

The Effects of Oxidative Stress on Inducing Senescence in Human Fibroblasts

Shivani Chowdhary

South Carolina Governor's School for Science and Mathematics

Oxidative stress, specifically from hydrogen peroxide exposure, was performed to determine if it induced senescence in cell lines such as HeLa cells and primary human fibroblasts. The purpose of this experiment was to find the optimal stage, concentration, and time of exposure to induce the greatest number of senescent cells. After dividing cultures of both the HeLa and human fibroblasts cells in order to reduce confluency, the cells were placed in a six well plate and exposed to hydrogen peroxide for two hours at various concentrations. The plates were checked at twenty-four-hour intervals, and then fixed with senescence associated beta-galactosidase as a biomarker to observe the senescent cells in culture. It was hypothesized that hydrogen peroxide exposure would increase the number of senescent cells due to the accumulation of reactive oxygen species. The data indicated that, there was an increase in the number of senescent cells following 48 hours of treatment. The number of senescent cells peaked following 72 hours of treatment and did not change significantly as result of 96 hours of treatment supporting our hypothesis.

Introduction

There are many theories as to why aging occurs, but they ultimately fall under two categories. The first category is that aging is programmed, and the second considers the environmental effects and assaults to organisms that cause the damage which results in aging. The accumulation of biological waste that cannot be removed, dysregulation of regulatory pathways, telomere shortening, and reactive oxygen species, which play a role in the functioning of the mitochondria, are just a few of the many hypotheses that fall under the two major classifications (Sergiev, 2015). Our main focus was on the reactive oxygen species and free radicals involved with oxidative stress. We used cultured human fibroblasts which exhibit a limited proliferation due to several passages of the cell cycle. (Magalhaes, 2002). The passage number is the number of times the cell line has been allowed to grow to a certain confluency prior to re-plating (Masters and Stacey, 2007). The more passages and replication cycles a cell line undergoes, the more likely to senescence. Senescence occurs when cells reach their dividing capacity which eventually leads to apoptosis and possible necrosis (Burton, 2009). Senescent cells are identified by their morphological enlargement, reduced response to growth factors, and senescence-associated- β -galactosidase activity in the G1 phase of interphase. Earlier work has demonstrated that telomere shortening causes replicative senescence because short telomeres might activate a p53 DNA damage response pathway which leads to growth arrest (Zdanov, 2006). This evidence has also led to the suggestion that cellular senescence is the molecular basis of human aging (Burton, 2009). Senescent cells have been shown to accumulate with age in several different tissues observed in humans and rodents (Zdanov, 2006). Thus, cellular senescence can be regarded as a useful model for elucidating the molecular mechanisms that are involved in human aging.

In addition to replicative senescence, also known as passaging, cellular senescence can be induced by various stressors including oxidative stress. Oxidative stress is the accumulation of reactive oxygen species (ROS) at cytotoxic levels. Hydrogen peroxide (H_2O_2) is widely used to achieve oxidative stress-induced premature senescence within a short period of time (Chen, 2007). Induced premature senescent cells display many markers that are the same as the replicative senescent cells. Thus, oxidative stress-induced senescent cells serve as an excellent in-vitro model for aging research (Chen, 2007). Our work involves culturing primary human fibroblasts and exposing them to hydrogen peroxide for approximately two hours to determine if oxidative stress induces cellular senescence in these cells. Furthermore, the goal will be to standardize a protocol to induce senescence in human fibroblasts and determine whether cells that accumulate more reactive oxygen species become more senescent over time compared to untreated cells. We will use senescence-associated β -galactosidase (SA- β -gal) activity as the bio-marker specific only to senescent cells. This will help us to optimize an efficient protocol to induce premature senescence in response to oxidative stress. It will also provide an insight on how human fibroblasts react to oxidative stress and how their response can affect common processes such as the development of cancer and ageing.

Methods

Splitting of Human Fibroblasts

A human fibroblast cell culture was divided in order to reduce confluency and increase accuracy. The cells were incubated in 500 milliliters of fetal bovine serum and 5 milliliters of penicillin/streptomycin at 37° C and handled using sterile technique. The media was removed and discarded. The culture was rinsed with 5 milliliters of PBS. To remove the cells from the surface of the plate, 2 milliliters of trypsin was added and briefly rocked back and forth followed by three minutes of incubation at 37° C. Five milliliters of media was added to the trypsin in the plate and transferred with a pipette to sample tube. To collect the supernatant, the tube was centrifuged at 1700 rpm for two minutes and thirty seconds and the supernatant was discarded. Another 6 milliliters of fresh media was added, and 0.5 milliliters of this solution was plated. The plates were incubated in a 5% CO_2 environment at 37° C for approximately 24 hours. Following incubation, 1 milliliter aliquots of the cell solution were placed in six well plates. Hydrogen peroxide was added to each well in the following concentrations: 50 μ M, 100 μ M, 100 μ M, 150 μ M, and 200 μ M. The cells were then stored in an incubator for two hours at 37° C.

