

Organ-Specific Stem-Cell Conditioned Media Attenuate Oxidative Stress-Induced Senescence and DNA Damage in HT22 Hippocampal Neuronal Cells: A Combined SA- β -Galactosidase and γ H2A.X Immunofluorescence Study

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Citation: Skutella T, Chan MKS, Klokol D. Organ-Specific Stem-Cell Conditioned Media Attenuate Oxidative Stress-Induced Senescence and DNA Damage in HT22 Hippocampal Neuronal Cells: A Combined SA- β -Galactosidase and γ H2A.X Immunofluorescence Study. J Stem Cell Res. 8(1):1-22.

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Received: September 28, 2026 | **Published:** January 18, 2027

Abstract

Background: Cellular senescence is a hallmark of aging and age-related disease, characterized by irreversible cell-cycle arrest, accumulation of senescent cells, and secretion of pro-inflammatory factors collectively termed the senescence-associated secretory phenotype (SASP). Oxidative stress is a major driver of premature senescence and contributes to neuronal dysfunction and neurodegenerative disorders. Accumulating evidence indicates that many of the regenerative effects of stem cells are mediated through their secretome, raising interest in stem-cell-derived conditioned media as a cell-free therapeutic strategy for reducing cellular senescence and oxidative DNA damage.

Objective: This study evaluated the ability of tissue-specific stem-cell conditioned media to attenuate oxidative stress-induced senescence and DNA double-strand-break (DSB) formation in HT22 mouse hippocampal neuronal cells, and compared the relative efficacy of secretomes derived from six different tissue sources.

Methods: Cellular senescence was induced in HT22 cells by exposure to 800 μM hydrogen peroxide (H_2O_2) for 1 h at 37 °C. Conditioned media were collected from primary stem-cell cultures derived from retina, kidney, brain, testes, thymus, and heart tissues and applied to the cells for four days. Senescence was quantified by the senescence-associated β -galactosidase (SA- β -Gal) assay. Oxidative DNA damage was assessed in parallel by $\gamma\text{H2A.X}$ immunofluorescence using a rabbit polyclonal anti-phospho-histone H2A.X antibody (Cell Signaling Technology), with detection by a donkey anti-rabbit Alexa Fluor 546 secondary antibody and epifluorescence microscopy on a Zeiss Axiovert 200 ApoTome. Positive and negative controls were included with and without MEM- α carrier medium. Data were analyzed by one-way ANOVA with Tukey HSD post hoc testing and Welch's two-sample t-test for medium-control comparisons.

Results: H_2O_2 exposure induced robust senescence in HT22 cells (mean 535.7 SA- β -Gal⁺ cells vs. 2.3 in untreated controls) and produced abundant, discrete nuclear $\gamma\text{H2A.X}$ foci consistent with widespread DSB formation. All six tissue-specific conditioned media significantly reduced SA- β -Gal positivity relative to the positive control (one-way ANOVA $F = 20.1$, $p < 0.0001$; all treatment groups $p < 0.001$ by Tukey HSD). The largest reductions were observed for testes-derived (–52.5%) and kidney-derived (–50.4%) conditioned media, followed by brain-derived (–43.7%) and thymus-derived (–43.4%) preparations; retina-derived (–36.3%) and heart-derived (–32.4%) conditioned media produced smaller but still highly significant reductions. In parallel, $\gamma\text{H2A.X}$ immunofluorescence revealed a clear qualitative decrease in the number and intensity of punctate nuclear $\gamma\text{H2A.X}$ foci in all conditioned-media groups compared with the H_2O_2 positive control, with heart-, thymus-, testes-, retina-, kidney-, and brain-derived secretomes all showing markedly fewer $\gamma\text{H2A.X}^+$ foci than untreated stressed cells. MEM- α carrier medium alone had no significant effect on either control (positive control $p = 0.58$; negative control $p = 1.00$).

Conclusions: Tissue-specific stem-cell conditioned media significantly attenuated both oxidative stress-induced senescence and DNA double-strand-break formation in HT22 hippocampal neuronal cells. The convergence of reduced SA- β -Gal activity and reduced $\gamma\text{H2A.X}$ foci across all tested secretomes supports a coordinated cytoprotective effect of stem-cell paracrine factors on the oxidative-damage-to-senescence axis. Testes- and kidney-derived secretomes showed the highest activity and warrant priority mechanistic characterization. These findings support the therapeutic potential of cell-free stem-cell secretomes as modulators of neuronal senescence and oxidative DNA damage in aging-related neurological disorders.

Keywords

Cellular senescence; SA- β -galactosidase; $\gamma\text{H2A.X}$; DNA damage response; HT22 cells; Oxidative stress; Hydrogen peroxide; Stem-cell secretome; Conditioned media; Regenerative medicine; Neuroprotection; Healthy aging; Precursor stem cells.

Introduction

Cellular senescence

Cellular senescence is a state of stable, essentially irreversible cell-cycle arrest in which cells remain metabolically active but lose their capacity to proliferate [1]. Senescence can arise through replicative exhaustion due to telomere shortening or be induced prematurely by a variety of stressors, including oxidative stress, DNA damage, mitochondrial dysfunction, and oncogene activation [1]. Accumulation of senescent cells is a hallmark of aging and contributes to age-related tissue dysfunction. Senescent cells secrete a complex mixture of pro-inflammatory cytokines, chemokines, growth factors, and proteases collectively known as the senescence-associated secretory phenotype (SASP), which promotes chronic inflammation and contributes to age-related and neurodegenerative diseases [2]. Reactive oxygen species (ROS), particularly hydrogen peroxide (H_2O_2), are widely recognized as potent inducers of stress-induced premature senescence and are frequently used in experimental models to mimic oxidative damage associated with aging [1].

The senescence-associated β -galactosidase (SA- β -Gal) assay

Senescence-associated β -galactosidase (SA- β -Gal) is the most widely accepted and extensively used biomarker for the identification of senescent cells [3]. The assay is based on the observation that

senescent cells exhibit elevated lysosomal β -galactosidase activity that can be detected histochemically at pH 6.0, a condition under which non-senescent cells display minimal activity [3]. In the standard cytochemical staining procedure, the substrate 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside (X-Gal) is hydrolyzed by β -galactosidase, resulting in the formation of an insoluble blue-green precipitate within the cytoplasm of senescent cells [4]. This characteristic staining pattern allows senescent cells to be readily visualized and quantified by light microscopy. Due to its simplicity, low cost, and applicability to cultured cells and tissues, the SA- β -Gal assay remains the gold-standard first-line method for evaluating cellular senescence and serves as the primary quantitative endpoint of the present study [3,4].

γ H2A.X as a marker of oxidative DNA damage and senescence

DNA damage, and in particular DNA double-strand breaks (DSBs), is a central upstream trigger of cellular senescence. Upon DSB formation, the histone variant H2A.X is rapidly phosphorylated at serine 139 by the PI3K-like kinases ATM, ATR, and DNA-PK, generating the phosphorylated form γ H2A.X, which accumulates at DSB sites and is readily visualized as discrete punctate nuclear foci by immunofluorescence microscopy [5,6]. γ H2A.X foci are broadly used as a sensitive and quantitative marker of DSBs, oxidative genotoxic stress, and the persistent DNA damage response that accompanies stress-induced premature senescence [5,6]. In aged and senescent cells, γ H2A.X foci frequently persist as so-called DNA segments with chromatin alterations reinforcing senescence (DNA-SCARS), reflecting unrepaired oxidative damage. Combined assessment of SA- β -Gal activity and γ H2A.X foci therefore provides a more comprehensive readout of oxidative stress-induced neuronal senescence than either marker alone.

