

## QUANTUM 3D PEROXIDE MODEL FOR TREATING A SELECTIVE THERAPEUTIC SOLUTION FOR LIVER CANCER

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### ABSTRACT

Recent studies have demonstrated that reactive oxygen species (ROS) may be a plausible approach for treating cancer. However, the potential drawback is while many treatments halt carcinoma growth, they also have detrimental effects on normal tissue. Hence, creating a selective therapy would be advantageous. Our study utilized peroxide generating particles, sodium percarbonate (SPO), calcium peroxide (CPO), and magnesium peroxide (MPO), as an ROS delivery system, for targeting and killing hepatomas (HepG2), while having little to no effect on the normal healthy hepatic cells (hepatocytes). The relation between hydrogen peroxide and cell death was investigated in detail. All three peroxide delivery systems were able to reduce cell viability of the HepG2, while sustaining viability of the hepatocytes. All three systems also significantly reduced cell growth and colony formation of the HepG2 cells, whereas no significant change in the hepatocytes regarding morphological and growth patterns were observed. It was found that CPO was most effective at halting proliferative hepatomas. This data suggests that exploiting the intracellular hydrogen peroxide stress of hepatomas, may be a novel approach for targeting liver carcinomas in a selective manner. Also that peroxide is a beneficial tool for causing apoptosis of hepatomas.

**KEYWORDS :** Hepatoma, ROS, Calcium Peroxide, Magnesium Peroxide, Sodium Percarbonate

Cancer continues to be one of the leading causes of death throughout the world. The ideal therapy for treating cancer would be one that selectively eliminates cancer cells while at the same time minimizing damage to normal tissues. Many approaches have been explored to selectively target a wide variety of biochemical mechanisms that are unique to cancer cells. However, a potential drawback of targeting a particular cell pathway is that while it may be specific, it may also limit the utility of that therapy to a very specific type of cancer.

An alternative strategy would be to use therapies that exploit a characteristic weakness of many types of cancers while at the same time causing minimal damage to normal cells. These weaknesses are commonly associated with the deregulation of cancerous cells. For example, many cancerous cells have an overactive metabolism, DNA damage, replicative stress, proteotoxic stress, and oxidative stress (Lapse 2001). Hence, an effective therapeutic agent might exploit these weaknesses as a way to selectively target cancerous cells.

For this particular study we focused on the fact that cancerous cells generate an increased amount of reactive oxygen species (ROS) (Hoping. 1998). This is due to the increased metabolic activity in tumour cells which increases ATP requirements and therefore drives electron transport to produce excess superoxide and hydrogen peroxide. As a consequence of the increased metabolic

activity and hence increased production of ROS, the cancer cells are in a constant state of oxidative stress, but not at sufficiently high enough concentration for the ROS to kill the cancer cells. (Hoping. 1998) Therefore it seems reasonable that the addition of exogenous hydrogen peroxide can increase this stress even more. Studies substantiate this idea showing that hydrogen peroxide can induce apoptosis in tumor cells. It is also believed that cancer cells have reduced synthesis of antioxidants. Less antioxidants present, has the potential to lower the defence mechanism of these cells, making them more susceptible to death by peroxide. Hence, we postulate that cancer cells will be vulnerable to the addition of exogenous peroxide compared to normal cells.

If cancer cells are exposed to incrementally higher concentrations of ROS, then a point could be reached which selectively kills cancer cells while sparing normal cells. This may be possible because normal cells have a buffer capacity (antioxidants etc.) to counteract bursts of hydrogen peroxide. In contrast, the oxidant defences within the tumor cells have reduced buffer capacity to effectively deal with a modest increase in hydrogen peroxide thereby triggering cell death. This makes the use of hydrogen peroxide a novel targeted specific treatment. This concept has been demonstrated with Leucoma (an anti-cancer mitochondrial drug) to inhibit cancer cell growth in rats. This drug increased the hydrogen peroxide levels enough to trigger

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apoptosis. When hydrogen peroxide is prevented from reacting with the drug (in the presence of anti-oxidants), the drug was no longer effective in destroying the tumours.

Although many researchers have studied ROS as an approach for halting cancerous growth, the question of whether or not an ROS treatment can selectively halt hepatoma growth has not been answered in detail. Treatment for hepatocellular carcinoma has a very success rate compared to other cancer types and often leads to quick metastasis. Hence, finding an effective, selective pharmaceutical treatment for this disease proves to be advantageous.

While excessively high concentrations of hydrogen peroxide can have damaging effects on normal tissue growth and cellular function, having the ability to deliver sustained doses of hydrogen peroxide at modest levels, may provide an excellent way to provide extra oxidative stress to cancer cells while not harming normal cells. Therefore, studying materials which can provide a controlled or tenable release of hydrogen peroxide may provide a novel approach to treating cancer. Ideally, such materials would generate sufficient levels of hydrogen peroxide over time to trigger cell death in the already oxidatively stressed cancer cell.

