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Investigation of Genotoxic and Cytotoxic Effects of Hydrogen Peroxide

(The Importance of DNA Repair in Oxidative Stress)

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ABSTRACT

Oxidative stress-induced reactive oxygen species induce DNA damages that may result in cell death and DNA mutations. In this study, a genotoxic and cytotoxic potential of hydrogen peroxide as a ROS generator, and the effect of cell repair of oxidative stress in TK6 cells were investigated by in vitro micronucleus assay. Cell repair was inhibited by using cytosine arabinoside. Cellular responses to different concentrations of hydrogen peroxide were evaluated by generating dose-response curves.

The incidence of increased MN concentrations due to increased H_2O_2 concentrations did not allow the identification of a plateau region. A similar curve pattern was also observed with the repair inhibited group. However, damage caused by H_2O_2 was significantly higher ($p<0.001$) in the cells with repair inhibition. This demonstrates the importance of DNA repair in the observation of oxidative stress. Proliferative indexes did not change due to increasing H_2O_2 concentrations in both groups. But the curve of repair inhibited cells was significantly higher ($p<0.001$) than the curve of repair efficient cells. BN and multinucleate cell counts showed decreases with increasing ROS levels generated by H_2O_2. But these decreases were not reflected in proliferative index scores. This may be due to H_2O_2-induced apoptosis, which results in the overestimation of PI values in increasing H_2O_2 concentrations.

Key words: Toxicity, Oxidative Stress, Hydrogen Peroxide**,** TK6 Cells, Micronuclei.

1. INTRODUCTION

Damaging effects of oxidative stress generated by reactive oxygen species (ROS) occur if oxidant products in cells exceed the cell's natural chemical defense capacity. Once the damage is inflicted on DNA, enzymatic mechanisms are activated to repair oxidative DNA damage [1-4]. After repair, the remaining oxidized DNA damage may result in mutations, chain breaks, base damage, DNA-DNA, or DNA-protein cross-links. Recovery of cell viability after oxidative stress depends on cellular natural antioxidant levels and enzymatic repair.

To know the genotoxic potential of any chemical, dose-response relationships should be determined. From these dose-response relationships, it is necessary to define threshold levels at which chemical agents begin to show genotoxic effects [5-9]. Although there is evidence to support the presence of a threshold dose from a few dose-response curves for DNA-reactive agents, controversy persists regarding the determinability of threshold dose levels [10-12]. Using the micronucleus technique, Platel *et al.* showed that DNA-reactive agents such as hydrogen peroxide produced a non-linear dose-response relationship in TK6 cells with threshold values [8]. Determination of unobservable levels of genotoxic effect (threshold levels) from dose-response curves of genotoxic chemicals ensures the usage of these chemicals within safe boundaries. Nonetheless, in fields where the dose-response curves of the chemicals are linear (without threshold levels), it is told that chemicals do not have safe limits with having a genotoxic risk at even very low amounts of doses [13-14].

Examining DNA repair in genotoxic studies can supply data about the conformation of the dose-response curves. Threshold levels if there are maybe explained by the repair ability of the cells. There is a lack of data concerning the importance of DNA repair in genotoxic dose responses either with or without threshold levels. Therefore, in this study, the importance of DNA repair in oxidative stress was studied by determining dose-response curves. Hydrogen peroxide (H_2O_2) was used as ROS generating compound. Oxidative stress generated by hydrogen peroxide causes oxidative DNA bases, abasic regions, and DNA

strand fractures at close sites, leading to DNA damage [15-17]. Genotoxic and cytotoxic effects of H_2O_2 on TK6 cells of the human cell line and the role of DNA repair were measured by the micronucleus (MN) technique. This technique, based on micronucleus measurement in binucleate cells, is a good indicator of the genotoxic effects of aneugenic and clastogenic agents [18-22]. The MN test, where cytotoxic effects can also be analyzed, is easy to do and the results are accurate.

2. MATERIALS AND METHODS

2.1 Reagents and chemicals

Hydrogen Peroxide (30%, CAS No. 1.07209) was received from the Merck Pharmaceutical Company. Hanks' Balanced Salt solution (CAS No.H6648), Fetal Bovine Serum (heat-inactivated, CAS No. F-4135), RPMI-1640 medium (CAS No.R4130), Cytochalasin-B (CAS No.C-6762) and Cytosine β-D-arabinofuranoside (Ara-C, CAS No.C1768) were purchased from Sigma Chemical Corporation.

2.2 Cell culture

TK6 (ATCC CRL-8015) human B-lymphoblastoid cell line was obtained from the American Type Culture Collection (Manassas, VA 20108 USA). TK6 cells seeded in RPMI-1640 medium were kept in the culture at 37° in a humidified atmosphere with 5% CO_2. The culture medium was supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% (v/v) fetal bovine serum. To ensure continuous growth in culture, passages were made twice a week.

