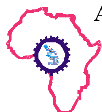
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Human Gingival Fibroblast and Raw Macrophage Cell Response to Steady State H_2O_2 Via GOX/CAT System

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Abstract

Periodontitis is one of the most prevalent forms of dental infections which are characterized by accumulation of subgingival bacteria resulting in chronic inflammation. Chronic dental infections do not always respond to antibiotics and often cause side effects. Therefore, treatment of dental infections with low dose hydrogen peroxide (H_2O_2) may offer a non-antibiotic alterative. Control of an effective dose of (H_2O_2) has limited control over cellular response and function. In this study, the glucose oxidase (GOX) and catalase (CAT) system was used to independently control the amount of H_2O_2 and oxygen in culture. The two aims of this study were: (1) to determine if steady state concentration of H_2O_2 (10µM) will alter Raw 264.7 macrophage like cell function, viability and morphology and (2) to determine if steady concentration of H_2O_2 (10µM) will alter CRL-2014 human gingival fibroblast like cell function, viability and morphology. Raw 264.7 cells were treated with 10µM H_2O_2 for duration of 24 hours. CRL-2014 cells were treated with 10µM H_2O_2 at 24, 48 and 72 hours. The experimental design for RAW 264.7 cells entailed the assessment of functional capacity through the measurement of malondialdehyde (MDA), nitric oxide (NO), and glutathione (GSH). Additionally, structural evaluation was performed through morphological and proliferation methods. To execute aim #2, functional activities assessed by measuring MDA, GSH and protein and structural change were determined as previously stated in aim I. The results revealed there were significant reductions in cell numbers and an increase in NO and GSH. These results suggest that RAW 264.7 cells were sensitive to steady state concentration of H_2O_2 and may maintain the capability to reduce oxidative stress damage to the cells via the redox system. CRL-2014 cells showed no significant difference in protein levels, MDA and GSH levels throughout the experiment. Overall, the cell response to low dose steady state of 10µM H_2O_2 suggests a cell specific response. Morphologic evaluation revealed that the majority of the cells have a single nucleus with rare multinucleated cells and occasional fused cytoplasm compared to control. Based on the observations of this study, future directions in research may include focus on various steady state concentrations of H_2O_2 effect on RAW 264.7 and CRL-2014 cells. These findings provide the literature with more insights regarding the interrelationship between the immune system and the inflammatory process with respect to dental infections.

Keywords: GOX/CAT, hydrogen peroxide, RAW 267.4 cells, CRL-2014 cells

1.0 Introduction

Periodontitis (PE) is a chronic disease which is characterized by an accumulation of subgingival dental biofilm, which induces an inflammatory response. Mixed gram-positive and gram-negative microorganisms initiate the inflammatory response; however, matrix metalloproteinase (MMP) driven by the host response are the major drivers of tissue destruction in the periodontium (Offenbacher, 1996). Pathogenic response to lipopolysaccharide (LPS) is known to recruit monocytes, macrophages and fibroblast. Consequently, this response triggers increase production of cytokines (IL-1, IL-6, IL-8, TNF-α) (Tani *et al*., 1997; McCoy *et al*., 2004). Fibroblast are the most prominent cell in the connective tissue of the periodontium and possess the capacity to maintain connective tissue and participate in wound healing (Hassell, 1993). Fibroblasts also have the capacity to produce proinflammatory

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cytokines in response to LPS. Several studies have documented that the specific fibroblast response to diverse LPS pathogens, peptidoglycans and CpG DNA includes production of IL-1â, IL-6, IL-8, and TNF-á, PGE_2 and MCP-1 (Hatakeyama, *et al.*, 2003; Jandinski *et al.*, 1991; Bickel, 1993; Gemmell, 1997; Offenbacher, 1993).

Current treatment of dental infection includes surgical reduction and systemic and local delivery of antibiotics. Traditionally antibiotic management could lead to resistance, side effects and increased biofilm formation. A recent review of periodontal oral biofilm and biofilm microbiology concluded periodontitis similar to other biofilm infections are refractory to host defenses and targeted antibiotic agents. The investigators determined non-targeted antibiofilm agents such as oxidative agents may be an effective treatment strategy on periodontal oral biofilms (Schaudinn, 2009).

Hydrogen peroxide is a reactive oxygen species (ROS) that causes damage to cells and bacteria. Hydroxyl radicals are generated from H_2O_2 derived from exogenous and endogenous production. The damaging effects of H_2O_2 include lipid peroxidation which leads to DNA damage and cell death (Halliwell, 1989). Oxidation defense include superoxide dismutase (SOD), catalase and GSH which determines the cell response to various concentrations of oxidants and signaling mechanisms for the antioxidant response. Investigators reported that H_2O_2 at a dosage of 700 μmol/L caused necrosis of immortalized rat embryo fibroblast, while a dosage of 150 μmol/L H_2O_2 induced apoptosis (Guenal *et al.*, 1997). In contrast, H_2O_2 exposure of BHK-21 fibroblast to lower levels of H_2O_2 (1 μM) actually stimulated cell proliferation (Burdon *et al.*, 1996).

At low controlled doses, H_2O_2 may eliminate the factors that support dental infections without altering cell function and structure. By eliminating bacteria supporting factors such as high bacterial concentrations, adherence of virulent bacteria into the biofilm matrix, and subsequent production of cytokines; tooth and implant tissue interface may be protected. The negative properties of antibiotic therapy provide a good argument to utilize a non-antibiotic such as H_2O_2. Due to the lack of conclusive evidence in the literature, this study was designed to provide more insights into this area.

