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The kinetics of the effect of manganese supplementation on SOD2 activity in senescent human fibroblasts

H.K. GHNEIM

Chair of Medical and Molecular Genetics Research, Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, King Saud University, Riyadh, Saudi Arabia.

Abstract. – OBJECTIVE: To investigate the effect of increasing Mn^{+2} concentrations on superoxide dismutase 2 (SOD2) activity in pre-senescent and senescent cultured fibroblasts, and to determine the Km Mn^{+2} values required to achieve maximal SOD2 activities in such cells.

MATERIALS AND METHODS: SOD2 activities, and superoxide anion (SOA) generation rates, were assayed in mitochondrial sonicates of young passage 5 fibroblasts sub-cultured in routine growth medium (MEM 1), and in an accurately identified senescent passage 20, 25 and 30 subcultures incubated with media containing supplemental Mn^{+2} increments equal to 60, 90, 120, 150 and 180 nM (MEM 2, 3, 4, 5 and 6 respectively).

RESULTS: Whereas SOD2 activity did not significantly change in any of the cells sub-cultured in MEM 1, the enzyme underwent progressive significant increases in early senescent passage 20 cells and senescent passage 25 and 30 cells. Such increases were relative to Mn^{+2} concentration and peaked in value in the senescent cells incubated with MEM 5 and MEM 6. Furthermore, whereas SOA generation rates underwent significant progressive increases in MEM 1-incubated senescent passage 20-30 cells, peaking in value at passage 30, the rates were gradually and significantly lowered in the cells incubated with MEM 2-MEM 6, and reached lowest values in those incubated with MEM 6 ($p<0.001$ for all comparisons). The computed Km values of Mn^{+2} with respect to SOD2 in senescent passage 20, 25 and 30 cells equalled 19.2, 39.6 and 54.4 nM respectively with corresponding SOD2 Vmax values of 37.6, 55.9 and 71.4 μmol/min/mg protein.

CONCLUSIONS: Senescent cells near the end of their replicative life span utilise more Mn^{+2} and achieve maximal SOD2 activities suggesting that the use of supplementary Mn^{+2} can help in combating oxidative stress.

Key Words:

Superoxide dismutase 2, Senescence, Superoxide anions, Manganese supplementation, Fibroblasts.

Introduction

The oxidative stress (OS) theory, is considered to be a feasible concept that clarifies the molecular mechanisms associated with the aging process. Production of reactive oxygen species (ROS), is a result of normal cellular oxidative metabolism mainly generated by the complexes of the mitochondrial respiratory chain[1-3], and other enzymes and pathways including dihydrolipoamide dehydrogenase in the α-ketoglutarate dehydrogenase complex, NADPH oxidase, xanthine oxidase, monoamine oxidase and cytochrome P450 proteins[4-10]. Whereas base-line levels of ROS are essential for redox signalling and cell survival[11], elevated levels are considered to execute damaging effects and contribute to aging and the pathogenesis of many age-related diseases[12]. It has been shown that a mild level of OS, can result in cellular responses useful for cell survival[11-13]. Nevertheless, OS characterised by significant increases in ROS generation causes indiscriminate alteration and destruction of the structure of macromolecules including DNA, proteins and lipids[14]. This can lead to cytotoxicity and cell death[11,13] which have been related to an alteration in the expression of genes including those associated with apoptosis[15].

To counteract the harmful oxidative effects of excessive ROS generation and limit the cellular damage, DNA lesions are repaired by several pathways including base and nucleotide excision, double-strand break and mismatch repair[16,17]. In addition, cells have evolved several antioxidant enzymes which act to neutralizes ROS. Superoxide anions are converted to non- reactive hydrogen peroxide by Cu/Zn – superoxide dismutase in the cytosol and Mn-superoxide dismutase in the mitochondria. Diffused hydrogen peroxide

Corresponding Author: Hazem K. Ghneim, Ph.D; e-mail: hghneim@hotmail.com

into the cytosol is, then, transferred to water by activities of glutathione peroxidase, catalase and thioredoxin peroxidase[18,19]. Cells also combat excessive ROS generation using non-enzymatic antioxidants including ascorbate, α-tocopherol, reduced glutathione, carotenoids, flavonoids, selenium, copper, zinc and manganese[19-22].

Superoxide anions (SOA), are considered to be the first step in the formation of ROS and precursor of most of these species. The bulk of SOA is generated as a result of electron leakage from the mitochondrial respiratory chain which reacts directly with oxygen[3]. Antioxidant defence of aerobic cells begins by dismutation of SOA through the activity of superoxide dismutase (SOD), which is considered to play a crucial first line defence against oxidant generation. There are three forms of SOD in mammals two of which (SOD1 and SOD3), have copper and zinc in their catalytic center and are located in cytoplasmic compartments (SOD1) or extracellularly (SOD3)[23]. A third SOD (SOD2), is mitochondrial and the human enzyme has manganese (Mn) in its reactive centre and active site, which functions as a metal cofactor. The enzyme is a nuclear-encoded polypeptide that must be transported to the mitochondrial matrix. The biologically active enzyme is a homotetramer with each subunit housing a single Mn^{+2} cofactor coordinated to amino acids Hist 107, Hist 198 and Asp 194[24]. SOD2 acts to scavenge toxic SOA and, thus, allows clearance of mitochondrial ROS and provides protection against cell death[25]. It lies in the vicinity of the SOA-generating mitochondrial respiratory chain and its deficiency has serious effects. The enzyme exhibits an anti-apoptic role against OS, ionizing radiation and inflammatory cytokines[26], and is an important constituent in apoptotic signaling of the mitochondrial death pathway and cardiac myocyte apoptosis thus making it play a cardioprotective role[27,28]. Its role in controlling ROS levels involves SOD2 in aging, cancer and neurodegenerative disease[29,30].

Replicative senescence is a process in which cells go into an irreversible growth arrest, and a finite number of cell divisions which contributes to organismal aging[31,32]. Studies including ours have shown that senescent cells, including fibroblasts, are associated with high levels of intracellular ROS and oxidative damage to DNA and proteins[20,33-35]. In addition, it has been reported that whereas increasing fibroblast oxidant levels by lowering antioxidant activity or altering oxygen concentration, can accelerate the onset of cellular senescence, increasing ROS scavenging can delay senescence and prolong life span[19,20,34-28]. To this end, it has been shown that transgenic *Drosophila melanogaster* overexpressing either SOD1[39], or SOD2[40,41], have extended life span by 20%. Studies have also demonstrated that fibroblast cell cultures established from transgenic mice where several antioxidant enzymes including SOD1, SOD2 and catalase were overexpressed had higher resistance against OS and lowered levels of oxidatively damaged macromolecules[30,42,43]. In the context of relating OS to senescence, we showed that proliferative serially subcultured senescent human fibroblasts, exhibited highly significant increases in SOD1 and SOD2 activities when the cells were subcultured in Cu, Zn and Mn supplemented media containing triple human plasma levels of cations[19]. Results also indicated that such enzyme activity increases significantly lowered the concurrent elevation in SOA generation observed in the senescent cells, and allowed extension of their life span by 4-6 population doublings. As a follow-up, the current work was undertaken to establish the optimal Km Mn^{+2} concentrations and incubation time, required to achieve maximal stimulation of mitochondrial SOD2 activities in senescent fibroblast at passages 20,25 and 30 of subculture incubated with media containing increasing supplemental Mn^{+2} increments.

