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Induction of renal cell line apoptosis by hydrogen peroxide

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Abstract

Background: Hydrogen peroxide is currently the most widely used as an apoptosis inducer due to its broad cytotoxic efficacy against nearly all cell types. cytotoxicity is achieved over a wide range of doses

Objective: To evaluate the cytotoxic effect of hydrogen peroxide on renal cell lines by detecting RIPK1.

Methods: In this study, we used a Vero cell line treated with H_2O_2 at concentrations 0.1 mM, 0.2 mM, 0.4 mM, 0.8 mM, and 1.6 mM and examined after 30 min,1hour,2hours,3hours, 4hours and 5 hours. by using MTT assay to detect cytotoxicity to cell line (by detecting cell viability). Spectrophotometrically measure the absorbance at a wavelength of 570 nm. Measure the background absorbance of multi-well plates at 690 nm and subtract from the 450 nm measurement. Sub-lethal dose to renal cell line is one treated with 1.6 mM for 5 hours. groups group 1: renal cell line as control which not treated by H2O2. group 2: Sub-lethal which renal cell line treated by concentration 1.6 mM of H2O2 for 5 hours. **Results:** H2O2 is cytotoxic to renal cell line by concentration from 0.1 mM to 1.6 mM. RIPK1 gene expressed in renal cell line treated by H2O2. The sublethal dose reached 1.6 mM for 5 hours. There is a significant difference between the 2 groups by detecting the expression of the RIPK1 gene.

Keywords: Hydrogen peroxide, cytotoxic, apoptosis, renal cell line, RIPK1 gene.

Introduction

Apoptosis is a form of programmed cell death that occurs in multicellular organisms. Biochemical events lead to characteristic cell changes (morphology) and death [1].

Cell apoptosis was first described as a cell death pathway unique from necrosis in 1972[2].

Thereafter, a plethora of apoptosis inducers was identified, such as hydrogen peroxide (H2O2), which has been the most widely used and studied at the mechanistic level. In many cases, transient exposure to H_2O_2 triggers apoptosis through the mitochondrial pathway involving sequential loss of mitochondrial membrane potential, cytochrome c release, and effector caspase-3 activation [3].

Hydrogen peroxide is used as an apoptosis inducer for many types of cells, including cell lines, tumor

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cells, primary cells, and highly differentiated cells [4],

Activation of NF-κB is usually caused by oxidative stress and may indicate DNA damage [5], RIP is a key mediator of necroptosis signaling pathways; therefore RIP can be used to distinguish apoptosis from necroptosis [6].

Receptor-interacting kinase 1 (RIPK1) functions in a variety of cellular pathways related to both cell survival and death. In terms of cell death, RIPK1 plays a role in apoptosis and necroptosis. Some of the cell survival pathways RIPK1 participates in include NF-κB, Akt, and JNK [7].

Material and methods

I. Maintenance of VERO Cell Line

Vero cell line (African green monkey kidney cells)

Cells were maintained in DMEM (Dulbecco,s Modified Eagle Medium) growth medium (Invitrogen-Gibco), supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen-Gibco) in 75 cm3 cell culture flask, flasks were incubated in 5% CO_2 incubator with 95% humidity at 37^{o}C

II. Trypsinization of Cells

1. Confluent monolayer of Vero cells in 75-cm2 flasks was examined under an inverted microscope to examine; the viability of cells and is free of contamination.
2. The old medium was aspirated off and 8 ml trypsin (1:250) pre-warmed at 37°C, added to each flask then incubated at 37°C for 10 min.
3. Cell culture was examined via an inverted microscope; if cells were detached; the action of trypsin was deactivated by the addition of equal amounts of growth medium supplemented with 10% FBS.
4. Transfer the cell suspension to the centrifuge tube with the medium and cells from step 1, and centrifuge at approximately 125 xg for 5 to 10 minutes. Discard the supernatant.
5. Resuspend the cell pellet in a fresh growth medium. Add appropriate aliquots of the cell suspension cells density (1.8×10^4 cells / well) in a volume of 100µl complete growth medium. Then Incubate cultures at 37°C for 24 hrs.

III. Cytotoxicity Study and sub-lethal dose of H_2O_2 Determination.

Wells growth medium added by 0.1mM, 0.2mM, 0.4mM, 0.8mM, and 1.6 mM of the H_2O_2 per well in a 2×3 well plate for 0.5 hr., 1 hr., 2 hr,3 hr.,4 hr., and 5 hr. to induce Vero cell destruction then evaluation by MTT assay to determine the sub-lethal dose

We use one well as normal control not treated by H2O2.