Staining of Human Fibroblasts

The staining of the cells was an essential step in the recognition of senescent cells based on color rather than morphology. After the cells had been treated with hydrogen peroxide, the culture media was aspirated, and the cells were washed two times with 500 μ l of PBS. After the last wash, the PBS was replaced with 250 μ l of 4% paraformaldehyde, PFA, for fixation. The cells were incubated for five minutes at room temperature. The 4% PFA was aspirated, and the cells were washed two times for five minutes each at room temperature with gentle shaking in 500 μ l PBS. Two hundred fifty microliters of SA- β -gal staining solution was added to each well. The cells were incubated in the dark at 37° C. The reaction was terminated by aspirating the staining solution, and the cells were rinsed two times with distilled water. After the last wash, 500 μ l of distilled water was added to each well. This procedure was repeated using multiple plates that were incubated at 37° C for 48, 72 and 96 hours prior to analysis. At

least one of the well plates for each twenty-four-hour interval was left untreated without staining procedure. Senescent cells were quantified based upon the presence/absence of stain.

Results

Peroxide treatment for 96 hours of 13FB cells resulted in a substantial increase in the number of senescent cells (Fig. 1).

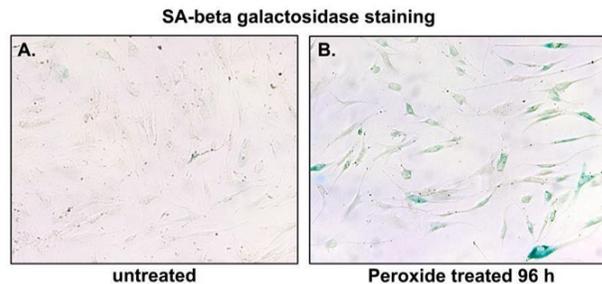


Fig. 1. SA- β -gal activity staining of 13BF cells treated with hydrogen peroxide. Cells were left untreated or treated with 100 μ m of hydrogen peroxide at 37° C for two hours. The hydrogen peroxide was removed and the cells were maintained in fresh complete DMEM media for 96 hours. Following incubation, cells were stained with SA- β -gal for 48 hours and then rinsed with PBS.

The untreated sample did not contain any senescent cells since there was no indication of blue color. However, in the 96 hour image, there were a number of cells that stained blue due to the activity of SA- β -gal that recognized senescent cells (Fig. 1). Table 1 indicates that at higher concentrations and at longer time intervals, the percentages of senescent cells increased and the percentages of untreated cells decreased.

Table 1. The percentage of blue cells and standard deviation of each concentration in 48,72, and 96 hours

48 Hours		
Concentration (uM)	%bluecells	stdev
untreated	3.172	0.8366
50 uM	34.5622	10.3923
100 uM	64.2004	11.8054
150 uM	67.6923	12.3612
200 uM	87.32394	17.224
72 Hours		
Concentration	%bluecells	STDev
untreated	16.5	3.44
50	45.0704	8.7101
100	80.4878	22.6715
150	79.777	16.078
200	86.764	12.6609
96 Hours		
Concentration	%bluecells	Stdev
untreated	3.3149	4.51663
50	44.6428	12.4526
100	84.98943	17.5499
150	89.8617	16.257
200	89.32394	17.328

As the concentration of hydrogen peroxide increased from the 50 μ M to 100 μ M, there was a significant difference in the number of senescent cells (Fig. 2).

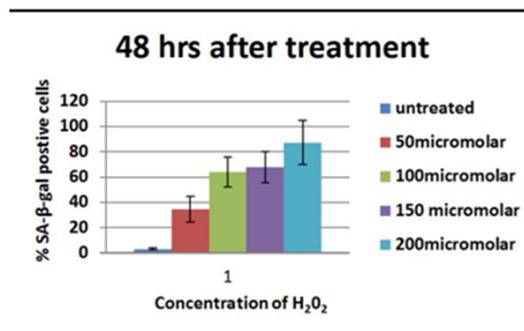


Fig. 2. Quantification of 13BF cells using SA- β -gal activity staining. The results are expressed as a percentage of the number of blue cells present 48 hours after the initial two hours of hydrogen peroxide treatment at various concentrations. Results are given as mean \pm SD of six different field count of each six well plate.