Materials and Methods

Preparation of Human Fibroblast Cultures

Primary human skin fibroblast cultures were established from eight forearm skin biopsies (~20 mg in weight) of normal adults volunteers with an average age of 21.1 ± 0.80 years (range, 19.8-22.3). Monolayer confluent cultures and sub-cultures were obtained by growing cells in routine Eagle's Minimum Essential Medium (MEM 1) containing 10% fetal calf serum and harvested by trypsinisation. Details regarding the culture process, contents and preparation of MEM 1 and the trypsinisation and harvesting media, are described by us elsewhere[19,20]. Eagle's MEM 1, culture flasks and all other culture reagents were purchased from Flow Laboratories (Mclean, VA, USA). Fibroblasts were cultured in a Gelaire BSB4 Lam-

inar-Flow cabinet at 37 °C in an atmosphere containing 18% O_2 and 5% CO_2.

Preparation and Identification of Senescent Fibroblast Cultures

Senescent fibroblast cultures were obtained by serial subculture of eight primary passage 1 cultures cultivated in MEM 1. Cells were sub-cultured up to passage 30 in 75 cm² flasks and those at passage 5, 10, 15, 20, 25 and 30 were examined for their growth and replication states and the activities of key glucose and glycogen degradative enzymes including phosphofructokinase, lactate dehydrogenase and glycogen phosphorylase in order to identify those that are senescent. In these studies fibroblasts were sub-cultured in MEM 1 containing 10% fetal calf serum as the only source of manganese (Mn^{+2}), and each passage number represented one population doubling. The growth and replication state of the cells were determined by investigating the rates of incorporation of radiolabeled leucine and thymidine into protein and DNA respectively. Details regarding this and the assays of the above-mentioned enzymes are documented by us elsewhere[20].

Preparation of Manganese Supplemented Culture Media and Experimental Design

The only source of manganese in routine MEM 1 is fetal calf serum and thus, a serum-free medium will be devoid of the trace element. In the present study, a set of serum free media (MEM 2, 3, 4, 5 and 6), were prepared by adding supplemental amounts of analytical grade manganese chloride dissolved in sterile deionized water to give final Mn^{+2} concentrations equal to 60, 90, 120, 150 and 180 nM respectively. To study the effect of increasing Mn^{+2} concentrations on SOD2 activity, routine growth MEM 1 from duplicate 75 cm^2 flasks of the eight confluent fibroblast subcultures at passages 5, 20, 25 and 30 was removed, and cells were incubated with MEM 2, 3, 4, 5 and 6 for ten hours. This incubation period was chosen based on the linearity studies of SOD2 activities in fibroblasts at different passages of subculture incubated with MEM 2 and MEM 6 for different periods of time (see results section). At the end of the ten-hour incubation period, Mn^{+2} supplemented media were removed and the cells were rinsed with phosphate buffered saline (3 ml, pH7.4), harvested and pelleted by centrifugation at 200 g for 3 minutes. Pellets were then used to prepare mitochondrial sonicates for the assay of SOD2 activity and SOA generation rates as described later.

Linearity of SOD2 Activities in Fibroblast Subcultures at Passages 5, 20, 25 and 30 Incubated with MEM 2 and MEM 6 with Respect to Time

To ensure maximum SOD2 activity with respect to incubation time, spent routine MEM 1 from confluent fibroblast cultures at passages 5, 20, 25 and 30 was replaced with serum-free MEM 2 and MEM 6 supplemented with Mn^{+2} at 60 and 180 nM respectively. The use of MEM 6 containing the highest levels of Mn^{+2} is justified, since it seemed to produce a maximal stimulatory effect on SOD2 activities in cells at passage 20, 25 and 30 (see results section). Duplicate flasks of the various subcultures were incubated with both media for 1, 2, 4, 6, 8, 10, 12 and 14 hours, at the end of which mitochondrial sonicates were used to measure SOD2 activities.

Preparation of Mitochondrial Fibroblast Sonicates

Pooled cells pellets from twelve flasks of passage 5, 20, 25 and 30 cells sub-cultured in MEM 1 and replaced later with Mn^{2+}-supplemented MEM 2-MEM 6, were homogenized using a Potter homogeniser (8-10 strokes) in 8 ml of 0.3 M mannitol solution containing 1mM EDTA and 10 mM Hepes buffer (pH 7.2). The homogenates were then centrifuged at 2000 rpm to 7 x 10^6 g/min. Supernatants were kept and unclear debris resuspended in mannitol (4 ml) and centrifuged as indicated above. The supernatants (4+ 8 ml) were then combined and centrifuged at 10,000 rpm to 2.95x10^8 g/min, and the mitochondrial pellets collected. Pellets were then sonicated for 15 seconds in ice using a Fisher Sonic Dismembrator Model 150 in 0.1 M phosphate buffer (pH 7) and appropriate aliquots were used to measure SOD2 activities and SOA generation rates.

Measurement of SOD2 Activity

SOD2 activity was assayed according to de Hann et al[44] and as modified by us[19]. Mitochondrial fibroblast sonicates (100 µl), were added to xanthine (25 µl, 1.142 mg/ml), hydroxyl ammonium chloride (25 µl), water (125 µl) and xanthine oxidase (75 µl, 0.1 U/ml). The mixture was then incubated at 25 °C for 20 mins, and sulphanilic acid (0.5 ml, 3.3 mg/ml) and α-naphthylamine (0.5 ml, 1 ng/ ml) were added and further incubated at room temperature for 20 mins and absor-

bance read at 530 nm. The addition of potassium cyanide (125 µl, 4 mM) in place of water specifically inhibits SOD1 activity and represents SOD2 activity.

SOA Assay

SOA generation was assayed by a modification of the method of Johnston et al[45]. Mitochondrial sonicates (50 µl) were incubated for 5 mins at 37°C with phosphate buffered saline (10 mM potassium phosphate and 150 mM sodium chloride pH 7.4, 1 ml) containing glucose (2 g/l) and fatty acid-free bovine serum albumin (2 g/l) with or without SOD (30 µg, 50 µl). To initiate the reaction ferricytochrome c solution (1.2 mM, 100 µl) was added, and the increase in absorbance was monitored at 550 nm. SOA generation was determined by calculating the difference between the sample without SOD and that with added SOD, and results were presented as the amount of reduced ferricytochrome c formed in µmol/min/mg protein.

Other Assays and Statistical Analysis

The Mn^{+2} concentration in routine MEM 1 was measured by Atomic Absorption Spectrophotometry (Perkin-Elmer 2380) using the appropriate standards, quality control samples and wavelengths as described by Mahoney et al[46]. Total protein content of mitochondrial fibroblast sonicates (20 µl) was measured according to Bradford[47].

Statistical Analysis

The statistical analysis was performed using the computer-based package of statistical product and service solution (SPSS, version 17.0). Analysis of variance followed by the post hoc Tukey-HSD test was done to evaluate statistical differences between mean ± SD values of SOD2 activities and SOA generation rates. Multiple comparisons were performed between sets of data of different cell passages cultured in different media. Values of $p < 0.05$ were considered to be statistically significant.

