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Original Article

Optimizing Se- methylselenocysteine concentration to enhance glutathione peroxidase 1 expression and mitigate oxidative stress in senescent human fibroblast

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Abstract

Glutathione peroxidase 1 (GPx1) activity, gene expression, and several oxidative stress (OS) marker levels were investigated in the senescent passage (P) 20, 25, and 30 fibroblasts cultured in media supplemented with increasing Se-Methylselenocysteine (MSC) increments. While GPx1 activity slightly increased in cells grown in standard culture medium (CM1) compared to primary P5 cells, the enzyme exhibited significant MSC-dose-dependent elevations in cells cultured in MSC-supplemented media (CM3-CM6) compared to CM1 ($p<0.001$). GPx1 activity in CM5-incubated P30, P25, and P20 cells equaled 5.99±0.62, 4.72±0.48, and 4.06±0.36 µmoles/min/mg protein respectively ($p<0.001$), with percentage increases of 250% in P30 cells compared to 190% in P20 cells when cultured with CM1. Similarly, GPx1 expression was markedly upregulated in CM2, CM4, and CM6-incubated cells compared to primary P5 cells ($p<0.001$), with fold change values of 1.51±0.12, 1.99±0.16, and 2.31±0.19 in P20 cells. Percentage upregulations were 50.0±3.68%, 89.5±7.11%, and 126.5±9.74% in CM2, CM4, and CM6-incubated P20 cells respectively, and reached 248.0±18.6% in P30 cells at the highest MSC concentration. Concurrently, OS marker levels were substantially higher in CM1-cultured P25 and P30 senescent cells compared to primary P5 cells ($p<0.001$). Furthermore, hydrogen peroxide levels were significantly reduced in CM3-incubated cells compared to CM1 ($p<0.01$), reaching the lowest values in CM6 ($p<0.001$), with reductions of approximately 11.5%, 40%, 57%, and 58% in P30 CM3, CM4, CM5, and CM6-incubated cells respectively. MSC-Km values for GPx1 were 0.87, 1.13, and 1.92 µM in P20, P25, and P30 cells, respectively, with corresponding Vmax values of 4.59, 5.68, and 7.94 µmole/min/mg protein. These findings suggest that senescent cells utilize higher amounts of MSC to upregulate GPx1 expression and maximize its activity, supporting using Se supplements to combat OS.

Keywords: Glutathione peroxidase1, Se-Methylselenocysteine supplementation, Senescent, Fibroblasts, Oxidative stress, MSC-Km values.

1. Introduction

Selenium (Se) is an essential cationic trace element with physiological importance [1]. In contrast to other trace elements, Se exerts its biological functions by being incorporated into selenoproteins as selenocysteine. Thus far, twenty-five selenoproteins have been identified, many exhibiting enzymatic activity [2]. Studies have shown that deficient Se and selenoproteins are linked to a large number of human pathologies, including muscle disorders, cardiovascular diseases, hepatopathies, renal failure, neurologic disorders, immune defense and inflammatory disorders, HIV, Diabetes, endocrine disorders, cancer, and aging-related disorders [3]. Selenoproteins form an integral part of the antioxidant defensome and regulate various signaling processes governing Ca^{2+} homeostasis and apoptosis [4, 5]. Examples include glutathione peroxidases, thioredoxin reductases, ER selenoproteins, and methionine sulphoxide reductase [6]. As oxidative injury, often referred to as oxidative stress (OS), is implicated in the pathologies mentioned above, selenoproteins are indispensable in maintaining the homeostatic redox state [7]. Significantly, selenoprotein synthesis is influenced by Se intake [8]. While mRNA levels of several selenoproteins, such as type 1 iodothyronine deiodinase and selenoprotein P, are not affected by Se deficiency, cellular Glutathione peroxidase expression is rapidly blunted when Se supply is low, highlighting the importance of selenoproteins in cellular homeostasis [9, 10]. In this context, it has been proposed that Se supplementation could be of therapeutic value [11]. However, excessive Se intake is toxic and may predispose to life-threatening conditions such as type 2 diabetes [12].

Glutathione peroxidases are antioxidant enzymes comprised of eight isoforms, five of which possess a seleno-

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cysteine residue and include cytosolic GPx1, gastrointestinal GPx2, plasma GPx3, phospholipid hydroperoxide GPx4, and the olfactory epithelium GPx6 [13]. GPx1 is a ubiquitous tetramer located in the cytoplasm and mitochondria of cells. It reacts with reduced glutathione (GSH) as a substrate for the reduction of hydrogen peroxide and organic hydroperoxides [13]. Fluctuations in Se levels and cellular redox state highly influence GPx1 activity [14]. Although synthesis of the enzyme is lowered by oxidative injury, GPx1 activity is quickly restored in comparison with other selenoproteins [15].

Aging is frequently modeled by cellular senescence. The number of senescent fibroblasts increases with age, and senescence-associated phenotypes have been considered to be features of age-related pathologies and longevity of humans [16, 17]. Moreover, replicative senescence has been associated with perturbed antioxidant mechanisms [18]. Nonetheless, the relationship between Se levels and aging must be better understood. Although plasma Se could remain unchanged with age, tissue distribution, and uptake may be altered [11, 15]. A correlation between Se, age, and longevity has only been suggested in very few studies. For example, Olivieri *et al.* demonstrated that Se-dependent GPX activity is significantly reduced in the elderly population over sixty years of age [19]. It was also shown that blood Se may be a biomarker of longevity in elderly subjects [20, 21]. Furthermore, Se enhances the replication of cultured bovine adrenocortical cells [22]. Congruently, we had previously reported that senescent human skin fibroblasts exhibited increases in GPx1 activity when the cells were subculture in a medium supplemented with sodium selenite at three times plasma levels [23]. Moreover, cellular hydrogen peroxide generation was very significantly lowered, and population doublings were extended [23]. Se exists naturally in inorganic and organic forms exhibiting different bioavailability. The organic forms include selenocysteine, selenomethionine, and their methylated derivatives, and process a high gastrointestinal absorption rate compared to other forms [20, 21]. As a follow-up to our previous work, this study examines the potential effect of supplemental MSC increments on GPx1 activity and its gene transcripts using primary and senescent human neonatal cultured fibroblasts. Moreover, Km Se values required to maximize GPx1 activity in such cells were also determined.