For this study, SPO, CPO and MPO were used as peroxide generators (Trivedi .2016). These particles were then incorporated into a 3D collagen gel and cells were sandwiched in between this matrices'. The sandwich system provided an optimal environment for the hepatic cells as well as a suitable drug screening model. The optimal concentrations were chosen for each oxidant (SPO 1mg/2.5ml gel, CPO 2.5mg/2.5ml gel and MPO 4mg/2.5ml gel). Optimized gels were then assessed and evaluated for their impact on hepatic carcinoma growth as well as normal hepatic growth. Experiments examined the effects of these peroxide gels on low and high cell density constructs.

## MATERIAL AND METHODS

### Preparation of Three-Dimensional Peroxide Generating Gels

1mg/ml of rat tail collagen type 1 (Invitrogen, Carlsbad, CA) was transferred into a 15-ml conical tube and

neutralized using 0.1N sodium hydroxide and sodium bicarbonate. F12 medium (Life Technologies, Carlsbad CA) was added as a pH indicator followed by the addition of phosphate buffered saline (PBS) to buffer the system. Following neutralization, peroxide generating particles (SPO, CPO or MPO) were incorporated into 2.5ml of collagen gel at various concentrations (SPO 1mg, CPO 2.5mg, MPO 4mg). The particles were thoroughly mixed into gel and 0.5ml was added into each well of a 96 good plate. The peroxide generating gel solidified after being incubated for 1hr at 37 °C. Desired cell type was then seeded on top of the gel. Following 1hr incubation, another collagen gel layer (no peroxide particles present) was added on top of the cells. Cells were monitored in this matrix during a 2-week period. All experiments conducted included control gels containing no peroxide generating particles.

### Hydrogen Peroxide Assessment

Quantification of hydrogen peroxide was assessed using titration. Samples were titrated with 0.0098M potassium permanganate. Potassium permanganate was standardized using oxalic acid and controls ran to confirm no cross reaction with organic matter. 4mg of peroxide particles (SPO, CPO and MPO) in separate 2.5ml sample of the gel was prepared inside of a 15ml conical tube and allowed to solidify for 30 minutes. At various intervals, 50 µL of gel was removed from the conical tube and transferred to a micro centrifuge tube. To the tube, 500 µL of water and 50 µL of 8M sulfuric acid were added. Samples were titrated with potassium permanganate, starting with 90% of the theoretical amount, followed by adding 10ul aliquots until reaction was complete (indicated by persistent pink colour). Triplicates used for all treatments (n=3).

### Cells Seeded in Three-Dimensional Peroxide Generating System

Hepatocytes and HepG2 seeded on separate 3D peroxide generating gels. Primary human hepatocytes were purchased from a commercial source (Invitrogen, Cell Z Direct). Hepatocytes were counted and  $5 \times 10^4$ - $1 \times 10^5$  cells were seeded on top of the solidified peroxide generating gels. Note: Following 1hr hepatocytes were washed with PBS to remove unattached cells from gel.  $5 \times 10^4$  HepG2 cells were seeded on the top of each peroxide generating gel.

After 1hr another layer of collagen gel (no peroxide material present) was added on top of the hepatocytes and HepG2. Top layer gel allowed to solidify for 1hr. 1ml of fresh KSFM medium (Life Technologies, Carlsbad CA) was added to all hepatocytes and 1ml of DMEM-high glucose (Life Technologies, Carlsbad CA) was added to the HepG2 cells. Morphological structure was assessed daily using bright field microscopy (Leica Inverted). During day 7 and 14, cell proliferation was measured quantitatively using an MTS assay. On day 14, cell viability was assessed qualitatively using live/dead assay (Life Technologies, Carlsbad CA).

Hepatocytes and HepG2 were also seeded in coculture.  $5 \times 10^3$  HepG2 tagged with Green Fluorescent Protein (GFP) and  $5 \times 10^3$  Hepatocytes were seeded together on top of peroxide generating gels. Cells were allowed to attach for 1 hour followed by a PBS wash. Next, a top layer of collagen gel (no peroxide material present) was added on top of cells and allowed to solidify for 1 hour. Williams E. Medium was used to feed cells during culture. On Day 7 cells were assessed for cell growth and morphological characteristics.

#### **Viral Transfection of HepG2**

To distinguish the HepG2 from the Hepatocytes grown together in coculture, HepG2 were tagged with GFP (cytoplasmic marker). 20,000 HepG2 cells were plated into a 6 well plate and allowed to grow to 70% confluence. 2ml of DMEM medium was aliquoted into a conical tube followed by the addition of 2ul of 8mg/ml Polybrene stock (Invitrogen). The solution was added into 1 well of 6 well plate. 10ul of GFP virus was pipetted into the well. Cells were incubated with the virus for 24 hours. The transfecting agent was removed from cells and fresh medium added. The virally transfected HepG2 were then expanded and used in the coculture experiment.

