells disrupt tissue homeostasis due to the inflammatory contents of the senescence-associated secretory phenotype and have local and systemic effects. Additionally, the differentiation of senescent progenitor cells is altered, with senescent MSCs more prone to adipogenesis than osteogenesis when compared with nonsenescent populations. However, isolation of senescent cells from human donors is fraught with significant donor variability, making the study of senescence challenging.

Replicative senescence has been the gold standard in generating senescent populations, but is time consuming and resource intensive, requiring repeated passages over a long and unpredictable duration. Stress-induced methods to prematurely induce senescence in MSCs *in vitro* have been investigated to overcome the burden of both biological variability and replicative senescence induction. However, there is no direct comparison between induction methods using the same battery of senescence-associated markers, making comparison of methods difficult. Therefore, we quantified and compared the senescent characteristics of MSCs upon replicative, H_2O_2, etoposide, and irradiative induction methods.

We observed growth arrest, spread morphology, and an increase in senescence-associated β-galactosidase across all induction groups. Treatment with etoposide and irradiation produced a similar secretion and gene expression profile as replicative induced senescence, but etoposide treatment resulted in a higher initial rate of apoptosis. These findings

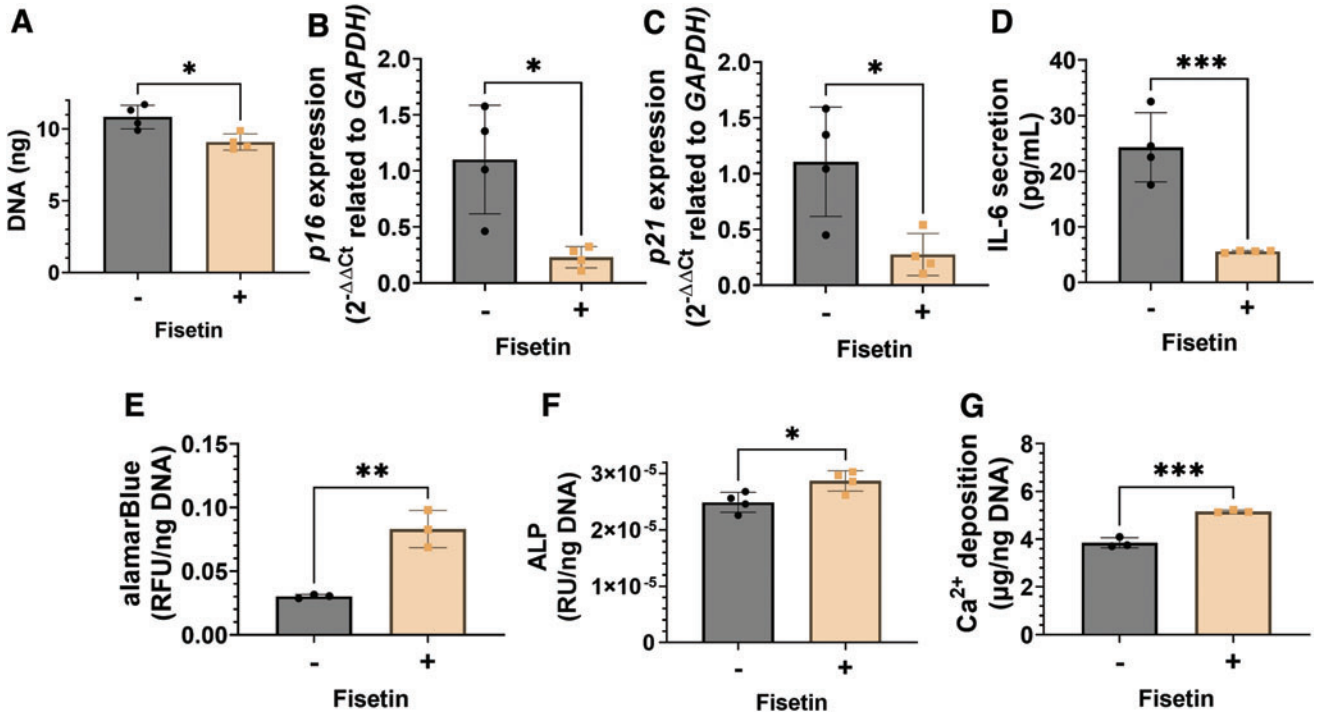


FIG. 5. Human bone marrow-derived cells entrapped in GelMA exhibit reduction in senescent markers and increased osteogenic differentiation upon fisetin treatment. **(A)** Cellular content was decreased with fisetin treatment. Expression of **(B)** *p16* and **(C)** *p21* were reduced when exposed to fisetin. **(D)** Secretion of IL-6 was decreased after application of fisetin treatment. **(E)** Fisetin treatment increased cell metabolic activity when normalized to DNA content. **(F)** Intracellular ALP activity and **(G)** calcium deposition increased with treatment of fisetin. Data are mean ± standard deviation, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. One donor, $n = 3$–4 replicates. Color images are available online.

demonstrate that irradiation of MSCs generates a senescent phenotype that is useful for continued study when compared with the other commonly used induction methods evaluated.

A more expansive study investigating the dose and treatment time may prove to also be effective at generating a senescent phenotype similar to replicative induced senescence. Additionally, MSCs are notoriously donor dependent, and these data are exclusive to bone marrow MSCs derived from one human donor. Further studies would benefit from including additional donors and expanding the study to MSCs sourced from other tissue compartments.