2. Materials and Methods

2.1. Preparation of primary and senescent cultures

Six ampules of cryopreserved primary human neonatal foreskin fibroblasts were purchased from Lonza Pharma and Biotech (Basel, Switzerland). Cells tested negative for mycoplasma, bacteria, yeast and fungi. Monolayer confluent primary cultures were established by growing cells in standard Eagle's culture medium (CM1). Cell collection, culture, and subculture processes (CM1) contents, as well as those of the trypsinization and harvesting media, have been previously described [24, 25]. Senescent cultures were obtained by serial subculture of CM1-cultivated primary cultures up to passage (P) 30, and fibroblasts at P5, P10, P15, P20, P25, and P30 were used for investigation. Senescent cultures were identified by studying the rates of incorporation of radiometric leucine and thymidine into protein and DNA, respectively, and by assaying oxidative stress markers at each of the above passages. Details of these methodologies have been previously documented in our earlier publications [23, 26, 27].

2.2. Culture media

The media utilized in this study comprised standard Eagle's culture medium (CM1) with 10% (v/v) fetal bovine serum (FBS), yielding an MSC concentration of approximately 0.6 µM. Additionally, five other media variants (CM2, CM3, CM4, CM5, and CM6) were prepared by supplementing the fetal bovine serum-free medium with MSC, achieving final concentrations of roughly 1.25, 2.5, 3.6, 4.8, and 6 µM, respectively. MSC concentration in CM1 approximately equaled normal plasma serum human Se levels. The Se concentrations in CM2 through CM6 were increased to reach maximal levels of 6 µM equivalent to six times the normal serum human level.

2.3. Experimentation

GPx1 activity and biochemical oxidative stress marker levels were examined in confluent CM1-grown cultures (n=6) at P5, 10, 15, 20, 25, and 30. Cells were harvested and pelleted by centrifugation at 200 g for 5 minutes. Appropriate volumes of pellet sonicates were used to measure various parameters. To investigate the effect of increasing MSC concentrations on GPx1 activity and hydrogen peroxide levels, spent CM1 was removed from primary (P5) and senescent (P20, P25 and P30) cultures, and cells were incubated with each of the BSA-free MSC-supplemented media for 10 hours. The 10-hour incubation period was chosen based on data obtained from the linearity studies of GPx1 activities when subcultures were incubated with CM2 and CM6 for increasing periods. Cells were then washed twice, harvested, and sonicated aliquots were assayed for GPx1 and hydrogen peroxide. To accurately compute MSC-Km values required for maximization of GPx1 activity in senescent P20, P25, and P30 cultures, CM6-cultured cells were further incubated with an additional medium (CM7) containing supplemental MSC at a concentration of 7 µM. CM1-CM6 pooled culture media were used throughout the study, and assays were performed within 4 h of harvesting the cells.

2.4. Linearity of GPx1 activity with respect to time in primary (P5), and senescent P20, P25 and P30 cultures incubated with CM2 and CM6

The spent CM1 medium from the different cultures was replaced with FBS-free CM2 and CM6, containing the lowest (1.25 µM) and highest (6 µM) MSC concentrations, respectively. Cells were incubated for up to 12 hours. After incubation, cell sonicates were assayed for GPx1 activity. The use of CM6 is justified as it showed the highest enzyme activity in P20 and P30 cells (see results section)

2.5. Biochemical assays

GPx1 activity was spectrophotometrically determined, as previously reported [23]. Generation of hydrogen peroxide (H_2O_2), as well as the levels of several oxidative stress markers, including lipid peroxides (LPO), superoxide anions (SOA), protein carbonyl content (PCC), and the Glutathione reduced/oxidized ratio (GSH/GSSG) in fibroblast cultures, was determined as previously documented [28]. Protein concentration was determined using cell sonicates (20 µl) as described by Bradford [29]. CM1

MSC levels were measured using an atomic absorption spectrophotometer (Perkin-Elmer, MA, USA) as documented elsewhere [30].

2.6. Gene expression profiling of GPX1 using Real-Time Quantitative PCR

Sample preparation, RNA extraction, cDNA synthesis, and PCR reaction mixture have been described elsewhere [31]. The amplification program and PCR amplicon specificity were performed and evaluated as previously reported by us [32]. 18S rRNA was used as an internal control [28].

2.7. Cell viability assessment by MTT assay

Cell viability was evaluated using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay, conducted in 96-well plates. Primary (P5) and senescent (P25, P30) fibroblasts were seeded at a density of 1×10^4 cells per well and allowed to adhere overnight. Cells were then incubated with culture media containing different MSC concentrations, specifically CM1 (0.6 µM) and CM6 (6 µM), for 24 hours at 37°C in a humidified atmosphere containing 5% CO2. Following the incubation period, the medium was discarded, and 100 µl per well of MTT solution (0.5 mg/ml dissolved in PBS) was added to the adherent cells, which were then incubated for an additional 3 hours at 37°C. The MTT solution was subsequently removed from each well, and 100 µl of isopropanol was added to solubilize the purple formazan crystals, followed by shaking for 2 hours at room temperature. Absorbance was measured at 549 nm using a microplate reader (ELX 800, BioTek Instruments, Winooski, VT, United States). All experiments were performed in triplicate, and cell viability was expressed as a percentage relative to control cells (cells grown in standard medium without MSC supplementation). No significant differences in cell viability were observed between cells cultured in CM1 and CM6, with viability remaining between 97-100% across all passages tested, confirming that the highest MSC concentration used in this study (6 µM) did not induce cytotoxicity.

2.8. Statistical analysis

One-way analysis of variance followed by Bonferroni correction was carried out to determine statistical differences between mean ± SD values of GPx1 and H_2O_2 levels in different fibroblast passages cultured in various media by the SPSS version 17.0 software (IBM, Armonk, NY, USA). Treatments were considered statistically different at $p < 0.05$. Data visualization and figure generation were performed using Python version 3.8. with the Matplotlib, Seaborn, and Plotly libraries.

3. Results

3.1. MSC levels in culture media

MSC concentration in pooled CM1 used in the study amounted to 0.59± 0.03 µM of (duplicate measurements). This approximately equals half the cation's level in adult human plasma. Pooled MSC-supplemented FBS-free media (CM2, CM3, CM4, CM5, CM6 and CM7) contained 1.25± 0.03, 2.48± 0.05, 3.65± 0.06, 4.81±0.05, 6.06±0.08 and 7.07±0.09 µM Se, respectively. Thus, CM2 contained near normal human plasma Se levels, whereas CM3, CM4, CM5, and CM6 contained Se equivalent to double, triple quadruple, and quintuple plasma levels, respectively.

