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The Effect of High Dose Methotrexate on Thiol/Disulfide Homeostasis in Pediatric Cancer Patients

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Abstract

Objectives: Current literature shows that there is a relationship between oxidative stress and Methotrexate (MTX). However, there are no clinical studies evaluating oxidant status in children treated with High Dose MTX (HDMTX) in the literature. Therefore, we aimed to evaluate the effects of HDMTX on thiol/disulfide balance in pediatric cancer patients.

Material and Methods: The study sample consisted of 19 patients with leukemia and lymphoma who had received 5 g/m^2 doses of MTX. All blood samples for hemogram, serum biochemistry, and thiol and disulphide analysis were collected at 0 and 48 h after starting the MTX infusion.

Results: Native thiol was 320.21 μmol/L (280.36 to 392.38) before treatment and 409.78 μmol/L (335.13 to 451.67) after treatment. The median post-treatment levels were found to be significantly higher than the median pre-treatment levels of native thiol (409.78 μmol/L and 320.21 μmol/L, respectively) and total thiol (441.61 μmol/L and 377.46 μmol/L, respectively) ($p<0.05$). A positive correlation was found between leukocyte, neutrophil, albumin, total thiol and native thiol levels before and after treatment.

Conclusion: This is the first study that highlights an association between thiol/disulphide homeostasis and HDMTX treatment. We think that these findings enhance our understanding about the relationship between oxidative stress and MTX.

Keywords: Children; Dynamic thiol/disulphide homeostasis; High dose methotrexate; Pediatric cancer; Leukemia; Lymphoma

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Introduction

Methotrexate (MTX) is a commonly used chemotherapeutic agent widely used in the treatment of some types of pediatric cancers, such as leukemia, lymphomas, osteosarcoma. It is a folate antagonist and can suppress the synthesis of DNA in the S-phase of the cell cycle, affects essentially the tissues that are growing most rapidly [1]. In addition, there are also numerous non-Dihydrofolate Reductase (DHFR) dependent mechanisms of MTX, such as anti-inflammatory effect, oxidative stress, cell differentiation, protein and DNA demethylation, protein acetylation and effects on protein kinases and other signaling proteins [2-5]. Nowadays, increasing evidence shows that there is a relationship between oxidative stress and efficacy of MTX *in vitro*; therefore, we evaluated redox status in pediatric leukemia and lymphoma patients having High-Dose MTX (HDMTX).

Thiols are essential and potent antioxidant molecules containing sulfhydryl group. Thiols are not only the redox tampon in the biologic systems but also a fundamental element of antioxidant defence system. Thiol structure is most commonly observed in plasma and other proteins. However, fewer amounts of thiol groups can also be seen in molecules containing cysteine, such as N-acetylcysteine, cysteinyl-glycine, glutathione, homocysteine, and γ-glutamylcysteine. In presence of oxidative stress, thiols react with oxidant molecules, and mediate the formation of reversible disulfide bonds. In this way, dynamic thiol/disulphide homeostasis is maintained. In physiological conditions, thiol/disulphide equilibrium is under balanced and protects protein structures [6]. There is also growing evidence demonstrating that an abnormal thiol/ disulphide homeostasis state is associated with the pathogenesis of different spectrum of disease [7-13]. One side of this double-sided balance has been measurable since 1979 [14]. But today a new automated method, developed

by Erel and Neselioglu, can measure two-sided of the dynamic thiol-disulphide balance [6]. There are no other reports that evaluate the relationship between dynamic thiol/disulphide balance and HDMTX by using a novel method which was described Erel and Neselioglu. Therefore, in this study, we evaluated the effects of HDMTX on thiol/disulphide homeostasis in pediatric cancer patients.

Population and Methods

This study was conducted between June 2018 and February 2019 at Pediatric Oncology Clinics of Dr. Sami Ulus Obstetrics and Pediatrics Training and Research Hospital and Ankara Yıldırım Beyazıt University. The study with a prospective design was carried out in 19 patients with ALL and NHL. The study was approved by the Ethics Committee of the Ankara Yıldırım Beyazıt University Faculty of Medicine (Report number: 2018/51).

Patients

The study sample consisted of 19 patients with ALL and NHL who had received 5 g/m^2 doses of MTX. Of our 19 patients, 14 were ALL and 5 were with NHL. The study population was composed of 13 females and 6 boys, and the median age was 61 months (ranged between 21 to 207 months). The clinical and laboratory findings of the patients are shown in detail in Table 1. All blood samples for complete blood count, urea, creatinine, albumin, protein, Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT), total bilirubin, thiol and disulphide analysis were collected at 0 and 48 h after starting the HDMTX infusion. Blood samples were centrifuged at 1500 g for 10 min without waiting. Serum samples were separated and kept at -80^{o}C until they were analyzed. Thiol/disulphide homeostasis tests were measured using a novel automatic and spectrophotometric method described by Erel and Neselioglu. Patients were assessed according to standard World Health Organization criteria for toxicity. Toxicity was evaluated on days 0, 2, 3 and 7 after the start of the HDMTX infusion on the grounds of mucositis, liver and kidney dysfunction, and hematologic status by oral inspection and determination of AST, ALT, total bilirubin, urea, creatinine, albumin levels and hemoglobin, platelet, leukocyte and neutrophil counts.

HDMTX administration

The first dose (1/10 of the full dose) was given Intravenous (IV) infusion over1 h. The remainder of the full dose was IV infused during the following 23 h. Prehydration was employed before HDMTX administration with alkalinization and lasted for 3 days. The standard Ca-folinate rescue was 15 mg/m^2 IV every 6 h at 36, 42, 48 and 54 h after starting the HDMTX infusion. The 0.9% saline mouths wash was administered to all patients three times daily during each HDMTX course.

