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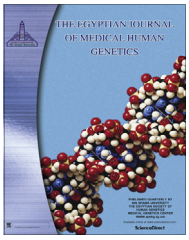

ORIGINAL ARTICLE

Evaluation of chromosomal aberrations induced by hydralazine in Chinese hamster ovary cells

Mozhgan Sedigh-Ardekani, Mostafa Saadat *

Department of Biology, College of Sciences, Shiraz University, Shiraz 71454, Iran
Institute of Biotechnology, Shiraz University, Shiraz, Iran

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KEYWORDS

Hydralazine;
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Polyploidy

Abstract *Background and purpose:* Hydralazine (HDZ) is a cardiovascular drug that is widely used to treat hypertension. The present study was done to assess the cytogenetic effects of HDZ on Chinese hamster ovary (CHO) cells.

Materials and methods: Methylthiazol tetrazolium (MTT) assay was carried out to determine the half maximal inhibitory concentration (IC_{50}) of the drug. The IC_{50} value for HDZ was 243.3 ± 16.9 μg/ml. To investigate the clastogenic effects of the drug, chromatid breaks and polyploidy in metaphases were analyzed. CHO cells were exposed to different concentrations of HDZ (20 and 40 μg/ml) for 24 h. The experiments were carried out in the presence and absence of metabolic activation system (S9 mix; 1 ml S9 mix contained: 0.3 ml phosphate solutions, 0.2 ml KCl, 0.2 ml $MgCl_2$, 0.1 ml S9 fraction, 0.1 ml G-6-P and 0.1 ml NADP), because HDZ is metabolized in the liver. Mitomycin-C and sodium arsenite were used as positive controls.

Results: In the absence of S9 fraction, the level of chromatid breaks statistically increased ($P = 0.011$) and mitotic index significantly decreased ($P < 0.001$) in CHO cells treated with HDZ. There was no significant difference between treated and untreated CHO cells with HDZ for the level of polyploidy ($F = 0.05$; df = 2, 6; $P = 0.945$). In the presence of S9 fraction, although the mitotic index elevated, still there was a significant difference between control and treated cells ($F = 50.53$; df = 2, 6; $P < 0.001$). There was no significant difference between 20 μg/ml of HDZ (+S9) and untreated cells for frequency of chromatid breaks. However, at the 40 μg/ml concentration of HDZ (+S9), there was a significant difference between treated and untreated cells.

Conclusion: HDZ have genotoxic effects on CHO cells in their non-toxic dose, but S9 mix addition decreased these effects.

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1. Introduction

Hydralazine (HDZ) is an antihypertensive drug used to treat hypertension, congestive heart failure, myocardial infarction and preeclampsia [1–5]. HDZ was found to create free radicals [6–8]. It has been reported that HDZ has mutagenic effect in bacterial test systems [9–12]. It is suggested that active oxygen

* Corresponding author. Tel.: +98 711 6134722.
E-mail addresses: saadat@shirazu.ac.ir, msaadat41@yahoo.com (M. Saadat).

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species generated by dihydralazine contribute to its mutagenicity [11].

It has been shown that HDZ increases the risk of cancers in mice and humans [13–15]. On the other hand, it has been reported that HDZ has anticancer effect on some cancer cells [16,17]. It should be noted that HDZ reactivated tumor suppressor genes [18]. HDZ was found to decrease apoptosis and free radical generation in both neurons and thymocytes [19]. Taken together, it is very likely that HDZ has interaction with DNA and chromosomes. The aim of the present study was to assess the effect of HDZ on chromosomes of Chinese hamster ovary (CHO) cells.

2. Materials and methods

2.1. Chemicals

Hydralazine HCl (hydrapres, 20 mg) was obtained from Rubio, nicotinamide adenine dinucleotide phosphate (NADP) was obtained from Sigma, Glucose-6-phosphate from Fluka, sodium arsenite (0.1 N) and mitomycine.

2.2. Cell culture

In the present study, experiments were carried out using cultured Chinese hamster ovary (CHO) cells. The CHO cells were maintained in RPMI-1640 medium (from GIBCO) supplemented with 10% inactivated fetal calf serum (from GIBCO), 2 mM L-glutamine and with the addition of penicillin (100 U/ml) and streptomycin (100 mg/ml).

This study was approved by the Shiraz University ethics committee. This work is carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving animal experiments.

2.3. Methylthiazol tetrazolium (MTT) assay

Cells were seeded into 96-well plates at 2×10^4 cells/well in 100 µl complete medium. After the cells clung to plates, the cells were treated with various concentrations of HDZ (25, 50, 100, 200, 500 and 1000 µg/ml) for 24 h. Final volume in each well was 200 µl. Subsequently, the cell viability was measured by MTT dye reduction assay [20,21]. The formazan dye was measured by ELISA reader. After obtaining the absorption, percentage inhibition of cell growth was calculated. The IC_{50} is defined as the cytotoxicity index that reduces the cell number to 50% compared with untreated-control CHO cells.