2.3 Adaptation of MN technique to TK6 cells

The Micronucleus assay system measuring chromosome aberrations are a good indicator of genotoxic and cytotoxic events. To score chromosome damage by MN technique, cells should be obtained as binucleate cells by preventing their cytokinesis with cytochalasin-B (cyt-B) at the end of the cell cycle. The appropriate concentration of cyt-B to achieve the optimal amount of binucleate cells in this study was found to be 3 µg/ml with the optimum culturing time of 24 hours. This method was applied to find out the effects of oxidative stress after H_2O_2.

2.4 H_2O_2 treatment

$0.25x10^6$ of TK6 cells in 5 ml of RPMI-1640 medium and FBS serum were added to the plastic culture flasks with 5% CO_2 in a humidified atmosphere at 37° for 72 hours in culture and treated with H_2O_2 for 30 minutes. Cells washed with HBSS were re-cultured in 3 µg/ml cyt-B medium and incubated for a further 24 hours. The cells were centrifuged at 200g for 10 min and swelled by adding 0.075M KCl solution and then rotated twice with a methanol-acetic acid fixative solution. As a result of this process, the fixed cells were dropped onto slides with a Pasteur pipette and binucleate cells were obtained. The dried slides were stained with 5% Giemsa dye for 4 minutes and covered with a coverslip to get ready for examination under a light microscope.

2.5 Ara-C application

Cell repair was studied by using cytosine arabinoside (Ara-C) that converts repairable DNA lesions to micronuclei by inhibiting the DNA excision repair mechanism. The MN technique was also optimized for the determination of oxidative stress in the presence of Ara-C [23]. After the doses of H_2O_2 applied to the growing cells in the culture, the washed cells were seeded in the presence of Ara-C (1 µg/ml) and incubated for 16 hours and then the washed cells were re-cultured in 3 µg/ml cyt-B medium and incubated for a further 24 hours. Subsequent fixation and staining procedures were repeated as described above and the slides were made ready for examination. Micronucleus frequencies (MN/BN) were scored by counting binucleate cells with and without micronuclei. To evaluate the cytotoxic effects of hydrogen peroxide, cells containing 1, 2, 3, and 4 nuclei were scored to determine proliferative indexes (PI).

Using this modified MN method, the cells in the culture were treated with 11 different concentrations of hydrogen peroxide ranging from 20µM to 100,000µM µM (in the presence and absence of Ara-C) to obtain dose-response curves.

2.6 Statistical analysis

Comparisons between MN frequencies and proliferative indexes after H_2O_2 treatment with and without Ara-C were made with paired t-test. The effects of H_2O_2 concentrations on MN frequencies and proliferative indexes were evaluated with regression analysis. Statistical analyses were performed with GraphPad Prism 6.0 (GraphPad Software, Inc., San Diego, CA).

3. RESULTS

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For the determination of dose-response curves, MN scores, and proliferative indexes after oxidative stress applied to cells in the growth phase by 11 different concentrations of H_2O_2 treatment are given in Table 1. MN and PI data were obtained by scoring a total of 158.485 cells. Values show the results of at least 10 different experiments.

Table 1. MN and PI values were obtained with different H_2O_2 concentrations

					Multiple nuclei				
No	**H_2O_2 (µM)**	**BN**	**MN**	**MN/BN**	**M1**	**M2**	**M3**	**M4**	**PI**
1	0	28590	150	0,005	37575	45	735	165	1,02
2	20	6360	60	0,009	3805	400	460	165	1,10
3	50	2445	30	0,012	14360	25	140	40	1,02
4	100	835	20	0,024	12825	60	20	5	1,01
5	200	530	20	0,038	18240	190	30	-	1,01
6	500	225	10	0,044	5940	45	10	-	1,01
7	1000	150	15	0,100	10755	90	-	-	1,01
8	2000	295	15	0,051	530	65	20	-	1,09
9	5000	235	15	0,064	9455	65	55	15	1,02
10	10000	90	10	0,111	340	-	30	-	1,11
11	50000	310	30	0,097	500	10	40	-	1,08
12	100000	345	35	0,101	750	30	45	-	1,08

BN; binucleate, MN; micronucleus, M1; 1 nucleated cell, M2; 2 nucleated cells, M3; 3 nucleated cells, M4; 4 nucleated cells, PI; proliferative index.

