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HYDROGEN PEROXIDE IMPEDES NK CELL AND T CELL MEDIATED CYTOTOXICITY AGAINST COLON CANCER CELL

Baskar Subramani[1], Chithra Ramanathan Pullai[2], Ezzati Radzuan[3], Arulvasu Chinnasamy[4], Sellamuthu Subbanna Gounder*[5].
[*1,2,3,5] *Nichi-Asia life Science Sdn Bhd, 478100, Selangor, Malaysia.*
[4]*Department of Zoology, University of Madras, Guindy campus, Chennai-600025, India.*

[*]***Corresponding author:-***
Email: ssellamuthu@gmail.com, Tel: +6036142 1920 / 21

Abstract:-

Although hydrogen peroxide (H_2O_2) is an admirable treatment for many cancer types, but the clinical impact of H_2O_2 on immune cells is not known. In the present study, we have demonstrated that H_2O_2 is a crucial factor to determine the cell mediated cytotoxicity of immune cells to the cancer cells. Natural killer (NK) cells and T cells were isolated from healthy donors and were activated and enriched the population at ex-vivo condition. At day14, activated NK and T cells were exposed to colon cancer cell at the ratio of 10:1 (Effector: Target) with or without H_2O_2 (50 µM and 100µM) treatment for 6 h and 12 h. Cytotoxicity assay showed that cytotolytic ability of NK and T cells was strongly suppressed in the presence of H_2O_2. H_2O_2 induced NK and T cell dysfunction was analyzed and results demonstrated that NK and T cells were lose their viability on dose and time dependent manner. Also, flow cytometry analysis revealed that H_2O_2 significantly suppresses the activation and proliferation CD8 T cell population. Whereas, NK cell subset ($CD56^{bright}$ and $CD56^{dim}$) was dramatically altered by H_2O_2. When exposed to H_2O_2, $CD56^{dim}$ population was considerably increased at different time points. Hence, we conclude that H_2O_2 directly or functionally defect the NK and T cell mediated cytotoxicity and however, further studies are warranted to confirm the concept with mild dose of H_2O_2 with different cancer cell lines.

Keywords:-*Hydrogen peroxide, Immune cell, NK cell, T cell and immunotherapy.*

INTRODUCTION

Despite hydrogen peroxide is not considered as a primary cancer treatment for any cancer patient but still is extensively applied as an efficient supplemental treatment. H_2O_2 is a strong oxidant and acting as an antiviral, antifungal and antibacterial agent. Other than cancer treatment, H_2O_2 can also be used for many common diseases such as typhoid fever, cholera, ulcers, asthma, whooping cough, and tuberculosis. There are different statements had been made on the mechanistic action of H_2O_2 on cancer cell eradication that (1) H_2O_2 was liberating pure oxygen and modulating the hypoxic cancer environment, (2) H_2O_2 provoked oxygen based metabolism, whereas cancer cell apparently used non-oxygen (anaerobic) based metabolism, (3) H_2O_2 directly kill the microbes inside the cancer cells and revert them into normal cell (Liou and Storz 2010; Rieber and Strasberg-Rieber 2012) and (4) Increased intracellular levels of H_2O_2 could reduce the cell proliferation, promote the apoptosis, and inhibit the invasion, metastasis and angiogenesis (Lopez-Lazaro 2006). With these unique phenomena, there are many chemotherapeutic drugs (eg. doxorubicin, etoposide, paclitaxel, cisplatin) are extensively used in nature of fully or at least partially induces the intracellular level of H_2O_2 (Lee et al. 2000; Alexandre et al. 2006). However, it is not clear whether or not H_2O_2 influence the immune cells function against cancer cells.