The MTT method of monitoring in vitro cytotoxicity is well suited for use with multiwall plates. For best results, cells in the log phase of growth should be employed and the final cell number should not exceed 106 cells /well. Each test should include a blank containing a complete medium without cells.

1. Reconstitute each vial of MTT [M-5655] to be used with 3 ml of medium or balanced salt solution without phenol red and serum. Add reconstituted MTT in an amount equal to 10% of the culture medium volume.
2. Return cultures to an incubator for 2-4 hours depending on cell type and maximum cell density. (An incubation period of 2 hours is generally adequate but may be lengthened for low cell densities or cells with lower metabolic activity.) Incubation times should be consistent when making comparisons.
3. After the incubation period, remove cultures from the incubator and dissolve the resulting formazan crystals by adding an amount of MTT Solubilization Solution [M-8910] equal to the original culture medium volume.

4. Gently mixing in a gyratory shaker will enhance dissolution. Occasionally, especially in dense cultures, pipetting up and down [trituration] may be required to completely dissolve the MTT formazan crystals.
5. Spectrophotometrically measure the absorbance at a wavelength of 570 nm. Measure the background absorbance of multi-well plates at 690 nm and subtract from the 450 nm measurement. Tests performed in multi-well plates can be read using the appropriate type of plate reader or the contents of individual wells may be transferred to the appropriate size cuvette for spectrophotometric measurement.

From the MTT assay, the well-treated 1.6mM by H_2O_2 for 5 hours is the sub-lethal dose.

Vero Cells have been cultured for 24hr. then exposed to a sub-lethal dose of H2O2 for 5 Hr.

IV. Gene expression analysis of P53 gene, RIPK1 gene, and EGFR gene (RT-PCR)

II.4.1. RNA isolation

In gene expression analysis, it is very important to prepare high-quality RNA because RNA can be contaminated by genomic DNA. To reduce contamination of genomic DNA from RNA, proper RNA isolation and cDNA synthesis procedures need to follow. RNA isolation was carried out with the RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions.

1- Cells were transferred to a centrifuge tube, and pellet by centrifugation at 300 x g for 5 min. Completely aspirate supernatant.

2- Buffer RLT was added to monolayer cells. the cell lysate was collected with a rubber policeman. Vortex to mix until no cell clumps should be visible

3- One volume (350 µl) of 70% ethanol was added to the homogenized lysate and mixed well by pipetting without centrifugation.

4- The volume applied up to 700 µl of the sample, including any precipitate which may have formed, to a RNeasy mini spin column sitting in a 2-ml collection tube, and centrifuged for 15 sec at ≥8000 x g (≥10,000 rpm)

5- 700 µl Buffer RW1was pipetted onto the RNeasy column and centrifuged for 15 sec at ≥8000 x g (≥10,000 rpm) to wash.

6- RNeasy column was transferred into a new 2-ml collection tube (supplied). 500 µl Buffer RPE was pipetted onto the RNeasy column and centrifuged for 15 sec at ≥8000 x g (≥10,000 rpm) to wash.

7- 500 µl Buffer RPE was pipetted onto the RNeasy column, then centrifuged for 2 min at maximum speed to dry the RNeasy membrane**.**

8- RNeasy column was transferred into a new 1.5-ml collection tube (supplied) and pipetted 30–50 µl of RNase-free water directly onto the RNeasy membrane. Finally centrifuged for 1 min at ≥8000 x g (≥10,000 rpm) to elute.

II.4.2. cDNA synthesis

1- 1 µg of total RNA was used to synthesize cDNA using iScriptTM One-Step RT-PCR Kit with SYBR® Greenis a convenient and highly sensitive solution for real-time quantitative PCR of RNA templates.

2- cDNA synthesis and PCR amplification are carried out in the same tube. This kit is optimized to deliver maximum RT-PCR efficiency, sensitivity, and specificity without compromising fluorescent signals.

3- The obtained cDNA was used to determine the mRNA expression levels of Bcl-2, P53 by RT-PCR analysis.

4- GAPDHwas used as an internal control. (**Stagliano et al., 2003; Zhao et al., 2017).**

The primers sequences used for the amplification of RIP, P53, EGFR, and GAPDH were as follows:

Target Gene	Primer Sequence
RIPk1	F 5′-GAGAGCTGGTAGTTAGTAGCATGA -3′ R5′- AATTCCAATAATGAACCCAATAGATTAGTT -3′
GAPDH	F 5′- GTCTCCTCTGACTTCAACACGC -3′ R5′- ACCACCCTGTTGCTGTAGCCAA-3′

All primers were synthesized by (Sangon Biotech, Shanghai, China).