3.2. Identification of primary and senescent cultures

3.2.1 Growth and Replication Studies.

The growth and replication investigations of the cultures (not presented here for lack of space) were consistent with previous reports on fibroblast cultures [23, 26]. Briefly, present results indicated that CM1-incubated P5 cultures needed 24 hours to acquire maximal rates of L-(U-14C) leucine and (methyl-3H) thymidine incorporation into protein and DNA, respectively, thus reaching confluence. However, P15, P20, P25, and P30 fibroblast subcultures required 48, 96, and 120 hours, respectively, to do so. In addition, identical data was acquired for all cell passages cultured in CM2-CM6. Hence, different passage cells were harvested at the appropriate times after confluence and replenished with fresh CM when cultured beyond 48 hours.

3.2.2 Biochemical oxidative stress markers as indicators of Senescence.

Results indicated that H_2O_2 production levels and GSH/GSSG values did not significantly change in CM1-cultured P5 and P10 fibroblasts (Figure 1). In contrast, there were slight but significant H_2O_2 level rate elevations and similar decreases in P15 fibroblast's GSH/ GSSG ratios compared to P5 cells ($p<0.05$) for both parameters). These changes, however, were highly significant in P20, P25, and P30

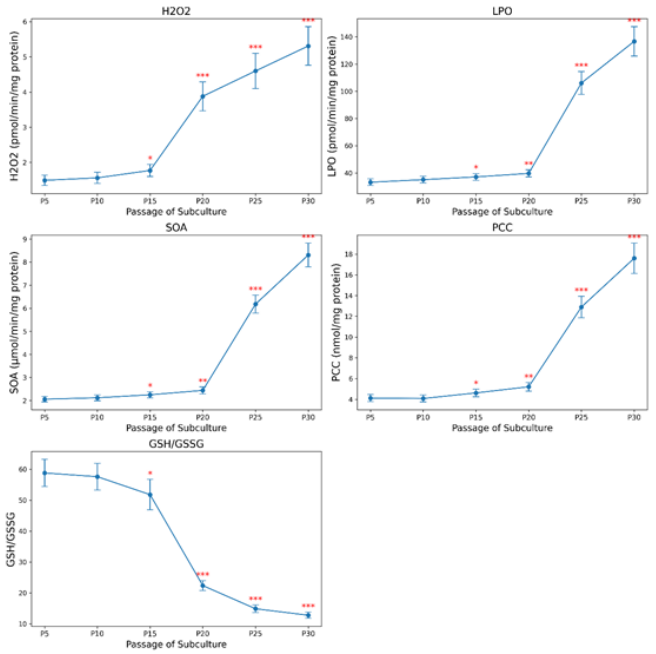

Fig. 1. Biochemical oxidative stress markers in primary and senescent human fibroblasts cultured in standard medium (CM1). Levels of oxidative stress markers in CM1-serially cultured fibroblasts, H_2O_2= hydrogen peroxide, LPO = lipid peroxides, SOA = superoxide anions, PCC = protein carbonyl content, GSH/GSSG = reduced/oxidized glutathione ratio. CM1 Se concentration is indicated in the text. Values shown are means ± SD of duplicate determinations. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$: Comparison of values for P15, P20, P25, and P30 cultures against those at P5. The figure illustrates the changes in various oxidative stress markers over different passages of subculture fibroblasts. Each subplot represents one marker, showing the progression of oxidative stress through passages P5, P10, P15, P20, P25, and P30. Statistical significance is highlighted with corresponding stars. For the interactive figure, visit https://interactive-sciences.github.io/Selenium/.

senescent cells compared to P5 cells ($p<0.001$). Data also showed that although LPO and SOA production rates and PCC levels were not significantly different in CM1-cultured P5 and P10 cells, they were slightly but significantly elevated in P15 cells compared to P5 cells ($p<0.05$). Furthermore, these elevations were more significant in P20 cells ($p<0.001$) and markedly significant in P25 and P30 cells ($p<0.001$) when compared against P5 levels (Figure 1). This data proved that P20, P25, and P30 cells underwent oxidative stress, a characteristic of senescence.

The above growth, replication, and oxidative stress parameter results made it possible to identify P5 cultures as primary cells and those at P20, P25, and P30 as senescent.

3.3. Effect of increased incubation time of CM2 and CM6-incubated primary and senescent cultures on GPx1 activity

It is evident from Figure 2 that incubation of primary P5 fibroblasts with both CM2 (1.25 µM MSC) and CM6 (6.06 µM MSC) required 4 hours to achieve maximal GPx1 activity. In contrast, senescent P20 and P30 cultures required 6 and 8 hours, respectively, to do so. Furthermore, GPx1 activities were similar for CM2 and CM6-incubated P5, P20, and P30 cells for up to 12 hours. Following the above results, cells at all passages throughout the study were harvested after incubation with all media for 10 hours. Additional results revealed that CM6-incubated senescent P20 cells showed a maximal GPx1 activity of 3.92± 0.35 µmole/min/mg protein 6 hours post-incubation. In contrast, senescent P30 cells yielded a much higher activity 8 hours post-incubation with the same medium equal to 6.11± 0.61 µmole/min/mg protein ($p<0.001$). Both these activity values were also very significantly higher compared to those recorded when cells at the same passage were incubated with CM2 for the same periods (2.48± 0.22 and 2.68± 0.26 µmole/min/mg protein for P20 and P30 cells, respectively; $p<0.001$).