Immune cells play a key role in early host immune response against pathogens and cancer cells. In particular, natural killer (NK) cells and T cells are the primary innate and acquired immune system which is actively participating in cancer cell formation and/or eradication. Based on the surface expression protein CD56, NK cells are subdivided into two major subsets such as $CD56^{dim}$ NK cell, which is responsible for cell-mediated cytotoxicity and $CD56^{bright}$ NK cell, which is responsible to regulate the immune responses by producing various levels of cytokines (Harlin et al. 2007). Cytolytic property of NK cell ($CD56^{dim}$) is highly depending on the ratio between activating and inhibitory signals, which will decide whether the target cells are susceptible to NK cell mediated lysis or not (Moretta and Moretta 2004; Lanier et al. 2000). In contrast, T cells recognize their target cells through processed antigenic peptides presented via MHC. Cytotoxicity of T cell is depending on their subset population of CD8 T cell, which is responsible for direct lysis of target cells bearing antigen and CD4 T cell, which is responsible for producing cytokines that can be directly destroy the target cells or can be stimulate the other effectors cells such as B cells (Shafer-Weaver et al. 2004; Broere et al. 2011). Both immune cells are using granule-mediated and Fas ligand (FasL) activated pathways to eradicate the target cell.

There were limited studies carried out in conjunction with the role of H_2O_2 and immune cell dysfunction. In this present study, we have attempted to address whether H_2O_2 influence the cytolytic property of NK and T cells against colon cancer cells. Our data suggest that H_2O_2 significantly alter the NK cell subset and potently affect the cytolytic property of NK and T cells to the colon cancer cells. We would recommend, further studies are require to find out the reason behind the alteration of NK cell subset and poor cytolytic activity of NK and T cells against colon cancer cells.

Materials and Methods

Preparation and culture of NK and T cells Peripheral blood samples (25 – 30 ml in heparin collection tubes) were collected from healthy donors, after written informed consent. Peripheral blood mononuclear cells (PBMC) were isolated by lymphoprep (AXIS-SHIELD PoC AS, Oslo, Norway) density gradient centrifugation at 2000 rpm for 20 mins. Isolated PBMCs were washed twice in phosphate buffered saline and finally resuspended in culture media containing 10% autologous plasma and IL2. Suspended PBMCs were cultured as per the protocol described by Takada et al. 2011 and Ratnavelu et al. 2013. The cells were maintained for 14 days with routine media nourishment.

Colon cancer cell culture

HT-29 cells were cultured in DMEM/F12 media with 10% fetal bovine serum and 1% penicillin/ streptomycin. Cells were maintained under optimal environment that 5% CO_2 at 37˚C. Media was refreshed weekly twice. Trypsinization (0.025% trypsin) was performed to dislodge the cells for passage or experiment.

H_2O_2 treatment and cytotoxicity assay

Target cells (HT-29) were seeded in 96 well microplates at density of $5x10^3$ cells/ well in 200 µl culture media and were allowed to attach. Similarly, effector cells (NK and CTL) were seeded at density of $5x10^4$ cells/well in 200 µl culture media, which equally 10 fold higher than target cells (i.e 10:1 of effector: target cells). Followed by, both target and effector cells were treated with 50 µM and 100 µM of H_2O_2 (30% w/w) for 6 h and 12 h to determine the cytotoxic effect of H_2O_2 on target and effector cells (triplicates were maintained). For cytolytic assay, effector cells were co-cultured with target cells at ratio of 10:1 (E:T) with or without H_2O_2 (50µM and 100µM) for 6 h and 12 h. After treatment, added 20 µl of working MTT solution and incubated the plates at 37˚C for 4 h. Following incubation, plates were centrifuge at 5000 rpm for 5 mins and then supernatant was aspirated. The formazan was dissolved with 100 µ1 of DMSO. Plates were read at 570 nm in ELISA reader. The percentage of cytolysis was calculated using the following formula: % cytolysis 100-(E:T+ H_2O_2/[E+ H_2O_2]+ [T+ H_2O_2]*100.

Flow cytometry analysis for effect of H_2O_2 on NK and CTL cells

Expanded NK and T cells were cultured with or without H_2O_2 (50µM and 100µM) for 6 h and 12 h. After incubation time, cells were stained with fluorochrome-conjugated mAbs specific for the lineage markers of NK cells (anti-CD56,

anti-CD3) and CTLs (anti-CD4, anti-CD8, anit-CD69), as previously described (Subramani et al. 2014). Analyses were performed using flow cytometer (FC- 500 Beckman Coulter, USA).