#### **Peroxide Effect on Rapidly Proliferating HepG2 Hepatomas**

HepG2 cells were seeded at  $1 \times 10^4$  cells/well in a 96 well plate. Cells were left to grow in culture for 24 hours, until 95% confluence. Following 24 hours, 50  $\mu$ L of peroxide gels were added on top of cells. Morphology was assessed every 24 hrs. MTS was used to measure cell proliferation on day 1, 3, 7 and 14.

#### **Cell Proliferation Assay**

MTS colorimetric assay (Cell Titer 96 Aqueous cell proliferation kit; Promega, Madison, WI) was used as a quantitative method for assessing cell proliferation. The medium was aspirated from each well and 120  $\mu$ L of fresh MTS containing solution (20  $\mu$ L MTS reagent + 100  $\mu$ L of cell culture medium) was added to each well, and the samples were incubated at 37 °C for 1 hr. A sample of solution was removed from each well and transferred to a new 96 well plate. Samples were read at an absorbance of 490 nm using a plate reader.

#### **Imaging Analysis**

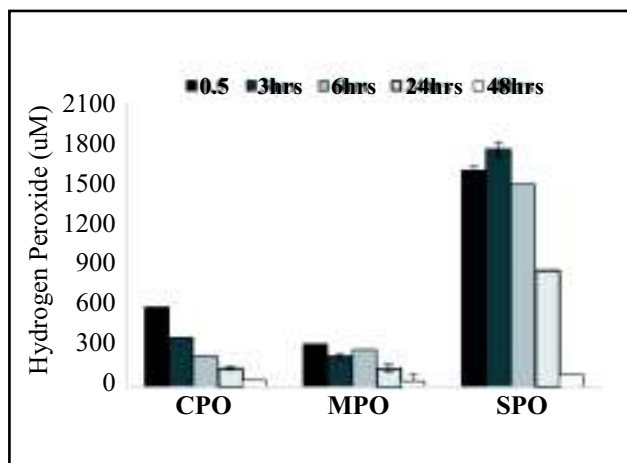
During the culture of cells in 3D gels, morphological structure was assessed on a daily basis using an inverted microscope (Leica Axiovert) at total magnifications of 100X and 200X. Components observed on the HepG2 cells included: colony formation (cluster of HepG2 cells), colony dispersion and migratory patterns. Hepatocytes were observed for membrane rigidity and liver cord formation.

#### **Cell Viability Assay**

A Live and Dead assay was used as a qualitative method for assessing cell viability. Media was removed from all wells and the gels washed with PBS twice for 5 minutes. After washing to remove excess medium, samples were incubated with reagents from a live and dead cell staining kit (Live/Dead Viability/Cytotoxicity, Life Technologies). Antibody was diluted in PBS using a 1:500 ratio. Cells were incubated in reagent for 1hr at room temperature. Following incubation samples were washed three times with PBS for 5 minutes each to remove excess antibody and the samples were later fixed in 10% formalin for 20 minutes. After fixation, the samples were viewed under a microscope (Leica Inverted) to assess live (green fluorescence) and dead (red fluorescence) cells.

#### **Quantitative Image Analysis**

HepG2 were seeded separately and in coculture with the hepatocytes. HepG2 colonies (cluster of HepG2) were measured by picking ten colonies and using phase and contrast image to calculate the area. HepG2 growth seeded in the coculture experiment was measured by quantifying the GFP marker. HepG2 tagged with GFP (fluorescent cytoplasmic marker), were imaged at 4X magnification



**Figure 1. Peroxide content was measured in gels using titration method. Samples were run in triplicates to obtain an n=3. Results indicate that a burst of peroxide is released during the first 24hrs followed by a gradual degradation over time. Error bars represent standard deviation. Note: Some samples contain no error bars because variance below the limit of detection.**

using the GFP fluorescent filter. The green fluorescent intensity of live cells was then quantified using Image J Software. A decrease in GFP intensity, when compared to the control, indicated a decrease in HepG2 growth.

#### Statistical Analysis

Data shown in the bar graphs are mean  $\pm$  standard deviations, unless otherwise noted. Student's paired t-test was used to determine significance of difference between means. A p value of less than 0.05 was interpreted as a significant difference between data means. N=3 was utilized for all assays.

## RESULTS

### Hydrogen Peroxide Production

Titration with potassium permanganate has a sensitivity level up to 3uM [19]. Our gels generated approximately 300-1800uM of peroxide. Pure peroxide gels (hydrogen peroxide incorporated into gels) were evaluated to determine optimal peroxide levels for this system. Pure peroxide gels were loaded with various hydrogen peroxide concentrations and cell proliferation as well as morphological structure was measured. It was determined that hydrogen peroxide concentrations of 1000  $\mu$ M and

above induced significant cell loss/death (data not shown.) The morphological analysis confirmed that cells are able to proliferate in a 500  $\mu$ M or less of hydrogen peroxide, making this range of peroxide desirable to use during culture. Once the concentrations exceeded 1000  $\mu$ M, cells were unable to proliferate in culture. From a phenotypical perspective, the HepG2 cells became dispersed in culture and lost their colony formation indicative of cell death. Following Peroxide dose curve, peroxide generating gels (CPO, SPO, and MPO gels) were assessed for hydrogen peroxide release in a time trial.