The approach of GOX/CAT system used in this study was to independently provide and control the amount of H_2O_2 and oxygen in culture. This follows the principle of Muller *et al.* (2009) in which GOX generates H_2O_2 by consuming oxygen and CAT degrades H_2O_2 into water and ½ molecule of oxygen. As CAT activity removes H_2O_2 in equal amounts, a steady state concentration of H_2O_2 is achieved (Mueller,*et al.*, 2009).

2.0 Methods

Cell Lines: Two cells lines were used in this study. (1) CRL-2014 (gingival fibroblast, human) was obtained from the American Type Culture Collection (ATCC) Rockwell, MD. The cell line was maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with fetal bovine serum (10%) and 1% multi-dose antibiotics (10,000 IU/mL penicillin, 10,000 μg/mL streptomycin and 25 μg/mL amphotericin B). (2) Raw 264.7 cells were obtained form ATCC. The RAW 264.7 cells were cultured and plated in 24-well tissue culture plates. The plates were loaded with RAW 264.7 cells at a density of 1.0×10^6 cells/well in 1.0 mL of Roswell Park Memorial Institute (RPMI) 1640 medium with 10% heated inactivated fetal calf serum and 1% antibiotics. The RAW cells were incubated in 24-well plates for the specified time. Medium renewal was done 2 to 3 times per week. Both cell lines were maintained in a humidified 5% CO_2/95% air atmosphere at 37°C.

Cell Count: A cell count was performed using standard laboratory protocols to examine the cell lines by microscopy for cell proliferation (Webster *et al.*, 2000; Coleman *et al.*, 2000). Following each experimental period, the cells were collected and resuspended in medium. The suspended cells were centrifuged at 1000 rotations per minute for 10 minutes. The media was removed by aspiration and resuspended in 300 μL PBS. The cells were mixed with a 1:1 ratio of cells and trypan blue (20 μL) of each. Ten microliters of 1:1 trypan and cell solution were added to the hemocytometer and then cells were counted in four corners and center squares. The hemocytometer was placed on the stage of an

inverted microscope and focus was determined by cell type. The cells were counted with the hemocytometer at the end of each experimental session to determine cell proliferation. The numbers of cells, per milliliter, were calculated using the following formula: [cells/mL = # of cells counted x 10^4 X dilution factor divided by # squares counted].

Morphological Evaluation: Hematoxylin and Eosin (H&E) staining procedure was performed according to standard laboratory procedure (Webster *et al.*, 2000; Coleman *et al.*, 2000). Cells were covered with (50%) ethanol for 1 minute, and then covered with (95%) ethanol for 1 minute, followed by (50%) ethanol two times at 1 minute each. The cells were then covered for 1 minute with distilled water then hematoxylin was allowed to cover cells for 3 minutes. Stain was removed by rinsing 5 times for 3 minutes each with distilled water. Cells were washed with (50%) ethanol twice for 1 minute followed by (95%) ethanol twice for 1 minute. Eosin stain covered cells for 5 minutes and then washed with (95%) ethanol twice for 1 minute each followed by (100%) ethanol twice for 1 minute each. Cover slips were removed and mounted with glue and glass cover slips. Image Pro Digital Software was used for slide imaging. Criteria for morphological evaluation included nucleus and cellular shape, size, color and background, as well as, cytoplasm appearance and response, and N/C ratio measurement.

Protein Assay: The Thermo Scientific Pierce BCA Protein Assay Kit was utilized in this study to determine protein concentration in CRL-2014 cells. One standard was prepared per kit instructions. Serial dilutions for the standard included 100 µL of BSA/PBS mixture (2 mg BSA powder plus 1 mL PBS) placed in tube 1 and 2, and then 100 µL of PBS was added to tube 2-8. Twenty microliters of standard was placed in each standard line, while 20 µL of sample was placed in wells of the 96-well plate. Then, 200 µL of mixed reagent A & B (reagent A; 300 µL plus reagent B; 15 mL) was added to each standard and sample well. The plate was incubated for 30 minutes. Sample absorbance was read using a wavelength of 540 nm using the WinSelect program.

Malondialdehyde Determination (MDA): MDA was the method used to determine damage of the cell membrane (Del Rio *et al.*, 2005). Trichloroacetic acid (TCA) 20%, 0.5 mL was combined with 100 µL of supernatant. After allowing the precipitate to sit for 1 minute, 0.5 mL of 67% Thiobarbituric acid (TBA) in 2.0 M NaOH was added to the precipitate. Then, samples were heated for 45 minutes at 100°C water bath. Then, the samples were cooled for 5 minutes and centrifuged at 2500 rpm for 10 minutes. From top of solution, 100 µL were added to a 96-well plate. Supernatant absorbance was read at a wavelength of 492 nm. The MDA levels were compared to a standard curve for final analysis.

Glutathione (GSH) Assay: Glutathione assay was used to determine intracellular damage (Camera and Picardo, 2002; Rahman *et al.*, 2006 and Baker *et al.*, 1990). The samples (50 µL) and standard (50 µL) were added to a 96-well plate. Afterwards, 100 µL of mixed reagent was added to both standards and samples. The reagent was a mixture of 5 mL of DNTB, 5 mL of NADPH, 5.75 mL of buffer (EDTA + buffer), and 100 µL of glutathione reductase. Sample absorbance was read at a wavelength of 492, and then the 96-well plate was incubated for 30 minutes and read again to determine decrease in absorption. Results were compared to a standard curve for final analysis.