The acute exposure of cells to reactive oxygen species, chemotherapeutics, small molecule treatment, UV exposure, and gamma irradiation have all been investigated to prematurely induce senescence.[15,17,19] These methods primarily rely upon DNA damage, producing errors in initiation of apoptosis. There is no single method to identify senescent cell populations, thereby requiring a battery of tests to determine senescent phenotype. Cells are commonly stained to detect the production of senescence-associated β-galactosidase as a marker of senescence, yet the role of the culture microenvironment is often overlooked.

Herein, we observed that replicative, H_2O_2, etoposide, and irradiative stress halt the proliferation of MSCs in culture and is a necessary metric when identifying senescent populations. All but the H_2O_2 induction method yielded a spread cell morphology by day 7, limiting the use of morphology as an indicator for senescence.

Additionally, senescence-associated β-galactosidase production is temporally and cell density dependent, with differences seen only 1 week postinduction and increased presentation in the LP groups as cell density increased. Increases in γ-H2AX were immediately apparent across stress induction methods but were not identified in replicative induced groups. This indicates that γ-H2AX prevalence is a good indicator for stress-induced premature senescence but may be limited for replicative induced senescence.

Beyond changes in cytochemical staining or cell morphology, senescent cells are frequently identified by the expression of senescence-associated genes such as *p16* and *p21*. *p16* is accumulated with repeated replication in culture and has been used as a hallmark for replicative senescence. Furthermore, *p16* expression is increased in aged mice and human tissue as well as progeria populations.[38] *p21* is a downstream target of *p53* activation, halting the cell cycle in the G0 phase. Expression of *p21* is transient with an early increased expression followed by a dramatic decrease in expression.[13] We found that expression of *p16* is increased only in replicative senescent MSCs at early time points, and *p21* expression is likewise temporally limited. While trends persist, the extent of *p16* and *p21* are limited as indicators of senescence of MSCs in culture and are likely only useful at early time points.

The SASP affects tissue homeostasis and is characterized by increased secretion of proinflammatory cytokines, such as IL-6, IL-8, and IL-1β, *in vitro* and in biological tissues.[12,34] In these studies, stress-induced methods increased the production of cytokines in the SASP similar to that of replicative induced senescence in monolayer culture. However, the secretion profile of these factors is temporal, with differences noted after extensive culture periods. The high concentration of proinflammatory cytokines secreted in the SASP alters nonsenescent cell phenotype and reinforces senescence.[39] Due to the influence of this secretome, it is important to evaluate the SASP when assessing senescent load.

