In Vitro Evaluation of Hepg2 Cell Proliferation Altered by Reactive Oxygen and Nitrogen Species

Abstract
This study was to investigate cell proliferation regulated by reactive oxygen and nitrogen species and their scavengers. Earlier conclusions are paradoxical on roles that reactive oxygen and nitrogen species and their scavengers enhance or inhibit cancer cell proliferations. This study employed the MTS assay to evaluate proliferations of HepG2 cells treated with various concentrations of two different nitric oxide donors, hydrogen peroxide and their scavengers for 24 hours. The MTS assay revealed that low concentrations of SNP, SNAP and H2O2 can significantly enhance HepG2 cell proliferation, and high concentrations of cPTIO significantly inhibited HepG2 cell proliferation. Additionally, the assay also indicated that 100 µM H2O2, 100 µM cPTIO or 100 U/mL catalase did not have evident synergisms with NSP or SNAP. However, due to reactions of the chemicals with reagents of kits of the MTS assay, the data were not inferred that low concentrations of NO and H2O2 enhance proliferation, whereas high concentrations inhibit proliferation, and whether there are synergistic effects with the combination of SNP (or SNAP) and H2O2, cPTIO or catalase.

Keywords: Hepatocellular carcinoma; Reactive oxygen species; Reactive nitrogen species; Cell proliferation; Synergism

Introduction
The hallmark of hepatocellular carcinoma (HCC) is uncontrolled proliferation of tumour cells, which ignore inhibitory signals to cell proliferation, promote growth of blood vessels and invade other organs [1]. The detailed mechanisms for this are still unclear [2,3]. Mounting evidence suggests that reactive oxygen species (ROS) and reactive nitrogen species (RNS) generated through chronic liver inflammation are involved in the process of HCC development [4-6]. Therefore, a precise understanding how ROS and RNS regulate cell proliferation will help decipher the enigma of hepatocarcinogenesis.

Epidemiology has revealed that most of HCCs develop in chronic inflammatory conditions of the liver [1]. In these conditions, various activated inflammatory and immune cells are recruited to the site of inflammation and generate ROS and RNS. Local concentrations of ROS and RNS have been shown to be elevated in chronic liver inflammation [7-10].

As described in literature reviews, ROS and RNS can interact with nucleic acids, proteins and other biological macromolecules to abnormally regulate cell proliferation. For example, ROS and RNS are able to oxidize DNA to induced genetic instability [11-13]. This may randomly induce mutations of key genes in hepatocytes like tumour suppressor genes or oncogenes resulting in a growth advantage for affected cells that ultimately leads to abnormal regulation of cell proliferation and development of HCCs [14,15]. ROS and RNS may also regulate cell proliferation through changes in key amino acids in proteins such as oxidation [16], S-nitrosylation of thiols of cysteines [17-19] or nitration of tyrosine [20,21].

Cell proliferation is perhaps influenced by concentrations of ROS and RNS, with the potential for synergisms effects between NO and ROS. Reports revealed that low concentrations of ROS and RNS can enhance cell proliferation, whereas high concentrations have been shown to inhibit cell growth [5,22-24]. For example, this dual role of H2O2 has also been observed in previous studies with keratinocytes [25], HeLa cells [26], lens epithelial cells [27], human prostate cancer cells [28] and cardiomyocytes [29]; with cell viabilities assessed using the MTS or MTT assay. Likewise, NO also exerts the dual role that low concentrations of NO enhance...
proliferations of head and neck squamous cell carcinoma FaDu cells [30] and ovarian carcinoma cell growth [31], whereas high concentrations inhibit the cell proliferations, through assessing cell viability using a MTT assay. These data have stimulated interest into anti- or pro-cancer properties of ROS and RNS [22,32]. In clinical trials, inhibition of ROS generation, or delivery of high concentrations of NO via donor drugs or gene therapy has been attempted for cancer therapies [33,34] However, the roles of ROS and RNS on cell proliferation are debated [35-37].

