Vitamin B12 protects against DNA damage induced by hydrochlorothiazide

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ABSTRACT

DNA damage induced by hydrochlorothiazide was previously reported in cultured human lymphocytes. In this study, we aimed to investigate the harmful effects of hydrochlorothiazide on DNA by measuring the spontaneous frequency of sister chromatid exchanges (SCEs) in cultured human lymphocytes. We also aimed to investigate the possible protection of that damage by vitamin B12. The results showed that hydrochlorothiazide (5 μg/mL) significantly increased the frequency of sister chromatid exchanges (P < 0.001) in human lymphocytes in comparison with control. Additionally, the frequency of hydrochlorothiazide-induced SCEs was significantly decreased by co-treatment with vitamin B12 at concentration of 13.5 μg/mL (P < 0.001). In conclusion, hydrochlorothiazide is genotoxic to human lymphocytes and its toxicity is reduced by vitamin B12.

1. Introduction

Hydrochlorothiazide (HCT) is a famous first-line antihypertensive drug that belongs to the thiazide diuretics class. HCT is also used to reduce edema associated with heart failure or cirrhosis, nephrotic syndrome, pregnancy, and nephrogenic diabetes insipidus (Herman and Bhimji, 2017). The main site of action of HCT is the distal convoluted tubule (Kondrack and Mohiuddin, 2009). The mechanism of action of HCT involves reduction in cardiac output and peripheral resistance (Neutel and Smith, 2013).

Previous studies have reported a genotoxic effect for HCT. For example, a study that was conducted on cultured human lymphocytes of 32 healthy adults showed that HCT enhanced the frequency of micronucleus production via a mechanism that principally includes chromosomal delay, and chromosomal breakage to a lesser extent (Andrianopoulos et al., 2006). In addition, HCT enhanced UVA-induced DNA damage (Kunisada et al., 2013).

Vitamin B12 (Cobalamin) is a water-soluble vitamin that has a significant role in the normal function of the brain and nervous system and the biosynthesis of important blood components (Gille and Schmid, 2015). It is one of the famous eight B vitamins. It plays a major part in numerous biological processes, especially the ones affecting DNA synthesis and regulation. Vitamin B12 works as a methionine synthase coenzyme, where it is crucial for folate metabolism and nucleotides biosynthesis (Roth et al., 1996).

Various studies have shown that vitamin B12 plays a significant role in protecting and maintaining the stability of the human genome. As an example, the protective effects of B12 were tested against the DNA damage induced by ribavirin (Joksic et al., 2006), pioglitazone (Alzoubi et al., 2012), Paclitaxel (Alzoubi et al., 2014), thimerosal (Waly et al., 2016), and in hemodialysis patients (Stopper et al., 2008). All of these studies and many more have given positive promising results that support the growing role of vitamin B12 in preventing DNA damage. Therefore, the aim of the current investigation was to evaluate the effect of HCT on DNA by examining the SCEs frequency in human lymphocytes. In addition, the potential protective effect of cobalamin (vitamin B12) on the level of DNA damage after exposure to HCT was also investigated.

2. Materials and methods

2.1. Subjects

Samples of whole venous blood were obtained from 5 healthy male donors under aseptic conditions in heparinized tubes. The
age range for the donors was 21–30 years (mean age = 24.66 ± 2.13). The exclusion criteria for subjects were alcohol use, medications and supplement consumption in the past 3 months and cigarette smoking. The study protocol was in accordance with Declaration of Helsinki and all its amendments. Informed consent was obtained from each volunteer according to Institutional Review Board of Jordan University of Science and Technology.

2.2. Blood cultures

Lymphocyte cultures were set up by adding 1 mL of heparinized freshly withdrawn whole blood to 9 mL of PB max media (RPMI 1640 medium with 15% fetal bovine serum, 1% penicillin/streptomycin and 3% phytohemagglutinin). Cultures were incubated in the dark at 37 °C for 72 h in CO₂ incubator. The cultures were treated with 10 μL of 10 μg/mL colcemid (final concentration in culture media was 10 μg/mL) two hours before harvesting of cells, which works as spindle inhibitor increasing the mitotic index (Azab et al., 2017).

2.3. Treatment of blood cultures

HCT powder (5 mg) was weighted separately to be used freshly for blood cultures and was dissolved in 1 mL of Dimethylsulfoxide (DMSO), 10 μL of this solution was used to make a final concentration in culture media equal to 5 μg/mL (Andrianopoulos et al., 2006). Vitamin B12 powder (13.5 mg) was dissolved in distilled water (1 mL), 10 μL of this solution was used to make a final concentration in culture media equal to 13.5 μg/mL (Alzoubi et al., 2012; Joksic et al., 2006). Study experimental groups are shown in Table 1.