Senolytic agents are a class of small molecules that target senescent cells by inducing apoptosis. Fisetin, a flavonoid with antioxidative properties, has been used as a senolytic agent both *in vitro* and upon systemic administration *in vivo*.[27,28] However, the systemic clearance of senescent cells may be detrimental given the utility of senescence in distinct circumstances, such as arrest of tumorigenesis or liver fibrosis.[40]

The extraction and expansion of autologous cells for cell-based therapy or tissue engineering is one approach to address tissue damage in elderly patients, but the vitality of cells from the elderly represents a significant challenge, perhaps due to the increased frequency of senescent cells. Having established irradiation as an effective means to induce senescence in MSCs, we tested the potential of fisetin to clear senescent MSCs and promote osteogenic differentiation *in vitro*. When entrapped in GelMA, we did not observe differences in senescent burden or osteogenesis for mixtures of senescent and healthy MSCs over 2 weeks.

Interestingly, we failed to detect a significant decrease in osteogenic behavior of constructs titrated with senescent MSCs, suggesting that the ratio of senescent MSCs used was insufficient to impair osteogenesis. Alternatively, the healthy MSCs may have outgrown the senescent cells, thereby minimizing their influence at later time points. Future studies would benefit from a titration study of senescent MSCs to identify the minimum number of cells that decreases osteogenesis, along with experimental groups designed to confirm the effect of fisetin treatment on senescent MSCs. Furthermore, the dosing regimen of fisetin may be insufficient to reduce senescent load in these conditions, as repeated doses of fisetin *in vivo* reduced senescent load across a variety of tissues and improved life span.[28]

BMDCs are a promising heterogeneous population for musculoskeletal tissue regeneration due to their relative ease of isolation and generation of robust, vascularized tissue.[41] The response to senolytic treatment is cell type dependent,[26,27] and this variability of response is important to consider when applying senolytic treatments in complex tissues or systemically.

Herein, fisetin treatment did not result in a reduction of senescent characteristics or osteogenic markers of MSCs alone after 14 days of culture. However, when applying fisetin to more clinically relevant BMDCs in GelMA, we observed a reduction in the prevalence of senescent markers, improved BMDC metabolic activity, and increased osteogenic differentiation. These results indicate that other cell populations within the bone marrow space may suffer from increased senescent load, thereby affecting the osteogenic potential of the population.

In agreement with prior work,[41,42] these data emphasize the importance of the diverse heterogeneous cell population within bone for cell-based approaches in bone tissue engineering. However, this work would benefit from additional donors spanning different ages as well as techniques to identify baseline senescent cell phenotype without relying upon conventional cell culture methods. To better understand the impacts of the heterogeneous population, a thorough investigation of the senescent load of each cell type and their response to fisetin treatment is merited.

In conclusion, we assessed the efficacy of multiple common methods to prematurely induce senescence in human MSCs. We selected irradiation-induced senescence as our preferred model due to the similarity to replicative induced senescence and lack of relative cytotoxicity. We then interrogated the potential of the senolytic agent, fisetin, to target senescent cells in 3D GelMA hydrogels. Fisetin treatment did not affect the senescent or osteogenic phenotype of MSCs alone, but we observed significant reductions in senescent markers and corresponding increases in osteogenic differentiation when examining a more complex, heterogeneous population of human BMDCs.

The study of the influence of senescent cells on musculoskeletal health will be improved by establishing an efficient method of senescence induction in controlled environments. Furthermore, these approaches will aid in the identification and testing of senolytic agents to effectively target and clear senescent cells *in vivo* or for the development of improved tissue-engineered bone grafts for subsequent implantation.

Disclaimer

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. The funders had no role in the decision to publish or preparation of the article. All schematics in this work were created using BioRender.

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No competing financial interests exist.

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