Cell proliferation is usually evaluated, based on the metabolic activity of viable cells [38]. The MTT assay and the MTS assay are widely employed to assess cell proliferation in a range of situations, including investigations of the effects of ROS and RNS, and for evaluating new anti-cancer drug candidates [39,40]. The principle of the MTT assay is that tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) is converted into a formazan by mitochondrial enzymes [41,42]. It is assumed that mitochondria of viable cells produce these enzymes, but those of dead cells do not, and that the amount of formazan formed is directly proportional to the number of metabolically active cells in the culture [43,44].

One of the limitations of the MTT assay is that the formazin product produced is initially water insoluble. The sample needs to be dissolved in organic solvents, and then dissolved in water for a measurement with a spectrophotometer. The MTS assay is an improved version of the MTT assay, which formazan product is an aqueous soluble product that generated in the presence phenazine methosulfate (PMS) [45,46]. PMS is used as an electron coupling reagent [47]. The principle of the MTS assay is that 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxophenyl)-2-(4-sulfophenyl)-2H-tetrazolium can be transformed to formazan by mitochondrial enzymes in the inner mitochondrial membrane of cells [46]. The aim of this study was to investigate the potential effects of H2O2 and NO on HepG2 cell proliferation; and to determine whether synergistic effects occurred between NO and H2O2, cPTIO or catalase.

Materials and Methods

Materials

Sodium nitroprusside dehydrate (SNP, S0501), S-Nitroso-N-Acetyl-Penicillamine (SNAP, N-3398), 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt (cPTIO, C221), catalase (C-3515) and trypsin blue (T8154) were purchased from Sigma (St. Louis, MO, USA). Dulbecco’s Eagle minimal essential medium (DMEM) (11995-115), foetal bovine serum (0405), penicillin-streptomycin (0021) and trypsin (0187) were from Invitrogen (Mulgrave, Vic, Australia). Fungizone (list 43760) was from APOTHECON (Princeton, NJ, USA). Sodium hydrogen carbonate (NaHCO3, A475-500G) was from Analytical UNIVAR Reagent Ajax Finechem (Mt Wellington, New Zealand). Phosphate buffered saline (PBS) without Ca2+ and Mg2+ was from Queensland Institute of Medical Research (Brisbane, Australia).

HepG2 cells (a human hepatocellular carcinoma cell line, ATCC, HB8065) were obtained from Dr Grant Ramm’s laboratory at the Queensland Institute of Medical Research and originally sourced from the American Type Culture Collection (Manassas, VA, USA). CellTiter 96® AQcous Non-Radioactive Cell Proliferation Assay kit (G5421) was purchased in Promega Corporation (Madison, WI, USA).

Drug stock solutions

SNAP was dissolved in sterile DMSO, whereas SNP and cPTIO were dissolved in PBS to make stock solutions, then stored at -20°C [48]. Solutions of H2O2 were freshly made at the time of use. 42 mg of MTS powder from the CellTiter 96® AQcous Non-Radioactive Cell Proliferation Assay kit was completely dissolved in 21 mL of Dulbecco’s phosphate buffered saline to prepare MTS solution. The MTS solutions were stored at -20°C protected from light.

Cell culture

Cryovials containing HepG2 cells stored in a liquid nitrogen Dewar were removed to a water bath at 37°C to defrost the cells for 2 minutes. The cryovials were wiped with 70% ethanol and the contents of the tubes were poured into a 50 mL falcon tube containing warm complete medium. The falcon tube was spun at 500 × g for 5 minutes to form a small cream coloured pellet. The supernatant was poured off. Pellets were washed in fresh medium and resuspended in 10 mL of fresh complete medium. Pellets were separated by drawing through a sterile 21-gauge needle. Concentrations of the cells were determined using the trypan blue exclusion method, which cells were stained with trypan blue, and then, cells were counted in a hemocytometer under a microscope. Cell concentrations were adjusted to 1 × 106 cells/mL. 10 mL of this cell solution was transferred into a 75 cm2 tissue culture flask. The cells were cultured at 37°C in a humidified 5% CO2 and 95% air incubator. Cell cultures were checked microscopically daily to ensure that cells were healthy and growing as expected. Cultured medium was renewed every 3 days.