Statistical analysis

Statistical analyses of results were performed using the mean ± SD t test. The percentage of cytotoxic effect of H_2O_2 and cytolytic activity of NK cells and CTL cells were significant when the value of $*p$ was < 0.05.

Results

Effect of H_2O_2 on effector and target cells

First, we have examined whether H_2O_2 induce cancer cell death and they could do so, whether the death was dose or time dependent manner. To addressing this issue, we used HT-29 (colon) cancer cell line and treat them with 50 µM and 100 µM H_2O_2 for 6 h and 12 h. Cytotoxicity assay showed that H_2O_2 was considerably kill the cancer cell in a dose dependent manner but not effective in time dependent manner. However, the cytotoxic ability was not therapeutic consequence, because the cytotoxic ability of 50 µM and 100 µM H_2O_2 was 20 and 10%, respectively (Figure 1A). Next, we have analyzed whether H_2O_2 induce the proliferation or cytolytic activity of NK and T cells, if they could do to, whether the cytolytic action was dose or time depend manner. We produced maximum purity (>75%) of NK and T cells from healthy donor peripheral blood. Both immune cells were treated with different concentration (50 µM and 100 µM) of H_2O_2 for 6 h and 12 h. Cytotoxicity analysis showed that both immune cells were significantly suffered by H_2O_2 in dose dependent manner but not time dependent manner. Cytotoxic effect of 50 µM and 100 µM of H_2O_2 on NK cells were 56±1.5 and 60±3 percentage, respectively. Whereas, T cells were lose their viability 52±2 and 63±1 percentage on treatment with 50 µM and 100 µM of H_2O_2, respectively (Figure 1B). This data clearly demonstrated a dominant role for H_2O_2 in immune cells, which mean H_2O_2 remarkably reduce the viability of immune cell at even low (50µM) and moderate (100µM) concentration.

H_2O_2 de-promote the cytolytic action of NK and T cell

Further, NK and T cell function was examined as specific cytotoxicity against HT-29 colon cancer target cells. When the NK and T cells were co-cultured with colon cancer cells in the presence of H_2O_2, the cytolytic activity was diminished in dose dependent manner than those of control group, effector and target cells alone (without treatment). In the absence of H_2O_2, the cytolytic activity of NK and T cells were 54±0.5% and 47±0.4%, respectively. When treated with 50 µM H_2O_2, cytolytic activity was 47±1.3% and 40±0.06%, respectively. While treated with 100 µM of H_2O_2, cytolytic activity was 38±3% and 30±2.8%, respectively (Figure 2). Microscopic analysis also supporting the finding that different dose of H_2O_2 considerably reduce the cytolytic ability of NK cells and T cells (Figure 3). Number of cancer cell appearance in control group (NK cell or T cell and cancer cell) was comparably lesser than the treated group (NK cell or T cell and cancer cell with H_2O_2). Overall the result suggest that NK cell and T cell cytolytic action was significantly modulated by H_2O_2 in dose dependent manner but not time dependent manner.

H_2O_2 induce NK cell and T cell dysfunction

We examined whether H_2O_2 alter the phonotypical character of NK and T cell, if could do to, whether the alteration affect the effector cells activation or proliferation and cytolytic activity. Phenotypes of NK and T cells were determined by fluorochrome-conjugated antibodies specific for NK and T cell subsets. Human NK cell has two different subpopulation that based on expression pattern of CD56, which is $CD56^{dim}$ NK cell (responsible for cytolysis) and $CD56^{bright}$ NK cell (responsible for cytokine production). In accordance with our finding, H_2O_2 treated NK cell shows two distinct population of NK cells that $CD56^{dim}$ and CD56 bright , whereas H_2O_2 untreated NK cells shows single/homologous population of $CD56^{bright}$. We therefore asked whether H_2O_2 induce the differentiation of $CD56^{dim}$ from $CD56^{bright}$. The reason behind the skewed CD56 ^{bright} to $CD56^{dim}$ NK cell is not known. In contrast, there were no dramatic changes observed between treated and untreated T cells subsets of CD4 and CD8. However, administration of H_2O_2 significantly suppresses the activation and proliferation CD8 population (Figure 4). Altogether, this finding may explained that H_2O_2 considerably alter phenotypic feature of NK and T cell population, which directly influence the cytolytic activity against cancer cells.