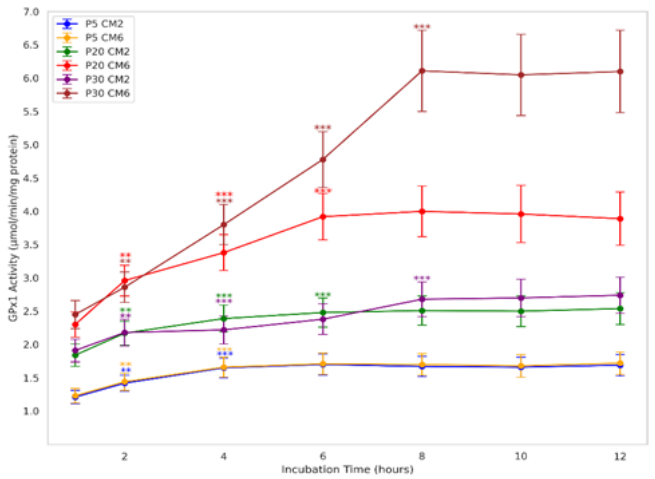

Fig. 2. Time-dependent GPx1 activity in primary (P5) and senescent (P20, P30) fibroblasts incubated with low (CM2) and high (CM6) MSC concentrations. GPx1 activity at increased incubation periods. GPx1 = Cellular glutathione peroxidase. Values shown are means ± SD of duplicate determinations. CM2 contains 1.25 µM MSC, and CM6 contains 6 µM Se. ** = $p < 0.01$, *** = $p < 0.001$: Comparison of GPx1 activities in CM2 and CM6-incubated cells for 2 and 4 hours (at P5), 2, 4, and 6 hours (at P20), and 2, 4, 6, and 8 hours (at P30) against their respective activities at 1 hour. ### = $p < 0.001$: Comparison of GPx1 activities in CM6-incubated P20 and P30 cells for 6 and 8 hours, respectively, against those recorded for the CM2-incubated cells for the same periods. The graph illustrates the changes in GPx1 activity over various incubation periods for primary (P5) and senescent (P20, P25, P30) fibroblast cultures incubated with selenium-supplemented media CM2 and CM6. Significant differences in GPx1 activity are highlighted between different media and cell passages. For the interactive figure, visit https://interactive-sciences.github.io/Selenium/.

3.4. Effect of increasing MSC increments on GPx1 activity in P5, P20, P25 and P30 cultures

Figure 3 results indicated no statistically significant changes occurred in GPx1 activity in P5, P10, and P15 fibroblasts regardless of the MSC concentration of the incubation medium. However, in senescent P20 cultures, GPx1 underwent highly significant and progressive activity increases when cells were incubated in each of CM2-CM6 compared against CM1 ($p<0.001$). Furthermore, GPx1 activity peaked in CM5-cultured cells and was maintained at similar values in those cultured in CM6. Additionally, data indicated a highly significant elevation in GPx1 activity in CM3-incubated P20 cells compared to CM2 ($p<0.001$). Such increases became gradually lower in magnitude and less significant in CM4-incubated cells compared to CM3 and in those incubated in CM5 against CM4 ($p<0.01$, $p<0.05$ respectively). Results also indicated that for CM2-incubated P25 and P30 cells, there were significant increases in GPx1 activities when compared to those incubated with CM1 ($p<0.01$). However, such increases became more pronounced and significant in both

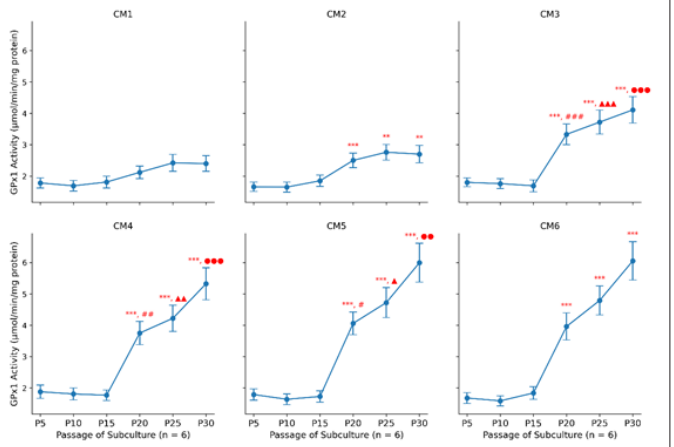

Fig. 3. Dose-dependent effects of Se-Methylselenocysteine supplementation on GPx1 activity across progressive fibroblast passages (P5-P30). Effect of MSC supplementation on GPx1 activity in P5, P10, P15, P20, P25, and P30 cultures. GPx1 = Cellular glutathione peroxidase. Values shown are means ± SD of duplicate determinations. CM1 - CM6 Se content as documented in the text. *** = $p < 0.001$, ** = $p < 0.01$. Comparison of GPX1 activities in P20, P25, and P30 cells incubated with CM2-CM6 against those incubated with CM1. ###, ▲▲▲ = $p < 0.001$, ##, ▲▲ = $p < 0.01$, #, ▲ = $p < 0.05$. Comparison of GPX1 activities in P20 and P25 cultures incubated in CM3 against CM2, CM4 against CM3, and CM5 against CM4 respectively. ●●● = $p < 0.001$, ●● = $p < 0.01$. Comparison of GPx1 activities in P30 cultures incubated in CM3 against CM2, CM4 against CM3, and CM5 against CM4. Comparisons between CM6 and CM5-incubated P20, P25, and P30 cells were insignificant. For the interactive figure, visit https://interactive-sciences.github.io/Selenium/.

cultures incubated in CM3- CM6 against CM1 ($p<0.001$). Furthermore, as observed for P20 cells, GPx1 activity elevations in P25 and P30 cells were gradual, proportional to the Se concentration of the incubation medium, and peaked at similar magnitudes in CM5 and CM6-incubated fibroblasts. Moreover, much higher GPx1 activities were achieved in CM5 and CM6-incubated P30 cells compared to P25 cells, which were much higher than those recorded in P20 cells incubated in the same media. For example, activities in CM5- incubated P30, P25, and P20 cells equaled 5.99± 0.62, 4.72± 0.48, and 4.06± 0.36 µmoles/min/mg protein respectively ($p<0.001$ upon comparisons between activities). In percentage terms, such increases amounted to 250% in P30 cells compared to only 190% in P20 cells when both cultures were incubated with CM1. This suggested that increasing the MSC concentration enhanced GPx1 activity, which increased as the cultures passed from P20 to P25 and P30.