Stem-cell secretomes as a cell-free rejuvenation strategy

Targeting cellular senescence has emerged as a promising strategy in regenerative and anti-aging medicine because reducing the burden of senescent cells can improve tissue function and extend healthspan in preclinical models [7]. Increasing evidence indicates that many of the therapeutic effects of stem cells are mediated through paracrine signaling rather than direct cellular engraftment. Stem-cell secretomes released into the extracellular environment contain a diverse array of bioactive molecules, including growth factors, cytokines, antioxidant enzymes, microRNAs, and extracellular vesicles such as exosomes. These secreted factors can influence cellular survival, proliferation, inflammation, oxidative-stress responses, and DNA-damage repair in neighboring cells.

Cell-free conditioned media derived from stem-cell cultures therefore represent a promising therapeutic approach capable of delivering regenerative and rejuvenating signals without the risks associated with live-cell transplantation. By reducing oxidative stress, promoting DSB repair, modulating inflammatory pathways, and attenuating senescence-associated signaling, stem-cell secretomes may help preserve cellular function and resilience. Consequently, evaluating whether tissue-specific stem-cell conditioned media can simultaneously reduce SA- β -Gal-positive cells and γ H2A.X-positive nuclear foci in an oxidative stress-induced neuronal senescence model provides a direct and quantitative assessment of their anti-senescent and DNA-protective potential.

Objectives of the study

In previous work we investigated regenerative activity and biological safety of organ-specific PSC-derived products [8,9] and performed a comparative proteomic analysis of PSC cultures that defined a shared core proteome and specific molecular markers associated with distinct cellular phenotypes [10].

The primary aim of the present study was to investigate the anti-senescent and DNA-protective effects of tissue-specific stem-cell-derived conditioned media on oxidative stress-induced neuronal senescence. To establish an in vitro model of cellular aging, HT22 mouse hippocampal neuronal cells were exposed to 800 μM H_2O_2 as a well-characterized inducer of oxidative stress and premature cellular senescence. Senescence was assessed using the SA- β -Gal assay, and DNA double-strand-break formation was assessed in parallel by $\gamma\text{H2A.X}$ immunofluorescence.

Following oxidative stress induction, HT22 cells were treated with conditioned media obtained from stem-cell primary cultures derived from six different tissue sources (retina, kidney, brain, testes, thymus, heart). The study aimed to determine whether bioactive factors within these secretomes could attenuate senescence-associated cellular changes, reduce the proportion of SA- β -Gal-positive cells, and diminish the abundance of $\gamma\text{H2A.X}$ nuclear foci compared with untreated stressed controls.

A secondary objective was to compare the relative efficacy of the six tissue-specific conditioned media, since stem-cell secretomes contain distinct combinations of growth factors, cytokines, extracellular vesicles, and antioxidants whose anti-senescent and DNA-protective activity may vary according to tissue origin.

Ultimately, this study sought to assess whether stem-cell-derived secretomes can counteract oxidative stress-induced senescence and DNA damage in neuronal cells and to identify promising cell-free therapeutic candidates for future applications in regenerative medicine, healthy aging, and the prevention of age-related neurodegenerative disorders.

Materials and Methods

Cell model and induction of oxidative stress-induced senescence

The murine hippocampal neuronal cell line HT22 was used as an in vitro model of neuronal oxidative stress and senescence. HT22 cells are an immortalized mouse hippocampal neuronal line widely employed in studies of neurodegeneration, oxidative injury, aging, and cellular senescence because of their sensitivity to ROS-mediated damage and reproducible response to H_2O_2 -induced stress [11–14].

To induce premature cellular senescence, HT22 cells were exposed to 800 μM H_2O_2 for 1 h at 37 °C. Oxidative stress induced by H_2O_2 is a well-established experimental model that mimics age-related ROS accumulation and promotes senescence-associated phenotypes, including growth arrest, increased lysosomal activity, mitochondrial dysfunction, DNA double-strand-break formation, and altered cellular morphology [11–14]. Following H_2O_2 exposure, cells were cultured under recovery conditions to allow the development of a stable senescent phenotype prior to analysis.

SA- β -galactosidase assay

Cellular senescence was evaluated using the SA- β -Gal assay, the most widely accepted histochemical marker of senescent cells [3,4,15]. SA- β -Gal-positive cells were identified by the presence of a characteristic blue cytoplasmic precipitate generated by X-Gal hydrolysis at pH 6.0 and quantified by light microscopy. The percentage of senescent cells was calculated as:

$$\text{SA-}\beta\text{-Gal-positive cells (\%)} = (\text{Number of blue-stained cells} / \text{Total number of cells counted}) \times 100.$$

A minimum of five randomly selected microscopic fields per well were analyzed, and the mean percentage of SA- β -Gal-positive cells was calculated for each replicate.

For figure preparation and visualization purposes only, all micrographs underwent identical digital processing. Images were converted to the CIELAB color space, and a fixed linear gain factor of 2.0 was applied exclusively to negative b* values (blue–yellow axis) to enhance the existing blue SA- β -Gal signal. The lightness (L) channel, a channel, and near-neutral background pixels were preserved to prevent artificial color shifts. All quantitative analyses and cell counting were performed on the original, unprocessed images.

γ H2A.X immunofluorescence

Oxidative DNA damage was assessed by immunofluorescence detection of the DSB marker γ H2A.X. Cells were seeded on coverslips and treated with 800 μ M H₂O₂ for 1 h at 37 °C (positive controls) or left untreated (negative control), after which supernatants from the different tissue-specific primary stem-cell cultures were added to the corresponding experimental wells. After four days of culture, cells were washed in PBS, fixed, and blocked/permeabilized with 10% Normal Donkey Serum, 1% BSA, and 0.1% Triton X-100 in PBS for 1 h at room temperature.

The primary antibody, anti-phospho-histone H2A.X (Ser139) rabbit polyclonal (Cell Signaling Technology), was applied at 1:200 in 10% Normal Donkey Serum, 1% BSA, and 0.05% Triton X-100 in PBS overnight at 4 °C. After three washes in PBS, cells were incubated with donkey anti-rabbit IgG Alexa Fluor 546 secondary antibody in 5% Normal Donkey Serum and 0.5% BSA in PBS for 1 h at room temperature. Following three additional washes in PBS and one wash in H₂O, coverslips were mounted with Mowiol and analyzed on a Zeiss Axiovert 200 microscope equipped with an ApoTome structured-illumination module. Nuclei were counterstained with DAPI. Two independent control conditions were included on every plate: an omission control in which the primary antibody was omitted (secondary-only control) and an untreated control without H₂O₂ exposure. γ H2A.X-positive nuclei were identified by the presence of discrete punctate nuclear foci in the Alexa Fluor 546 channel and evaluated qualitatively across matched microscopic fields.

Experimental design and study groups

To evaluate the anti-senescent and DNA-protective effects of tissue-specific stem-cell conditioned media, HT22 cells were allocated to four categories of experimental groups.

Negative control groups: Untreated HT22 cells served as baseline controls representing physiological levels of senescence. Two negative control groups were included:

- Negative Control (+MEM): cells maintained in the presence of MEM- α medium.
- Negative Control (–MEM): cells maintained without MEM- α supplementation.

Positive control groups: Oxidative stress-induced senescence controls were generated by exposing HT22 cells to 800 μ M H₂O₂ without conditioned-medium treatment:

- Positive Control (+MEM): H₂O₂-treated cells cultured in MEM- α -containing medium.