At each incubation time there was a notable difference in the number of blue cells in the treated wells when compared to the untreated cells (Figure 3). There was a larger difference between the 48 hours and 72/96 hour trials, but the 72 and 96 hour trials were very similar (Figure 3). In addition, the maximum amount of SA- β -gal activity staining was observed at 72 hours and 96 hours (Figure 3). In Figure 4, there was no significant difference in the 100, 150, and 200 micromolar treatments at 72 hours.

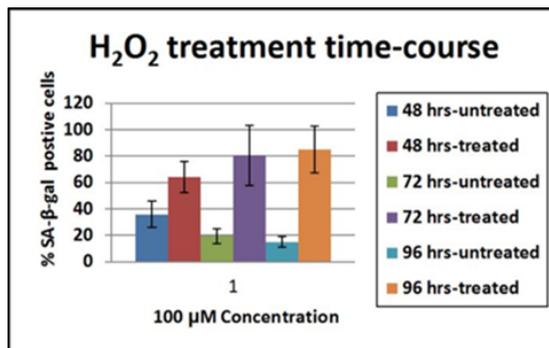


Fig. 3. Quantification of 13BF cells using SA- β -gal activity staining. The results are expressed as a percentage of the number of blue cells counted at various timepoints after initial two-hour treatment with hydrogen peroxide. Results are given as mean \pm SD of six different field count of each six well plate.

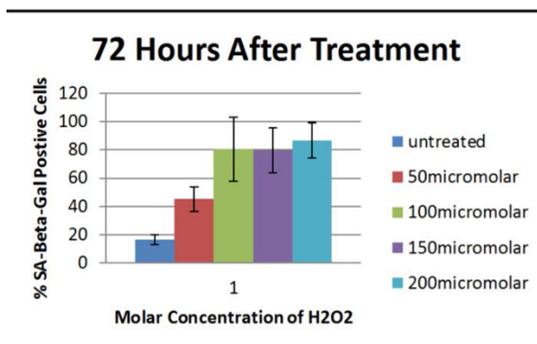


Fig. 4. Quantification of 13BF cells using SA- β -gal activity staining. The results are expressed as a percentage of the number of blue cells present 72 hours after the initial two hours with hydrogen peroxide at various concentrations. Results are given as mean \pm SD of six different field count of each six well plate.

Discussion

The optimal time and concentration to induce senescence in cells was at 96 hours and with 100 to 150 micromolar concentrations of hydrogen peroxide. Ultimately, the higher concentrations of hydrogen peroxide and longer exposure times resulted in the highest percentage of senescent cells. Our findings agreed with others who stated that reactive oxygen species (ROS), does contribute to senescence at higher concentrations (Davali, 2016). Their results indicated that oxygen concentration limits cellular lifespan *in vitro*. Davali (2016) also found that dysregulated ROS and senescence results in aging and other diseases. Dysregulated ROS occurs when it cannot complete its function as an important regulator of intracellular signaling pathways. We also observed that the morphology of the senescent cells appeared to be flat and large as previously reported in the literature (Davali, 2016).

Future work could include a 24-hour treatment of hydrogen peroxide on 13FB cells and compare the results to the 48, 72 and 96 hour exposures. This would help determine whether there are any effects of hydrogen peroxide on the morphology of cells within a one-day period or whether more time is needed to induce senescence in cells. In addition, the next step could be to observe the effects of H₂O₂ treatment on other bio-markers of senescence such as p53 activation and determine the effect of salubrinal (an eIF2- α phosphate inhibitor) on cellular senescence after oxidative stress using H₂O₂ on 13FB cells. This would further the study of human fibroblast's response to oxidative stress, which plays a role in cancer and aging. Despite numerous experiments aging and cancer remain prevalent problems in today's society, but the field of cellular senescence leads us to finding a strategy capable of decelerating the aging process.

Acknowledgements

I would like to acknowledge the doctor in charge of the lab, Dr. Rekha Patel. I would like to acknowledge Benedicth Ukhueduan my graduate student supervisor, the Patel lab, Dr. Glenn Morrow, and Josh Witten. I would also like to thank the South Carolina Governor's School for Science and Mathematics, the Summer Program for Research Interns (SPRI), and ASPIRE II awarded from USC to RCP for providing the funding for this research.

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