3.5. Effect of increasing MSC increments on H_2O_2 production in P5, P20, P25, and P30 cultures

It is evident from Figure 4 results that the rate of H_2O_2 production did not change in P5 and P10 fibroblasts regardless of the MSC-supplemented medium compared to those cultured in routine CM1. In CM5 and CM6-incubated P15 cells, however, there were moderate but significant decreases in H_2O_2 generation compared to those cultured in each of CM1– CM4 ($p<0.01$). No significant changes were recorded for CM4, CM3, and CM2-incubated P15 cells against those incubated with CM1. Figure 4 data also showed moderate but significant decreases in CM3-incubated P20 cultures compared against CM2 ($p<0.05$). However, such reductions became significant in the CM4-6 incubated cultures ($p<0.01$). In addition, although there was a markedly significant drop in H_2O_2 generation in CM4- incubated P20 cells against those incubated in CM3 ($p<0.001$), this drop was less significant in CM5-incubated cells compared to those incubated in CM4 ($p<0.05$), and not significant in those that were CM6- incubated against CM5 or CM3 against CM2. Figure 4 results also showed that both CM3- P25 and P30 cells underwent significant decreases in H_2O_2 generation compared to CM1-incubated cells ($p<0.01$) and highly significant decreases in each of those incubated with CM4 – CM6 ($p<0.001$). Furthermore, although such decreases were moderate and slightly significant in CM3-incubated P25 and P30 cells against those incubated in CM2 ($p<0.05$), they became highly significant in cells incubated in CM4 against CM3 and CM5 against CM4 ($p<0.001$), and not significant between those incubated in CM6 against CM5. All the above H_2O_2 generation rate reductions were progressive, higher in magnitude, proportional to the MSC concentration, and reached the highest values in CM5 and CM6-incubated P25 and P30 cells. The above reductions amounted to approximately 11.5%, 40%, 57% and 58% in P30 CM3, CM4, CM5 and CM6-incubated cells respectively when compared to CM1-incubated cells. Furthermore, the above percentage reduction values in CM5 and CM6-incubated senescent P25 and P30 cells were significantly higher when compared to those reported for P20 fibroblasts incubated with the same media (~ 48% and 49% in CM5 and CM6-incubated P20 cells respectively).

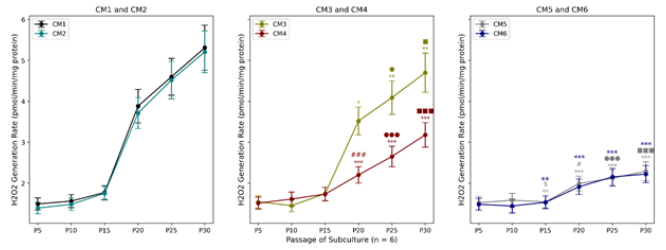

Fig. 4. Reduction of hydrogen peroxide production in primary and senescent fibroblasts following treatment with increasing MSC concentrations. Effect of MSC supplementation on H_2O_2 generation rates in different passage stages of fibroblasts. H_2O_2 = Hydrogen Peroxide. Values are presented as means ± SD of duplicate determinations. The MSC concentrations in CM1 - CM6 are as documented in the text. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. Comparison of H_2O_2 generation rates in P20, P25, and P30 cells incubated with CM2 - CM6 against those incubated with CM1. $ = p <0.01: Comparison of H_2O_2 generation rates in CM5 and CM6 P15 cells against CM4-incubated cells. # = $p < 0.05$, ### = $p < 0.001$. Comparison of H_2O_2 generation rates in P20 cells incubated in CM5 against CM4, and CM4 against CM3. ●,■ = $p < 0.05$, ●●●,■■■ = $p < 0.001$. Comparison of H_2O_2 generation rates in P25 and P30 cells incubated in CM3 against CM2, CM4 against CM3, and CM5 against CM4. Comparisons between CM6 and CM5-incubated P15, P20, P25, and P30 cells were not significant. For the interactive figure, visit https://interactive-sciences.github.io/Selenium/.

3.6. Effect of increasing MSC increments on *GPx1* gene expression in P5, P20, P25 and P30 cultures

It is clear from Figure 5 that *GPx1* gene expression levels did not undergo any significant changes when P5 fibroblasts were incubated in CM2, CM4, or CM6. However, in P20 cultures, *GPx1* gene expression levels underwent marked upregulation (fold change values equaled 1.51± 0.12, 1.99± 0.16, and 2.31± 0.19 when fibroblasts were incubated in CM2, CM4, and CM6 respectively relative to P5 cells incubated in the same media ($P<0.001$). Percentage upregulations amounted to 50.0± 3.68%, 89.5± 7.11%,

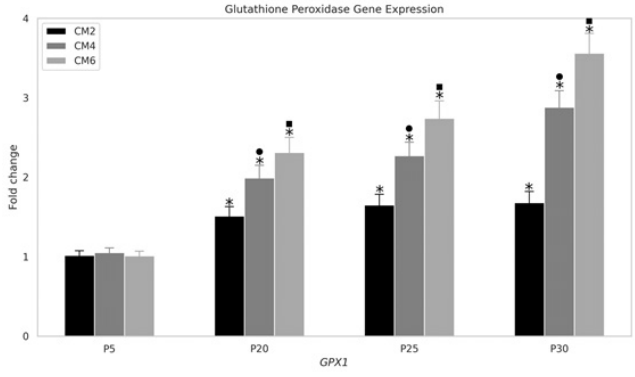

Fig. 5. Differential upregulation of *GPx1* gene expression in primary versus senescent fibroblasts in response to graduated MSC supplementation. Gene expression of *GPx1* in primary (P5) and senescent (P20, P25, and P30) fibroblasts cultured in CM2, CM4, and CM6. GPx1 = glutathione peroxidase 1. Values shown are means ± SD for duplicates of 6 cultures. CM2, CM4, and CM6 MSC contents are documented in the text. * = $p< 0.001$. Comparison of gene expression levels in P20, P25, and P30 cells cultured in CM2, CM4, and CM6 against those reported for P5 cells cultured in the same media. ●, ■ = $p< 0.001$. Comparison of expression levels of *GPx1* in P20, P25, and P30 cells cultured in CM4 against CM2 and those cultured in CM6 against CM4.

and 126.5± 9.74% in CM2, CM4, and CM6-incubated cells, respectively, compared against P5 cells incubated in the same media ($p<0.001$). In CM2, CM4, and CM6- incubated senescent P25 fibroblasts, results showed that the enzyme transcripts underwent even higher upregulation, equaling 64.3± 4.76%, 114.3± 9.00% and 168.6± 13.7%, respectively, relative to primary P5 cells cultured in the same media ($p<0.001$). Moreover, results indicated that the highest percentage upregulation of GPx1 transcripts was achieved in P30 senescent cells incubated with CM2, CM4, and CM6, where values equaled 66.3± 4.84%, 172.4± 13.7%, and 248.0± 18.6% respectively compared to P5 cells incubated with the same media. Further analysis of Figure 5 data indicated that fold change values and percentage upregulation in *GPx1* gene expression of P20, P25, and P30 cells were very significantly higher when cells were incubated in CM4 against CM2 and in CM6 against CM4 ($P<0.001$).