The peroxide generating gels showed good stability over time. The CPO, MPO, and SPO gels showed peak levels of 590, 320, and 1760  $\mu$ M, respectively, within the first three hours of gel formation. The SPO remained above 1000  $\mu$ M until approximately 24 hours, when the measured peroxide level dropped to 860  $\mu$ M. Because some of the CPO and MPO particles do not completely dissolve, the test samples were centrifuged to remove residual unreacted particles before measuring hydrogen peroxide concentration. If the residual particles were not removed, then the peroxide values measured would represent the total amount of hydrogen peroxide present, including that which was still bound within the particles. It is important to note that during this peroxide experiment all peroxide generating gels were assessed using 4mg/2.5ml gel. However, during cell culture experiments concentrations varied from 1mg-4mg/2.5ml gel, to not exceed desired peroxide levels. Hence normalizing the data to the desired concentration used in culture suggests that the peroxide gels generated 300-450uM of peroxide during culture. The peroxide generating gels showed a gradual release of peroxide during the first 48 hours.

Presumably, it would appear that such high levels of peroxide would prevent growth of cancerous and normal cells. However studies show that cells cultured in 3D can survive exposure to cytotoxic agents better than 2D [20]. Hence, the peroxide generating gels have the potential to produce enough peroxide to kill the already oxidatively stressed hepatic carcinoma cells, while having little to no effect on the healthy hepatic cells.

### Morphology and Growth Patterns

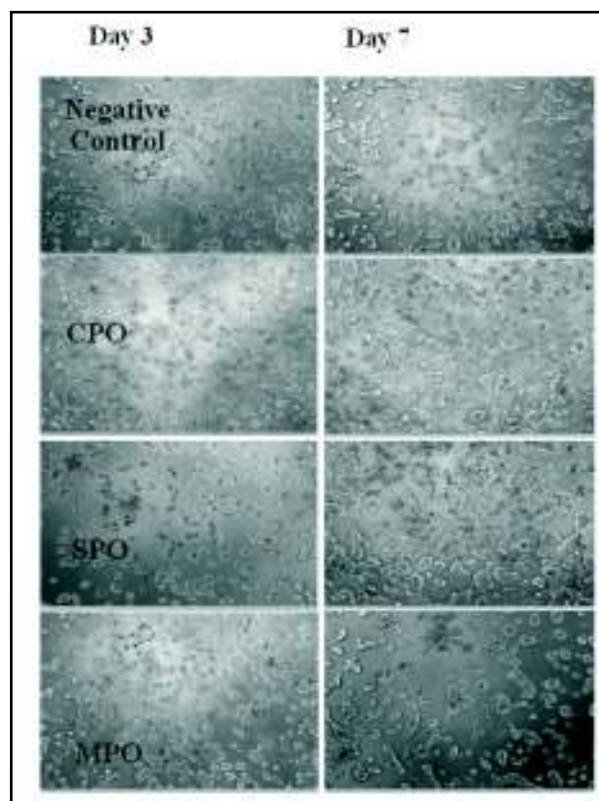
Primary human hepatocytes were seeded on the

surface of a three dimensional peroxide generating gel and cultured during a two week period. It was observed that the primary hepatocytes in all three gels maintained their cuboidal shape and structure in each of the control groups. The primary hepatocytes also displayed liver cord formation (cell to cell linkage). Cell growth stabilized throughout the two week period. Overall, the morphological patterns of the hepatocytes seeded on the peroxide gels displayed normal cell structure (Fig. 2). The HepG2 cells seeded on top of the 3D peroxide gels on the other hand displayed significant loss of structure (Fig. 3). The cells lost most of their colony formation (cluster of HepG2). Any colonies that did form were very loose, which is atypical for carcinoma cells. Of particular note, the number of colonies and size of colonies was significantly reduced in the gels containing the peroxide generating

