

Enalapril protect human lymphocytes from genotoxicity of Hydrochlorothiazide

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Abstract: Hydrochlorothiazide (HCTZ) belongs to the thiazide diuretics family that is used for the treatment of hypertension. Enalapril is another drug that is used for the treatment of hypertension. Recently, both drugs were combined in a single medication called vaseretic that showed a strong synergistic effect against hypertension. The aim of this investigation is to examine genotoxicity of HCTZ/enalapril on chromosomal damage by measuring the frequency of sister-chromatid exchanges (SCEs) in cultured human lymphocytes. Findings showed that HCTZ (5µg/mL) significantly increased SCEs frequency ($P<0.01$) in cultured cells relative to the untreated cells. The levels of SCEs induced by Enalapril (10µg/mL) was similar to the level detected in the untreated cultures ($P>0.05$). Interestingly, SCEs induced by combined treatment were significantly lower than HCTZ alone ($P<0.05$). Thus, enalapril seems to protect lymphocytes from genotoxicity induced by HCTZ. Neither HCTZ nor enalapril treatment (alone or in combination) induced changes in the mitotic index and the proliferative index ($P>0.05$). In conclusion, HCTZ increased SCEs in cultured lymphocytes, and this increase is reduced by enalapril.

Keywords: Enalapril, hydrochlorothiazide, DNA damage, sister chromatid exchanges, lymphocytes.

INTRODUCTION

Hypertension is a common condition worldwide with an estimate prevalence of about 30% among adults (Cifkova *et al.*, 2016). Uncontrolled hypertension contributes to the development of cardiovascular (such as heart attack and stroke) and renal diseases (Lackland and Weber, 2015; Textor, 2017). Hydrochlorothiazide (HCTZ) diuretic is considered among the most prescribed drugs in clinical practice and are mainstays in the therapy of hypertension (Cooney *et al.*, 2015). HCTZ decreases the extra cellular volume by interacting with a thiazide-sensitive Na-Cl co-transporter in the kidney (Mugellini and Nieswandt, 2012) and the subsequent interference with the reabsorption of Na in the kidney tubules. This promotes the excretion of Na-Cl and the amount of excreted water (Neutel and Smith, 2013). Hydrochlorothiazide induces several adverse effects like hypokalemic metabolic alkalosis and hyperuricemia, allergic reactions and hyponatremia, which is an important adverse effect of thiazide diuretics (Duarte and Cooper-DeHoff, 2010). At the cellular level, HCTZ is known to cause genotoxicity. For example, HCTZ has been shown to cause non-disjunction and crossing over in diploid strains of *Asperigillus nidulans* (Bignami *et al.*, 1974).

Enalapril, on the other hand, is an inhibitor of ACE (angiotensin converting enzyme) and used for oral management of hypertension (Wilde *et al.*, 1994). Among the side effects of enalapril include acute renal failure, elevation of potassium in the blood, cough and skin

swelling (Joshi *et al.*, 2010; Parish and Miller, 1992). Recently HCTZ and enalapril were combined in a single medication called vaseretic that showed a potent effect against hypertension (Guerrero *et al.*, 2008). In the current study, genotoxic effect of HCTZ/enalapril on human cultured lymphocytes using sister-chromatid exchange (SCE) was investigated. Studying the combined effects of drugs as they used in patients might be a better approach than investigating each drug alone.

MATERIALS AND METHODS

Five adult healthy males (age: 23-30 years) donated blood to be used in this study. Once obtained from subject, blood samples were placed in lithium heparinized tubes under aseptic conditions. All donors were non- alcoholic and non-smokers and were not taking drugs and vitamins in at least 3 months prior to the study (Khabour *et al.*, 2016). All donors gave written informed consent as indicated by the instructions of institutional ethics committee (IRB-JUST). Collected blood samples were immediately used (within 1-2 hours) of sampling.

Hydrochlorothiazide and enalapril, 5, Bromo-2,-Deoxyuridine, acetic acid, potassium chloride, buffers, Hoechst 32285 (bisbenzimidazole) dye and Giemsa powder were purchased from Sigma-Aldrich (St. Louis, MO, USA). Absolute methanol and acetic acid were obtained from Gainland Chemical Company (UK). Colcemid and PB max media (contains FBS, RPMI 1640, penicillin/streptomycin and phytohemagglutinin) were obtained from Gibco Invitrogen (UK).