3.7. GPx1 MSC-Km values in senescent P20, P25 and P30 cultures

Michaelis-Menten Curves and Hanes-Woolf Plots were constructed using Microsoft® Excel software and Figure 3 data for each P20, P25, and P30 cell cultures. The computed results as indicated below in Figures 6, 7, and 8 indicated that the Se-Km values for P20, P25, and P30 cells were 0.87, 1.13, and 1.92 µM, respectively. The corresponding Vmax computed values for GPx1 activity were 4.59, 5.68, and 7.94 µmole/min/mg protein, respectively.

4. Discussion

Selenium (Se) is a crucial micronutrient, serving as a

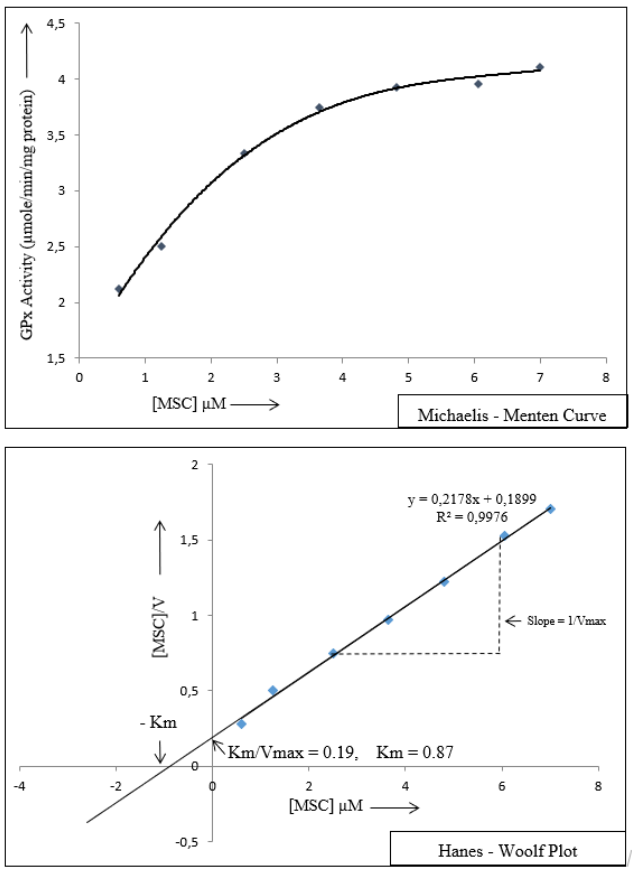

Fig. 6. Michaelis-Menten kinetics and Hanes-Woolf plot analysis of MSC-dependent GPx1 activity in early senescent (P20) fibroblasts (Se-Km = 0.87 µM).

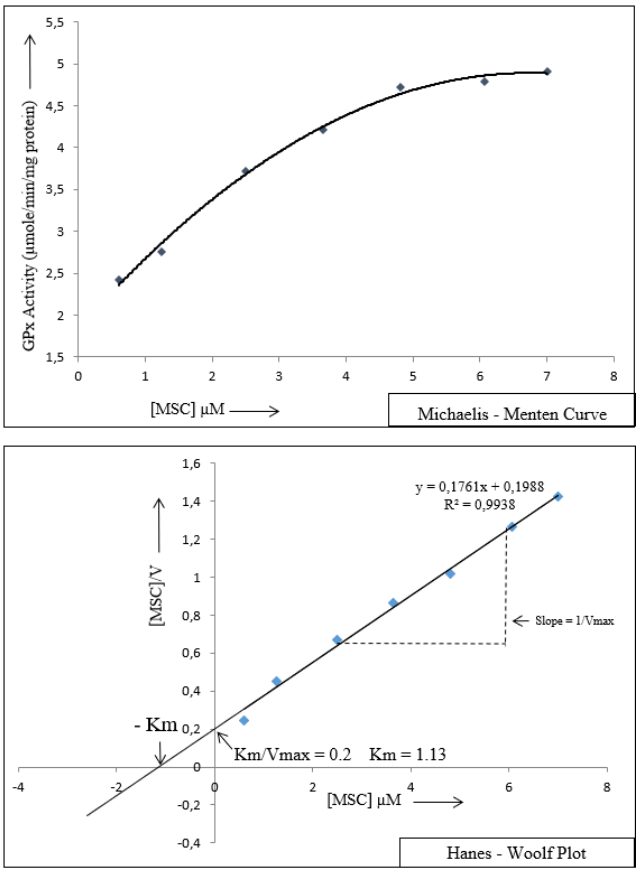

Fig. 7. Michaelis-Menten kinetics and Hanes-Woolf plot analysis of MSC-dependent GPx1 activity in mid-senescent (P25) fibroblasts (Se-Km = 1.13 µM).

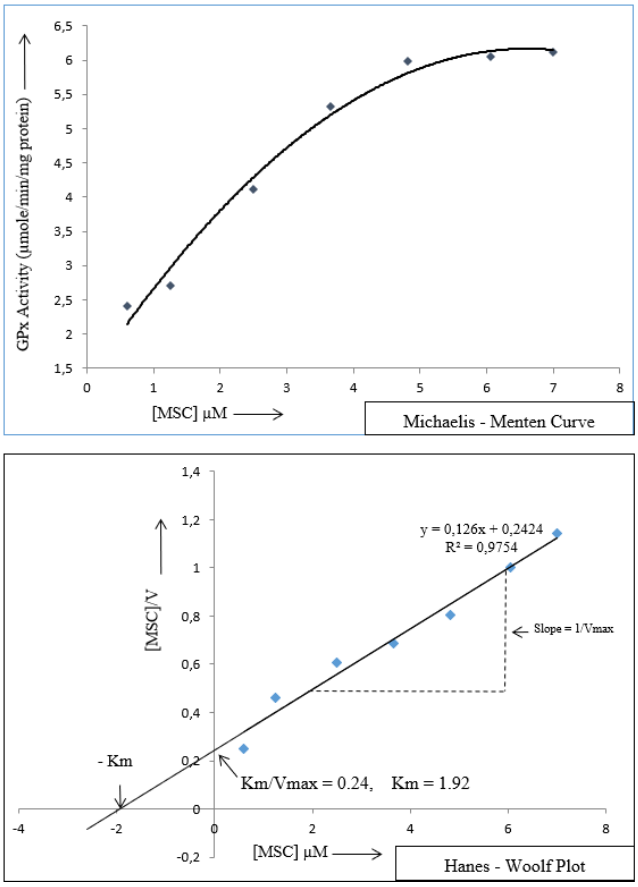

Fig. 8. Michaelis-Menten kinetics and Hanes-Woolf plot analysis of MSC-dependent GPx1 activity in late senescent (P30) fibroblasts (Se-Km = 1.92 µM).