Cell subculture

Cells were subcultured after being grown to 70% confluence. Trypsin, versene, Dulbecco’s, PBS and complete DMEM medium were pre-warmed. The adhering cell monolayer was washed twice with a small volume of PBS at 37°C. Two ml of warm trypsin solution was added to the cultures covering the adhering...
cell layer. The flasks were placed in a 37°C warming tray for 2 minutes to dislodge cells. 30 mL of warm complete DMEM medium was added in the flask to neutralize the trypsin. Cells were then pelleted as described above and a similar process was undertaken to culture cells in 75 cm² tissue culture flasks.

**Cell seeding**

When cells grew to 60% confluence in 75 cm² flasks, they were dislodged with trypsin as described above, washed and resuspended to make 50 mL of cell solution with a concentration of 2 × 10⁶ cells/mL. 100 µL of the cell solution was added into each well of 96-well plates. These cells were cultured for 48 hours at 37°C.

**Treatment**

Prior to use, working solutions of H₂O₂, cPTIO, SNP and SNAP were prepared in PBS to concentrations ranging from 10 µM to 10 mM. 10 µL of the appropriate working solution was added into each culture well containing 90 µL of cell solution by 10-fold serial dilution. In the first set of experiment, cells were exposed to concentrations of 0, 1, 10, 50, 100, 200 and 1000 µM SNP, SNAP, H₂O₂, or cPTIO. In a second set of experiments, cells were exposed to 0, 1, 10, 50, 100, 200 and 1000 µM SNP or SNAP with or without 100 µM of H₂O₂, 100 µM of cPTIO or 100 U/mL of catalase for 24 hours at 37°C, 5% CO₂, in a humidified incubator.

**Cell proliferation assay**

Immediately before cell viability was measured, 2 mL of MTS solution and 100 µL of PMS solution were removed from the freezer, thawed in a 37°C water bath and mixed evenly using aseptic techniques. Once cells were treated with the chemicals for 24 hours, 20 µL of the combined MTS/PMS solution were added into each well of the 96-well plate containing the cell suspension and then incubated for 2 hours at 37°C, 5% CO₂, in a humidified incubator. The absorbance was recorded at 490 nm using an ELISA plate reader. The viability of cells was calculated for each assay using the formula:

\[
\text{Percentages of cell proliferation} = 100 \times \frac{\text{Absorbance values of test sample}}{\text{Absorbance values of control sample}}
\]

**Equation 1**

**Statistical analysis**

All statistical analyses and graphs were performed using GraphPad Prism 6.0 from GraphPad Software, Inc (Avenida de la Playa La Jolla, USA). Statistical differences of proliferations rate of HepG2 cells treated with SNP, SNAP, H₂O₂, or cPTIO compared to those of HepG2 cells were not treated with these chemicals were determined using one-way analysis of variance (ANOVA) followed by Dunnett’s Multiple Comparison Test. Significant differences of proliferation rates of various concentration points between SNP (or SNAP) with 100 µM H₂O₂, 100 µM cPTIO or 100 U/mL catalase and SNP (or SNAP) alone were determined with Bonferroni and Šídák methods. P values<0.05 were considered significantly different. Each experimental condition was performed in triplicate. All experiments were repeated for three times. Data were expressed as mean % ± standard deviation (SD).