Results

Mn+2 concentration in Culture Media

The Mn^{+2} concentration in pooled batches of routine growth medium MEM 1 containing 10% FCs as the only source of Mn^{+2} and used throughout the subculture process equalled 30.1±0.15 nM (mean ± SD of triplicate readings). This concentration is approximately half the level of the cation in normal human plasma. Thus, Mn- supplemented serum-free media (MEM 2, 3, 4, 5 and 6) prepared to contain 60, 90, 120, 150 and 180 nM Mn^{+2} respectively, had levels of the cation equivalent to x1, x1.5, x2, x2.5 and x3 normal plasma human levels.

Identification of pre-senescent and Senescent subcultures

Details of data regarding the growth and multiplication rates, as well as the activities of key glucose and glycogen degradative enzymes, often used as biochemical markers of fibroblast senescence, will not be presented here for lack of space. Briefly, results indicated that MEM 1- sub-cultured fibroblasts at passages 5 and 10 required 24 hours post-subculture to achieve maximal rates of L-[U – ^{14}C] leucine incorporation into protein and [methyl – ^{3}H] thymidine into DNA to become confluent. Furthermore, whereas cells at passage 15 required 48 hours to reach such rates, those at passages 20 and 25 required 96 hours and those at passage 30 required 120 hours. Results also indicated that once reached, maximal rates were maintained at very similar values at all longer culture periods for cells at all passages. Data also showed that the specific activities of phosphofructokinase, lactate dehydrogenase and glycogen phosphorylase were not significantly changed in passages 5 and 10 cells, but underwent progressive significant increases in passage 15 cells, and extremely significant ones in passage 20 and 25 cells which peaked in value at passage 30. Furthermore, very similar results were obtained when all passage cells incubated with Mn^{+2}-supplemented media (MEM 2-MEM 6). Other findings of the present work showed that MEM 1-subcultured cells at passages 35-38 became at the end of their replicative life span as they did not undergo additional population doublings. All the above observations agree with those extensively documented by us in previous studies that examined the effect of replicative senescence on various metabolic activities[19,20,48-50].

Based on the above, passage 5 sub-cultured were considered in this study to represent young pre-senescent cells and were classified as controls. Furthermore, those at passage 20 were considered to be at an early phase of senescence, whereas those at passages 25 and 30 as senescent. Throughout the research, cells at the different passages were harvested for investigation at the appropriately indicated time periods post-subculture and were replenished with fresh MEM 1 when sub-cultured for longer than 48 hours.

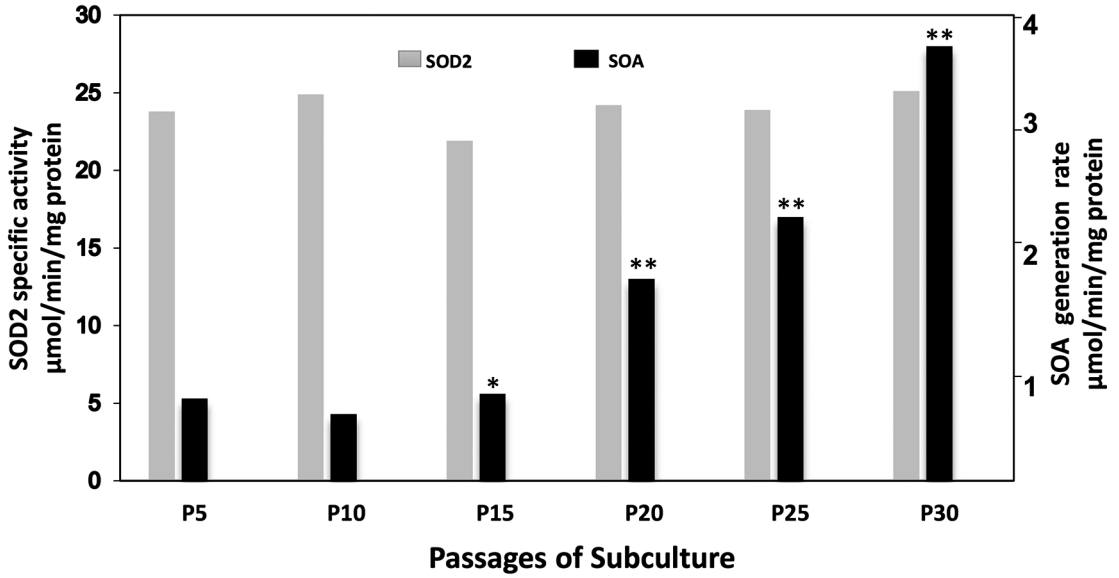


Figure 1. Effect of senescence on mitochondrial SOD2 activity and SOA generation in fibroblast subcultures cultivated in MEM 1. SOD2 = mitochondrial superoxide dismutase 2, SOA = superoxide anions. Values shown are means ± SD of duplicate determinations for 8 subcultures. MEM1 = routine growth medium. $^{*}p < 0.01$, $^{**}p < 0.001$ upon comparison of SOA generation rates in passage 15, 20, 25 and 30 cells against those generated in passage 5 cells.

Effect of senescence on mitochondrial SOD2 activity and SOA generation rates in MEM 1-sub-cultured fibroblasts

As evident from Figure 1, SOD2 specific activities were not significantly changed in mitochondrial sonicates of fibroblasts at all passages subcultured in MEM 1 (23.8±0.85, 24.9±0.94, 21.9±0.86, 24.2±1.03, 26.4±1.06 and 25.1±1.00 µmol/min/mg protein in cells at passages 5, 10, 15, 20, 25 and 30 respectively). In contrast, although mitochondrial SOA generation rates showed no significant variations in pre-senescent cells at passages 5, 10 there were statistically significant and progressive increases in rates beginning in passage 15 fibroblasts which peaked in value in senescent passage 30 cells (0.73±0.06, 0.69±0.07, 0.84±0.07, 1.66±0.09, 2.18±0.20, and 3.61±0.34 µmol/min/mg protein in cells at passages 5, 10, 15, 20, 25 and 30 respectively). See appropriate *p*-values in Figure 1 obtained upon the comparison of passage 15, 20, 25, and 30 rates against that recorded for passage 5 cells.

Effect of Increased Incubation Time of pre- Senescent and Senescent Fibroblasts with MEM 2 and MEM 6 on SOD2 Activity

As seen from Table I, incubation of pre-senescent passage 5 fibroblasts with MEM 6 containing the highest Mn^{+2} concentration (180 nM) required 4 hours to achieve maximal mitochondrial SOD2 activity. However, early senescent passage 20 cells required 6 hours to exhibit such rates, and senescent cells at passages 25 and 30 required 8 hours (activities equalled 24.3±0.99, 34.1±1.34, 46.6±1.72 and 54.8±2.08 µmol/min/mg protein in cells at passages 5, 20, 25 and 30 respectively). Also, evident from Table 1 results, fibroblasts at the different passages required the same time periods to achieve maximal SOD2 activities when cells were incubated with MEM 2 containing a Mn^{+2} concentration of 60 nM equivalent to normal human plasma levels of the cation (25.7±1.03, 27.8±1.24, 33.3±1.29 and 36.8±1.24 µmol/min/mg protein in cells at passages 5, 20, 25 and 30 respectively). In addition, very similar maximal SOD2 activities were maintained in all passage cells incubated with either MEM 2 or MEM 6 for periods up to 14 hours. Hence, to ensure maximal enzyme activity, cells at different passages in all subsequent experiments were incubated with MEM 2, 3, 4, 5 and 6 for 10 hours prior to harvesting. Closer examination of Table I data indicated that whereas early senescent passage 20 cells exhibited a maximal SOD2 activity equal to 34.1±1.34 µmol/min/mg protein 6 hours post-incubation with MEM 6, senescent cells at passag-

Table I. Effect of Mn+2 supplementation on mitochondrial SOD2 activity in pre-senescent P5 and senescent P20, 25 and 30 fibroblast subcultures.