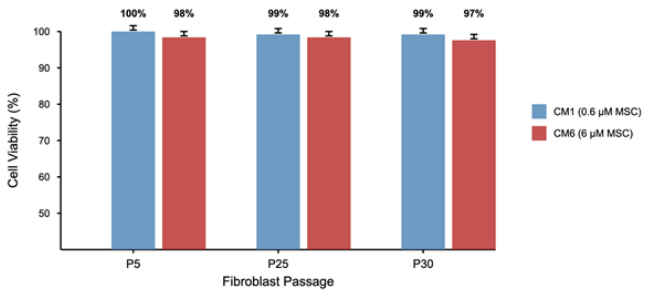

Fig. 9. Cell viability assessment of primary (P5) and senescent (P25, P30) fibroblasts.

cofactor for several antioxidant enzymes, and its role in cellular homeostasis has long been recognized. Despite the established significance of Se in cellular processes, the relationship between Se levels, selenoprotein expression, and aging remains a contentious topic. The interplay between Se and oxidative stress (OS) forms the crux of this debate, given that OS is both a cause and consequence of cellular aging.

Thus, investigating whether or not Se deficiency constitutes a risk factor for aging and consequently many aging-related disorders is of utmost importance to devise novel anti-aging therapeutic approaches. Furthermore, it is also important to establish if and how Se deficiency influences the transcriptional activity of selenoprotein genes, which may be identified as a possible cause for aging. In this study, we sought to answer these questions using serially subcultured human fibroblasts as an in vitro model of aging.

This study aimed to elucidate whether MSC deficiency is a risk factor for aging and its associated disorders and to understand how MSC influences selenoprotein gene transcription. Using serially subcultured human fibroblasts as an in vitro aging model, we have provided compelling evidence linking Se supplementation with enhanced antioxidant defense mechanisms in senescent cells. Human fibroblasts, a robust model for studying cellular aging, typically exhibit finite lifespans characterized by approximately 40 population doublings [6, 33, 34]. The replicative senescence of cultured cells has been shown to reflect many aspects of organismal aging, most notably the increase in senescent cells with age. In our laboratory, we have extensively employed this model to investigate aging-related metabolic alterations and the decline in antioxidant capacity [23-27, 33, 35]. Human fibroblasts are a successful model that has been extensively used in our laboratory to study cellular metabolic changes associated with the decline of the antioxidant capacity with age [23–27,35]. Skin fibroblast cultures typically have a finite lifetime of around 40 population doublings (Hayflick limit). The use of such cultures in the present study is advantageous since it was possible to cultivate cells in standard culture medium (CM1) in which FBS is the only source of Se at approximately half the metalloid concentration in human plasma. This also permitted incubating the subcultures with FBS-free media (CM2, CM3, CM4, CM5 and CM6) supplemented with increasing MSC concentrations up to 6x its normal human plasma levels. The MSC concentration in CM1 is lower than that of human plasma [18], which ensures structural stability of GPx1 and its activity. Se also enhances proliferation of serial subcultures, especially at senescence since it protects against reactive oxygen species (ROS) overproduction. ROS accumulation aggravates apoptosis and damages proteins, carbohydrates and nucleic acids [41,42]. Along those lines, Se supplementation of the medium has been shown to selectively modulate selenoprotein expression and increase population doublings, whereas low levels of the metalloid suppressed cell proliferation in culture [9]. One limitation in this study was the use of high, potentially toxic concentrations of Se (up to 6 µM). Although our data regarding protein and DNA synthesis showed gradually lowering rates of growth and replication with each subsequent passage, the percentage of cell death was consistently low (2%–6%) regardless of passage number or Se concentration. This has been currently investigated using the MTT assay, as shown in Figure 9. However, around 40 population doublings, cells became rounded, enlarged and ruptured independent of Se content. Significant loss of viability was observed at 9 µM Se, highlighting its cytotoxic potential.

Additionally, replicative senescence of serially cultured fibroblasts has been associated with a number of morphological and biochemical changes. These include accumulation of oxidized proteins, telomere shortening, cell enlargement, flattened cellular morphology, decreased growth and replication rates, elevated production of oxidative stress markers and an increase in the number of cells showing ß-galactosidase activity [23,24,26,27,35]. Moreover, such changes have been associated with a decrease in antioxidant defense mechanisms [18]. In the present study, care was taken to identify the culture passage at which senescence begins and continues. Data showed that CM1 P5- and P10-cultured cells needed 24 h to reach confluence and maximal rates of radiolabeled leucine and thymidine incorporation into protein and DNA. Additionally, P15, P20 and P25 and P30 subcultures required 48, 96 and 120 h, respectively. Hence, cells at different passages were harvested at such times post-culture. Harvesting times were identical with those obtained by us when using human skin fibroblasts in previous studies [23,24,26]. The above findings showed that whereas P5 and P10 cells were primary (pre-senescent), those at P20 were at early senescence, and those at P25 and P30 were senescent. Execution of the above findings ensured that protein and DNA yields of the cells could not be affected by senescence and that changes in GPx1 activity are attributed to senescence. Total protein and DNA yields of all investigated subcultures came to 119.8 ± 16.2 µg/100 µL and 8.12 ± 0.94 µg/100 µL, respectively, with protein/ DNA ratios of 14.6 ± 1.31 unaffected by the Se concentration of the medium. In addition, results of the MTT cell viability assay exhibited non-significant cytotoxicity (2%–4%) for all subcultures regardless of the Se concentration of the medium. Results in Figure 1 showed a very significant, progressive increase in oxidative stress markers (H2O2, LPO, SOA and PCC), all of which peaked at around 400% in P30 cells compared against P5 cells. Noted too was the very significant decrease in GSH/GSSG ratios reaching lowest levels in P30 cells compared to P5 cells. These findings provided further evidence that P5 and P10 fibroblast cultures were primary, those at P20 were early senescent whereas those at P25 and P30 were senescent.