**Results**

**Proliferation of HepG2 cells treated with SNP, SNAP, H₂O₂ or cPTIO**

HepG2 cells were treated with 0 to 1000 µM SNP, SNAP, H₂O₂, or cPTIO for 24 hours. Cell proliferations were assessed with the MTS assay. Statistical differences of proliferation rates of HepG2 cells treated with various concentrations of SNP, SNAP, H₂O₂, or cPTIO compared to those of negative controls that were not treated with these chemicals were determined using one-way analysis of variance followed by a Dunnett’s Multiple Comparison Test. A p value of less than 0.05 was considered statistically significant. Cell proliferation of HepG2 cells significantly increased from 1 to 50 µM SNP, but there was no significant difference from 100 to 1000 µM SNP compared to negative controls (Figure 1A). There was a significant increase in cell proliferation at doses of SNP between 1 to 100 µM SNP, but there was no significant difference from 200 to 1000 µM SNP compared to negative controls (Figure 1B). There was a trend that low concentrations (1 to 200 µM) of H₂O₂ enhanced cell proliferation, but there was no a statistically significant change from 500 to 1000 µM H₂O₂ compared to negative controls (Figure 1C). High concentrations (200 to 1000 µM) of cPTIO reduced absorbance values, in particular, 500 and 1000 µM cPTIO reduced 80% absorbance values (Figure 1D).

**HepG2 cell proliferation induced by SNP without or with H₂O₂, cPTIO, or catalase**

HepG2 cells were treated with 0 to 1000 µM SNP with or without 100 µM H₂O₂, 100 µM cPTIO or 100 U/mL catalase for 24 hours, before cell proliferation was evaluated with the MTS assay. A similar trend to SNP alone was observed when SNP at various concentrations was combined with 100 µM H₂O₂, 100 µM cPTIO or 100 U/mL catalase, which low concentrations of SNP enhanced cell proliferations (Figure 2). A Bonferroni and Šídák’s Multiple Comparison Test indicated that there was a statistically significantly differences between proliferations of HepG2 cells treated with 1 µM SNP alone or with 100 µM H₂O₂. This was not seen for other concentrations of SNP ± 100 µM H₂O₂. Moreover, at various concentration points of SNP, there was no significant difference between proliferation rates of HepG2 cells treated with SNP alone or when combined with 100 µM cPTIO or 100 U/mL catalase.

**HepG2 cell proliferation mediated by SNAP with or without H₂O₂, cPTIO or catalase**

HepG2 cells were treated with SNAP with or without 100 µM H₂O₂, 100 µM cPTIO or 100 U/mL catalase for 24 hours, with cell proliferation measured by the MTS assay. A Bonferroni and Šídák’s Multiple Comparison Test indicated that there were not statistically significant differences between proliferations of HepG2 cells treated with various concentrations of SNAP alone and those of HepG2 cells treated with SNAP plus 100 µM H₂O₂, 100 µM cPTIO or 100 U/mL catalase (Figure 3).
Discussion

This study did not support the hypothesis that low concentrations of NO enhanced proliferation, and that high concentrations inhibited proliferation. The MTS assay revealed that (1, 10, 50 µM) SNP and (1, 10, 50, 100 µM) SNAP significantly enhanced HepG2 cell proliferation, but (100 to 1000 µM) SNP and (200 to 1000 µM) SNAP showed a non-significant trend to inhibit cell proliferation (Figure 1A and 1B). These data indicated that SNP, in higher dose (100 µM), significantly enhanced cell proliferations, but 100 µM SNP did not. However, my research revealed that 100 µM SNAP released 37 ± 14 µM NO whereas 100 µM SNP released 5.8 ± 1.8 µM NO. SNAP released more NO than SNP, and both of them released NO in dose-dependent manner. A previous study also revealed that SNAP released more NO than SNP [49]. It is to say that the cell proliferations indicated by the MTS assay in this study were not relative to NO concentrations.

This study did not support the hypothesis that low concentrations of H₂O₂ enhanced proliferation, whereas high concentrations inhibit proliferation. The MTS assay revealed a trend that low concentrations of H₂O₂ enhanced HepG2 cell proliferation, and high concentrations of H₂O₂ inhibit cell proliferation, but there were no significant differences (Figure 1C). The result was different from that of a previous study that for keratinocytes exposed to up to 1000 µM H₂O₂ for 24 hours, where the relative absorbance increase to 102 to 115% of control for 100 to 500 µM H₂O₂, but reduced by 60% at 1000 µM H₂O₂ [25].