Discussion

Hydrogen peroxide plays a dual role in cancer development and therapy, when it is increased in cellular levels it has been linked to rapid proliferation (Polytarchou et al. 2005), apoptosis resistance (Del Bello et al. 1999), angiogenesis (Arbiser et al. 2002) and metastasis (Nelson et al. 2003). On other hand, accumulative evidence postulated that increased cellular level of H_2O_2 may be an efficient way to kill the cancerous cells by altering the microenvironment, which favors to induce apoptotic cell death in cancer cells (Ahmad et al. 2004; Hirpara et al. 2001; Clement et al. 2003). Furthermore, cancer cells are more susceptible to H_2O_2 induced cell death than those of normal cells. It has been proposed that cancer or cancer derived cells produced high concentration of H_2O_2, which is supported to their self-development and protect form host immune system by suppress immune cells. Such increased concentration of H_2O_2 is a hallmark of cancer cells and the level is close to threshold of cytotoxicity. Thus, the cancer cells cannot survive when even exposed to mild dose of exogenous H_2O_2, this might be a one possible mechanism that H_2O_2 kill cancer cell specifically (Chen et al. 2005; Nicco et al. 2005). Although, H_2O_2 has remarkable ability to eradicate the cancerous cells but the potential effect of H_2O_2 on cell-mediated cytotoxic immune cells is not known.

In the present study, we have demonstrated the effect of H_2O_2 in antigen independent and dependent cytotolytic immune cells of NK and T cells, respectively. NK and T cells are powerful effector cells for many solid and liquid tumor cells. The superficial cytolytic activity of NK cells is regulated by a balance between activating and inhibitory receptors, whereas the cytolytic activity of T cells mostly depending on the antigenic peptides presenting via MHC. To address our hypothesis, we have produced high quality of NK and T cells from healthy donor peripheral blood. Initially, H_2O_2 was tested on their cytotoxic effect on immune cells; accordingly both immune cells were subjected to H_2O_2 at different concentration (50 and 100 µM) for different time points (6 and 12 h). Our cytotoxic data showed that NK and T cells were significantly suffered by H_2O_2 in dose dependent manner but not time dependent manner. This result suggested that NK and T cells were more susceptible to H_2O_2 at even low concentration. With correlating our result, Fredrik and groups (2006) demonstrated that oxygen radicals induce lymphocyte cell death at dose dependent manner.

Next, we analyzed the degree immune cell dysfunction in the presence of H_2O_2 as specific cytotoxicity against HT-29, colon cancer target cells. In accordance with earlier reports (Betten et al. 2004; Hansson et al. 1999), the ability of effector (NK and T) cells to lyses their target cells were potentially decreased in the presence of oxidized environment. Our finding also demonstrated that cytolytic action of NK and T cells were significantly diminished on dose dependent manner (Figure 2). In agreement with cytotoxicity data, the microscopic image analysis clearly showed that cytolytic action of NK and T cells were considerably decreased on dose wise (Figure 3). These poor cytolytic actions may either due to insufficient or dysfunctional effector cells (Kono et al. 1996). As mentioned earlier in this study that NK and T cells were losing their viability when exposed to 50 and 100 µM of H_2O_2. Next, we analyzed whether H_2O_2 depress the cytolytic function of NK and T cell. Cytolytic function of NK and T cells was depending on their unique surface receptors. Flow cytometry analysis demonstrated that the total population of NK cells was still constant, but the NK cell subset was considerably altered by H_2O_2. NK cell can be classified into two sub-population based on their level of CD56 expression, which is called $CD56^{dim}$ and $CD56^{bright}$ NK cell. $CD56^{dim}$ NK cell population was increased considerably when exposed to H_2O_2. Although it is a good sign that increased cytolytic responsive $CD56^{dim}$ NK cell population, but the overall cytolytic nature of NK cell was depleted. The reason behind the skewed $CD56^{bright}$ to $CD56^{dim}$ NK cell is not clear. Recently, it has been reported that $CD56^{bright}$ NK cells were more resistant to H_2O_2 due to their strong antioxidant capacity (Harlin et al. 2007). Whereas, $CD56^{dim}$ NK cells were highly sensitive to H_2O_2 because of their poor antioxidant system. In connecting with this study, one possible explanation for skewed $CD56^{bright}$ to $CD56^{dim}$ NK cell is H_2O_2 may induce the differentiation of $CD56^{dim}$ NK cell and subsequently make them as a H_2O_2 sensitive cells, which ultimately leads to apoptotic cell death. In contrast, T cell subpopulation (CD4 and CD8) was unchanged during the course of H_2O_2 treatment. However, cytolytic responsive CD8 population was suffered in state of activation and proliferation (Figure 4). To supporting our data, several investigators have demonstrated that oxygen or free radicals are robustly inhibited the anti-tumor cytolytic activity and other function of T cells. Typically, this tumor killing effector cells are functionally concealed by relative low concentration of exogenous oxygen radicals (Hansson et al. 1999; Kono et al. 1996).