Nitric Oxide Assay: Standard laboratory procedure was used to determine the amount of NO in RAW 264.7 cell culture supernatants (Dirsch *et al.*, 1998). Cell culture supernatants and standard (100 µL) were plated into a 96 well plate in duplicates. Standard solution was prepared with 0.069 g $NaNO_2$/500 mL distilled water. The standard solution (1 ml) was mixed in 9 mL of distilled water. Reagent 1 (0.1 g of N-1(1-naphthyl) ethylenediamine dihydrocholide (NED)/100 mL H_20) and reagent 2 (1.0 g sulfanilamide/100 mL added to 5% phosphoric acid). Twenty five milliliters of reagent 1 and 25 mL of reagent 2 were combined as a mixed reagent. One hundred microliters of mixed reagent were added to each of the standards and samples in the 96-well plate. After 5 minutes at room temperature, the plate was read at 492 nm and 540 nm.

Oxidizing Agent (Hydrogen Peroxide): Glucose oxidase (GOX) from *Aspergillus niger* was obtained from Sigma-Aldrich (GOX 542-50 KU).

Catalase (CAT) 1 MU was obtained from MP Biomedicals, LLC. The GOX/CAT system was used to independently provide and control the amount of H_2O_2 and oxygen in culture (Mueller *et al.*, 2009). A 1:10,000 GOX dilution was made by mixing 9.999 mL distilled H_2O with 10μL of stock GOX solution. Then, 10μL of 1:10,000 mixed solution was added to 9.999 ml of distilled H_2O resulting in a 1:100,000 GOX dilution. Using a 24-well plate, 1.6 mL of medium was added to each well containing GOX/CAT. Then, 400 μL of 1:100,000 GOX dilution was added to each experimental well for a final volume of 2 mL per well (1:500,000). Catalase stock solution was used to prepare a CAT solution of 1:400,000. The CAT solution was prepared by mixing 5 mL of distilled H_2O with 100 μL of stock CAT (1:200,000) solution. CAT (10 μL) was placed in each experimental well that contained GOX. After treatment of experimental wells, the 24-well plate was incubated for specific number of hours for each experiment.

Experimental Design Aim I: The first phase of this study was done to determine if steady state concentration of H_2O_2 (10 μM) will alter RAW 264.7 macrophage like cell function, viability and morphology after 24 hours. RAW 264.7 cells were plated at a density of 1 x 10^6 wells/time onto one 24-well plate to serve as a 24 hour group. A total of 24 wells/time was plated with RAW 264.7 cells and then divided into 2 groups. The two groups were treated as follows: Group I: control (no treatment); Groups II was treated with 10μM H_2O_2. After 24 hours, the cells and supernatant were removed from each well and stored for future biochemical analysis (MDA, glutathione assay, and nitric oxide). Cells were collected after rinsing with 1 mL PBS by scraping the walls of the culture tubes with a 1mL disposable pipette. Cells from each well were then counted using a hemocytometer and trypan blue. Cells were maintained in a cultured environment and plated on coverslips for H&E staining. Wells that were representative from each group were used for morphological evaluation. Cell morphology was analyzed using 400X magnification after experimental period of 24 hours.

Experimental Design Aim II: The second phase of this study was done to determine if steady concentration of H_2O_2 (10 μM) will alter CRL-2014 human gingival fibroblast like cell function, viability and morphology after 24, 48, and 72 hours. CRL-2014 cells were plated at a density of 1 x 10^6 wells/time onto six 24-well plates to serve as 24, 48 and 72 hour groups. A total of 48 wells/time was plated with CRL-2014 cells and then divided into 2 groups. The two groups were treated as follows: Group I: control (no treatment); Group II was treated with 10 μM H_2O_2. After each experimental period of 24, 48 and 72 hours, the cells and supernatant were removed from each well and stored for future biochemical analysis (MDA, glutathione assay, and protein). After supernatants were collected, the cells were harvested after treatment with 20 μL trypsin followed by incubation for 2 minutes, and then 1 mL of medium was added to stop the enzyme effect of trypsin. Cells were then harvested by lightly scraping the walls of the wells with a 1mL disposable pipette. Cells from each well were then stored for future analysis of protein levels. Cells were maintained in a cultured environment and plated on coverslips for H&E staining. Representative wells from each group were used for morphological evaluation. Cell morphology was analyzed using 400X magnification after experimental period of 24, 48 and 72 hours.

Statistical Analysis: T-tests were performed to assess whether the means of two groups were statistically different from each other. The Mann-Whitney Test was used as a non-parametric option

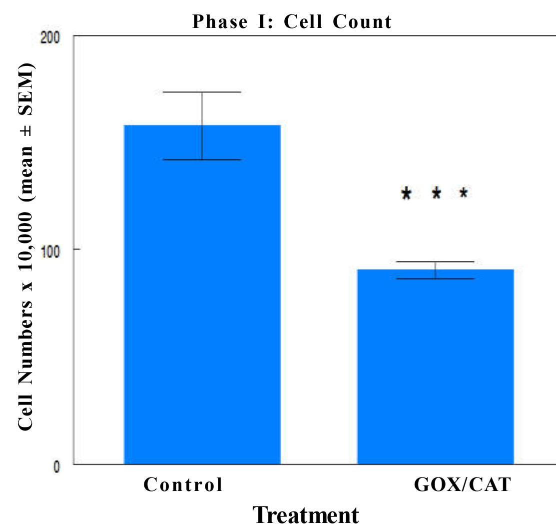


Figure 1: The effects GOX/CAT (10μM H_2O_2) on cell numbers of RAW 264.7 cells after 24 hours incubation. Values are expressed as cell numbers x 10,000 (mean ± SEM).
***P<0.001

for t-test. This test does not assume that the data is normally distributed. This method tested the equality of 2 medians (two medians of the population are equal). Descriptive data was expressed as mean ± SEM. The Jandel Company Statistical Analysis Software (Sigma STAT) was used for statistical analysis.