This study first proposed that cPTIO interfered with the MTS assay. The MTS assay revealed that 500 and 1000 µM cPTIO reduced absorbance values to 15.2 and 13.4% of that of negative controls respectively (Figure 1D). Thus, it appears that in excess of 80% of HepG2 cells treated with 500 or 1000 µM cPTIO died. However, my pervious experiments demonstrated that the modified trypan blue method and the LDH assays did not identify major toxic effect of 500 and 1000 µM cPTIO on HepG2 cells. This suggests that the low MTS assay read out with concentrations of cPTIO was due to an effect of cPTIO on the MTS assay rather than HepG2 cell death. Previous studies have shown that cPTIO...
is an oxidant [48,50] and has a similar structure to MTS and MTT [46,51]. Thus, at high concentrations cPTIO competes with MTS and MTT for electrons, leading to reduced transformations of MTS and MTT to formazan, leading to decrease of absorbance values. There are similar observations in previous studies. For example, 500 µM cPTIO significantly decreased absorbance values of human breast carcinoma MDA-MB-468 cells after a 48 hour treatment, determined using the MTT assay [52]. According to above discussion, these data cannot be inferred that high concentrations of cPTIO induce large cell death.
This study did not support the hypothesis, that there was adequate significant evidence of a synergistic interaction between NO and H₂O₂, cPTIO or catalase on HepG2 cell proliferation. Figure 2 did not reveal significant difference between SNP and SNP plus 100 µM H₂O₂, 100 µM cPTIO, or 100 U/ml catalase (except 1 µM SNP plus 100 µM H₂O₂). Figure 3 did not reveal significant difference between SNP and SNP plus 100 µM H₂O₂, 100 µM cPTIO, or 100 U/ml catalase either. At 1µM SNP, there was significant difference between SNP and SNP plus 100 µM H₂O₂, but at a large amount of other concentrations of SNP (or SNAP), there was no significant difference between SNP (or SNAP) and SNP (or SNAP) plus100 µM H₂O₂.

This study has several limitations. Theoretically, the tetrazolium salts of the MTT and MTS assay are reduced to colorimetric formazan by succinate dehydrogenase, with absorbance being proportional to the number of viable cells. However, accumulated data have shown that a number of parameters interfere with the MTT and MTS assays.

1. The conversion of tetrazolium to formazan is oxidoreduction, but release of NO from SNP or SNAP is also oxidoreduction. Therefore, transfer of electrons can occur between these agents, potentially interfering with the assay. Moreover, the mechanisms of the bio-reduction of tetrazolium [53] and the release of NO by SNP [54,55] or SNAP [56,57] are not completely clear.

2. SNP, SNAP and H₂O₂ decay at different rates. It has been reported that 100 µM H₂O₂ decays rapidly to zero within 1 hour in the presence of cells in the media, while NO released by SNAP increases in a time-dependant manner [58]. This means that H₂O₂ may not react with NO before it completely decays.

3. Some chemical agents may interact with the MTT and MTS assays without affecting actual cell viability. Recent reports have demonstrated that cholesterol [59], plant extracts [60], emodin [61], ascorbic acid [62] and flavonoids [63] chloroquine [38] all may interfere with the MTT assay.

4. In addition to mitochondrial dehydrogenases and oxidoreductases, enzymes in the cytoplasm and in non-mitochondrial membranes can also reduce tetrazolium to formazan [43,64]. Many other reductants, e.g., NADH, NADPH, succinate and pyruvate, are also capable of reducing tetrazolium in the presence [53] or absence of enzymes [65,66].

Summary

The MTS assay in this assay revealed that low concentrations of SNP and SNAP can significantly enhance HepG2 cell proliferation, whereas the changes with high concentrations were not statistically significantly different from control cells. H₂O₂ exerted a trend that low concentrations enhanced the cell growth whereas high concentrations inhibited cell growth, but there was no statistically significant difference. 100 µM H₂O₂, 100 µM cPTIO or 100 U/mL catalase did not have evident synergisms with NO. However, these data should be carefully explained due to interferences of a number of parameters with the MTS assays. Furthermore, due to the interference, conclusions in earlier studies on pro-cancer or anti-cancer roles of reactive oxygen and nitrogen species and their scavengers are uncertain.

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