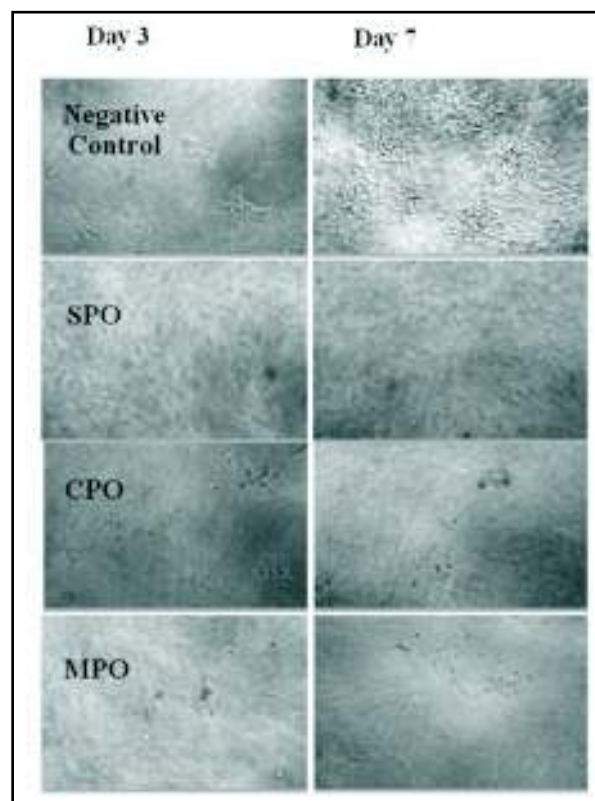
material as shown in Fig. 3 and Fig. 4. This suggests that the 3D peroxide gels were able to reduce colony size significantly and reduce growth of carcinoma HepG2, while maintaining the morphology of the primary hepatocytes.

#### Cell Proliferation Assay

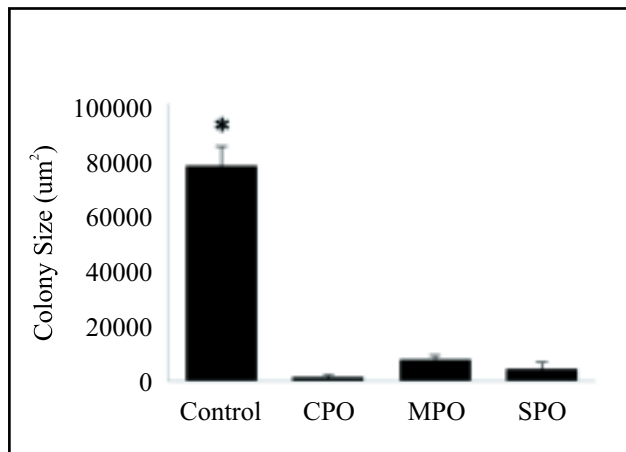
For further quantification of cellular survival, the cell growth of Hepatocytes and HepG2 were measured. To estimate the cell growth in peroxide generating gels, MTS assays were performed at various time points. The results shown in Fig. 5A demonstrate that in all three peroxide generating gels (CPO, SPO & MPO), there was no significant difference in hepatocyte growth when compared to the control. However, for the peroxide generating gels containing HepG2 cells (Fig. 5B), the cell growth was significantly reduced. This suggests that the peroxide gels are supplying hydrogen peroxide at levels that halt HepG2



**Figure 2. Morphology of Hepatocytes seeded on peroxide gels were assessed using the Lecia Inverted at a magnification of 200X. All samples compared to the negative control (no treatment)**



**Figure 3. Morphology of HepG2 seeded on peroxide gels were assessed using the Lecia Inverted at a magnification of 200X. All samples compared to the negative control (no treatment).**



**Figure 4.** Colony size of HepG2 cells were calculated using Image J. Software. Samples were captured using phase contrast microscopy at 100X. Size of colonies within a 100X field of view were counted. \*p<0.001 indicating significantly larger colonies than treated groups.

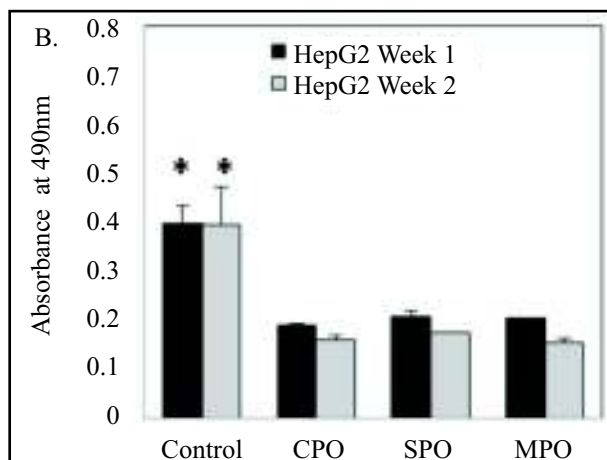
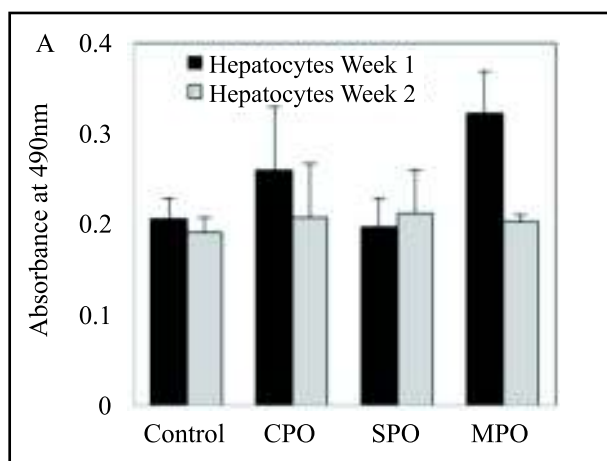
growth, but not hepatocyte growth. It is very plausible to believe that HepG2 are vulnerable to exogenous peroxide, because they are already oxidatively stressed, hence the addition of even a small amount of peroxide, can lead to apoptosis.