Passage of subculture (n = 8)	SOD2 specific activity µmol/min/mg protein					
	MEM 1	MEM 2	MEM 3	MEM 4	MEM 5	MEM 6
P 5	23.9±0.85	25.7±1.03	23.9±1.05	25.0±1.07	22.9±1.07	24.2±1.10
P 20	24.2±1.03^{a}	27.7±1.24^{b}	31.0±1.06^{c}	32.1±1.05^{cd}	33.8±1.17^{de}	34.3±1.09^{e}
P 25	26.3±0.86^{a}	33.3±0.88^{b}	37.1±0.91^{c}	41.6±1.17^{d}	44.7±0.98^{e}	46.6±1.08^{f}
P 30	25.1±0.84^{a}	36.8±1.24^{b}	45.9±1.15^{c}	49.2±1.01^{d}	51.7±0.71^{e}	53.8±0.95^{f}

SOD2 = Superoxide dismutase 2. Values shown are means ± SD of duplicate determinants for 8 subcultures MEM 1 = Routine growth medium. MEM 2, 3, 5 and 6 = Serum free media supplemented with Mn^{+2} as detailed in text. Mean ± SD values followed by the same letter (a,b,c,d,e,f) are not statistically different. See Results section for p-values obtained upon comparisons of SOD2 activity between subcultures incubated with different media.

es 25 and 30 showed higher maximal enzyme activities 8 hours post- incubation with the same medium equal to 46.6±1.72 and 54.8±2.08 µmol/min/mg protein respectively. These values were significantly higher when the same cells were incubated with MEM 2 (27.8±1.24, 33.3±1.29 and 36.8±1.24 µmol/min/mg protein in passages 20, 25 and 30 respectively), and suggested that increasing Mn^{+2}concentration lead to stimulation of SOD2 activity which seemed to progress in value as the passage of subculture increased from 20 to 25 and 30. Details of such results including statistical analysis are presented below.

Effect of increasing Mn^{+2} concentrations on SOD2 activity and SOA generation rates in pre-senescent and senescent fibroblast subcultures

As evident from Table II, there were no significant changes in mitochondrial SOD2 activity of pre-senescent passage 5 fibroblasts subcultured in routine MEM 1 or any of the Mn^{+2}-supplemented media MEM 2-MEM 6. However, in early senescent passage 20 cells, analysis of variance showed that the enzyme underwent progressive very significant activity increases in subcultures incubated with MEM 2, 3, 4, 5 and 6 when all values were compared to that recorded in cells sub-cultured in MEM 1 (27.7±1.24, 30.7±1.06, 32.1±1.05, 33.8±1.17 and 34.3±1.09 against 24.2±1.03 µmol/min/mg protein; p <0.001). In addition, although there were very significant SOD2 activity increases when comparisons were performed between passage 20 subcultures incubated with MEM 2 and MEM 3 (27.7±1.24 against 31.0±1.06 µmol/min/mg protein; p <0.001), the increases were not statistically significant when comparisons were made for cells incubated with MEM 4 against MEM 3 and MEM 6 against MEM 5 (32.1±1.05 against 31.0±1.06 and 34.3±1.09 against 33.8±1.17 µmol/min/mg protein respectively; p = 0.062 and 0.391 respectively). Nevertheless, comparisons of

Table II. Effect of Mn^{+2} supplementation on mitochondrial SOA generation rates in pre-senescent P5 and senescent P20, 25 and 30 fibroblast subcultures.

Passage of subculture (n = 8)	SOA generation rate µmol/min/mg protein					
	MEM 1	MEM 2	MEM 3	MEM 4	MEM 5	MEM 6
P5	0.73±0.06	0.76±0.08	0.68±0.05	0.75±0.07	0.69±0.06	0.74±0.08
P20	1.74±0.09^{abc}	1.69±0.08^{bcd}	1.72±0.10^{cd}	1.64±0.08^{de}	1.61±0.08^{e}	1.52±0.07^{f}
P25	2.18±0.2^{abc}	2.06±0.14^{bcd}	2.00±0.12^{cd}	1.95±0.11^{d}	1.76±0.09^{e}	1.56±0.08^{f}
P30	3.61±0.26^{a}	2.62±0.22^{b}	2.38±0.20^{c}	2.12±0.19^{d}	1.83±0.10^{e}	1.49±0.07^{f}

SOA = Superoxide anion. Values shown are means ± SD of duplicate determinants for 8 subcultures. MEM 1 = Routine growth medium, MEM 2-MEM 6 = Serum free media supplemented with Mn^{+2} as detailed in text. Mean ± SD values followed by the same letter (a,b,c,d,e,f) are not statistically different. See Results section for p-values obtained upon comparisons of SOA generation rates between subcultures incubated with different media and between cells at different passages of subculture incubated with the same medium.

the enzyme activities in passage 20 subcultures incubated in MEM 4 against MEM 2, MEM 5 against MEM 3 and MEM 6 against MEM 4 (32.1±1.05 against 27.7±124, and 33.8±1.17 against 31.0±1.06 and 34.3±1.09 against 32.1±1.05 µmol/min/mg protein), were all very significantly higher; $p < 0.001$ for the first two comparisons and p = 0.001 for MEM 6 against MEM 4. It is also evident from Table II data that in senescent passage 25 subcultures, SOD2 activities exhibited very significant statistical increases when cells were incubated in MEM 2, 3, 4, 5 and 6 (33.3±0.88, 37.1±0.91, 41.6±1.17, 44.7±0.98 and 46.6±1.08 µmol/min/mg protein respectively, compared against 26.4±0.86 µmol/min/mg protein recorded when cells were sub-cultured in routine MEM 1; p < 0.001 for all comparisons). Such increases were progressive in nature and seemed to peak in value when the cells were incubated with MEM 6. In addition, the noted enzyme activity increases were of equal statistical significance when comparisons were made between subcultures incubated with all the Mn^{+2} supplemented media (p < 0.001), except between MEM 6 and MEM 5 where $p = 0.002$. A similar trend of change was obtained for senescent passage 30 fibroblasts. SOD2 activity increases equalled 36.8±1.24, 45.9±1.15, 49.4±1.57, 51.7±0.71 and 54.8±0.95 µmol/min/mg protein for subcultures incubated with MEM 2, 3, 4, 5 and 6 respectively, against 25.1±0.84 µmol/min/mg protein recorded for cells sub-cultured in MEM 1; $p < 0.001$ for all comparisons. Similarly, the enzyme activity increases were equally statistically significant when comparisons were performed between subcultures incubated in all Mn^{+2} supplemented media, $p < 0.001$. Further analysis of Table II data indicated that although there were highly significant SOD2 activity increases in senescent passage 20, 25 and 30 subcultures incubated with all Mn^{+2} supplemented media compared to those sub-cultured in MEM 1, maximal enzyme activity was achieved in passage 30 subcultures incubated with MEM 6 (53.8±0.95 against 25.1±0.84 µmol/min/mg protein, an increase of ~114%). However cells at passages 20 and 25 incubated with the same medium could only achieve increases of ~42% and ~77% respectively.