The in vivo relationship between Se levels and aging is not well established because of the paucity in relevant data. In one study, subjects over 60 years of age showed a drop in plasma GPx1 activity and Se levels [19], whereas

two others suggested that blood Se could be used as a predictor of longevity [20,21]. Conversely, supplementing growth medium with Se enhanced GPx1 activity, protected against oxidative injury by lipid hydroperoxides and promoted calcium signaling in endothelial cells [36,37]. In another study, selenite and selenometionine augmented the antioxidant response of trophoblasts from the placenta of pre-eclamptic patients, at least in part by upregulating GPx1 gene expression [38]. One more study demonstrated that Se-supplemented medium significantly improved the antioxidant capacity of oxidatively stressed bone marrow stromal cells [39]. Results of the present study were in general agreement with the above findings. However, what characterizes our study is that cells at different passages of culture were incubated with media containing increasing Se concentrations. This allowed for detailed results and made possible the calculation of Se-Km values for achieving maximal GPx1 activity in both early senescent (P20) and senescent (P25 and P30) fibroblasts, which was not reported elsewhere. Data in Figure 3 showed very similar GPx1 activity in CM1-cultured primary P5 and P10 cells indicating that 0.59 μM Se was sufficient for attaining normal activity of the enzyme in primary cells. However, slight elevations in GPx1 activity were noted in CM1-cultured senescent P20, P25 and P30 cells compared to primary P5 cells. These moderate activity increases could have been an outcome of senescence where GPx1 activity increased to neutralize the high H_2O_2 generation rates observed for P20, P25 and P30 cultures (Figure 1 and 4), and could also be associated with the relatively low Se concentration in CM1. Concurrent with this, Figure 3 data demonstrated marked gradual increases in GPx1 activity in senescent P25 and P30 cells incubated with CM2–CM6 supplemented with increasing Se increments, which was not observed in CM1-incubated fibroblasts. Furthermore, these increases were proportional to the Se concentration, and reached maximal values in CM5- (4.8 μM MSC) and CM6 (6.0 μM MSC)-incubated senescent cells. These observations suggested that senescent cells needed to quench oxidative stress by utilizing higher Se amounts for stimulation of GPx1 activity. More results (Figure 4) showed that CM1-cultured senescent P20, P25 and P30 cells produced around 2.6, 3.1 and 3.5 times more H_2O_2 compared against CM1-cultured primary P5 cells. Moreover, such rates were markedly and gradually reduced when the cells were incubated with media containing increased Se concentrations, and reached lowest levels in P25 and P30 cells incubated in CM5 and CM6. These results further indicated that senescent cells are more combative against oxidative stress when incubated with media of higher Se concentration.

In congruence with the enhanced GPx1 activity in senescent P20, P25 and P30 fibroblasts, we found (Figure 5) significant upregulation in GPx1 gene expression upon incubation in media containing supplemental Se. To this end, previous studies reported increases in GPx1 transcripts in oxidatively stressed human endothelial cells [36,37], bone marrow stromal cells [40] and placental trophoblasts of pre-eclamptic patients [38], all of which were cultured in media containing supplemental Se. The current study additionally showed that Se increases GPx1 expression in a dose-dependent manner (CM2, 4 and 6), and that GPx1 transcriptional activity was progressively augmented in senescent P25 and P30 cells compared to early senescent P20 cells incubated in the same media (Figure 5). Furthermore, we showed that Se positively influences GPx1 at both the transcriptional and translational levels (Figure 5). These findings predict that the oxidatively stressed P20, P25 and P30 cells were able to combat oxidative injury through upregulation of GPx1 expression levels, thereby maximizing the enzyme's catalytic activity when incubated in CM5 (4.8 μM Se) and CM6 (6.0 μM Se).

Although cellular Se uptake is not a central issue of the present study, Figure 2 results showed that primary P5 cells incubated in CM2 and CM6 required 4 h to reach maximal GPx1 activity, whereas senescent P20 cells required 6 h and senescent P25 and P30 cells required 8 h. Moreover, maximal enzyme activities were of very similar magnitude when the cells were incubated for up to 12 h in the same media. In addition, Figure 2 data showed that P20 and P30 cells incubated with 6 μM Se for 6 and 8 h, respectively, exhibited much higher GPx1 activities than those incubated with 1.25 μM MSC for the same time periods. All the above results seem to indicate that MSC uptake by cultured fibroblasts is time- and dose-dependent. These findings strongly suggest uptake and utilization of MSC is significantly increased in P20 fibroblasts and reach maximal levels in those at P25 and P30. In this context, results of the present study (Figures 6–8) reported increasingly higher MSC-Km values and corresponding GPx1 Vmax activities as cells moved from early senescence (P20) to senescence (P30). The calculated Km values of MSC with respect to GPx1 activity in P20, P25 and P30 fibroblasts were 0.87, 1.13 and 1.92 μM, respectively, with computed Vmax corresponding activities of 4.59, 5.68 and 7.94 μmol/min/mg protein.

In conclusion, the present study demonstrates that senescent fibroblasts require and utilize higher concentrations of Se-Methylselenocysteine to upregulate *GPx1* gene expression and maximize the enzyme's catalytic activity compared to primary cells. Our findings reveal progressively increasing MSC-Km values (0.87, 1.13, and 1.92 μM) and corresponding Vmax values (4.59, 5.68, and 7.94 μmole/min/mg protein) in senescent P20, P25, and P30 cells, respectively. This novel observation suggests an age-dependent shift in selenium requirements at the cellular level. Importantly, MSC supplementation effectively mitigated oxidative stress in senescent cells, as evidenced by significant reductions in hydrogen peroxide levels and other oxidative stress markers. The dose-dependent nature of *GPx1* upregulation and the enhanced transcriptional responsiveness to MSC with advancing senescence suggest that the molecular machinery regulating selenoprotein expression undergoes age-related modifications. These findings provide a mechanistic basis for considering personalized selenium supplementation strategies in older individuals to combat oxidative stress and potentially delay the onset of age-related cellular dysfunction. Future studies should focus on elucidating the precise molecular mechanisms underlying MSC's protective effects and exploring their translational implications for aging populations. We envisage future studies related to our work to include an in vivo study aim different age groups of male and female volunteers. Such a study should include sufficient numbers and candidates to allow for valid statistical analysis.

Conflicts of interest

None.

Consent for publications
The author read and approved the final manuscript for publication.

Ethics approval and consent to participate
No human or animals were used in the present research.

Informed consent
The authors declare that no patients were used in this study.

Availability of data and material
The data that support the findings of this study are available from the corresponding author upon reasonable request

Authors' contributions
Conceptualization, YAA, HKG; methodology, YAA, HKG, MA; validation, HKG, MA; formal analysis, YAA, HKG, FA; investigation, YAA, HKG, AMA; resources, YAA, HKG; writing—original draft preparation, YAA; writing—review and editing, FA, AMA, RF; visualization, FA, RF, SA, AMA; supervision, YAA, HKG; project administration, YAA; funding acquisition, YAA, HKG.

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