#### Cell Viability

Additional qualitative characterization was assessed using the cell viability Live and Dead assay. Primary human hepatocytes seeded on peroxide gels and control gels displayed same viability in relation to control (data not shown). In contrast, HepG2 cells seeded on the peroxide gels showed high cell death (Fig. 6). Very few HepG2 remained viable in the peroxide gels.

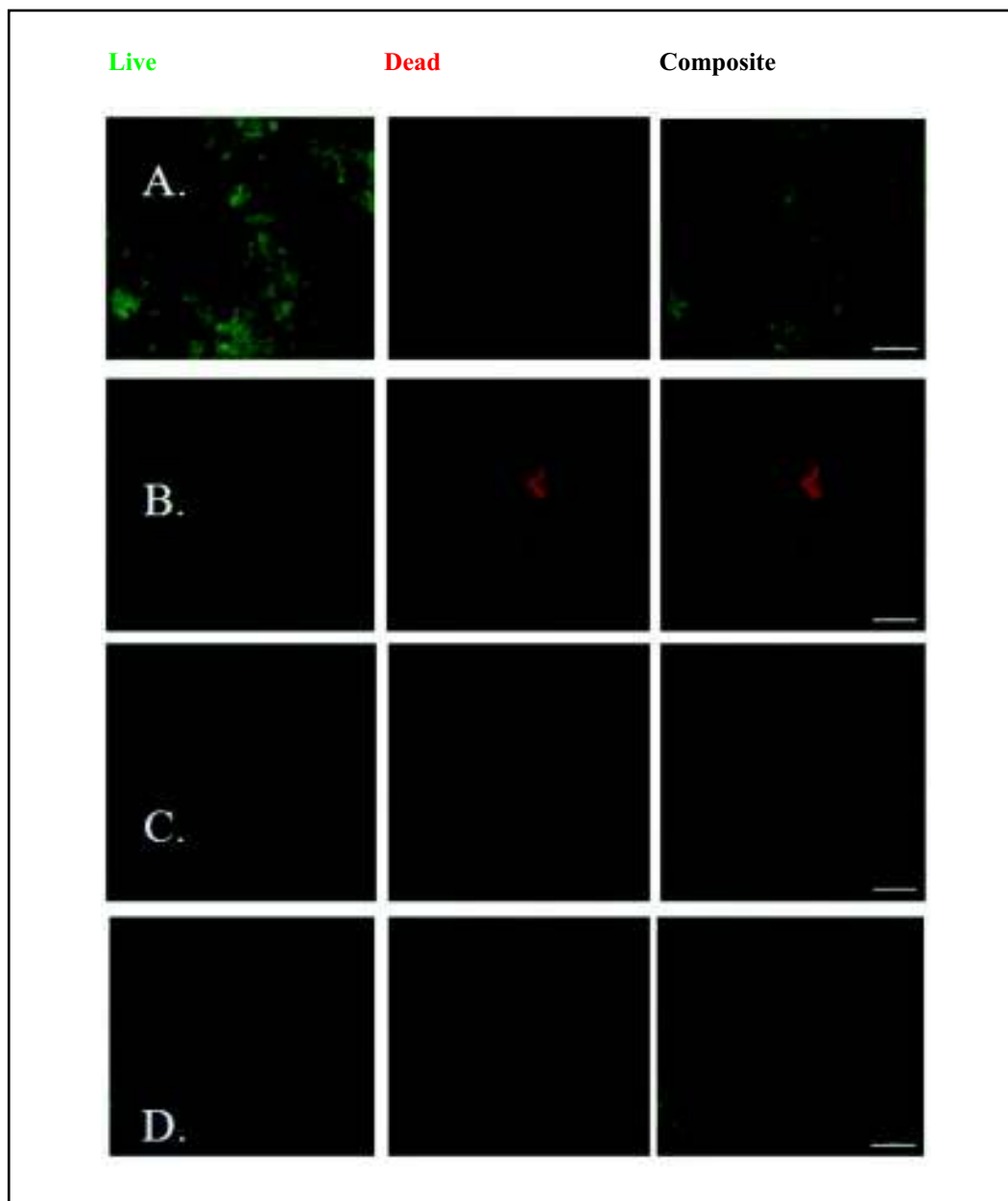
This data suggests that hepatocytes are more resistant to the elevated hydrogen peroxide environment. It is highly plausible that, HepG2 hepatomas are more susceptible to death because they have increased metabolic activity, which causes excess ROS production [6]. Hence the addition of exogenous peroxide adds to the already oxidatively stressed HepG2 environment. It is also plausible to believe that the HepG2 hepatomas are more sensitive to peroxide, because they have depletion of antioxidants; hence they are more affected by the

exogenous hydrogen peroxide. Studies substantiate this theory in that the deregulation of cancerous cells can cause reduced synthesis of antioxidants [6], [10], [11], [21]. However there is also data published that suggests some carcinoma cells generate an ample supply of antioxidants [22], [23], protecting them from oxidative damage. This suggests that the sensitivity of HepG2 hepatomas may be caused from excess ROS, reduced antioxidant capacity or both acting in conjunction. In any case, this illustrates that intracellular redox state is crucial in regulating hepatoma apoptosis.