- Positive Control (–MEM): H₂O₂-treated cells cultured without MEM- α .

The paired controls were included because all stem-cell supernatants were collected in MEM- α medium; this design enabled discrimination between biological effects attributable to stem-cell secreted factors and those potentially attributable to the carrier medium itself.

Treatment groups: Six treatment groups were established by exposing H₂O₂-stressed HT22 cells to conditioned media derived from stem-cell primary cultures originating from different tissues:

- Retina (Code 1/4)
- Kidney (Code 2/4)
- Brain (Code 4/4)
- Testes (Code 7)
- Thymus (Code 6)
- Heart (Code 2)

Each conditioned medium was evaluated independently for its ability to reduce the proportion of SA- β -Gal-positive cells and the abundance of γ H2A.X nuclear foci relative to the positive control.

Replication: All experimental conditions were performed using three independent biological replicates ($n = 3$). Statistical comparisons and percentage reductions in senescence were calculated relative to the positive control (+MEM), which served as the primary reference group.

Preparation of tissue-specific stem-cell conditioned media

Conditioned media (supernatants) were generated from six independent primary stem-cell cultures derived from retina, kidney, brain, testes, thymus, and heart tissues. Stem cells were maintained in Minimum Essential Medium Alpha (MEM- α) supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids (NEAA), 15 mM HEPES buffer, and 1% penicillin-streptomycin.

Upon reaching approximately 80–90% confluence, culture medium was replaced and cells were maintained for an additional 48 h to allow accumulation of secreted bioactive factors. Conditioned media were subsequently collected and centrifuged at 1,500 rpm for 10 min to remove detached cells and debris. Supernatants were sterile-filtered through 0.22 μ m pore-size membranes and stored at 4 °C until use. This preparation yielded cell-free conditioned media enriched in soluble secreted factors, including growth factors, cytokines, extracellular vesicles, antioxidant proteins, and other paracrine signaling molecules collectively referred to as the stem-cell secretome [1,7].

Treatment protocol

HT22 cells were seeded at a density of 2×10^5 cells per well and cultured for 48 h under standard conditions (37 °C, 5% CO₂). Prior to oxidative-stress induction, each tissue-specific conditioned medium was mixed at a 1:1 ratio with standard HT22 growth medium consisting of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS and 1% penicillin-streptomycin.

Cells were pre-treated with the conditioned-medium mixture for 12 h to allow uptake and cellular response to secreted factors. Oxidative stress was then induced by exposure to 800 μ M H₂O₂ for 1 h at 37

°C. Following treatment, H₂O₂-containing medium was removed and cells were washed with PBS. Cells were subsequently cultured for an additional 3 days in the corresponding conditioned-medium mixture to facilitate recovery and modulation of stress responses, and then maintained for a further 3 days in standard HT22 medium without conditioned media prior to senescence analysis.

At the conclusion of the culture period, cells were subjected to SA- β -Gal staining according to established protocols [15,16] and the percentage of SA- β -Gal-positive cells was determined microscopically. Parallel coverslips prepared according to Section 2.3 were used for γ H2A.X immunofluorescence analysis.

The HT22 cell line

The HT22 cell line is an immortalized murine hippocampal neuronal cell line that has become one of the most widely utilized in vitro models for studying neuronal oxidative stress, excitotoxicity, neuroprotection, and neurodegenerative disease mechanisms [17–19]. HT22 cells are derived from the mouse hippocampus and represent a subclone of the parental HT-4 cell line, which was originally immortalized using a temperature-sensitive SV40 large T-antigen [17,18]. Unlike mature hippocampal neurons, HT22 cells lack functional ionotropic glutamate and cholinergic receptors, which makes them unsuitable for studies of synaptic transmission, learning, and memory but allows investigators to isolate oxidative stress-related mechanisms of neuronal injury without the confounding effects of receptor-mediated excitotoxic signaling [18].

Morphologically, HT22 cells exhibit an adherent epithelial-like appearance and proliferate rapidly under standard culture conditions, with a reported doubling time of approximately 15 h [17]. A major advantage of the HT22 model is its high sensitivity to glutamate-induced oxidative stress: excess extracellular glutamate inhibits cystine uptake through the cystine/glutamate antiporter (System Xc⁻), leading to glutathione depletion, accumulation of ROS, mitochondrial dysfunction, and oxidative neuronal death [18,19]. HT22 cells are cultured in DMEM supplemented with 10% FBS, 4.5 g/L glucose, 4 mM L-glutamine, and 1.0 mM sodium pyruvate under standard incubator conditions (37 °C, 5% CO₂) and Biosafety Level 1 (BSL-1) containment [17]. To ensure experimental reproducibility and minimize genetic drift, low-passage, authenticated HT22 stocks were used; commercially authenticated HT22 cell lines are available from recognized biological resource suppliers, including Cytion (Product No. 305158) [17].

LC-MS/MS proteomic profiling of tissue-specific conditioned media

To begin identifying candidate mediators of the observed anti-senescent and DNA-protective activity, the six tissue-specific stem-cell conditioned media (brain, heart, kidney, retina, testis, thymus) were subjected to shotgun proteomic profiling by liquid chromatography–tandem mass spectrometry (LC-MS/MS) with label-free quantification (LFQ). Peptides were prepared from concentrated conditioned-medium fractions and separated by nano-LC, and MS/MS spectra were acquired on a high-resolution Orbitrap-class instrument. Raw spectra were processed with MaxQuant (label-free quantification enabled) against the mouse UniProt reference proteome; protein identification and quantification used default MaxQuant thresholds (peptide and protein FDR $\leq$ 1%). Reverse database hits, common contaminants, and proteins identified only by modification site were removed prior to analysis. LFQ intensities for each of the six tissues (columns: Brain, Heart, Kidney, Retina, Testis, Thymus) were used for tissue-comparative analyses.

Downstream analysis was performed in Python 3 (pandas, NumPy, matplotlib). LFQ intensities were log₂-

transformed (zeros treated as not detected). Candidate proteins were assigned to seven functional classes with plausible mechanistic links to the anti-senescent / anti-DSB phenotype observed in HT22 cells: (i) antioxidant / redox defense (peroxiredoxin, thioredoxin, glutathione peroxidase, PARK7/DJ-1, MSR families); (ii) DNA-damage response and DSB repair (H2A.X, ATM/ATR/DNA-PK, MRN, HR, NHEJ, PARP, HMGB1); (iii) chaperones / proteostasis (HSP60/70/90 families, TRiC/CCT, PDI); (iv) anti-inflammatory / SASP-modulating factors (TIMPs, SERPINS, IGFBPs, GDF11/15, decorin, biglycan, follistatin); (v) canonical extracellular-vesicle (EV) markers (CD9/63/81, TSG101, ALIX/PDCD6IP, syntenin-1/SDCBP, flotillins, 14-3-3 family); (vi) neurotrophic factors (BDNF, NGF, GDNF, S100 family, VEGFA, IGF1/2, clusterin); and (vii) mesenchymal-stem-cell (MSC) / matrix-associated proteins (THY1, VIM, NES, collagens I/III/V/VI, fibronectin, SPARC, POSTN, decorin, biglycan). For each class, per-tissue mean $\log_2$ (LFQ) and the number of detected candidates were computed. All analyses used the MaxQuant proteinGroups.txt output file (26F055_MaxQuant_260623).