As shown in Table III, there were no statistically significant changes in SOA generation rates in pre-senescent passage 5 fibroblasts subcultured in routine MEM 1 or any of the Mn^{+2} supplemented media MEM 2-MEM 6. However, in early senescent passage 20 subcultures incubated with MEM 4 and MEM 5, generation rates were moderately but significantly lowered ($p < 0.05$) and more significantly lowered in MEM 6-incubated subcultures ($p < 0.001$) upon comparison with that documented for MEM 1-incubated cells (1.64±0.08, 1.61±0.08 and 1.52±0.07 µmol/min/mg protein respectively against 1.74±0.09 µmol/min/mg protein). Further analysis of data showed that the SOA generation rate in early senescent passage 20 subcultures incubated with MEM 6 was significantly lower than those recorded for all other Mn^{+2}-supplemented media MEM 2-MEM 5 ($p < 0.001$ for all comparisons). In addition, although the rate in subcultures incubated with MEM 5 (1.61± 0.08 µmol/min/mg protein) was significantly lower than those recorded for MEM 2 and MEM 3 (1.69± 0.08 and 1.72± 0.10 µmol/min/mg protein respectively; $p < 0.001$), no significant changes were observed when rate comparisons were

Table III. Effect of increased incubation time of pre-senescent P5 and senescent P20, 25 and 30 subcultures incubated with MEM 2 and MEM 6 on SOD2 activity.

Incubation Time (Hours)	Fibroblast subculture passages SOD2 Activity (µmol/min/mg protein)							
	P5		P20		P25		P30	
	MEM2	MEM6	MEM2	MEM6	MEM2	MEM6	MEM2	MEM6
1	18.3±0.72	17.7±0.71	17.1±0.75	15.6±0.73	13.3±0.63	12.9±0.52	9.31±0.55	10.6±0.44
2	21.1±0.85	20.4±0.78	20.2±0.80	18.9±0.78	16.8±0.75	20.3±0.91	14.4±0.67	23.3±1.01
4	25.7±1.03	24.3±0.99	23.6±1.09	26.5±1.08	22.3±0.88	32.2±1.29	23.8±0.92	35.1±1.37
6	24.8±1.01	24.0±1.11	27.8±1.24	34.1±1.34	28.6±1.10	41.8±1.68	31.5±1.09	44.1±1.76
8	23.9±1.07	23.2±1.03	28.1±1.29	34.6±1.38	33.3±1.29	46.6±1.72	36.8±1.24	54.8±2.08
10	25.0±1.11	22.8±1.05	26.9±1.18	33.2±1.31	34.0±1.30	44.9±1.71	36.1±1.34	54.3±2.12
14	24.3±1.05	23.1±0.95	27.1±1.25	33.8±1.29	32.9±1.32	45.8±1.75	37.1±1.38	53.7±2.15

SOD2 = Mitochondrial superoxide dismutase. Values are means ± SD of duplicate determination for eight subcultures. MEM 2 and MEM 6 = Serum-free culture media containing 60 and 180 nM Mn^{+2}, respectively.

performed for the early senescent subcultures incubated in MEM 5 and MEM 4 (1.61± 0.08 against 1.64± 0.08 μmol/min/mg protein). Similarly, there were no significant changes in SOA generation rates of these cells upon comparisons against MEM 2, 3 and 4 values (1.69±0.08, 1.72±0.10 and 1.64±0.08 μmol/min/mg protein respectively). In senescent passage 25 subcultures incubated with MEM 3 and MEM 4, SOA generation rates were significantly lower when compared to those recorded in fibroblasts subcultured in routine MEM 1 (2.00±0.12 and 1.95±0.11 μmol/min/mg protein respectively, against 2.18±0.20 μmol/min/mg protein; $p < 0.05$). However, when passage 25 cells were incubated with MEM 5 and MEM 6, SOA generation rates became significantly lower upon comparison with those incubated in MEM 1 (1.76±0.09 and 1.56±0.08 μmol/min/mg protein respectively, against 2.18± 0.20 μmol/min/mg protein; $p < 0.001$). Table III data also indicated that rates in passage 25 subcultures were significantly lowered when comparisons were performed between cells incubated with MEM 6, 5 and 4 ($p < 0.001$ for MEM 6 against MEM 5 and MEM 4 and $p = 0.002$ for MEM 5 against MEM 4). In contrast, no significant changes were observed in senescent passage 25 rates when cells were incubated with MEM 2, 3 and 4. Moreover, the significant decreases in SOA generation observed MEM 4-incubated cells were progressive and reached lowest levels in those incubated in MEM 6. Rates in MEM 4, 5 and 6-incubated cells were equivalent to ~89%, 81% and 72% respectively of the rate observed in those subcultured in routine MEM 1. In senescent passage 30 cells, SOA generation underwent significant decreases upon incubation of the subcultures in all Mn^{+2}-supplemented media (2.62±0.22, 2.38±0.20, 2.12±0.19, 1.83±0.10, and 1.49±0.07 μmol/min/mg protein) in subcultures incubated with MEM 2, 3, 4, 5 and 6 respectively against 3.61±0.26 μmol/min/mg protein recorded in MEM 1-incubated cells ($p < 0.001$ for all comparisons). Such rate decreases were progressive, high in magnitude and significant when comparisons were made between passage 30 subcultures incubated with MEM 2, 3, 4, 5 and 6 ($p < 0.001$ for all comparisons), amounting to ~73%, 66%, 59%, 51% and 41%, respectively, of the value recorded when cells were incubated with MEM 1.

Computation of the Km Values of Mn^{+2} with Respect to SOD2 Activity Senescent Fibroblast Subcultures.

Table II data of the effect of Mn^{+2} concentration on SOD2 activity in early senescent passage 20 cells and senescent passage 25 cells and 30 cells was used to construct Michaelis- Menten curves and Hanes-Woolf plots using the Dynafit version 4.0 software program obtained from Biokin Ltd, Watertown, MA, USA. The results obtained are shown in Figures 2, 3, and 4 for cells at passages 20, 25 and 30 respectively. As evident the Michaelis- Menten curves indicated that the effect of increased Mn^{+2} concentration on SOD2 activity exhibited a saturation kinetics phenomenon in all studied cells. Furthermore, the computed Mn^{+2}-Km values equalled 19.2, 39.6 and 54.4 nM for cells at passages 20, 25 and 30 respectively with corresponding maximal SOD2 activities equal to 37.6, 55.9 and 71.4 μmol/min/mg protein.