Conclusion

Overall our data may explain that H_2O_2 considerably suppress the anti-tumor cytotolytic activity of T cell and NK cells. Further researches are warranted to explain the mechanistic action of H_2O_2 to inhibit the cytolytic action of T cell and NK cells.

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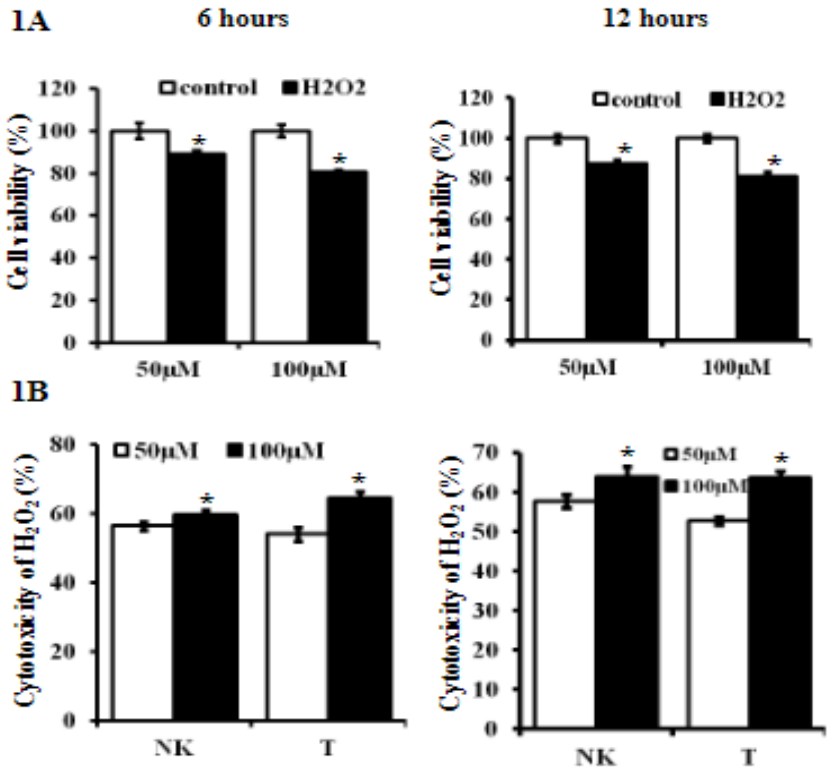
Legends

Figure 1: Cytotoxic effect of H_2O_2 in target (HT-29) and effector (NK and T) cells. A.
Colon cancer cells were incubated in the absence or presence of 50 and 100 µM of H_2O_2 for 6 and 12 hrs. The percentage of live cells were assessed by MTT assay. **B.** Purified NK and T cells were incubated in the absence or presence of 50 and 100 µM of H_2O_2 for 6 and 12 hrs. The percentage of the cytotoxicity was assessed by MTT assay. These results were obtained on paired samples (n=3). Data are expressed as mean±SD. **P*<0.005.