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Lymphocyte cultures

Blood/lymphocyte cultures were started by adding one mL of whole blood into screw capped polypropylene tubes containing nine mL of PB-Max medium. Bromodeoxyuridine (10 µg/mL media) was added right after culture initiation. To minimize photolysis of BrdU, culture vials were covered with aluminum foil and incubated in a CO₂ incubator for 72 hours at 37°C (Azab *et al.*, 2016). HCTZ and Enalapril were prepared each time fresh in Dimethyl sulfoxide (stock solution: 5mg of /mL and 20 mg/mL respectively) and were in cultures at final concentrations of 5µg/mL and 10µg/mL respectively. All drugs were added in the last 20 hours of the 72 hours incubation period (Alzoubi *et al.*, 2012).

Cell harvesting and preparation of metaphase slides

Cell harvesting was started by adding colcemid (100 µL of 10µg /mL stock) to cultures in the last 2 hours of the 72 hours incubation period (Al-Sweedan *et al.*, 2012). Cultures were then centrifuged at 1000 xg for 5 minutes. Using a pre-warmed hypotonic solution (0.56% KCl), the pellet was resuspended and incubated at 37°C for 30 minutes. Using centrifugation at 1000 xg for 5 minutes, the swollen lymphocytes were obtained. The lymphocytes were then fixed (one part methanol: three parts acetic acid, prepared immediately before use and was added drop-by-drop). Fixed lymphocyte were kept for 30 minutes in the room (Alzoubi *et al.*, 2014). Cells were then centrifuged (1000 xg), and washed with the fixative (3 times). Finally, 2 mL of fixative were added to the tube for pellet suspension. To obtain metaphase spreads, the pellet was dropped on microscope slides, which was pre-chilled. The slides were then kept until dried at room temperature and then exposed to florescence/Giemsa procedure (see below) to assay SCEs (Azab *et al.*, 2009).

Florescence-plus giemsa staining

Using Hoechst dye solution (10 µg/mL), slides were stained for 20 minutes. Slides were, rinsed with distilled H₂O, mounted in appropriate buffer (McIlvian, pH 8.0) and exposed to 350 nm UV light for 35 minutes at 45°C. Slides were then rinsed with distilled H₂O and stained for about 8 minutes using Giemsa solution (5%, pH 7.4) as previously described (Khabour *et al.*, 2015).

Analysis of sister chromatid exchange

Stained slides were examined at 1000x using Nikon medical microscope (Japan) to M2 metaphase spreads for evaluation of sister chromatid exchanges. For each treatment, 250 M2 metaphases (42-46 chromosomes each) were examined representing all donors (50 from each) as previously described (Alzoubi *et al.*, 2013).

Cell kinetic assessment

The mitotic Index, which indicates the cytotoxicity of examined drugs was determined via analysis of at least one thousand cells per donor and counting metaphases as

described earlier (Khabour *et al.*, 2013). The formula used for calculating mitotic the index is as follows:

MI= (number of metaphases / total number of cells) * 100
For evaluation of proliferation index (PI), one hundred metaphases from every subject were examined. The proliferation index and average generation times were determined via counting the percentages of M1, M2 and ≥ M3 and using the equations described elsewhere (Mhaidat *et al.*, 2016).

STATISTICAL ANALYSIS

Statistics were done via version 5 of Prism statistical program (La Jolla, CA, USA). Multiple groups were compared using ANOVA and Tukey's post-hoc Test. When the P value was <0.05, the difference between groups was considered significant.

RESULTS

Sister-chromatid exchanges (SCEs) were scored after 20 hours of treatment of cultures with either HCTZ (5µg/mL) or enalapril (Enal, 10µg/mL) or a combination of both (fig. 1). Significant elevation in the frequency of SCEs was observed after treatment of cultures with HCTZ as analyzed using ANOVA and Tukey post hock test (P< 0.01, fig. 1). Enalapril, on the other hand, did not induce any increase in the level of SCEs (P>0.05, fig. 1). When both HCTZ and Enalapril were used, the increase in SCEs induced by HCTZ was significantly reduced (P<0.05, fig. 1). This indicates that Enalapril protected lymphocytes from the genotoxic effect of HCTZ.