Statistical analysis

Data are presented as mean $\pm$ standard deviation (SD). Statistical analyses were performed using GraphPad Prism software (GraphPad Software, San Diego, CA, USA).

Differences among the positive control (+MEM) and the six conditioned-medium treatment groups were evaluated by one-way analysis of variance (ANOVA). When a significant overall effect was detected, pairwise comparisons were performed using Tukey's Honestly Significant Difference (HSD) post hoc test to control the family-wise error rate.

To evaluate the independent effect of the MEM- α carrier medium, positive-control and negative-control groups with and without MEM- α supplementation were compared separately using Welch's two-sample t-test, which does not assume equal variance between groups. Statistical significance was defined as $p < 0.001$, $p < 0.01$, $p < 0.05$; ns = not significant. γ H2A.X immunofluorescence images were analyzed qualitatively as a corroborative readout of the SA- β -Gal quantification.

Results

SA- β -Gal staining of HT22 cells after oxidative stress and treatment

The morphological features of SA- β -Gal staining are presented in Figure 1. In the H₂O₂-treated positive control group, numerous HT22 cells exhibited the characteristic blue-green cytoplasmic and perinuclear staining associated with elevated lysosomal β -galactosidase activity, indicating successful induction of cellular senescence. In contrast, cells treated with tissue-specific stem-cell conditioned media displayed visibly fewer SA- β -Gal-positive cells, whereas the untreated negative control groups exhibited minimal or no detectable staining.

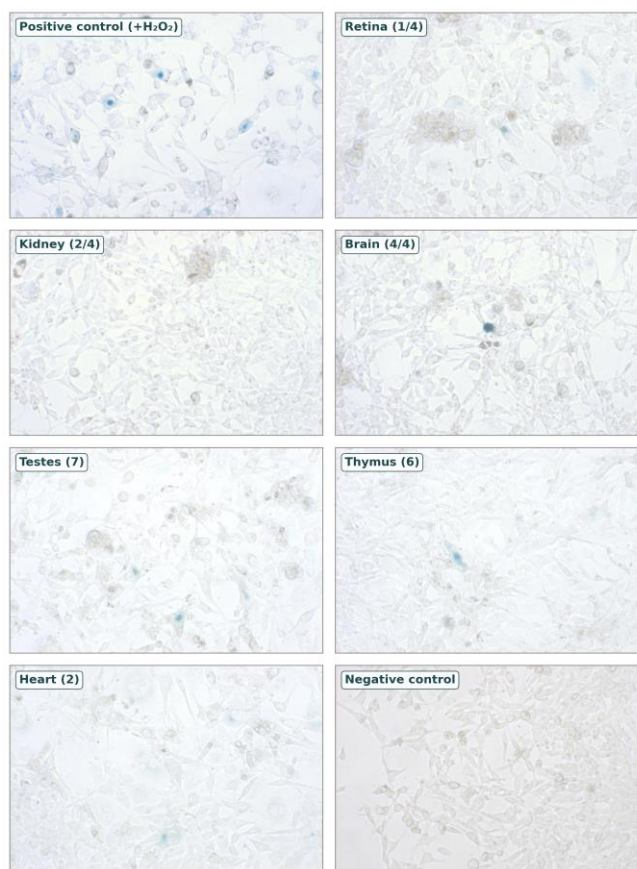


Figure 1: Representative SA-β-Gal cytochemical staining of H₂O₂-treated HT22 cells. Senescent cells are identified by blue-green cytoplasmic SA-β-Gal precipitate. Panels: positive control (HT22 + 800 μM H₂O₂), six stem-cell-conditioned-medium groups (retina 1/4, kidney 2/4, brain 4/4, testes 7, thymus 6, heart 2), and negative control.

The positive control shows the most abundant SA-β-Gal⁺ cells; conditioned-media groups show clearly fewer stained cells. For visualization, the blue signal was enhanced uniformly across all panels by a single fixed linear gain applied to the negative b* (blue) channel in CIELAB space, with the background left unchanged; quantification was performed on the original images. Tissue / code key: 1/4 = Retina · 2/4 = Kidney · 4/4 = Brain · 7 = Testes · 6 = Thymus · 2 = Heart · +MEM / no MEM = positive and negative controls run with or without MEM-α carrier medium · reference = positive control (+MEM); all percentage changes and post-hoc p-values are relative to this group.

Quantification of senescent cells

Quantitative results, including individual replicate values, group means ± SD, percentage changes relative to the positive control (+MEM), and statistical comparisons, are summarized in (Table 1).

Group	Code	Replicates	Mean	SD	Δ vs ref	p (vs ref)
Positive control (+MEM)	—	497, 508, 602	535.7	57.7	reference	—
Positive control (no MEM)	—	461, 520, 549	510.0	44.8	−4.8%	ns
Retina	1/4	345, 304, 374	341.0	35.2	−36.3%	< 0.001 (***)
Kidney	2/4	224, 279, 294	265.7	36.9	−50.4%	< 0.001 (***)
Brain	4/4	301, 265, 338	301.3	36.5	−43.7%	< 0.001 (***)
Testes	7	271, 260, 233	254.7	19.6	−52.5%	< 0.001 (***)
Thymus	6	270, 292, 347	303.0	39.7	−43.4%	< 0.001 (***)
Heart	2	379, 348, 359	362.0	15.7	−32.4%	< 0.001 (***)

Group	Code	Replicates	Mean	SD	Δ vs ref	p (vs ref)
Negative control (+MEM)	—	2, 4, 1	2.3	1.5	-99.6%	< 0.001 (***)
Negative control (no MEM)	—	3, 1, 3	2.3	1.2	-99.6%	< 0.001 (***)

Table 1: SA- β -Gal⁺ cell counts by group (n = 3). Δ vs ref = percentage change in mean SA- β -Gal⁺ cell count relative to the reference (positive control with MEM- α). p-values are Tukey HSD adjusted versus the positive control (+MEM); the MEM / no-MEM control comparisons were assessed by Welch t-test (ns).

A graphical representation of the quantitative analysis, including the MEM- α carrier medium controls, is shown in (Figure 2).

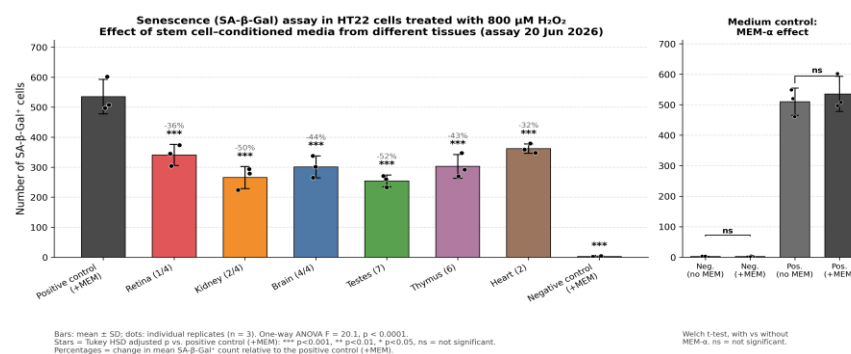


Figure 2: Quantification of SA- β -Gal⁺ cells. Left: number of SA- β -Gal⁺ cells per group; bars show mean $\pm$ SD and dots show individual replicates (n = 3). One-way ANOVA across the positive control (+MEM) and the six treatment groups: F = 20.1, p < 0.0001; stars indicate Tukey HSD adjusted p versus the positive control (+MEM) (***) p < 0.001. Right: medium (MEM- α) control. Adding MEM- α had no significant effect on either the positive or the negative control (Welch t-test, both ns), confirming that the carrier medium itself does not alter senescence.