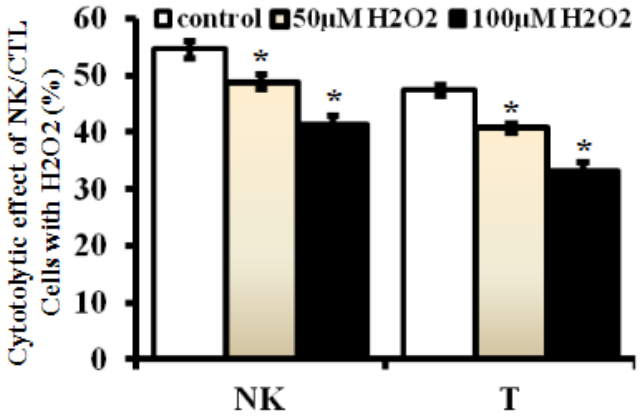
Figure 2: Hydrogen peroxide exacerbate cytolytic activity of NK and T cells to colon cancer cell.
Purified NK and T cells were co-cultured with colon cancer cells (HT-29) in the absence or presence of 50 and 100 µM of H_2O_2 for 6 and 12 h. The percentage of cytolysis was examined using MTT assay. These results were obtained on paired samples (n=3). Data are expressed as mean±SD. **P*<0.005.

Figure 3: Hydrogen peroxide extinguish NK and T cell cytolytic activity to colon cancer cells.
The rate of cytolytic activity of NK and T cells were monitored in the absence or presence of 50 and 100 µM of H_2O_2 for 6 and 12 h. The images were captured by inverted microscope at 100x magnification. The arrow denoting NK (White), T (Black) and HT-29 (Red and Blue) cells.

Figure 4: NK and T cells exhibit an unique susceptibility to H_2O_2.
Purified NK and T cells were incubated in the absence or presence of 50 and 100 µM of H_2O_2 for 6 and 12 h and stained with respective NK and T cell linage anybodies. Immunophenotyping of NK and T cells (before and after H_2O_2 treatment) were analyzed using flow cytometry. Results from one representative experiment of three are shown.



6 hours



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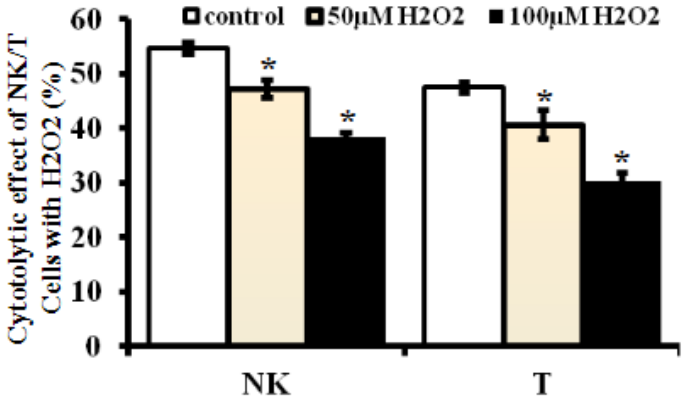