2.4. Metabolic activation system

Liver S9 fraction was obtained from Wistar male rats induced with phenobarbital. The livers from three rats were removed, pooled and S9 fraction (10,00*g*) supernatant was prepared following the standard procedure. Immediately before use, a S9 mix (S9 fraction with cofactors) was prepared: 1 ml S9 mix contained: 0.3 ml phosphate solutions, 0.2 ml KCl, 0.2 ml $MgCl_2$, 0.1 ml S9 fraction, 0.1 ml G-6-P and 0.1 ml NADP. The S9 mix was added (50 µl) to the cultures.

2.5. Chromosomal analysis

Chromosomal aberration assay was performed to investigate the cytogenetic effects of HDZ (in the presence and/or absence of S9 mix). The CHO cells were seeded at the density of 1.8×10^6 cells/ml in the volume of 10 ml. After 48 h, the cells were treated with different doses of HDZ (20 and 40 µg/ml) for 24 h. The cells were treated also with 20 and 40 µg/ml of HDZ in the presence of 50 µl of S9 mix. After 6 h of incubation, the medium was changed and the cells were incubated for 18 h. Mitomycin-C (0.06 µg/ml) and sodium arsenite (1 µgM) were used as positive controls [22–25]. Chromosomes were conventionally stained with Giemsa. In each slide, 100 metaphase spreads were analyzed in order to determine the chromosomal aberrations. Chromosome aberrations were classified to chromatid breaks and polyploidy. To determine the mitotic index (percentage of cells in mitosis), 1000 cells in each slide were observed.

2.6. Statistical analysis

All experiments (for determining either cytotoxicity or chromosomal aberrations) were performed in triplicate for each concentration. The level of the chromatid breaks, polyploidy, and mitotic index are presented as mean ± standard deviation (SD). The half maximal inhibitory concentration (IC_{50}) is a value indicating the concentration needed to inhibit cell proliferation by half. Comparisons of the mean values of the studied indices were done using one way analysis of variance (ANOVA). We used Duncan test as a Post hoc test. Statistical analysis was performed using SPSS statistical software package (version 11.5) for windows (SPSS Inc., Chicago, IL, USA). A probability of $P < 0.05$ was considered statistically significant. All *P*-values were two-tailed.

3. Results

We analyzed the effect of HDZ on proliferation of CHO cells using MTT assay (Fig. 1). The percent of cell growth inhibition was increased as a function of HDZ concentration

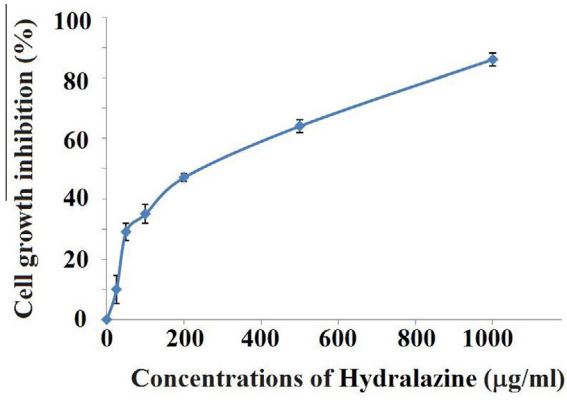

Figure 1 Effect of hydralazine on cell growth of Chinese hamster ovary (CHO) cell line for 24 h. The results are presented as percent of inhibition of cell growth obtained by MTT assay. Data are mean ± SD of triplicate experiments.

($P < 0.001$). The inhibitory concentration IC_{50} for HDZ was estimated to be 243.3 ± 16.9 μg/ml.

The effects of HDZ on chromosomal aberrations in treated cells are shown in Table 1. In the positive control cells treated with sodium arsenite and Mitomycin-C either chromatid breaks or polyploidy increased and mitotic index decreased compared with the untreated control cells ($P < 0.001$).

In the absence of S9 fraction, the level of chromatid breaks statistically increased ($F = 10.44$; df = 2, 6; $P = 0.011$) and mitotic index significantly decreased ($F = 68.58$; df = 2, 6; $P = < 0.001$) in CHO cells treated with HDZ. There was no significant difference between treated and untreated CHO cells with HDZ for level of polyploidy ($F = 0.05$; df = 2, 6; $P = 0.945$).