2.4. Harvesting and metaphases slides preparation

Treated blood cultures were centrifuged at 1000 × g for 5 min. Then, pellet was re-suspended in pre-warmed 0.56% KCl hypotonic solution and incubated for 30 min at 37 °C. The swollen lymphocytes were centrifuged at 1000 × g for 5 min, and fixed by drop-wise addition of freshly prepared Absolute methanol: acetic acid in ratio 3:1 (V:V), which works as a fixative, then incubated at room temperature for 30 min. After that, the cells were spun (1000 × g for 5 min), and washed with the same fixative for three times. Finally, a small amount of the fixative (around 2 mL) was used to re-suspend the cells. Using microscopic slides, which were pre-chilled and soaked with fixative, the cellular suspension was dropped at the after-mentioned slides to obtain metaphase spreads.

2.5. SCE assay

BrdU (30 μg/mL) was added to the culture media prior to incubation. All cultures were maintained in total darkness to minimize photolysis of BrdU at 37 °C for 72 h in CO₂ incubator (Khabour et al., 2013). The slides were dried using air, then, differentially stained using 10 μg/mL of Hoechst dye applied for 15 min. The slides were then rinsed with water and soaked in pH 8.0 McIlvian buffer. After that, the slides were irradiated using a 350 mm UV lamp and two 15 W tubes lamps at a distance of 7 cm for 35 min at 40 °C. Finally, the slides were rinsed with distilled water, re-stained for 6–8 min with 5% Giemsa stain and then air dried (Khabour et al., 2014; Mhaidat et al., 2016).

2.6. Analysis of sister chromatid exchange

Using light microscope (at high-resolution; final magnification: 1000× from Nikon, Japan), all slides were scored for SCE. A minimum of fifty-clearly differentiated metaphases containing 42–46 chromosomes were scored for every cell culture as previously described (Azab et al., 2015).

2.7. Mitotic index

The Mitotic Index (MI) reflects the degree of cytotoxicity of a certain drug (de Souza et al., 2016). It was calculated via examining a minimum of 1000 cells for every subject and metaphase cells scoring. MI values represent the percentage of metaphase cells appeared in each sample. It was calculated as, MI = number of metaphases/total number of cells × 100 (Alqudah et al., 2017).

2.8. Proliferative index

The Proliferative Index (PI) reflects the number of cells that are actively growing; i.e. a low proliferative index means a slower growth rate. For the cell proliferation index, at least 100 metaphase cells from each donor were scored. Pls were determined using the following formula:

\[
\text{PI} = \left[1 \times \text{M1}\% + 2 \times \text{M2}\% + 3 \times \text{M3}\%\right]/\text{total number of scored metaphases}
\]

2.9. Statistical analysis

Version 5 of Graphpad Prism Statistical Software (La Jolle, CA, USA) was used for data analysis. The comparisons of parameters were performed using one-way ANOVA test followed by Tukey's multiple comparison. Significant differences were at P < 0.05. Data were expressed as mean ± standard error (SE).

3. Results

There was significant differences in SCEs frequencies between the control group and the hydrochlorothiazide group (HCT versus Control: P = 0.00019, Fig. 1). Treatment of cultures with vitamin B12 significantly lowered level of SCEs induced by HCT (Vit. B12+HCT versus HCT: P = 0.0037). In addition, no significant difference was detected between control group and vitamin B12 group (P = 0.54). High frequency SCEs cell analysis was also performed. In this analysis, cells were divided into three categories: cells with <5 SCEs, cells with 5–7 SCEs and cells with >8 SCEs. The results showed that HCT caused an increase in the frequency of SCEs in cells by shifting the cells population from low frequency to high frequency (P = 0.027, Table 1). Treatment with vitamin B12 prevented this shift.

The mitotic index and proliferative index were measured to estimate cytotoxicity of HCT in cultured human lymphocytes. The MI was similar in control, Vit. B12, HCT and Vit. B12+HCT groups (P = 0.56, Fig. 2). Similarly, as shown in Fig. 3, there was no significant difference in the proliferative indices of cells in culture among all groups (P = 0.67). Thus, HCT was not cytotoxic to cultured human lymphocytes.

### Table 1

| Group       | NO # scored cells | Distribution of high frequency cells |
|-------------|-------------------|-------------------------------------|
|             |                   | <5       | 5–7     | >8  |
| Controls    | 250               | 51.6%    | 40%     | 8.4% |
| Vit. B12    | 250               | 52.8%    | 33.3%   | 13.3% |
| HCT         | 250               | 14.0%    | 44.4%   | 41.6% |
| Vit. B12+HCT| 250              | 32.4%    | 45.6%   | 22.0% |
| P-value     | <0.01             | <0.01    | <0.01   |
4. Discussion

In the current study, the possible protection for vitamin B12 on DNA damage induced by HCT was examined in human blood lymphocytes via SCEs assay. SCEs assay is a sensitive indicator of DNA damage. The result showed significant induction of SCEs by HCT and the increase was diminished by co-treatment of cultures with vitamin B12.