Exposure of HT22 hippocampal neuronal cells to 800 μ M H₂O₂ induced a robust senescence response, resulting in a marked increase in SA- β -Gal-positive cells compared with untreated controls. The positive control group containing MEM- α exhibited a mean SA- β -Gal-positive cell count of 535.7, whereas the corresponding negative control demonstrated only 2.3 positive cells on average. This greater than 200-fold separation between positive and negative controls confirmed efficient senescence induction and provided a broad dynamic range for evaluating anti-senescent interventions.

Treatment with conditioned media derived from all six tissue-specific stem-cell populations significantly reduced the number of senescent cells relative to the H₂O₂-treated positive control. The magnitude of senescence suppression ranged from approximately 32% to 53%, demonstrating substantial protection against oxidative stress-induced cellular aging. Among the tested secretomes, the most pronounced anti-senescent activity was observed with testes-derived conditioned medium, which reduced SA- β -Gal-positive cell numbers by 52.5% relative to the positive control. Kidney-derived conditioned medium demonstrated a comparable effect, reducing senescence by 50.4%. Intermediate levels of protection were observed with brain-derived and thymus-derived conditioned media (43.7% and 43.4% reductions, respectively), and retina-derived and heart-derived conditioned media produced more modest but still highly significant reductions of 36.3% and 32.4%, respectively.

To determine whether the carrier medium itself contributed to the observed effects, paired control groups with and without MEM- α supplementation were compared using Welch's two-sample t-test. No statistically significant differences were detected between positive control groups cultured with or without MEM- α ($p = 0.58$), and no differences were observed between the corresponding negative control groups ($p = 1.00$). These results demonstrate that MEM- α alone does not significantly influence senescence levels under the experimental conditions employed and strengthen the interpretation that the reductions in SA- β -Gal-positive cells observed in the treatment groups are attributable to bioactive molecules present within the stem-cell conditioned media.

γ H2A.X immunofluorescence: reduced DNA double-strand breaks after conditioned-media treatment

γ H2A.X immunofluorescence was performed as an independent, orthogonal readout of oxidative DNA damage in the same experimental system (Figure 3). In the negative control (HT22 cells without H₂O₂ exposure), nuclei showed uniform DAPI staining with essentially no discrete γ H2A.X foci in the Alexa Fluor 546 channel, consistent with a low physiological level of DSBs. The secondary-only control (H₂O₂-treated cells stained without primary antibody) showed only weak, diffuse background fluorescence without punctate signal, confirming the specificity of the primary antibody.

Exposure to 800 μ M H₂O₂ for 1 h produced a strong, punctate nuclear γ H2A.X signal in the vast majority of HT22 nuclei, with numerous bright discrete foci per nucleus and additional bright nuclear aggregates in a subset of severely damaged cells. This pattern is characteristic of widespread H₂O₂-induced DNA double-strand-break formation.

In marked contrast, HT22 cells that were exposed to H₂O₂ and then cultured for four days in the presence of tissue-specific stem-cell conditioned media (heart, thymus, testes, retina, kidney, and brain) showed a clear qualitative decrease in the number and intensity of punctate nuclear γ H2A.X foci compared with the H₂O₂ positive control. In all six treatment conditions, the density of γ H2A.X foci per nucleus was markedly lower and the very bright nuclear aggregates observed in the positive control were largely absent, while DAPI staining and overall nuclear morphology remained comparable across groups. This pattern indicates that the tissue-specific stem-cell secretomes attenuated the burden of oxidative DNA damage or accelerated its resolution over the four-day post-stress recovery period.

Importantly, this qualitative reduction in γ H2A.X foci mirrors the quantitative decrease in SA- β -Gal-positive cells observed in the same treatment groups (**Table 1, Figure 2**), providing convergent evidence that stem-cell conditioned media protect HT22 cells along the oxidative-DNA-damage-to-senescence axis rather than affecting only a single downstream marker.

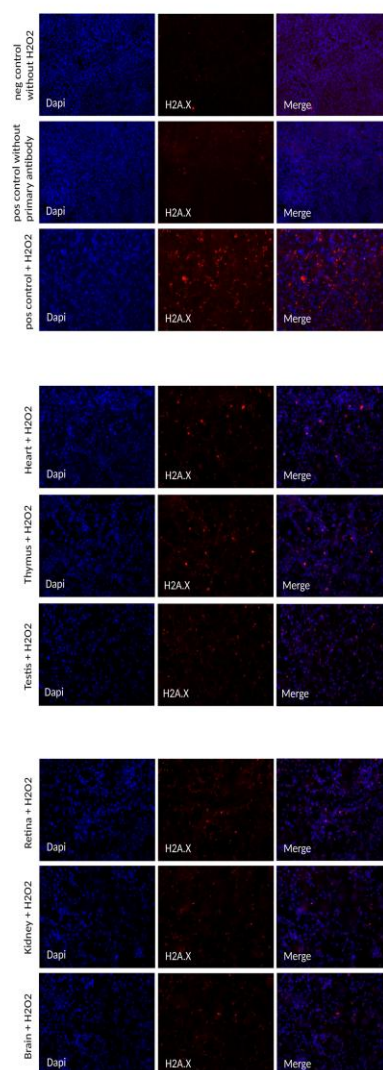


Figure 3: Representative γ H2A.X immunofluorescence in HT22 cells after H_2O_2 -induced oxidative stress and treatment with tissue-specific stem-cell conditioned media. Cells were treated with 800 μM H_2O_2 for 1 h at 37 $^\circ\text{C}$ or left untreated (negative control), and supernatants from primary stem-cell cultures were added for four days. Cells were fixed and stained with a rabbit polyclonal anti-phospho-histone H2A.X (Ser139) primary antibody (Cell Signaling Technology; 1:200) and a donkey anti-rabbit Alexa Fluor 546 secondary antibody; nuclei were counterstained with DAPI. Images were acquired on a Zeiss Axiovert 200 ApoTome microscope. Top rows: controls — negative control (no H_2O_2), primary-antibody omission control (secondary only), and H_2O_2 positive control show, respectively, essentially no γ H2A.X foci, weak diffuse background, and abundant discrete punctate nuclear γ H2A.X foci. Middle and lower rows: HT22 cells treated with H_2O_2 + conditioned media from heart, thymus, testes, retina, kidney, and brain primary stem-cell cultures show a clear qualitative reduction in the number and intensity of punctate nuclear γ H2A.X foci relative to the H_2O_2 positive control. Left column: DAPI; middle column: γ H2A.X (Alexa Fluor 546); right column: merge.

Proteomic profiling of the six tissue-specific conditioned media

To generate an evidence-based, testable hypothesis for the molecular basis of the anti-senescent and anti-DSB effects observed in HT22 cells, the six tissue-specific stem-cell conditioned media were profiled

by LC-MS/MS with label-free quantification. After removal of reverse hits, common contaminants, and identifications supported only by a single modification site, 754 proteins were retained across the six secretomes (Brain 548, Heart 539, Kidney 566, Retina 520, Testis 549, Thymus 525). A large conserved core of 359 proteins (48%) was detected in all six tissues, and a further 169 proteins were detected in four or five tissues, indicating a shared paracrine “core secretome” that is complemented by tissue-selective components.