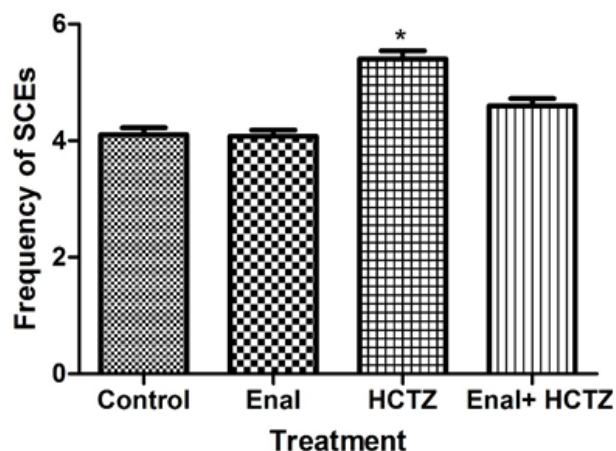


Fig. 1: The average of SCEs/cell in the different groups. Data are expressed as mean ± SEM. A total of 250 cells (50 per donor) were scored. Hydrochlorothiazide (HCTZ, 5µg/mL) induced significant increases in SCEs. Enalapril (10 µg) did not affect basal SCEs. SCEs in Enal+HCTZ group was significantly lower than that of HCTZ group and similar to that of observed in control and Enal groups. Thus, Enalapril significantly decreased the elevation induced by HCTZ. *indicates significant difference (P < 0.05) from all other groups.

To confirm the analysis performed using ANOVA described above, high frequency cells analysis was performed (table 1). In this analysis, M2 metaphase cells were further divided into three categories: M2 with ≤ 3 SCEs/cell, M2 with 4-7 SCEs/cell and M2 with ≥ 8 SCEs/cell as previously described (Bonassi *et al.*, 1999). The results showed that HCTZ increased the percentage of M2 with ≥ 8 SCEs/cell and addition of Enalapril significantly normalized to levels comparable to that showed in the control group (table 1). Thus, high frequency cells analysis confirmed ANOVA findings.

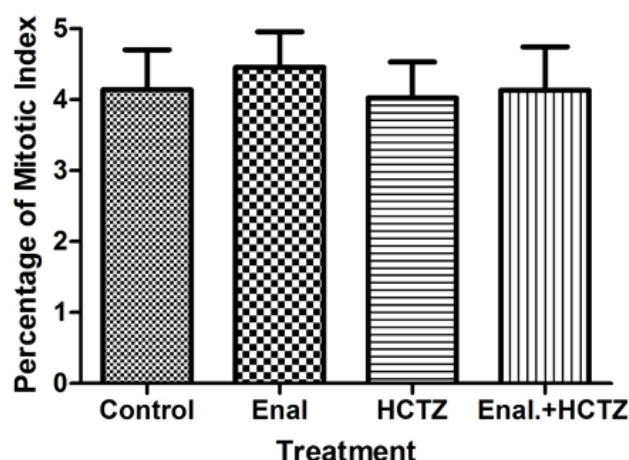


Fig. 2: Changes in the mitotic index in the different groups.

There is no statistical difference in the mitotic indices among all groups ($P > 0.05$). MI= number of metaphases / total number of cells*100. Data are expressed as mean $\pm$ SEM.

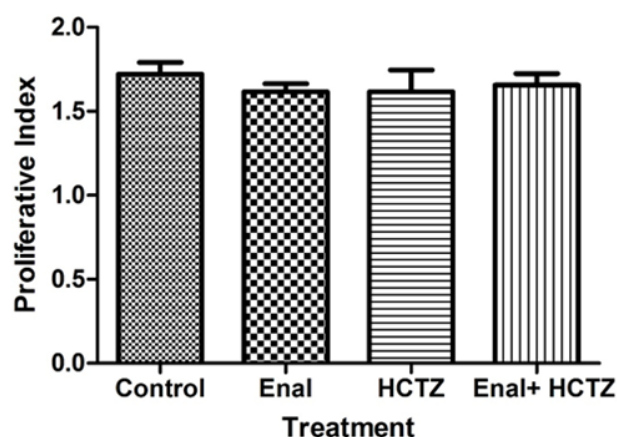


Fig. 3: Changes in the proliferative index in the different groups.

There is no statistical difference in the proliferative index among all groups ($P > 0.05$). Data are expressed as mean $\pm$ SEM.

The mitotic index was similar in control, HCTZ, Enalapril and combination of HCTZ/Enalapril ($P > 0.05$, fig. 2). In addition, there was no significant difference in the proliferative indices of all groups ($P > 0.05$, fig. 3).

DISCUSSION

In this study, we showed that HCTZ induced increases in the levels of SCEs of cultured human lymphocytes and enalapril reduced this increase to levels comparable to that observed in the controls.