3.0 Results

Cell Count (Phase I): Figure 1 shows the effects of 10µM H_2O_2 on cell numbers of RAW 264.7 cells after 24 hours incubation. After 24 hours, 10µM H_2O_2 treatment significantly reduce cell numbers by 44% when compared to control (P<0.001) according to Mann-Whitney non parametric test.

Normalized NO (Phase I): The nitric oxide levels are shown in Figure 2. Nitric oxide levels of RAW 264.7 cells treated with 10 µM H_2O_2 increased by 71% after 24 hours experimental period. According to Mann-Whitney non parametric test, results indicated a significant difference in the treatment group compared to the control (P<0.05).

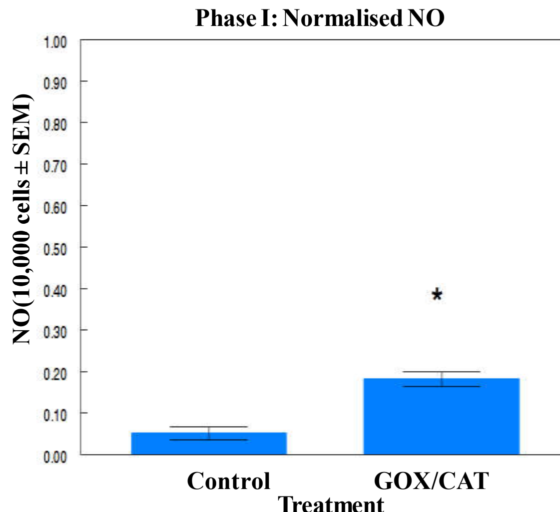


Figure 2: The effects of GOX/CAT (10µM H_2O_2) on nitric oxide levels of RAW 264.7 cells after 24 hours experimental period. Values are normalized with average cell count. Values are expressed mean NO ± SEM. *P<0.05

Normalized MDA (Phase I): The level of MDA appeared to increase after 24 hours as shown in Figure 3. However, a t-test was performed and results indicated no significant difference in MDA levels of RAW 264.7 cells treated with 10 µM H_2O_2 compared to control (P=0.626). Results suggest no damaging effects on cell membrane were evident at 24 hours with steady state concentration of 10 µM H_2O_2.

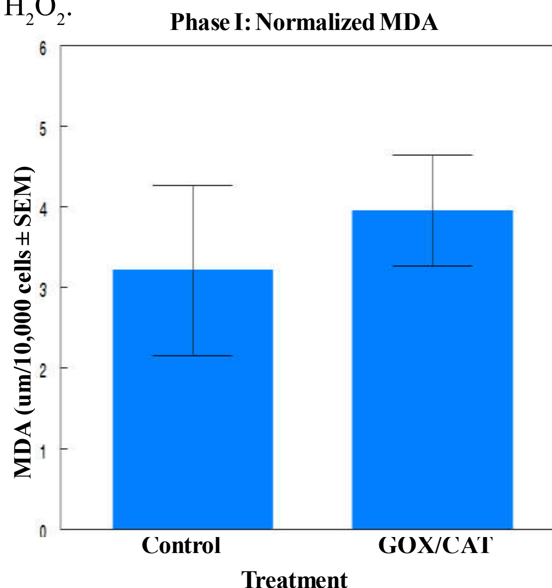


Figure 3: The effects of GOX/CAT (10µM H_2O_2) on MDA levels of RAW 264.7 after 24 hours experimental period. Values are normalized with average cell counts and expressed as MDA mean ± SEM.

Normalized GSH (Phase I): Figure 4 show the effects of 10 µM H_2O_2 on glutathione levels of RAW 264.7 cells after 24 hours experimental period. T-test was performed and results indicated a significant difference between GSH levels in cells treated with GOX/CAT (10 µM H_2O_2) compared to control (P<0.05). Glutathione levels increased by 49.2% compared to control. Significantly increased GSH levels at 24 hours indicated possible intracellular injury.

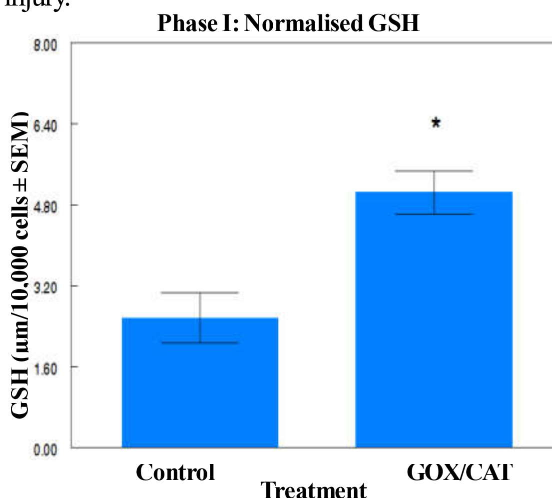


Figure 4: The effects of GOX/CAT (10µM H_2O_2) on GSH levels of RAW 264.7 cells after 24 hours experimental period. Values are normalized with cell counts and expressed as GSH mean ± SEM. *P<0.05