A candidate-class analysis was performed against seven functional groups with defined mechanistic relevance to the SA- β -Gal / γ H2A.X readouts (Methods, section 2.8). The mean $\log_2$ (LFQ) heatmap of these classes across all six tissues is shown in (Figure 4A). Anti-inflammatory / SASP-modulating factors, canonical EV markers, and MSC / matrix-associated proteins were consistently high across all secretomes (mean $\log_2$ (LFQ) $\approx$ 22–25), demonstrating that all six preparations qualify as bona fide extracellular-vesicle-containing stem-cell secretomes rather than random supernatants. The chaperone / proteostasis and antioxidant / redox-defense classes were also uniformly abundant ($\approx$ 21.5–22.5), consistent with a shared cytoprotective machinery. In contrast, the neurotrophic-factor class was markedly enriched in kidney (26.4), testis (24.6), and thymus (24.3), while the DNA-damage-response class was represented almost exclusively by HMGB1, which reached its highest signal in brain- and kidney-derived preparations. The number of individual protective-pathway proteins detected per tissue is summarized in (Figure 4B).

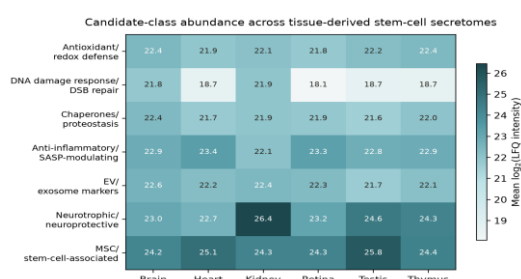


Figure 4A: Mean $\log_2$ (LFQ) intensity of seven functional candidate classes with plausible mechanistic links to the anti-senescent and DNA-protective activity, shown across the six tissue-specific stem-cell secretomes. Colour intensity encodes mean abundance within each class; “n.d.” indicates that no protein of the class was detected in that tissue.

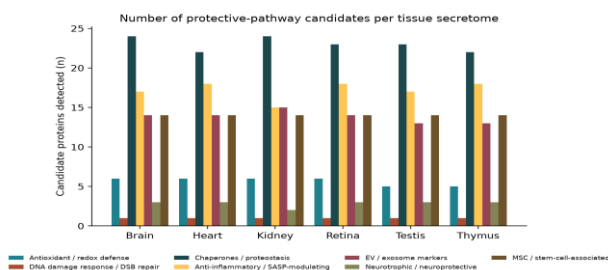


Figure 4B: Number of candidate proteins detected (LFQ > 0) per tissue for each functional class. Chaperones/proteostasis and anti-inflammatory/SASP-modulating factors provide the broadest per-tissue coverage, whereas the DNA-damage-response class is dominated by a single detected component (HMGB1).

A focused inspection of the individual proteins driving these signals identified a compact set of highly

abundant, tissue-conserved candidates (Figure 4C). The top-ranked proteins by mean $\log_2(\text{LFQ})$ across the six secretomes included the antioxidant peroxiredoxins Prdx1 and Prdx2 and the redox-active proteins Park7 (DJ-1), Txnrc5 and Gpx3; the chaperones Hspa8 (HSC70), Hsp90aa1/ab1, Hsp90b1 (GRP94), Hspa5 (BiP), Hspb1, Calr and the ER-resident disulfide isomerases P4hb, Pdia3 and Pdia6; the anti-inflammatory / SASP-modulating factors Timp2, Serpine1 (PAI-1), Serpinf1 (PEDF), Thbs1/2, Fstl1, Follistatin (Fst), Igfbp2/4/5/7 and Igf2; the DNA-damage-associated non-histone chromatin protein HMGB1; the neurotrophic / anti-apoptotic factor clusterin (Clu); and the canonical EV cargo/biogenesis proteins Pdc6ip (ALIX), Sdcbp (syntenin-1), and the 14-3-3 family (Ywhab/e/g/h/q/z). The complete per-tissue LFQ table for these candidates and a top-40 abundance heatmap are provided in (Figure 4C).



Figure 4C: Focused heatmap of the top-40 protective-pathway candidate proteins (ranked by mean $\log_2(\text{LFQ})$ across the six secretomes). Rows show individual proteins (gene name and short description); columns show tissues; colour encodes $\log_2(\text{LFQ})$. Prdx1/2, Park7 (DJ-1), HSP70/90-family chaperones, IGFBP2/4/5/7, TIMP2, PAI-1 (SERPINE1), PEDF (SERPINF1), thrombospondins (THBS1/2), follistatin (FST/FSTL1), clusterin (CLU) and the EV markers PDCD6IP (ALIX), SDCBP (syntenin-1) and the 14-3-3 family are detected across all or nearly all six tissues, defining a conserved anti-senescent / anti-DSB “core cargo”.

Taken together, the proteomic profile provides a coherent molecular framework for the biological effects observed in the HT22 assays. All six conditioned media contain, at high abundance, (i) redox-buffering enzymes capable of degrading H_2O_2 and secondary lipid peroxides (Prdx1/2/6, Gpx3, Txnrc5, Txnrd1,

Park7/DJ-1), (ii) extracellular and EV-associated chaperones that support proteostasis under oxidative stress (HSPA8, HSP90AA1/AB1, HSP90B1/GRP94, HSPA5/BiP, HSPB1, HSPD1/HSPA1A, calreticulin, PDIs), (iii) secreted SASP- and NF- κ B-modulating factors that antagonize the senescence-associated secretory phenotype (TIMP2/3, SERPINE1/PAI-1, SERPINF1/PEDF, IGFBP2/4/5/7, follistatin/FSTL1, thrombospondins, decorin, biglycan, IGF2), (iv) the DNA-damage-associated chromatin factor HMGB1, which extracellularly acts as a redox-sensitive DAMP but intracellularly promotes DSB repair, and (v) a canonical EV signature (PDCD6IP/ALIX, SDCBP/syntenin-1, 14-3-3 family, HSPA8, HSP90AA1, ANXA2/5) that identifies extracellular vesicles as a plausible delivery vehicle for the intracellular cargo (chaperones, peroxiredoxins, 14-3-3 proteins) required to reach recipient HT22 cells.

Overall pattern across readouts

Comparison of the six conditioned media revealed a clear hierarchy of anti-senescent activity by SA- β -Gal quantification: testes- and kidney-derived secretomes consistently produced the largest reductions in senescence, brain- and thymus-derived preparations showed intermediate effects, and retina- and heart-derived preparations produced smaller but still highly significant reductions. γ H2A.X immunofluorescence corroborated the SA- β -Gal data by showing that all six conditioned media reduced punctate nuclear γ H2A.X foci compared with the H₂O₂ positive control, consistent with attenuation of upstream oxidative DNA damage.

Although all conditioned media significantly attenuated oxidative stress-induced senescence and reduced γ H2A.X foci, the differences in efficacy observed across tissues suggest that tissue-specific stem-cell populations produce distinct secretome profiles with varying concentrations of growth factors, cytokines, extracellular vesicles, antioxidant proteins, and DNA-damage-response modulators. Such differences may influence their capacity to modulate oxidative stress responses, cellular repair pathways, and senescence-associated signaling networks.

The particularly strong activity observed for testes- and kidney-derived secretomes identifies these preparations as leading candidates for future mechanistic studies aimed at identifying the molecular mediators responsible for their anti-senescent and DNA-protective effects.