The reaction included 1 µl cDNA, 2 µl 10X Taq Buffer, 1.2 µl 25 mM $MgCl_2$, 0.4 µl 10 mM dNTP, 0.8 µl 1U/µL Taq polymerase, 1 µl each primer and DEPC water up to 20 µl. The PCR conditions were as follows: 95°C for 3 min and 30 cycles at 95°C for 30 sec, 56°C for 40 sec, and 72°C for 40 sec. **(Bustin, 2005 and kubista et al., 2006).**

Statistical analysis:

Results were statistically analyzed by SPSS version 22(SPSS Inc., Chicago, IL, USA). Tests of normality were performed. A student test was used for comparison between 2 means of normally distributed variables.

P-value was considered significant if <0.05.

Results

This study was carried out at Genetic Engineering and Biotechnology Research Institute, (GEBRI) Sadat city University. The research work evaluates the hydrogen peroxide effect on renal cell lines to induce apoptosis.

It was found that hydrogen peroxide at a concentration of 1.6 mM at 5 hours showed maximum apoptosis (sub-lethal dose) in the renal cell line. The renal cell line was divided into two groups; Group 1: control, Group 2: sub-lethal group which is renal cell line treated with H_2O_2, RIP-K gene was measured in two groups of renal cell line by RT-PCR

Cytotoxicity to renal cell line by hydrogen peroxide

Hydrogen peroxide has a high efficiency leading to cell death.

Hydrogen peroxide causes apoptosis and necroptosis cells at a concentration ranging from 0.1mM to 1.6 mM.

We reach a sub-lethal dose of hydrogen peroxide at a concentration of 1.6 mM for 5 hours.

The cell lines used in this study were sensitive to hydrogen peroxide which causes stress to the renal cell line.

We use the MTT assay to detect cytotoxicity to cell lines (by detecting cell viability).

Spectrophotometrically measure the absorbance at a wavelength of 570 nm. Measure the background absorbance of multi-well plates at 690 nm and subtract from the 450 nm measurement.

Table 1 and figure 1 show percentage cytotoxicity to renal cell lines by different concentrations of hydrogen peroxide at different times.

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Table 1: percentage of cell line toxicity by different hydrogen peroxide after 0.5hour, 1hour, 2hours,3 hours, 4hours and 5hours

H2O2 conc.	0.5 hour	1 hour	2 hours	3 hours	4 hours	5 hours
H2O2 0.1 mM	2 %	4 %	7 %	8%	9 %	12 %
H2O2 0.2 mM	3 %	5 %	8 %	9%	11 %	15 %
H2O2 0.4 mM	8 %	14 %	22 %	26%	32 %	38 %
H2O2 0. 8mM	12 %	16 %	28 %	34%	40 %	52 %
H2O2 1.6 mM	18 %	26 %	38 %	43%	54 %	85 %