Discussion

Fibroblast subcultures provide a feasible experimental model whereby human tissue maintained in vitro under optimal conditions, has been successfully and extensively used by us to study intracellular metabolism[19,20,50]. In this study it was possible to subject the cells to increasing Mn^{+2} concentrations since the only source of the latter in routine MEM 1 culture medium is fetal calf serum. Hence, it was possible to incubate fibroblast subcultures with a series of serum-free media (MEM 2-MEM 6) containing supplemental Mn^{+2} increments up to triple the normal human plasma levels of the metal cation. This ensured that fibroblasts were subjected to sufficient amounts of Mn^{+2} for stabilising the structural integrity of SOD2 and for achieving maximal rates of its activity. To this end, it has been reported that routine culture medium (containing 10% v/v fetal calf serum), does not have sufficient amounts of trace elements required for optimal expression of antioxidant activity[51]. In the current study, routine MEM 1 contained ~50% of the mean normal human plasma Mn^{+2} levels. In addition, other optimal conditions were provided to allow fibroblasts to grow, multiply and metabolize at maximal rates. These included the provision of sufficient volumes of culture medium (20 ml/75 cm^2 flask) which was frequently changed, the addition of HEPES buffer (pH 7.2) in both culture and trypsinization media and the use of streptomycin and penicillin for combating bacterial infection. As a result, the percentage of cell death was always constantly low (3-7%), unaffected by the subculture passage number (senescence) or the highest Mn^{+2} concentration (180 nM) (data not shown). However, after ~35 population doublings, cells

became rounded, enlarged and ruptured, regardless of whether maintained in MEM1 or MEM2-MEM6. Preliminary data of the current study indicated that cell death occurred at a Mn^{+2} concentration of 300 nM, thus depicting Mn^{+2} toxicity. No data is available in our lab as to weather apoptosis is a feature of late senescence in cultured human fibroblasts, or was induced by high (300 nM) Mn^{+2} concentrations.

As confirmed by us and others, the onset and continuation of replicative senescence in cultured fibroblasts is accompanied by a number of histological and biochemical changes that can be used as markers for the identification of senescent cells. These include an increase in population doubling time, an increase in cell size, flattened cellular morphology, gradual and progressive lowered rates of cellular growth and replication as the passage of subculture is increased, an increase in glucose and glycogen degradative enzyme activities and accumulation of cells exhibiting senescence associated β-galactosidase activity[52]. In the present study, every effort was taken to identify the passage of subculture at which senescence sets in and continuous. It was evident that MEM1-subcultured fibroblasts at passages 5 and 10 required 24 hours to become confluent and achieve maximal rates of radiolabelled leucine and thymidine incorporation into protein and DNA. However, those at passages 15, 20 and 25 and 30 required 48, 96 and 120 hours respectively and were harvested accordingly. These harvesting times were in agreement with those documented by us previously[19,20,48-50], and provided evidence that passage 20 cells enter senescence which progresses into passages 30 and 35. This also ensured that protein and DNA yields of harvested cells were not affected by senescence and indicated that any changes noted in SOD2 activity could be interpreted as a cause of senescence. Mitochondrial fibroblast

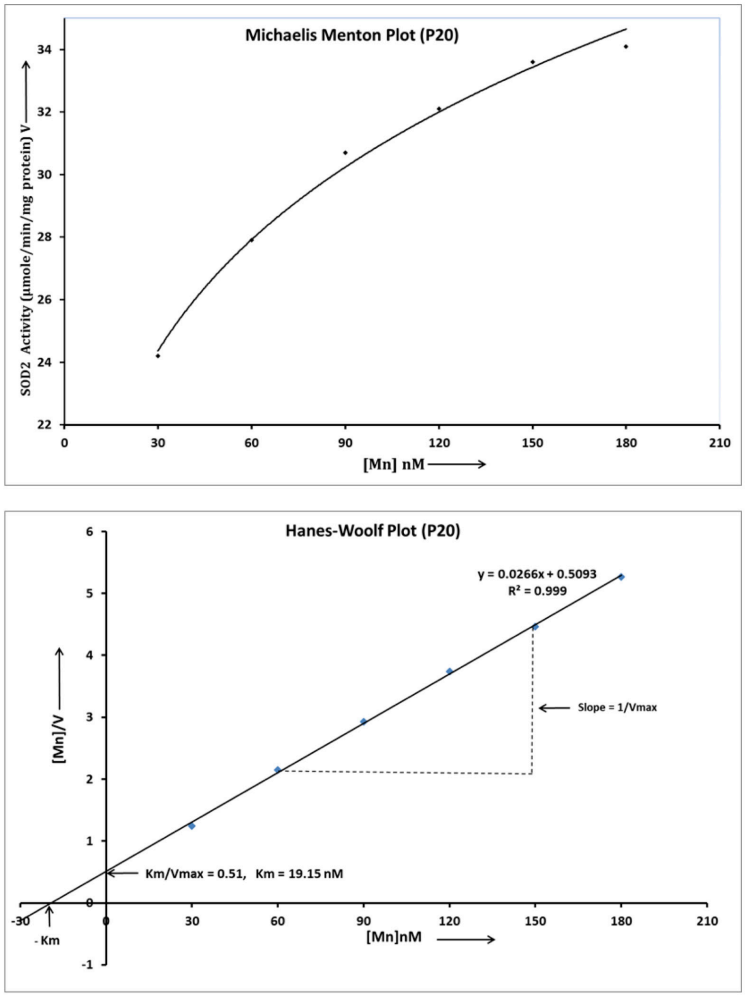
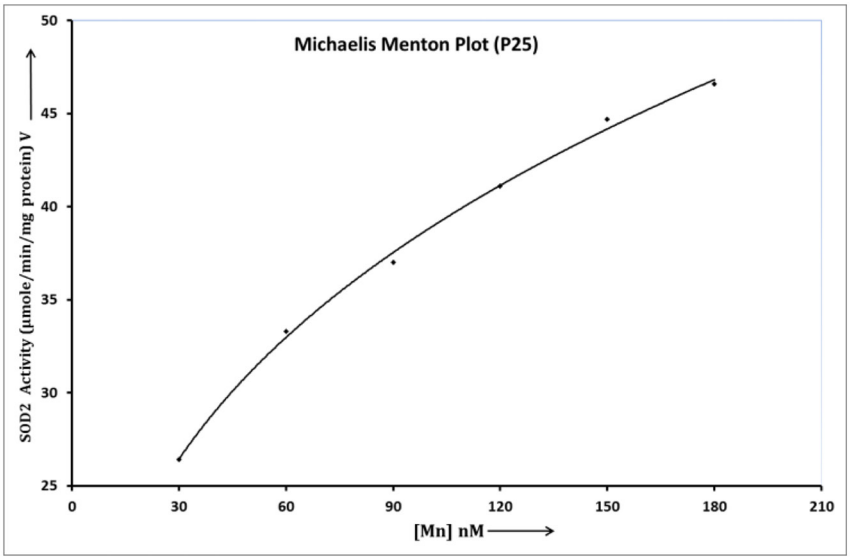


Figure 2. The Km value of Mn^{+2} with respect to SOD2 activity in passage 20 early senescent fibroblast subcultures.