Protein Levels (Phase II): CRL-2014 cells strongly adhere to the tissue culture plate and either scraping or deattaching the cells enzymatically does not allow for accurate cell numbers to be obtained. Therefore, we assessed cellular protein levels for viability and compared cell morphology of cells grown on cover slips for cytotoxicity. Figure 5 shows the effect of 10µM H_2O_2 on the protein levels of CRL-2014 cells after 24, 48, and 72 hours. After 24 hours, protein levels increased by 16% over control, while protein levels decreased by 6% at 48 hours and 13% after 72 hours in response to 10µM H_2O_2. However, results of Mann-Whitney indicated no significant difference between the mean protein values compared to control after 24 hours (P = 0.396), 48 hours (P = 0.557) and 72 hours (P = 0.744). These results suggest no alteration in cellular protein concentration hence viability in response to a steady state dose of 10µM H_2O_2.

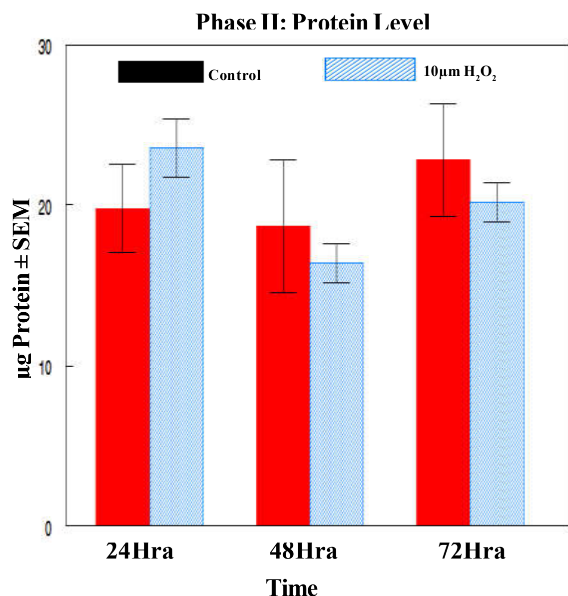


Figure 5: The effects of 10µM H_2O_2 on protein levels of CRL-2014 cells after 24, 48 and 72 hours experimental period. The values are expressed as protein mean ± SEM.

Normalized MDA (Phase II): MDA shows a slight decrease of 19% after 24 hours compared to control value. After 48 hours, MDA levels decreased by 35% compared to control value. In contrast, MDA levels increased slightly by 31% after 72 hours compared to control value (see Figure 6). Although MDA displays a gradual decrease after 24 and 48 hours and slight increase after 72 hours, there was no statistical significant cell membrane damage in cells treated with 10µM H_2O_2 after each treatment period 24 hours (P=0.498), 48 hours (P=0.976) and 72 hours (P=0.372).

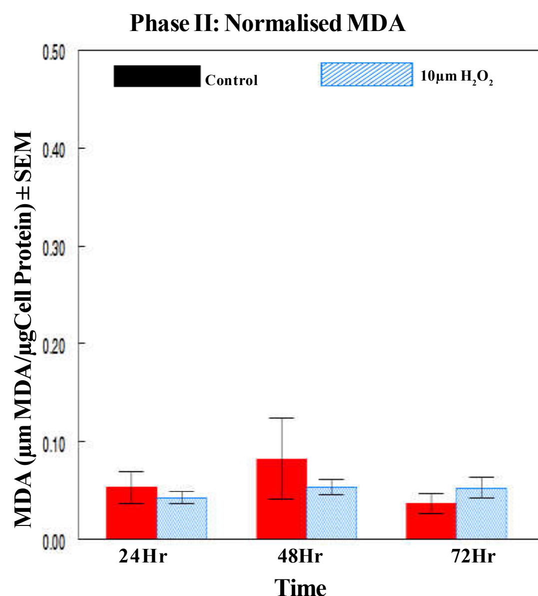


Figure 6: The effects of 10µM H_2O_2 on MDA levels of CRL-2014 cells after 24, 48, 72 hours incubation. Values are normalized with protein and expressed as MDA mean ± SEM.

Normalized GSH (Phase II): GSH levels (Figure 7) showed an increase of 43% in response to 10µM H_2O_2 compared to control at 24 hour, while GSH levels decreased by 37% compared to control after 48 hours. No change in GSH was shown after 72 hours. While the GSH levels displayed a slight change after 24 and 48 hours, results indicated no significant intracellular damage after 24 hours (P=0.396), 48 hours (P=0.286) and 72 hours (P=0.711) compared to control.

Morphology of RAW 264.7 Cells Treated with 10µM H_2O_2 after 24hours

Figure 8 shows photomicrographs which represent the effects of 10µM H_2O_2 on RAW 264.7 cells at 24 hours (400X magnification). Cells in the control group (A) appear healthy with major features that include: mostly spindle cells with occasional round shapes, mitotic figures, minimal clustering and a hy-

perchromic appearance. The cells in the treatment groups (B) and (C) are similar in size, color, and N/C ratio and cytoplasm presentation. The cells are medium in size, round in shape with occasional spindle cells, and display a hyperchromic appearance with occasional mitotic figures. All cells contain a single nucleus with occasional multinucleated cells. The N/C ratio is 2:1. Clustering of cells and irregular cytoplasm are seen occasionally compared to control. Cells in image (D) have a spindle shape with less clustered appearance than cells in image (B) and (C).