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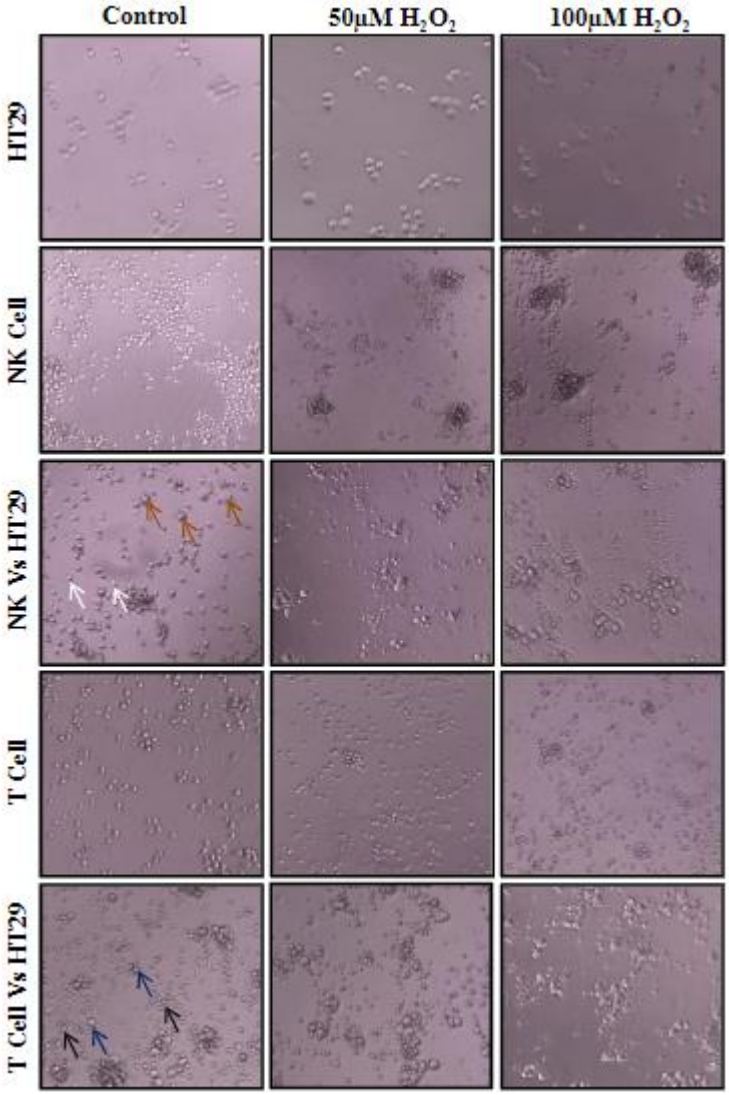
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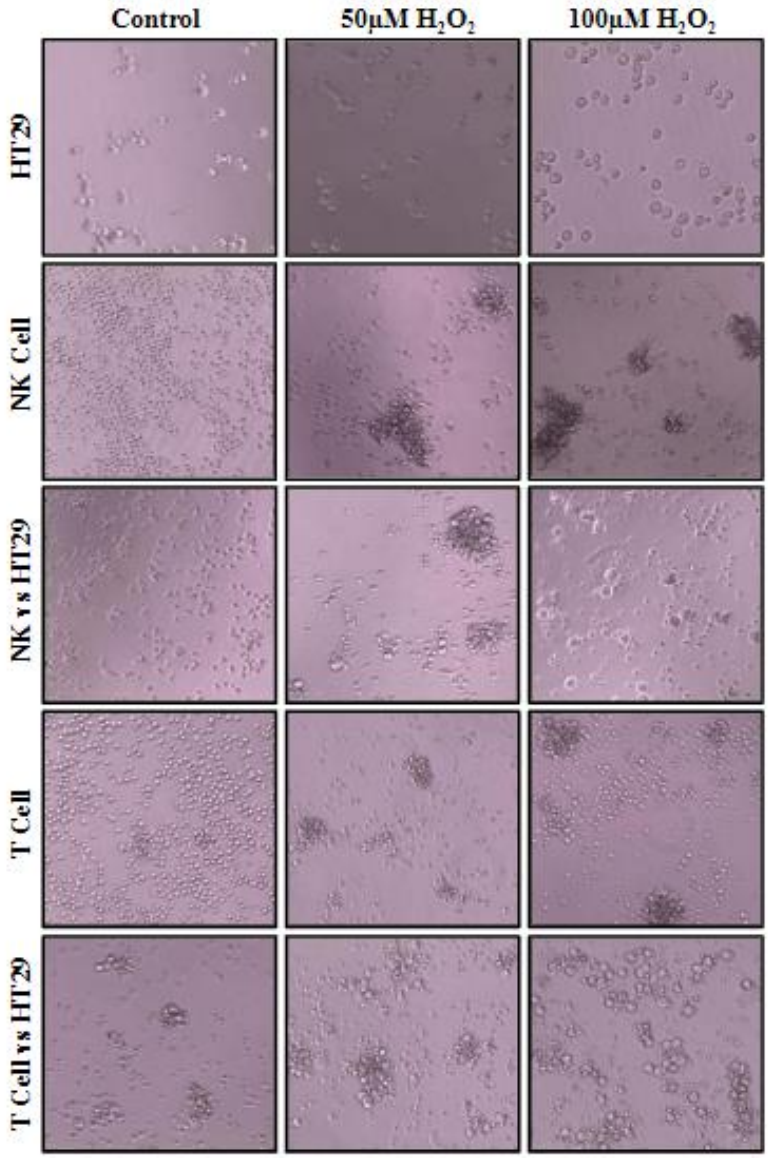
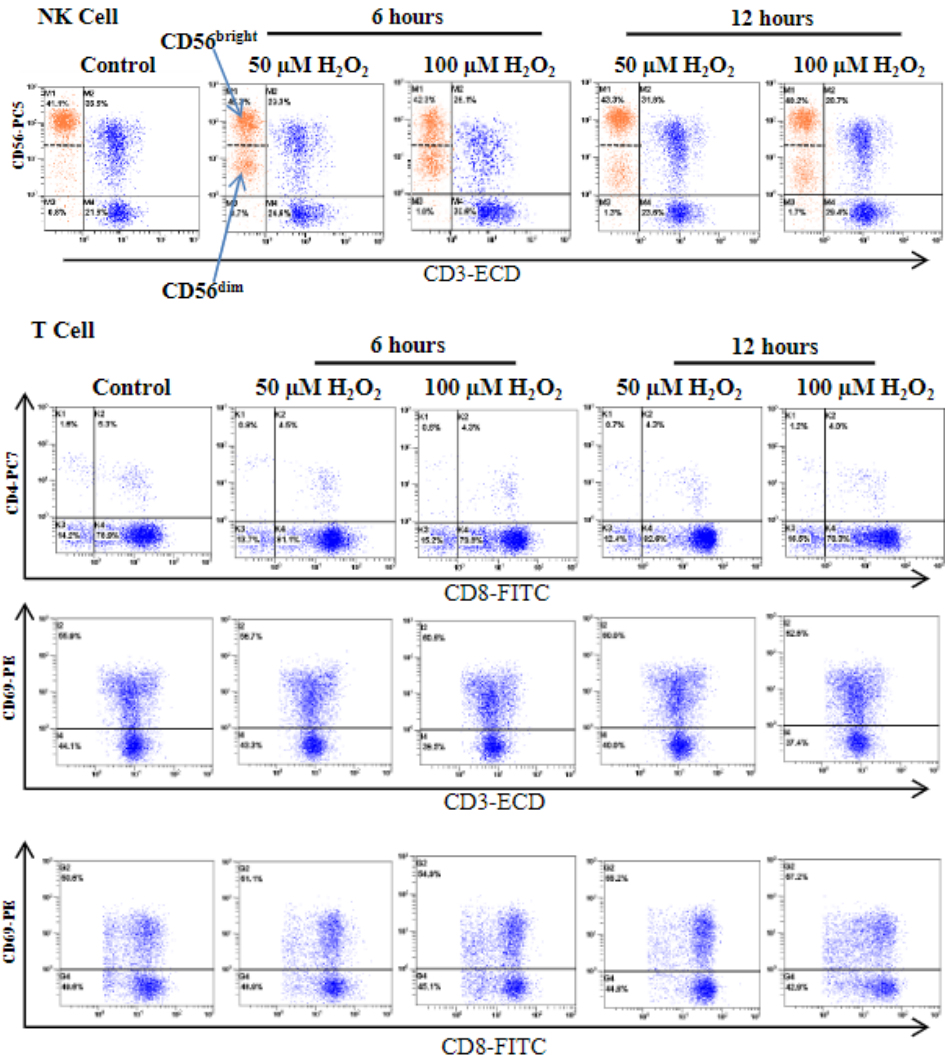
100µM H_2O_2
HT29
NK Cell
NK Vs HT29
T Cell
T Cell Vs HT29

12 hours


Control
50µM H_2O_2
100µM H_2O_2
HT29
NK Cell
NK vs HT29
T Cell
T Cell vs HT29


NK Cell
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12 hours
CD56bright
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100 μM H2O2
CD56-PC5
CD3-ECD
CD56dim
T Cell
6 hours
12 hours
Control
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100 μM H2O2
50 μM H2O2
100 μM H2O2
CD4-PC7
CD8-FITC
CD69-PE
CD3-ECD
CD69-PE
CD8-FITC