BN cells have 2 nuclei like M2 cells, but because their nuclei are similar in size and regular shapes, they have been used in micronuclei scoring. The frequency of MN/BN refers to the MNs observed in these BN cells. With the increase in hydrogen peroxide dose, there was a significant decrease in the number of BN cells. This fall has also been observed in mononucleate and multinucleate cells. Cells with 1 nucleus (M1) were scored most at each concentration, whereas M2, M3, and M4 were observed less often. PI values were calculated by the formula (M1+2M2+3M3+4M4)/(M1+M2+M3+M4). The values remained similar.

To determine the role of cell repair capacity in chromosomal damage caused by oxidative stress, excision repair inhibitor cytosine arabinoside was given to the cells. Micronucleus damage and proliferative capacity results are given in Table 2. MN and PI data in this experimental group were obtained by scoring a total of 60.150 cells. In addition to falling BN amounts with Ara-C application alone (1st row in Table 2), decreases in both BN cells and the number of cells with 1 and multiple nuclei are also observed in this group due to increasing H_2O_2 concentrations.

Table 2. MN and PI values were obtained with different H_2O_2 concentrations in the presence of Ara-C.

					Multiple nuclei				
No	**H_2O_2 (µM) +Ara-C**	**BN**	**MN**	**MN/BN**	**M1**	**M2**	**M3**	**M4**	**PI**
1	0	19605	510	0,026	11175	315	4890	6180	1,46
2	20	720	20	0,028	370	130	60	140	1,31
3	50	375	30	0,080	165	30	30	75	1,30
4	100	1005	30	0,030	1530	180	150	255	1,30
5	200	770	30	0,039	1690	110	130	260	1,31
6	500	305	15	0,049	150	45	25	30	1,22
7	1000	280	25	0,089	500	35	60	40	1,23
8	2000	540	60	0,111	5355	225	120	180	1,14
9	5000	280	40	0,143	595	40	85	35	1,24
10	10000	60	10	0,167	70	10	10	20	1,39
11	50000	130	20	0,154	165	10	30	25	1,30
12	100000	155	25	0,161	130	15	25	30	1,30

BN; binucleate, MN; micronucleus, M1; 1 nucleated cell, M2; 2 nucleated cells, M3; 3 nucleated cells,

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M4; 4 nucleated cells, PI; proliferative index.

MNs scored in binucleate cells were increased in the presence of Ara-C. A smaller number of M2, M3, and M4 were observed with increasing H_2O_2 concentration.

Increasing MN frequencies were observed with increasing H_2O_2 concentrations in both groups. The relationship between different H_2O_2 doses and MN frequencies which increase depending on these varying dose levels is determined by using regression analysis and is given in Figure 1.

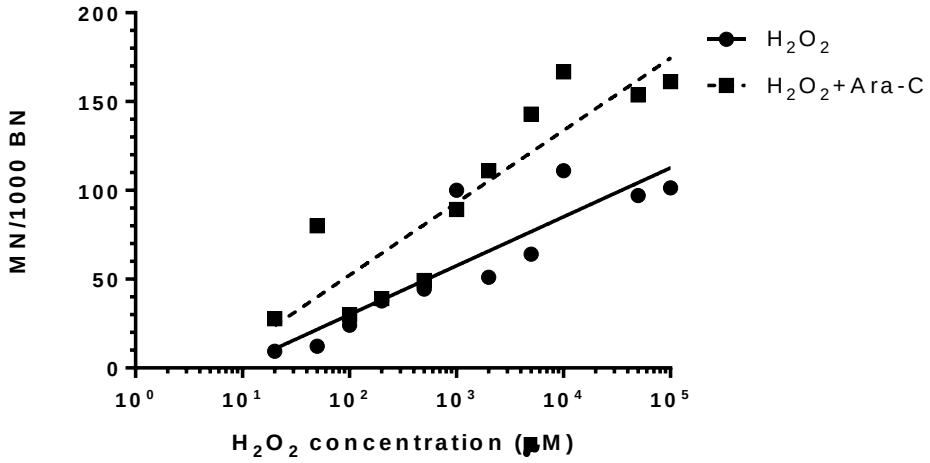

Figure 1. MN dose-response curves obtained with H_2O_2 and H_2O_2 + Ara-C.

The MN dose-response curve obtained by the Ara-C application showed a significant difference ($p < 0.001$) from the curve where H_2O_2 was applied alone. The comparison between the two groups in terms of proliferative indexes is given in Figure 2.