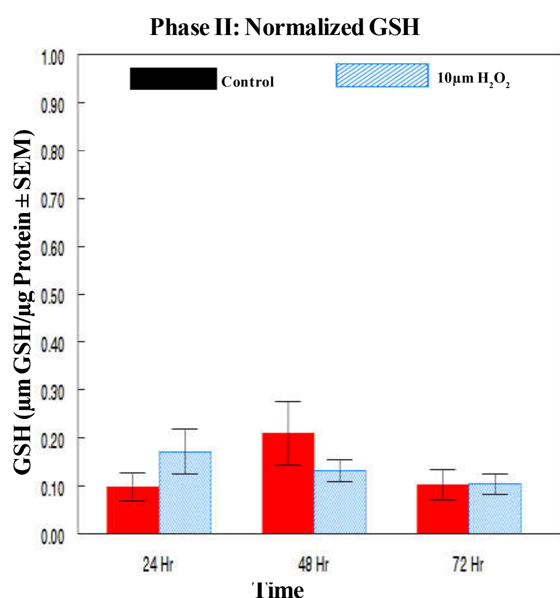
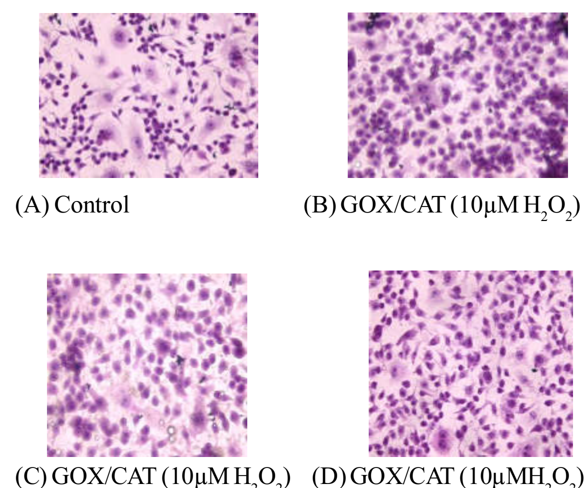


Figure 7: The effects of 10µM H_2O_2 on glutathione levels of CRL-2014 cells after 24, 48, 72 hours incubation. Values are normalized with protein and expressed as GSH mean ± SEM.

Morphology of RAW 264.7 Cells Treated with 10µM H_2O_2 after 24hours

Figure 8 shows photomicrographs which represent the effects of 10µM H_2O_2 on RAW 264.7 cells at 24 hours (400X magnification). Cells in the control group (A) appear healthy with major features that include: mostly spindle cells with occasional round shapes, mitotic figures, minimal clustering and a hy-perchromic appearance. The cells in the treatment groups (B) and (C) are similar in size, color, and N/C ratio and cytoplasm presentation. The cells are medium in size, round in shape with occasional spindle cells, and display a hyperchromic appearance with occasional mitotic figures. All cells contain a single nucleus with occasional multinucleated cells. The N/C ratio is 2:1. Clustering of cells and irregular cytoplasm are seen occasionally compared to control. Cells in image (D) have a spindle shape with less clustered appearance than cells in image (B) and (C).



(A) Control (B) GOX/CAT (10µM H_2O_2)

(C) GOX/CAT (10µM H_2O_2) (D) GOX/CAT (10µM H_2O_2)

Figure 8: H&E of RAW 264.7 cells with treatment of 10µM H_2O_2 after 24 hour period (400X magnification). For more details see results section.

Morphology of CRL-2014 Cells Treated with 10µM H_2O_2 after 24, 48 and 72 hours

Figure 9 shows the representative images of H&E stained CRL-2014 cells treated with 10µM H_2O_2 after 24 hours. Control cells (A) appear healthy with major characteristics that include: cells appeared hyperchromic with primarily spindle shaped cells and occasional round cells, clear background with clustered arrangement and some visible mitotic figures. Similar features are seen in (B), (C) and (D) images. Cells treated after 24 hours appeared to have cluster formation, hyperchromic features, spindle shapes with occasional round cells. The majority of the cells have a single nucleus with rare multinucleated cells and occasional fused cytoplasm compared to control.

Figure 10 shows the representative images of H&E stained CRL-2014 cells treated with 10µM H_2O_2 after 48 hours. Control cells (A) appear healthy, which include the following features; round and spindle shapes, occasional mitotic figures, single arrangement, prominent nucleus and single arrangement. CRL-2014 cells treated with 10µM H_2O_2 showed a simular morphological response in images (B), (C) and (D). The major features include; cells in image (B) are more hyperchromic and clustered

compared to control, otherwise simular to control. Cells in (C) and (D) images are less hyperchromic than control, otherwise cells appear healthy.

Figure 11 shows the representative images of H&E stained CRL-2014 cells treated with 10µM H_2O_2 after 72 hours. Control cells (A) at 72 hours are simular to control at 48 hours. Cells treated with 10µM H_2O_2 (B), (C), and (D) showed fewer cells with occasional clustering compared to control, otherwise cells appear healthy.