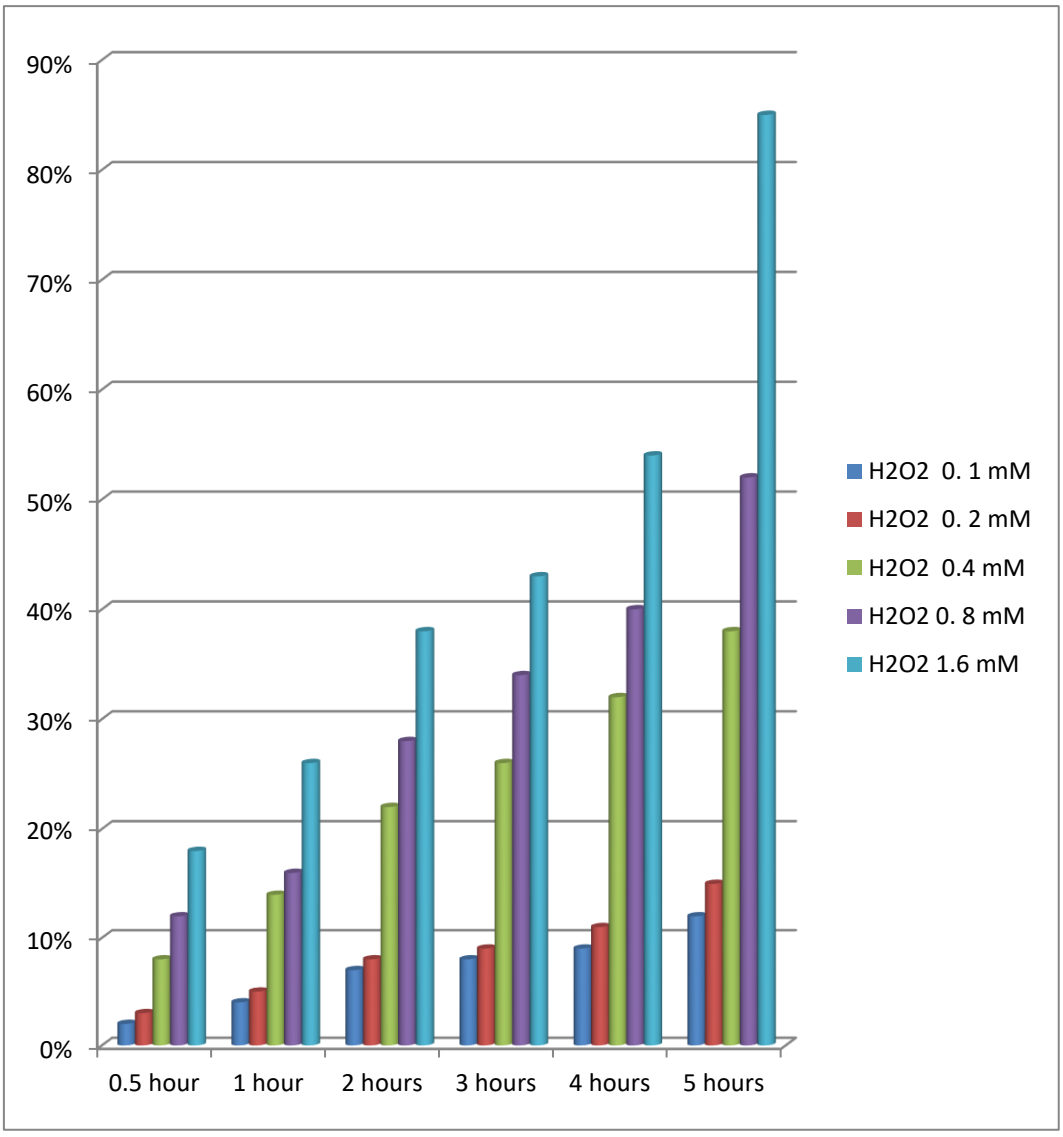

Figure (1): comparison between different concentrations of H_2O_2on renal cell line after 0.5h, 1hour, 2hours, 3hours, 4hours, and 5hours.

Table 2: showed the RIPK gene levels of the studied group. RIBK was significantly increased in renal cell lines treated with H_2O_2 Compared to other groups denoting apoptosis.

Table 2: Comparison of the studied groups regarding RIPK1:

	Group 1 Control (n=20)	**Group 2 Sublethal (n=20)**	**ANOVA (P-value)**	**Post hoc test**
	Mean ±SD (Range)	Mean ±SD (Range)		
RIPK 1	2.77 ±0.39 R=1.66-3.89	6.02±0.85 3.61-8.44	F=101.64 P<0.001*	P <0.001*

*: significant P: Controls vs. sub-lethal,

Discussion

In this research, we studied the cytotoxic effect of hydrogen peroxide on a renal cell line which causes apoptosis and necroptosis We use an MTT assay to detect cytotoxicity to cell line (by detecting cell viability)

By detecting the RIPK1 gene by using RT PCR as an index to apoptosis and necroptosis. We reached a sub-lethal dose of 1.6 mM of H2O2 after 5 hours.

Certain indices can reveal specific aspects of the cell death process. For example, caspase-9 can be used to monitor the initiation of apoptosis [8], activation of NF-*κ*B is usually caused by oxidative stress and may indicate DNA damage [9], and P53 can indicate dysregulation of the cell cycle and proliferation [5].

there is still no study that systematically studied different susceptibility to H_2O_2-induced apoptosis among cell types, which is critical for determining if H_2O_2 is a suitable apoptosis inducer in a specific context. Indeed, whether apoptosis or necroptosis is induced under different dosages of H_2O_2 is often unconfirmed [10].

In this study, we reached the sub-lethal dose of hydrogen peroxide concentration in a renal cell line which was used in different researches.

Conclusion

Hydrogen peroxide has a high efficiency leading to cell death Hydrogen peroxide causes apoptosis and necroptosis in a concentration ranging from 0.1mM to 1.6 mM. The cell lines used in this study Vero cell line as a renal cell line which sensitive to hydrogen peroxide.

The maximum concentration of hydrogen peroxide which causes sub-lethal necrosis is 1.6 mM after 5 hours

MTT assay is used to detect cytotoxicity to cell lines (by detecting cell viability).

Spectrophotometrically measure the absorbance at a wavelength of 570 nm. Measure the background absorbance of multi-well plates at 690 nm and subtract from the 450 nm measurement.

RIPK1 is used as a marker for apoptosis as they detected by RT-PCR

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No conflict of interest

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