Discussion

The present study investigated whether tissue-specific stem-cell conditioned media can mitigate oxidative stress-induced cellular senescence and DNA damage in HT22 mouse hippocampal neuronal cells. Using a well-established H₂O₂-induced senescence model, SA- β -Gal staining as the primary quantitative endpoint, and γ H2A.X immunofluorescence as an orthogonal marker of DNA double-strand breaks, the study demonstrated that conditioned media derived from all six stem-cell sources significantly reduced the burden of senescent cells and produced a clear qualitative reduction in oxidative DNA-damage foci. These findings support the hypothesis that stem-cell secretomes exert protective paracrine effects capable of modulating cellular responses to oxidative stress along the DNA-damage-to-senescence axis.

The successful induction of senescence by exposure to 800 μ M H₂O₂ confirms the suitability of the HT22 model for studying oxidative stress-associated neuronal aging. Oxidative stress is recognized as a major driver of cellular senescence through the generation of ROS, DNA damage, mitochondrial dysfunction, and activation of senescence-associated signaling pathways. In the present study, H₂O₂ treatment

produced a pronounced increase in SA- β -Gal-positive cells and abundant discrete nuclear γ H2A.X foci compared with untreated controls, generating a wide assay window and providing a robust platform for evaluating potential anti-senescent interventions. The marked separation between positive and negative controls confirms both the effectiveness of the senescence-induction protocol and the sensitivity of both assay systems.

A key finding of this study is that all tested stem-cell conditioned media significantly reduced SA- β -Gal positivity in stressed HT22 cells (32–53% relative to the positive control) and that this reduction was mirrored by a clear qualitative decrease in punctate nuclear γ H2A.X foci. The convergence of these two independent readouts strengthens the interpretation that secreted factors present within the conditioned media protect neuronal cells not only by attenuating the downstream senescence program but also by reducing the upstream oxidative DNA-damage burden. These results are consistent with the growing body of evidence suggesting that many regenerative effects traditionally attributed to stem-cell transplantation are mediated by the secretome rather than by direct cellular engraftment. Stem-cell secretomes contain complex mixtures of growth factors, cytokines, extracellular vesicles, antioxidant enzymes, and nucleic acids that can influence cellular survival, stress resistance, DNA damage response signaling, and inflammatory responses.

An important observation was the tissue-dependent variation in anti-senescent efficacy. Among the six tested preparations, testes-derived conditioned medium demonstrated the strongest protective effect (52.5% reduction in SA- β -Gal-positive cells), closely followed by kidney-derived conditioned medium (50.4%). Brain- and thymus-derived secretomes exhibited intermediate activity, whereas retina- and heart-derived preparations produced smaller but still highly significant reductions. γ H2A.X immunofluorescence showed a qualitatively parallel effect, with all six conditioned media reducing DSB foci relative to the H₂O₂ positive control. These findings suggest that stem-cell populations from different tissues secrete distinct combinations of biologically active molecules, resulting in measurable differences in their ability to modulate oxidative stress and senescence pathways.

The particularly strong activity observed for testes-derived conditioned medium is noteworthy. Testicular tissue contains highly proliferative stem-cell populations that support continuous germ-cell renewal throughout life and may therefore produce secretomes enriched in growth-promoting, cytoprotective, and anti-apoptotic factors. Kidney-derived stem-cell secretomes are known to contain factors involved in tissue repair, metabolic regulation, and protection against oxidative injury. The superior performance of these two preparations across both SA- β -Gal and γ H2A.X readouts is consistent with the proteomic profile (**Figure 4A–C**, section 3.4), which shows that both testis- and kidney-derived conditioned media are strongly enriched for neurotrophic factors (kidney mean log₂(LFQ) 26.4; testis 24.6) and carry high signals of core protective proteins such as clusterin, IGFBP2/5/7, IGF2, follistatin, TIMP2, PAI-1, HSPA8, HSP90 and the peroxiredoxins.

A hypothesis for the molecular combination underlying the protective effect

Because the SA- β -Gal and γ H2A.X readouts converge on the same DNA-damage-to-senescence axis, and because all six conditioned media reduce both endpoints, a plausible working hypothesis is that the observed protection is not attributable to a single “active ingredient” but to a conserved multi-component cargo delivered largely via extracellular vesicles. Integrating the proteomic profile with the biological

readouts, we propose that the anti-senescent and anti-DSB activity of the six stem-cell secretomes is driven by the combined action of four converging protein modules that are co-detected in all six preparations (**Figure 4C**):

1. **A redox-buffering module** — Prdx1, Prdx2 and Prdx6 (thiol-based peroxidases that reduce H_2O_2 and organic peroxides), Gpx3 (extracellular selenoperoxidase), Txnrc5/Txnrd1 (thioredoxin system) and Park7/DJ-1 (H_2O_2 -inducible cytoprotectant and redox sensor). Delivery of these enzymes — either as soluble proteins or as EV cargo internalized by recipient cells — would directly degrade residual H_2O_2 and the secondary reactive species that drive $\gamma\text{H2A.X}$ focus formation, providing a mechanistic explanation for the observed reduction of DSB foci without invoking direct enhancement of repair.
2. **A proteostasis / chaperone module** — HSPA8 (HSC70), HSP90AA1/AB1, HSP90B1 (GRP94), HSPA5 (BiP), HSPB1, HSPD1/HSPE1 (mitochondrial chaperonin), calreticulin, and the protein disulfide isomerases P4HB, PDIA3, PDIA6. Extracellular and EV-delivered chaperones are increasingly recognized as cytoprotective; they refold oxidatively damaged proteins, buffer ER stress, and dampen the unfolded-protein response, all of which are upstream triggers of oxidative-stress-induced senescence.
3. **A SASP- and growth-factor-modulating module** — Timp2 and Timp3 (MMP inhibitors that also suppress SASP amplification), SERPINE1/PAI-1 and SERPINF1/PEDF (secreted anti-apoptotic and anti-angiogenic factors implicated in senescence modulation), IGFBP2/4/5/7 (regulators of local IGF/insulin signalling and, for IGFBP7, of oncogene-induced senescence), IGF2, follistatin (FST) and FSTL1 (activin/TGF- β pathway modulators), thrombospondins (THBS1/2), decorin and biglycan. This module is expected to counteract paracrine amplification of senescence, reduce SASP-associated inflammatory signalling, and support neuronal survival.
4. **A DNA-repair-supporting and neurotrophic module** — HMGB1 (the only DDR-class protein consistently detected across the six secretomes, with the highest signal in brain- and kidney-derived preparations), together with clusterin (an extracellular anti-apoptotic chaperone with documented neuroprotective activity) and — where enriched — S100A10/A11 and VEGFA. Intracellular HMGB1 is required for efficient DSB repair and chromatin remodelling around $\gamma\text{H2A.X}$ foci; transfer of HMGB1 via EVs offers a candidate mechanism for direct support of DSB repair, complementing the upstream redox-buffering effect.

Delivery of this combined cargo is plausibly mediated by extracellular vesicles, as evidenced by the robust and consistent detection of canonical EV biogenesis and cargo markers across all six secretomes (PDCD6IP/ALIX, SDCBP/syntaxin-1, HSPA8, HSP90AA1, ANXA2/5, and the entire 14-3-3 family YWHAB/E/G/H/Q/Z). This EV signature is compatible with MISEV2018/2023 criteria for the presence of small EVs in the preparations and suggests that at least part of the observed activity is EV-borne rather than exclusively due to soluble factors. Importantly, the four modules act in a temporally and mechanistically complementary manner: modules 1 and 2 protect proteins and DNA from ongoing oxidative attack (upstream of $\gamma\text{H2A.X}$), module 4 supports repair of DSBs that have already formed, and module 3 prevents secondary paracrine reinforcement of the senescence program (downstream of SA- β -

Gal induction). This combined-cargo model provides a coherent, testable explanation for the observed convergent reduction of both DNA-damage foci and SA- β -Gal positivity across all six tissue-specific secretomes and predicts that selective depletion of any single module (for example, immuno-depletion of clusterin, IGFBP7 or HMGB1, or inactivation of the peroxiredoxin/thioredoxin system by hyperoxidation) should attenuate but not abolish the effect.