**Figure 5.** Cell proliferation of HepG2 hepatoma cells and primary hepatocytes assessed using MTS Assay. (A). Hepatocytes. (B) HepG2 cells.

\* p<.05 indicates treated groups significantly less than controls.



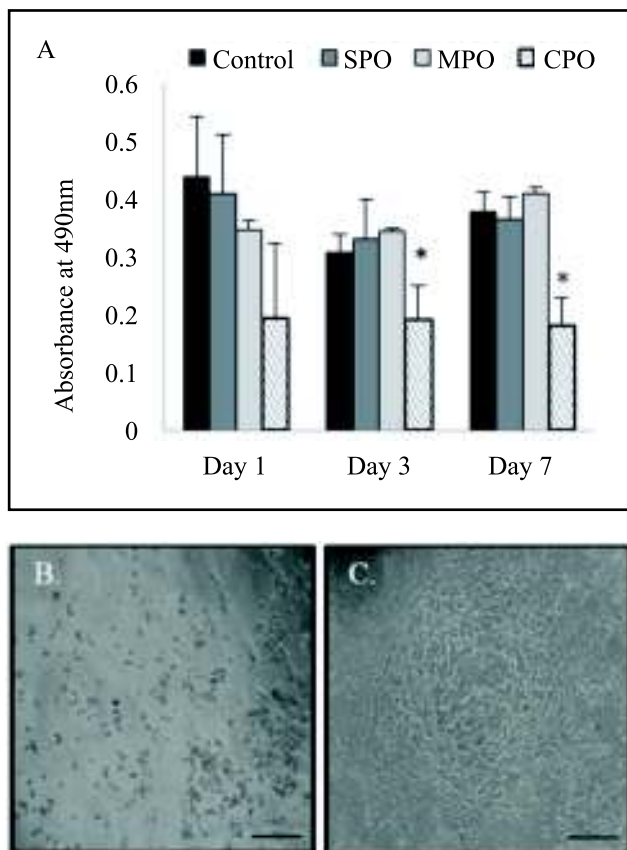
**Figure 6 : Viability of HepG2 Cells Monitored Using Live Dead Assay.**

**A. Control B. SPO C. CPO D. MPO. Red (Dead Cells) Green (Live Cells).**

**There are Significantly More Dead Cells Present Within the Peroxide Gels When Compared to the Control as Well as Less Viable Cells Present.**

**Note : Multiple Non-Viable HepG2 Were Washed Away During Media Change.**

**Peroxide Effect on Proliferative HepG2**



**Figure 7. Cell Growth Using MTS Assay and Morphological Characteristics Were Observed.**

**(A) Cell Growth of Peroxide Gels and Controls**

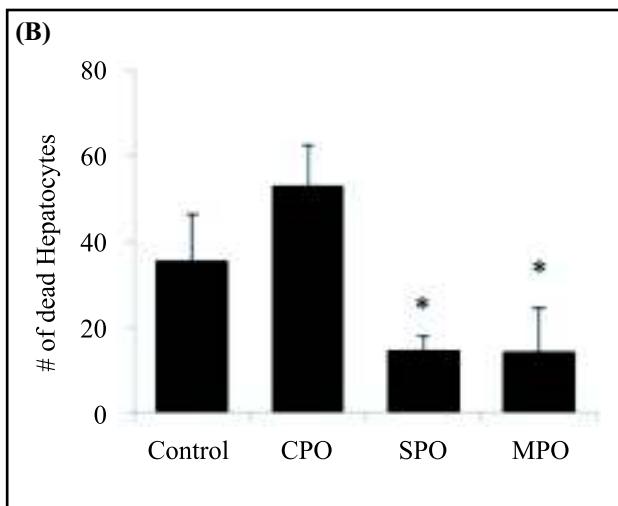
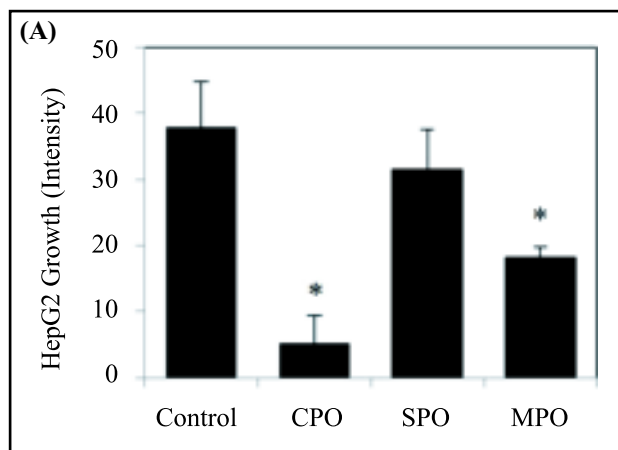
**(B) Morphology of CPO (C) Morphology of**