In the presence of S9 fraction compared to the absence of S9 fraction, the mitotic index elevated in treated CHO cells with HDZ (Table 1). However, there was a significant difference between control and treated cells ($F = 50.53$; df = −2, 6; $P < 0.001$). In the presence of S9 fraction, the level of chromatid breaks showed statistically significant differences between the experimental groups ($F = 23.87$; df = 2, 6; $P = 0.001$). For frequency of chromatid breaks, there was no significant difference between 20 μg/ml of HDZ (+S9) and untreated cells ($P > 0.05$). However, at the 40 μg/ml concentration of HDZ, there was a significant difference between treated and untreated cells ($P < 0.05$). It should be noted that there were significant differences between treated cells in the presence and absence of S9 fraction (Table 1; $P < 0.05$).

4. Discussion

In the present study we found that HDZ inhibits the cell growth of the CHO cells (Fig. 1). This finding is in good agreement with previous report of Song and Zhang where they used three cancer cell lines (Hela, CaSki, SiHa) and a normal cell line (ECV304) [16].

Our present findings demonstrated that HDZ (−S9 mix) induced the chromatid breaks. This finding also might be interpreted with the formation of free oxygen radicals by HDZ which was previously reported [6,7]. It should be mentioned that the present data are in agreement with previous studies reporting the mutagenic effect of HDZ in bacterial test systems [9–12].

Interestingly, the present results indicated that in cells treated with HDZ and presence of S9 mix compared with the cell treated with HDZ without S9 fraction, the level of chromatid breaks was reduced and level of mitotic index increased. This finding might be explained by: (1) The S9 mix fraction has many enzymes such as NAT (N-acetyl transferase). NAT may acetylate HDZ and subsequently form two metabolites (N-acetyl hydralazine and 3-methyl-triazolo-phthalazine). These products may be less toxic than the original drug and cause reduction in chromosome aberrations. (2) We know that the S9 mix also contains catalase (an important antioxidant enzyme). It has been shown that catalase detoxifies the active free radicals specially hydroxyl radicals [26]. These suggest that HDZ metabolites are less toxic than the original drug. It should be noted that same effect of S9 fraction was previously reported in relation to HDZ [27] and other drugs such as propranolol [24].

In conclusion, HDZ can cause chromatid breaks in CHO cells, and the metabolic activation system (S9 mix) plays an important role in the drug cytotoxicity reduction. Some studies have revealed plasma levels of HDZ vary among individuals [28]. It has been reported that HDZ is subject to polymorphic acetylation; slow acetylators generally have higher plasma level of HDZ and require lower doses to maintain control of blood pressure [29,30]. The U.S. Food and Drug Administration (FDA; an agency responsible for the control and safety of food and drugs) has established five categories (A–E) to indicate the potential of a drug to cause birth defects if used during pregnancy. The categories are determined by the reliability

Table 1 Induction of chromatid breaks, polyploidy and mitotic index in CHO cells by Hydralazine in the presence and absence of S9 mix.

Treatment	Aberrations per 100 metaphases		Mitotic index (%)
	Chromatid breaks	Polyploidy	
Hydralazine (μg/ml)			
0 – S9mix	4.7 ± 1.52[a]	8.0 ± 1.0[a]	7.7 ± 0.52[a]
20 – S9mix	16.3 ± 2.51[a]	7.6 ± 0.57[a]	3.9 ± 0.62[b]
40 – S9mix	35.0 ± 13.89[b]	8.3 ± 4.04[a]	2.06 ± 0.64[c]
Statistical analysis			
F (df = 2, 6)	10.44	0.05	68.58
P-value	0.011	0.945	<0.001
0 ± S9 mix	4.3 ± 0.57[a]	6.6 ± 2.51[a]	7.6 ± 0.32[a]
20 ± S9mix	4.6 ± 1.52[a]	5.3 ± 0.57[a]	5.7 ± 0.26[b]
40 ± S9mix	14.6 ± 3.21[b]	6.6 ± 1.52[a]	5.08 ± 0.38[b]
Statistical analysis			
F (df = 2, 6)	23.87	0.59	50.53
P-value	0.001	0.582	<0.001
Positive controls			
Mitomycin-C (0.06 μg/ml)	19.0 ± 1.0	20.0 ± 3.60	3.4 ± 0.40
Sodium arsenite (1 μM)	20.0 ± 2.64	12.3 ± 2.51	3.7 ± 0.62

Note: The results are average of three independent experiments.
F, df, *p* same alphabets means no statistically significant difference between groups ($P > 0.05$).

of documentation and the risk to benefit ratio. HDZ has been assigned to pregnancy category C by the FDA. The category C means that animal reproduction studies have shown an adverse effect on the fetus and there are no adequate and well-controlled studies in humans, but potential benefits may warrant use of the drug in pregnant women despite potential risks.

Finally it should be mentioned that HDZ must be used cautiously in general and particularly in children and pregnant women. HDZ should only be given during pregnancy when benefit outweighs risk. However, further experiments are necessary to clarify the significance of the present findings, particularly in humans with respect to genetic polymorphisms of the genes involved in metabolism of HDZ.

Disclosure statement

The authors declare no conflict of interest.

Acknowledgements

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