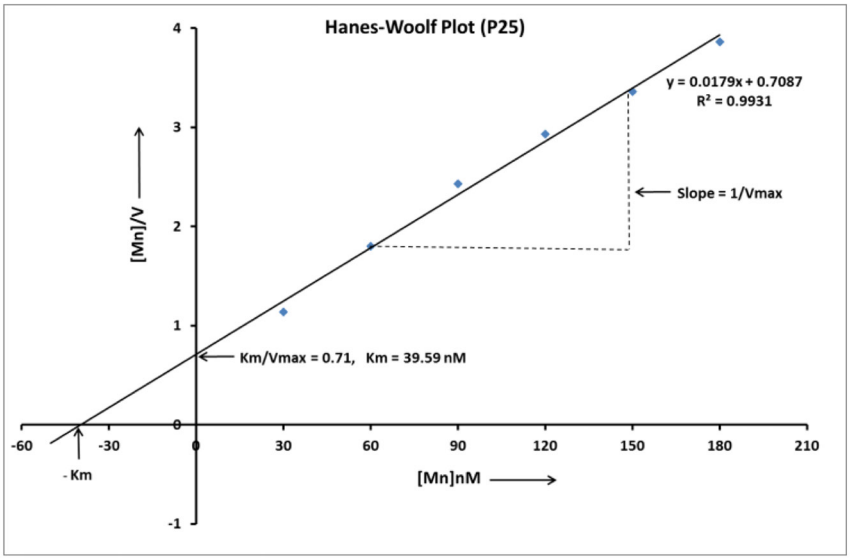


Figure 3. The Km value of Mn^{+2} with respect to SOD2 activity in passage 25 senescent fibroblast subcultures.

sonicates of the eight subcultures had protein and DNA contents of 64.8±7.81 and 4.36±0.52 µg/100 µl respectively with a protein/DNA range ratio of 14.8±1.42 for the eight subcultures at all passages. Other data not shown but documented by as previously[19,20,48,49], indicated that phosphofructokinase, lactate dehydrogenase and glycogen phosphorylase activities, underwent significant increases which began in cells at passage 20 and progressed to very significant high values at passages 25 and 30. Furthermore, these results were not changed when all subcultures were rocked to improve oxygenation[20] suggesting that the enhanced glucose and glycogen catabolism was not due to hypoxia and is a feature of senescence. More data used to identify senescent cells was related to SOA generation (Table III). These rates underwent significant increases in passage 15 and 20 cells reaching very high 4 and 5-fold levels in passage 25 and 30 cells sub-cultured in routine MEM1 upon comparison with passage 5 cells. In light of all the above findings, fibroblasts at passage 20 were considered to have entered replicative senescence and were very likely at an early phase of the process. However, cells at passages 20 and 25 were considered to be senescent and those at passages 5 as pre-senescent young controls. Furthermore, fibroblast subcultures ceased to divide around passage 35 (35 population doublings) although cells were refed with culture medium for seven consecutive days. Hence, cells at the highest passage 30 currently investigated would not have reached the Hayflick limit.

Many studies have described age-related changes in SOD1 and SOD2 activities and corresponding mRNA abundance using cultures of young and senescent WI-38 fetal lung fibroblasts, skin fibroblasts established from young and old donors and human fibroblasts at different passages of subculture. All these studies[19] reported highly controversial results. Such SOD1 and SOD2 activity discrepancies could be due to the types of investigated fibroblasts or donors of different ages and their different genetic and environmental backgrounds. Other factors could have included the provision of optimal culture and subculture conditions, accurate identification of senescent cells and the availability of sufficient Zn^{+2}, Cu^{+2} and Mn^{+2} concentrations for achieving maximal SOD1 and SOD2 activities especially in oxidatively stressed cells. To this end fetal calf serum from different sources and different batches from the same source are likely to contain significantly variable levels of the metal cations. In the current report, all the above factors were minimized, and results indicated similar SOD2 activities when pre-senescent passage 5 fibroblasts were sub-cultured in MEM 1 and incubated with MEM 2, 3, 4, 5 or 6 containing increasing supplemental Mn^{+2} increments (Table II). This suggested that near human plasma concentrations of the cation (30.1 nM) was sufficient for the expression of normal SOD2 activity in young pre- senescent cells. However, there were statistically significant increases in SOD2 activities in early senescent passage 20 and senescent passage 25 and 30 cells incubated with all the Mn^{+2} supplemented media which could not be seen when the cells were sub-cultured in routine MEM1. Such increases progressed to higher values that were relative to Mn^{+2} concentrations, and peaked when cells were incubated with MEM5 and MEM6 containing highest supplemental Mn^{+2} increments (150 and 180 nM). Furthermore, early senescent passage 20 cells and senescent passage 25 and 30 cells cultured in MEM1 generated ~1.4, 2 and 4 times more SOA respectively than pre- senescent passage 5 cells sub cultured in the same medium (Table III). This indicated that senescent fibroblasts become vulnerable to endogenous OS and need to combat this by utilizing higher Mn^{+2} concentrations required to stimulate their SOD2 activity. Indeed, the increases in SOD2 activities of senescent fibroblasts can be attributed to the presence of higher Mn^{+2} concentrations in MEM 2- MEM6. Table III also indicated that SOA generation rates significantly and maximally decreased in MEM 6-incubated early senescent passage 20 cells and senescent passage 25 and 30 cells by ~13%, 29% and 59% respectively when compared with those generated in the cells incubated in MEM1. Although these lowered rates were still significantly higher in senescent cells than those produced by pre-senescent passage 5 cells also incubated in MEM 6, they further indicate that cells towards the end of their replicative life span become resistant to endogenous OS in the presence of Mn^{+2} concentrations equal to 180 nM. To this end, Mn^{+2} was shown to protect cultured human fibroblasts against oxidative injury induced by hydrogen peroxide and UVA radiation[53]. We were able to demonstrate[19] a significant stimulation of SOD1 and SOD2 activities in senescent fibroblasts subcultured in a medium containing triple human plasma levels of Zn^{+2}, Cu^{+2} and Mn^{+2} which enabled the cells to more efficiently quench high SOA generation rates. It has been shown[21] that Zn^{+2} supplementation significantly increased plasma and erythrocyte total SOD activity in healthy old subjects, and in rats[54] placed on a diet containing 10 times the RDA of the cation resulting in increases in SOD1 activity and mRNA expression levels. However, studies related to optimal Mn^{+2} concentrations required for maximal SOD2 activation in senescent cells are not available which marks the importance of the current report.