Hydrochlorothiazide is a diuretic drug that has been approved for treatment of uncomplicated hypertension. Enalapril is an ACE inhibitor which also used for treatment of hypertension. Findings of the present study showed that HCTZ significantly increased the frequency of SCEs in cultured human lymphocytes. This is in accordance with previous study that showed significant elevations in the frequency of SCEs after treatment of Chinese hamster ovary (CHO) cells (National Toxicology, 1989). HCTZ has also been shown to increase chromosomal instability and to induce micronucleus (MN) formation (Andrianopoulos). Finally, HCTZ has been shown to induce increases in the chromosomal non-disjunction in *Aspergillus nidulans* (Bignami M. *et al.*, 1974). Thus, our result and those of others indicate that HCTZ is genotoxic.

The results showed no increases in the levels of SCEs after treatments of culture lymphocytes with enalapril. On the contrary, enalapril significantly reduced the increases in SCEs induced by HCTZ in cultured lymphocytes to levels comparable to that observed in the control group. A study by Ghorbanihaghjo *et al.*, showed that elevation in the oxidative DNA damage biomarker 8-hydroxy deoxyguanosine after renal transplantation is significantly lowered by Enalapril (Ghorbanihaghjo *et al.*, 2008). In rat model, enalapril has been shown to protect against age-related changes in rat liver mitochondrial DNA content and gene expression (de Cavanagh *et al.*, 2008). Similarly, enalapril has been shown to ameliorates the DNA damage in the heart, kidney, liver and germ cells of the streptozotocin-induced diabetic rat (Kushwaha and Jena, 2012; Kushwaha *et al.*, 2012). Enalapril also prevented the increase in DNA damage induced by nicotine in the body of streptozotocin-induced diabetic rat (Kushwaha and Jena, 2014). Moreover, Cisplatin induced DNA damage and nephrotoxicity was prevented by treatment of rats with enalapril (Rani *et al.*, 2016). Thus, enalapril seems to exert genoprotective effect against genotoxic agents including cisplatin, streptozotocin and HCTZ.

The mechanism by which enalapril exerts its genoprotective properties is required more investigation. One mechanism could be due to the anti-oxidant property of enalapril (Rani *et al.*, 2016). Enalapril has been shown to improve the balance between reactive oxygen intermediates and antioxidant enzymes in the kidney cortex of the rats exposed to cisplatin. Since SCEs can be induced by diseases and agents that cause oxidative stress,

Table 1: Distribution of high frequency cells among different groups

	≤ 3 Exchanges*	4-7 Exchanges*	≥ 8 Exchanges*
Control	45.6 ± 8.5	47.2 ± 5.4	7.2 ± 3.8
Enalapril	42.4 ± 3.5	53.6 ± 2.7	4.0 ± 1.1
HCTZ	23.6 ± 6.4 ^{\$}	58.8 ± 4.0	17.6 ± 4.8 [^]
HCTZ+ Enalapril	33.2 ± 5.4	57.6 ± 3.4	9.2 ± 2.4
ANOVA P value	< 0.01	0.1203	< 0.01

HCTZ: Hydrochlorothiazide, *Mean ± SEM, \$ indicates significant difference from other groups (P<0.01), ^ indicates significant difference from other groups (P<0.01)

it is possible enalapril protected human lymphocytes from genotoxicity of HCTZ via its anti-oxidant properties. In support of this, HCTZ has been shown to induce oxidative stress in the liver and brain of rats (Ribeiro *et al.*, 2013; Ribeiro *et al.*, 2009). Other mechanisms could involve enhancement of gene expression that involved in DNA damage repair. More studies are required to pin down the exact mechanism.

To evaluate the cytotoxicity of hydrochlorothiazide and enalapril, cell kinetics analysis was performed, using MI and PI. The results of this study showed absence of any cytotoxic effect of hydrochlorothiazide and enalapril at the examined concentrations. These results are consistent with previous studies that showed the absence of cytotoxicity of hydrochlorothiazide (Devlin *et al.*, 1995) and enalapril (Jurima-Romet and Huang, 1993).

It is worth to mention that recently both drugs are combined in a single medication for the treatment of hypertension. Based on the results of this study, we recommend the prescription of such combined medication because of its medical importance in protecting the body from oxidative stress and genotoxicity of hydrochlorothiazide.

Among the limitations of this study is that we did not perform dose response curve for the investigated drugs. Therefore, we recommended in future studies to do dose response curve in order to determine the optimum dose of enalapril that completely normalize the genotoxicity induced by hydrochlorothiazide.

CONCLUSION

HCTZ increased SCEs in cultured lymphocytes, and this increase is reduced by enalapril, which adds further justification for combining these two drugs together.

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