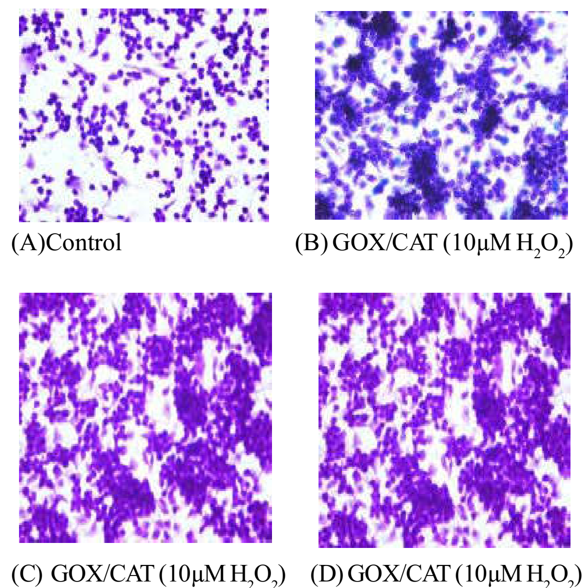


Figure 9: H&E of CRL-2014 cells with treatment of 10µM H_2O_2 after 24 hour period (400Xmagnification). For more details see results section.

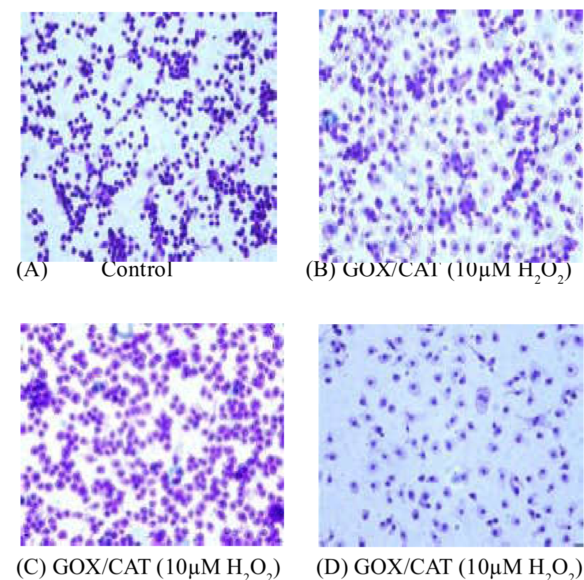


Figure 10: H&E of CRL-2014 cells with treatment of 10µM H_2O_2 after 48 hour period (400Xmagnification). For more details see results section.

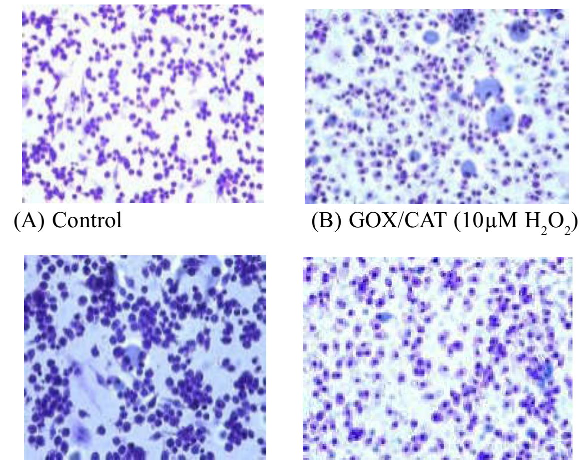


Figure 11: H&E of CRL-2014 cells with treatment of 10µM H_2O_2 after 72 hour period (400Xmagnification). For more details see results section.

4.0 Discussion

RAW 264.7 Cell Response to Steady State Concentration of H_2O_2 (10µM)

Results of this study suggest RAW 264.7 cell proliferation was altered by the treatment of a steady state concentration of 10µM H_2O_2 after 24 hours. At 24 hours, 10µM H_2O_2 significantly reduced cell numbers by 44% when compared to control. In contrast to significant reductions in cell numbers, results from this study suggest no cell membrane damage was evident after 24 hours. According to the literature, previous studies emphasized the use of high doses of H_2O_2. Studies using high dose H_2O_2 have revealed important signal pathways and gene activation, decreased cell growth and cellular damage (Weiss, 1989; Thannickal and Fanburg, 2000). These studies are in contrast to our results which showed a significant decrease in cell numbers.

Low dose H_2O_2 exposed to RAW macrophage like cells revealed significant findings. The results of this study may be explained by the fact that cell lines such as macrophages could be more sensitive to low dose H_2O_2 and activate pathways which induce cellular apoptosis. Low dose signaling pathways are not fully understood while high doses of H_2O_2 were found to function as key messengers that modulate

protein phosphorylation through cysteine oxidation denoting a new signaling pathway for ROS (Guy *et al.*, 1993). The findings of this study are in agreement with studies that found macrophages treated with low dose H_2O_2 resulted in decreased cell numbers (Tang *et al.*, 2007).

Morphological evaluation of RAW 264.7 cells treated with 10µM H_2O_2 suggests minimal structural changes. The major features included cells that appeared round and somewhat clustered with occasional irregular cytoplasm compared to mostly spindle shaped cells with prominent nucleus and regular shaped cytoplasm of control cells. Overall, the cells were able to maintain viable structural activities.

A significant increase by 71% in NO was observed after 24 hours. These findings are supported by the classic response of macrophage cells to products that cause oxidative stress. For example, nitric oxide is released by macrophages in response to LPS and other toxic stimulants over several hours to provide defense against oxidative damage and pathogens (MacMicking *et al.*, 1997).