The HCT is the most widely used thiazide-type diuretic (Herman and Bhimji, 2017). The results showed that HCT (5 μg/mL) is genotoxic as measured using SCEs assay. This is consistent with a previous study by Andrianopoulos et al. (2006), who indicated that HCT is able to induce DNA damage in a dose-dependent manner in cultured human lymphocytes. The genotoxicity of hydrochlorothiazide was evaluated in their study using cultured human lymphocytes at (5 and 40 μg/mL) concentrations via micronucleus assay FISH analysis. This genotoxicity was attributed mainly to chromosome delay (aneugenicity). In addition, genotoxicity of HCT was previously reported in vitro in CHO cells using SCE assay and mouse lymphoma cell assay with concentrations of HCT starting from 43 and up to 1300 μg/mL (Brambilla and Martelli, 1990). HCT might increase SCEs via induction of oxidative stress (da Silva, 2016). The evidence in literature regarding the relationship between HCT and oxidative DNA damage is variable and sometimes contradicting. HCT along with high-fat diets was found to increase the oxidative stress in the liver (Ribeiro et al., 2013) and the brain of rats (Ribeiro et al., 2009) in two separate studies. The levels of some enzymatic and non-enzymatic antioxidants were decreased in rats fed a high fructose diet (HFD) with or without supplementation of HCT (Ribeiro et al., 2013). In addition, increased cortical oxidative stress after HCT treatment in human was seen in another study (Reungjui et al., 2007). The evidence in these studies suggests that oxidative DNA damage can be a possible mechanism explaining the genotoxicity of HCT observed in the study.

Vitamin B12 plays a major role in numerous biological processes, especially the ones affecting DNA synthesis and regulation (Lawrence and Roth, 1996). The results of our study revealed that vitamin B12 has significantly reduced HCT-induced SCEs, indicating the ability of vitamin B12 to reduce DNA damage induced by HCT. This finding is consistent with the results of other studies that have shown that vitamin B12 plays a significant role in protecting and maintaining the stability of the human genome against mutagenicity induction by some genotoxic drugs such as ribavirin (Joksic et al., 2006), pioglitazone (Alzoubi et al., 2012), Paclitaxel (Alzoubi et al., 2014), thimerosal (Waly et al., 2016), and in hemodialysis patients (Stopper et al., 2008). The evidence in our study illustrate that vitamin B12 is important determinant of DNA damage. Vitamin B12 ability to regulate inflammatory cytokines suggests that it may have antioxidative properties (Birch et al., 2009), which can be a possible explanation for vitamin B12 protection against HCT mediated DNA damage. Cobalamins, including methylcobalamin were found to be effective antioxidants in vitro (Waly et al., 2016).

To evaluate the cytotoxicity of HCT and vitamin B12, cell kinetics analysis was done, which includes MI and PI. Our results showed that the difference in the values of MI and PI were insignificant among all groups, which indicates that HCT did not have a significant cytotoxic effect on these parameters at the used concentration with 20 h of exposure. The plasma elimination half-life of HCT in humans ranges between 3.2 h and 13.1 h (Sica et al., 2011), in patients with decreased renal function, the half-life of elimination is around 11.5 h in case of mild renal impairment; (endogenous creatinine clearance between 30 and 90 mL/min), and around 20.7 h in case of endogenous creatinine clearance below 30 mL/min (Niemeyer et al., 1983), which makes the latter case the closest to the time of exposure in our experiment. Additionally, cell kinetics for vitamin B12 showed that MI and PI in blood cells derived after vitamin B12 treatment were similar to control, confirming that vitamin B12 did not have any cytotoxic effect at the used concentration (13.5 μg/mL). This finding is consistent with the findings of Alzoubi et al. (2012) who used the same concentration.
Among the limitations of the study is that only one concentration of HCT was used in cultured human lymphocytes. While this concentration was used previously to examine genotoxicity of HCT (Andrianopoulos et al., 2006), it is considered low relative to other ones (Brambilla and Martelli, 2007; Bucher et al., 1990). In addition, the used B12 concentration is higher than that used orally by humans. Thus, more studies are needed to confirm the present finding including the use of more concentrations and the inclusion of different cell systems/genotoxicity assays.

Results of this study could have some applications; HCT is a widely used drug for a large population of patients, current results could implicate the possibility of co-administration of vitamin B12 supplements along with HCT. The study findings, on top of that, support a promising role of vitamin B12 in DNA protection against genotoxins.

5. Conclusions

Results of this study have shown a genotoxic effect of HCT at 5 μg/mL and a protective effect for vitamin B12 (13.5 μg/mL) against this genotoxicity. However, what is less clear is if HCT can induce genotoxicity at lower doses, indicating that dose titration is needed for a future study. Finally, the use of different forms and concentrations of cobalamin might be needed.

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