**MPO, SPO and Controls. \*p<.05 Indicates Treatment Group Significantly Less Than the Control.**

To determine whether peroxide gels can halt growth of carcinoma cells in growth phase, an experiment was conducted to simulate an aggressively proliferating status. HepG2 cells were grown to confluence. Once cells began proliferating rapidly, the cells were treated with the peroxide gels. The morphology of HepG2 seeded with MPO, SPO and control gels were very similar in that they maintained confluence. Hence, HepG2 seeded with MPO and SPO gels did not reduce cell growth of the proliferative hepatomas. However, the CPO gels reduced cell growth significantly (Fig. 7). Furthermore, we can gather from this data that CPO was the most effective material tested in that

it halted hepatoma growth, after the cells had already proliferated uncontrollably. This is important because the time liver tumors become symptomatic; they have progressed to an aggressively proliferative status. So having a treatment which can halt growth of cells in growth phase is critical for a therapy. One possibility for the increased effectiveness of CPO may be due to alkaline properties of CPO. PH levels were tested for all gels and CPO gels possessed a higher pH which may create a less hospitable localized alkaline environment for the HepG2 cells. In fact research suggests that carcinoma cells cannot withstand alkaline environments (2). For this reason it is plausible to believe that the peroxide levels in conjunction with the pH allowed CPO to be more effective in halting the HepG2 growth.

**Observation of HepG2 and Hepatocytes in 3D Co-Culture**





**Figure 8 : HepG2 and Hepatocytes Were Observed  
in a 3D Co-Culture System**

**(A) HepG2 Growth Indicated by Live HepG2**

**Fluorescing With Green GFP tag.**

**(B) Dead Hepatocytes Were Counted Manually Using**

**Phase and Contrast.**

**\*p<.05 Indicates Significantly Less Than Control**

To determine whether peroxide gels can selectively kill HepG2 cells in a mixed system, indicative of a human model, we created a peroxide gel and seeded both HepG2 and Hepatocytes in co-culture. HepG2 were tagged with a green GFP marker to distinguish between the two cells. Cells were co-cultured together for 7 days on peroxide gels followed by assessment. Growth of the hepatomas was assessed using Image J. The cell growth data shown in Fig. 8 suggests that once the cells are co-cultured together there is variability in the efficiency of the peroxide gels. CPO gels reduced HepG2 hepatoma growth substantially (Fig. 8A). MPO was also able to halt HepG2 growth, but to a lesser degree than CPO and no significant effect was observed from the SPO gels on HepG2 growth (Fig. 8A).

Observation of hepatocyte death was assessed by manually counting the dead cells (dark round cells). There was no significant difference in hepatocyte death when comparing the control gels with the peroxide generating gels (Fig. 8B). Morphology and infrastructure of the hepatocytes was also observed (data not shown) and there did not appear to be any atypical patterns. This data suggests that even in a co-culture or multi-celled environment, the CPO and MPO gels are still quite effective in halting HepG2 hepatoma growth, while having little to no effect on the viability of hepatocytes. Further studies needed to look at dosage effects.

This stable release of hydrogen peroxide creates a sustained environment for halting HepG2 hepatoma growth. Although hydrogen peroxide is highly toxic to the HepG2 cells, it is believed that the hepatocytes are more resistant to the ROS species being generated. We could measure no negative effects on the hepatocytes when exposed to these levels of hydrogen peroxide. The resistance to hydrogen peroxide seen in primary

hepatocytes along with the susceptibility of hepatoma cells to killing by hydrogen peroxide indicates some selective killing in regards to hepatomas and hepatic cells.

## CONCLUSION

In this study we have demonstrated suppression of HepG2 hepatoma growth as early as seven days following treatment with peroxide gels. Most importantly,

we have shown that our treatment had no negative effect on normal hepatic cells in regards to viability, cell growth patterns, proliferation and morphology. Hepatocytes exposed to peroxide gels withstood the highly oxidative environment. Furthermore, the results showed that peroxide gels were able to halt HepG2 growth, viability, and colony size. When peroxide gels were incorporated into a system of confluent, proliferative HepG2, CPO gels were most affective in halting the HepG2 growth. It is believed the alkaline environment in conjunction with the hydrogen peroxide may be creating a novel environment for halting hepatoma growth.

In summary, our studies confirm that the utilization of peroxide can in fact selectively kill the HepG2 while not harming the hepatocytes. Hence, there is an ROS level that primary hepatocytes can tolerate, but hepatomas cannot. Future studies entail testing functionality of cells within a 3D peroxide model. Different cellular constructs will be assessed including breast and prostate matrices for assessing breast and prostate cancer.

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