Findings from this experiment suggest possible intracellular damage was evident after 24 hours incubation with 10µM H_2O_2. A significant increase in GSH levels (49.2%) was evident at 24 hours. Interestingly, as GSH levels increased, NO levels increased. These finding are in agreement with a study where investigators reported moderate but significant increased glutathione levels were induced with low dose (1µM H_2O_2) while high dose (>10µM H_2O_2) substantially increased GSH levels in bovine pulmonary artery endothelial cells (Day *et al.*, 2003). Hydrogen peroxide detoxification by catalase and glutathione has been shown to protect macrophages from oxidant damage *in vitro* (Pietarinen *et al.*, 1995). The major defenses against H_2O_2 are catalase and the glutathione redox cycle. These enzymes reduce H_2O_2 to non toxic products. The reduction of H_2O_2 to H_2O occurs when glutathione peroxidase oxidizes glutathione to glutathione disulfide, while catalase converts H_2O_2 to H_2O and O_2. Recently, NO at low concentrations was found to also provide protection to RAW 264.7 macrophages from H_2O_2 toxicity by inducing the formation of catalase (Yoshioka *et al.*, 2006). Response to oxidative stress varies with cell density and cell type and redox imbalance. While an increase in the oxidative environment from high dose oxidants and ROS causes cells to stimulate transcriptional regulation of genes that regulate glutathione, there is limited knowledge about the signaling process in low levels of ROS (Day *et al.*, 2003 and Reth, 2002).

CRL-2014 Cell Response to Steady State Concentration of H_2O_2 (10 µM)

As expected, protein levels showed no significant difference in protein concentration in CRL-2014 cells treated with a steady state dose of H_2O_2 (10 µM) compared to control after each treatment point of 24, 48 and 72 hours. Results of this study suggest that cell proliferation was not altered by 10µM steady state dose of H_2O_2. It was interesting to note that no time dependent effect was evident in protein levels when challenged with a steady state dose of H_2O_2 (10µM) after 24, 48 and 72 hours treatment periods. Our findings are supported by studies that found dividing human cells grown in culture with low (3-15µM) concentrations of H_2O_2 and other oxidants stimulate cell growth and mitosis (Burdon *et al.*, 1989; Burdon, 1995).

Morphological evaluation of CRL-2014 cells after 24 hours showed fused cytoplasm in some cells compared to control, otherwise cells appear healthy. Similar morphological results were seen in cells treated at 48 and 72 hours. These findings suggest no alteration in CRL-2014 cell structure with respect to size, shape, nucleus and color after 24, 48 and 72 hours exposure with a steady state dose of H_2O_2.

Recently, studies that used low and high doses of H_2O_2 to investigate ROS effects on eukaryotic effects found biphasic results with respect to cell growth. Similar results to this present study with respect to cell growth were demonstrated in a study which found fibroblast treated with 3 to 15 µM of H_2O_2 showed growth and a significant mitogenic response. In addition, it was reported that cells showed temporary growth arrest with DNA repair at 120 to 150 µM and apoptosis at 0.5 to 1 mM dose (Davies, 1999). Even though this study used a different fibroblast cell line and different H_2O_2 delivery, the results indicate the difference in cell growth and mitotic response of fibroblast to low dose H_2O_2

compared to high dose H_2O_2 on cell proliferation.

Findings in this study suggest that CRL-2014 cells treated with steady state dose of 10μM H_2O_2 will not induce damage in cell membrane nor intracellular structure. In a recent study examining the effects of glutathione by oxidative stress in bovine pulmonary artery endothelial cells, researchers reported H_2O_2 bind to antioxidant response element (ARE) to regulate GSH and cell growth signaling (Day *et al*., 2002). Results from another study suggested exogenous addition of both stimulatory dose of 1μM H_2O_2 and inhibitory dose of >10μM H_2O_2 moderately and substantially increased glutathione levels respectively (Day *et al*., 2003). The results from Day's study are in contrast to our results in that CRL-2014 cells treated with a steady state dose of 10μM H_2O_2 showed an increase at 24 hours, decrease at 48 hours and no change at 72 hours in glutathione levels compared to each corresponding control. Differences in the results of this study compared to Day's study may be due to the use of a different cell line and H_2O_2 delivery compared to CRL-2014 cell line and GOX/CAT system. In a recent review, the investigators concluded that little is known about low dose ROS balance of the redox environment compared to high dose ROS cell response. In summary, this study found that response to cell growth; metabolism and cellular functions of detoxification were dependent on the dose of ROS and specific cell types (Day and Suzuki, 2005).

5.0 Conclusions

The results of this study demonstrate that significant reductions in cell numbers and significant increase in NO and GSH suggest that RAW 264.7 cells are sensitive to low dose steady state concentration H_2O_2 and may maintain capability to reduce oxidative stress damage to the cells via the redox system. In addition, morphological evaluation showed some clustering of cells and irregular cytoplasm seen occasionally compared to control. Findings from the data in this study showed no alteration in CRL-2014 cell proliferation, morphology, intracellular or cell membrane integrity after treatment with a steady state concentration of H_2O_2 (10μM) after 24, 48 and 72 hours. These findings suggest low dose steady state concentration of H_2O_2 (10μM) may indicate specific dose and cell type response to exposure to ROS. Based on the following observations, we propose that low dose H_2O_2 may have potential as an antibiotic alternative in the treatment of dental infections. Future studies are needed to determine alternative doses and chronic exposure to oxidative agents at low doses.

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