It is essential to maintain SOD2 activity at normal levels as the enzyme is important for eukaryotic growth and survival. Mice homozygous for SOD2 disruption die at a neonatal age with damage to the heart indicating mitochondrial dysfunction[55]. Similarly, SOD2 absence in Drosophila results in early death[56]. Mn^{+2} is essential for the activation of SOD2 as it acts as a cofactor that coordinates with each of the enzyme's four subunits. However, cellular pathways that result in active SOD2 assembly are unknown. The enzyme does not co-ordinate with Mn^{+2} during unfolding of the protein required for mitochondrial import[57]. It has been shown[58] that formation and loading of the Mn^{+2} active site of SOD2 occur within the mitochondrial matrix by metalation of the apoprotein during the folding of the protein thereby generating the biologically active enzyme. Mn^{+2} transport across the cell membrane of rat hepatocytes has been shown[59] to occur by a unidirectional saturable process exhibiting passive-mediated transport and has a Km of 1.2 μM. Furthermore, Mn^{+2} content of subcellular fractions of rat liver has been reported to be highest in the nuclear and mitochondrial fractions. Although translocation of Mn^{+2} into the mitochondria is still unclear, it seems

that it is transported via high-affinity metal transporters including DMTI (a member of the family of natural resistance-associated macrophage proteins NRAMP), ZIP 8 (a member of the solute carrier 39), the transferrin receptor (TfR) and store-operated Ca^{+2} channels[60]. Mn^{+2} uptake studies into the mitochondria of cultured fibroblasts are not available. However, one study reported that the cation accumulates in different compartments of cultured brain cells including mitochondria[61]. Although Mn^{+2} uptake by fibroblasts is not a central issue of this investigation, Table I data showed that whereas pre-senescent passage 5 fibroblasts incubated with MEM2 and MEM6 required 4 hours to achieve maximal SOD2 activity, early senescent passage 20 cells and senescent passage 25 and 30 cells required 6 and 8 hours respectively. Furthermore, very similar maximal enzyme activities were recorded when the cells were incubated with the same media for periods up to 14 hours. This suggests that the accumulation of Mn^{+2} into mitochondria of cultured fibroblasts is a time-dependent saturable process which seems to be enhanced in senescent cells that exhibited significantly higher SOD2 activities when incubated with MEM6 (180 nM Mn^{+2}) in comparison with those recorded when the cells were incubated with MEM 2

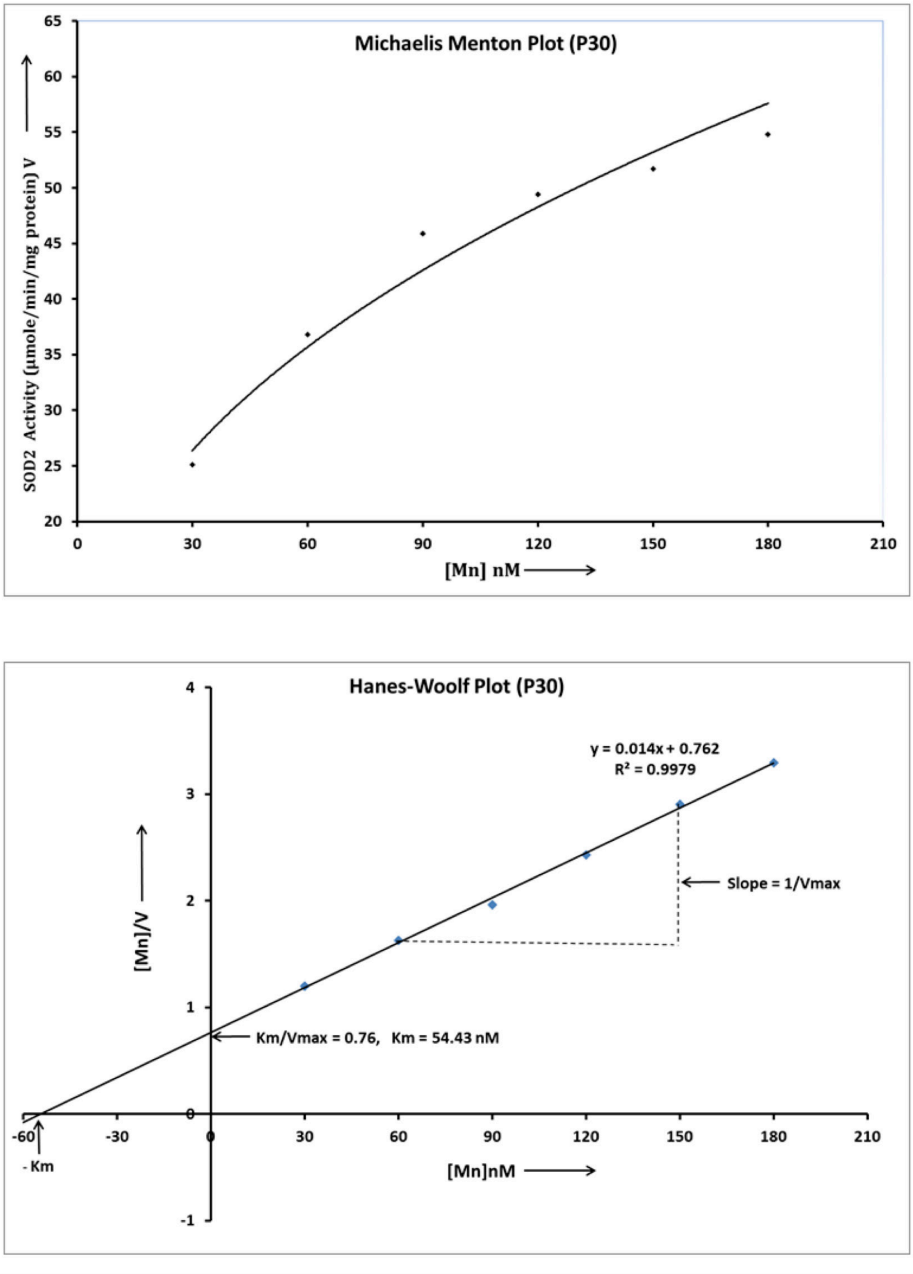


Figure 4. The Km value of Mn^{+2} with respect to SOD2 activity in passage 30 senescent fibroblast subcultures.

(60 nM Mn^{+2}) (Table I). More results of the present study regarding the Km values of Mn^{+2} with respect to SOD2 activity showed that whereas MEM 6-incubated passage 20 early senescent cells could only achieve maximal SOD2 activity increases equivalent to ~42% of the enzyme activity recorded when the cells were sub-cultured in MEM1, senescent cells at passage 25 and 30 achieved increases equivalent to ~76% and 114%, respectively. These findings also suggest that Mn^{+2} uptake and utilization is significantly increased as sub-cultured fibroblasts enter senescence, and are maximised when cells approach the end of their life span at higher passages. This phenomenon allows senescent cells to combat the elevated level of OS illustrated in this study. Indeed the Km values of Mn^{+2} with respect to SOD2 activity equalled 19.2, 39.6 and 54.4 nM for passage 20, 25 and 30 cells respectively, with corresponding SOD2 Vmax values of 37.6, 55.9 and 71.4 µmol/min/mg protein (Figures 2, 3 and 4). To this end, a study is underway in our laboratory for the investigation of radiolabelled^{54} Mn uptake and subcellular distribution in pre-senescent passage 5, early senescent passage 20 and senescent passage 25 and 30 fibroblasts subcultures.

Conclusions

Results of the current study demonstrated increasing Km values of manganese with respect to SOD2 activities in senescent passage 20, 25 and 30 fibroblasts. It is thus concluded that senescent cells near the end of their replicative life span utilized ore manganese to reach maximal SOD2 activity which allowed the cells to combat OS and caused a reduction in endogenous SOA generation.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

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