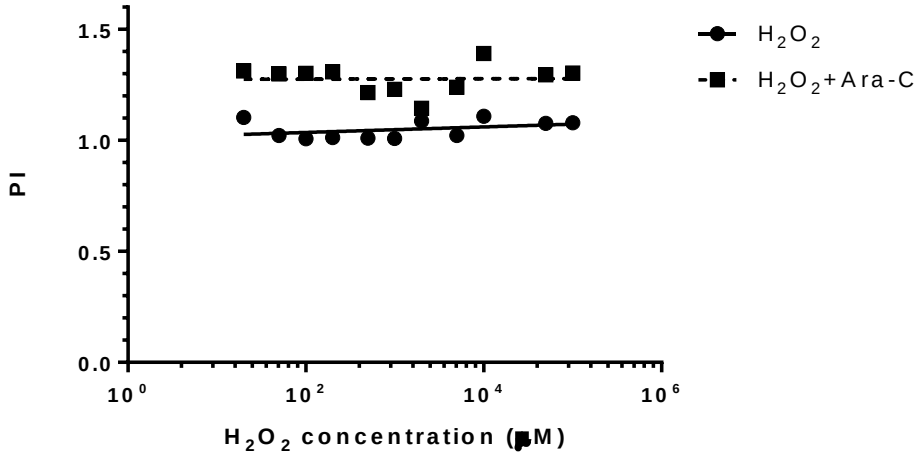

Figure 2. PI dose-response curves obtained with H_2O_2 and H_2O_2 + Ara-C.

When the PI differences in Figure 2 are considered, the curve obtained by the Ara-C application shows a significant difference ($p<0.001$) from the curve where H_2O_2 is applied alone.

4. DISCUSSION

The genotoxic effects of chemical agents in the environment we exist in and the substances we ingest should be recognized. The dose-response relationship of the chemicals should be determined to know the dose-dependent effects of the chemicals. From these dose-response relationships it is necessary to define threshold levels if there is at which the chemical agents begin to show genotoxic effects [5,8-9]. This is possible by determining dose-response curves and extrapolating to levels where no toxic effects are observed [24-25].

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In determining the dose-response relationship, intracellular oxidant levels and repair mechanisms responsible for repairing DNA damage are important. Radical scavengers such as GSH and thiol molecules in the antioxidant defense system, which is the first defense mechanism of the cell before the enzyme repair of DNA damage, have an important role in eliminating oxidative stress. Thus, the effect of oxidative stress on DNA should not be noticed until the antioxidant pool is exhausted.

In vitro MN technique is used in studies of the toxic effects caused by chemicals and radiation in the genome, determination of chromosomal irregularities, prediction of cancer risk and aging-related degenerative diseases [23,26-28]. In this work, chromosome damage caused by H_2O_2, which is one of the sources of oxidative stress, was determined by the MN test. The cell response determined by the broad spectrum of H_2O_2 concentrations as defined by the first-order equation which was determined according to the least-squares principle and gave the best fit to the data points ($r^2 = 0.78$; Figure 1). The Plateau region was not found on the linear dose-response curve. Since the curve tended to increase continuously, it didn't make it possible to define the threshold level where H_2O_2 had no genotoxic effect. This does not provide information about the size of the first defense mechanism, the antioxidant defense system.

The initial damage caused in DNA by ROS activity that overcomes the antioxidant defense system is observed to be chromosomal damage if it cannot be enzymatically repaired. The ability of DNA to repair itself is important in observing damages. So, to determine how repairable capacity varies with the amounts of oxidative stress, cell cultures in this study, treated with H_2O_2 were given Ara-C which inhibits excision repair. The cellular response in these repair inhibited cells against H_2O_2 was also defined by the first-order equation ($r^2 = 0.81$; Figure 1) with no plateau region. Both curves in Figure 1 tended to increase continuously, however, damage caused by oxidative stress was higher in the cells in which repair was inhibited. A significant difference ($p<0.001$) was observed when the curves were compared. This brings out the importance of DNA repair in the observation of oxidative stress.

Proliferative indexes did not change due to increasing H_2O_2 concentrations in both H_2O_2 ($r^2 = 0.13$) and H_2O_2 + Ara-C ($r^2 = 0.0004$) applications. However, when the curves were compared (Figure 2), the PI curve obtained by the Ara-C application shows a significant difference ($p<0.001$) from the curve where H_2O_2 is applied alone. Although it was observed that BN and MN values decreased with increasing H_2O_2 concentrations, it was confusing that PI values did not change in each of the 2 groups. May be, decreases in BN and multinucleate cell counts at higher ROS levels generated by H_2O_2 were due to H_2O_2-induced cytotoxicity, but this didn't reflect on PI values because H_2O_2-induced apoptosis leading to overestimation of PI values [29].

5. CONCLUSION

DNA excision repair is important in ROS induced DNA damage and cytotoxicity. H_2O_2-induced apoptosis may be important in cytotoxicity that is measured by proliferation index values.

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