The tissue-specific hierarchy of activity (testis $\approx$ kidney > brain $\approx$ thymus > retina $\approx$ heart) is compatible with quantitative rather than qualitative differences in this shared cargo. Testis- and kidney-derived secretomes combine high levels of neurotrophic factors (clusterin, IGF2, IGFBP2/5/7) with strong redox-buffering and chaperone signals, whereas heart-derived preparations, although rich in extracellular matrix and SASP-modulating factors (THBS1, TIMP2, follistatin), reach somewhat lower levels of the neurotrophic and DNA-repair-supporting modules. This is consistent with — but does not yet prove — the interpretation that the balance among the four modules, rather than the presence of any single dominant protein, defines the potency of a given secretome preparation.

The inclusion of paired control groups with and without MEM- α medium represents an important methodological strength of the study. Because all conditioned media were generated in MEM- α , it was necessary to determine whether the carrier medium itself contributed to the observed reductions in senescence. Statistical analysis demonstrated no significant differences between MEM-containing and non-MEM controls in either the positive or negative control groups. This finding confirms that the observed anti-senescent effects originated from biologically active components secreted by the stem cells rather than from the culture medium used during secretome preparation.

The results also provide support for the concept of cell-free regenerative therapy. Compared with live-cell transplantation, conditioned media and purified secretome products offer several potential advantages, including easier storage and standardization, reduced immunogenicity, elimination of risks associated with uncontrolled cell proliferation, and simpler regulatory pathways. The demonstration that cell-free conditioned media can significantly reduce both markers of neuronal senescence and markers of oxidative DNA damage suggests that secretome-based therapeutics may represent a promising strategy for combating age-related cellular dysfunction and promoting tissue resilience.

From a neurological perspective, these findings are particularly relevant because the accumulation of senescent cells, persistent DNA damage foci, and chronic oxidative stress are increasingly recognized as contributors to neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, and other aging-associated disorders. By simultaneously reducing SA- β -Gal positivity and γ H2A.X foci in hippocampal-derived neuronal cells, stem-cell secretomes may help preserve neuronal function, limit inflammatory signaling, and improve cellular resistance to age-related stressors. While the current study does not directly address disease models, the observed effects provide a rationale for further investigation in more complex systems.

Limitations and future directions

Several limitations should be considered when interpreting the findings. First, the study was conducted with a relatively small sample size ($n = 3$ biological replicates per group). Although statistically significant differences were observed across all treatment groups, validation in larger independent experiments

would improve statistical power and strengthen confidence in the observed tissue-specific effects.

Second, senescence was induced using a single oxidative stress paradigm consisting of exposure to 800 μM H_2O_2 for a fixed duration. Future studies should incorporate dose-response and time-course experiments using multiple H_2O_2 concentrations and exposure durations to determine the therapeutic range, potency, and temporal characteristics of the observed anti-senescent effects.

Third, the primary quantitative endpoint was the SA- β -Gal assay, supplemented by qualitative $\gamma\text{H2A.X}$ immunofluorescence. Although this combination captures both a downstream (SA- β -Gal) and an upstream (DSB) readout, future work should include quantitative image analysis of $\gamma\text{H2A.X}$ foci per nucleus, complementary markers of senescence such as p16^{INK4a}, p21^{CIP1/WAF1}, and SASP cytokines (IL-6, IL-8), and telomere-associated DNA damage foci to provide a more comprehensive characterization.

Fourth, although the present work provides an initial LC-MS/MS proteomic characterization of the six tissue-specific secretomes and derives a testable multi-module hypothesis (section 4.2), the study did not perform per-tissue biological replicates of the proteomic run, quantitative EV characterization (particle counts by NTA, size distribution, transmission electron microscopy, and full MISEV2018/2023-compliant marker panel), transcriptomic or metabolomic profiling, or functional loss-of-function experiments (e.g., immunodepletion of clusterin, IGFBP7 or HMGB1; hyperoxidation of the peroxiredoxin/thioredoxin system; heat inactivation vs. EV isolation vs. size-fractionation of the conditioned media). Such experiments will be required to move the combined-cargo hypothesis from association to causation. Priority validation should focus on the four candidate modules identified here (redox-buffering, proteostasis/chaperone, SASP/growth-factor-modulating, DNA-repair-supporting/neurotrophic) and on the testes- and kidney-derived preparations, which demonstrated the strongest protective effects.

Finally, the present work was performed exclusively in an immortalized mouse hippocampal neuronal cell line. Although HT22 cells provide a well-established and reproducible model of neuronal oxidative stress, additional studies in primary neuronal cultures, human-derived neuronal cells, three-dimensional organoid systems, and in vivo models of aging and neurodegeneration will be necessary to determine the translational relevance of these findings.

Conclusions

This study evaluated the ability of tissue-specific stem-cell conditioned media to attenuate oxidative stress-induced cellular senescence and DNA double-strand-break formation in HT22 mouse hippocampal neuronal cells using SA- β -Gal staining as the primary quantitative endpoint and $\gamma\text{H2A.X}$ immunofluorescence as an independent marker of oxidative DNA damage. Cellular senescence was successfully induced by exposure to 800 μM H_2O_2 , resulting in a substantial increase in SA- β -Gal-positive cells and abundant nuclear $\gamma\text{H2A.X}$ foci compared with untreated controls.

Conditioned media derived from stem-cell primary cultures of six different tissue origins significantly reduced the number of senescent cells relative to the H_2O_2 -treated positive control (one-way ANOVA $F = 20.1$, $p < 0.0001$; Tukey HSD $p < 0.001$ for all comparisons) and produced a clear qualitative reduction in punctate nuclear $\gamma\text{H2A.X}$ foci. The magnitude of the protective effect varied according to tissue source, with testes-derived and kidney-derived secretomes showing the strongest activity (52.5% and 50.4%

reductions in SA- β -Gal-positive cells, respectively), followed by brain- and thymus-derived preparations, and retina- and heart-derived preparations. The absence of significant differences between MEM-containing and non-MEM controls (positive control $p = 0.58$; negative control $p = 1.00$) confirmed that the carrier medium itself did not influence senescence levels.

Taken together, these results provide convergent evidence that stem-cell secretomes exert a measurable paracrine cytoprotective effect against oxidative stress-induced neuronal senescence and DNA damage. These findings support the growing concept that many regenerative effects of stem cells are mediated through secreted factors rather than direct cellular engraftment and highlight the potential of cell-free secretome-based approaches in regenerative medicine and healthy aging research. In particular, the pronounced activity observed in testes- and kidney-derived conditioned media warrants further mechanistic investigation to identify the molecular mediators responsible for their effects and to evaluate their application in aging-related neurological disorders.

Conflicts of interest

The study was sponsored by European Wellness Biomedical Group. The authors declare no other conflicts of interest.

Author contributions

Conceptualization: T.S., M.K.S.C., D.K.; methodology and experimental work: T.S.; formal analysis: T.S.; writing — original draft: T.S., D.K.; writing — review and editing: T.S., M.K.S.C., D.K.; supervision: M.K.S.C. All authors have read and agreed to the submitted version of the manuscript.

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