Statistics

Data analysis was performed using the Statistical Package for Social Sciences 21.0 (SPSS Inc. Chicago, IL, USA). Conformity of the data to a normal distribution was evaluated using the Kolmogorov-Smirnov test. The Mann-Whitney and Wilcoxon rank sum test was used to determine statistical significance. The parametric values were presented as Mean ± Standard Deviation (SD), and non-parametric values were presented as median (interquartile range). Wilcoxon test was used to compare changes in pre-treatment and post-treatment blood count parameters. The correlation was evaluated using Spearman's correlation test. P values <0.05 were considered statistically significant.

Table 1: Clinical and pre-treatment laboratory findings of patients.

	Patients (n:19)
Male/Female	13/13
*Age (months)	61 (21-207)
*Relative weight (%)	100 (76-150)
Diagnosis	
Acute lymphoblastic leukemia	14
Non-Hodgkin Lymphoma	5
*White blood cell count (/mm^3)	5900 (1970-27540)
*Absolute neutrophile count (/mm^3)	3210 (860-23990)
*Hemoglobin value (g/dl)	11.3 (7.9-13.3)
*Thrombocyte count (/mm^3)	370000 (211000-967000)
*Urea nitrogen (mg/dL)	8 (1-54)
*Creatinine (mg/dL)	0.41 (0-0.71)
*Albumin (g/dL)	4.0 (2.8-4.6)
*Total Bilirubin (mg/dL)	0.4 (0.1-2.4)
*AST (U/L)	26 (14-71)
*ALT (U/L)	25 (9-138)

*Data are expressed as median with ranges

Results

The clinical features and demographics of these patients are summarized in Table 1. No difference was detected between pre-treatment and post-treatment groups in terms of hemoglobin, platelet, leukocyte, neutrophil, urea, creatinine, albumin, protein, AST, and ALT values ($p>0.05$). There was a statistically significant increase in total bilirubin level after treatment ($p<0.05$). When the median pre-treatment and post-treatment native thiol and total thiol levels were compared, the post-treatment levels were found to be significantly higher than the pre-treatment levels ($p<0.05$). The median level of native thiol was 320.21 μmol/L (280.36 to 392.38) before treatment and 409.78 μmol/L (335.13 to 451.67) after treatment. Total thiol was 377.46 μmol/L (329.27 to 451.76) before treatment and 441.61 μmol/L (394.68 to 509.55) after treatment (Figure 1 and 2). When pre-treatment and post-treatment disulphide and percent thiol levels (disulfide/native thiol, disulfide/total thiol, and native thiol/total thiol) were compared, there was no statistically significant difference (Table 2). A positive correlation was found between leukocyte, neutrophil, albumin, total thiol and native thiol levels before and after

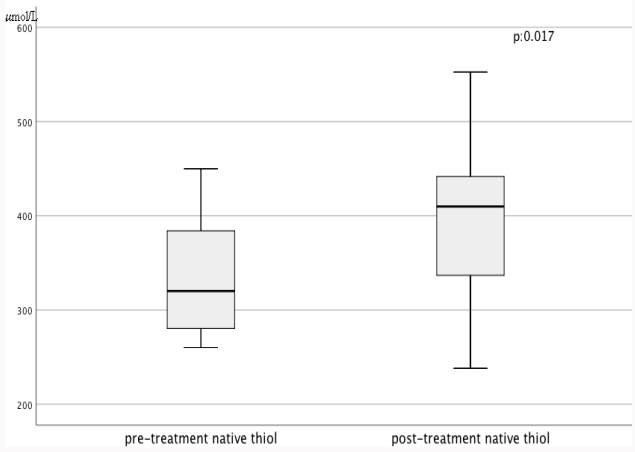

Figure 1: Pre-treatment and post-treatment native thiol levels.

Table 2: Comparison of thiol/disulphide homeostatic parameters between pre-treatment and post-treatment periods.

	Pre-treatment Median (Q1-Q3)	Post-treatment Median (Q1-Q3)	p
Native thiol (μmol/L)	320.21 (280.36-392.38)	409.78 (335.13-451.67)	0.017
Total thiol (μmol/L)	377.46 (329.27-451.76)	441.61 (394.68-509.55)	0.006
Disülfide (μmol/L)	32.07 (22.13-45.03)	28.63 (18.40-35.96)	>0.05
Albumin	4.14 (3.77-4.51)	4.15 (3.80-4.63)	>0.05
Disulphide/native thiol ratio (%)	9.56 (7.52-12.83)	6.89 (4.40-9.72)	>0.05
Disulphide/total thiol ratio (%)	8.02 (6.53-10.20)	6.05 (4.04-8.14)	>0.05
Native thiol/total thiol ratio (%)	83.94 (79.58-86.92)	87.88 (83.71-91.90)	>0.05

Table 3: Correlations between thiol parameters and other parameters.

Pre-treatment	**Native thiol**		**Total thiol**		**Disulphide**	
	r	**p**	**r**	**p**	**r**	**p**
White blood cell count	0.358	0.145	0.612**	0.007	0.498*	0.035
Absolute neutrophile count	0.315	0.203	0.579*	0.012	0.505*	0.033
Albumin	0.624**	0.006	0.525*	0.025	0.172	0.494
Post-treatment	**Native thiol**		**Total thiol**		**Disulphide**	
	r	**p**	**r**	**p**	**r**	**p**
White blood cell count	-0.002	0.993	0.108	0.68	0.494*	0.044
Absolute neutrophile count	-0.022	0.933	0.101	0.699	0.471	0.56
Albumin	0.139	0.596	-0.222	0.391	0.583*	0.014

*p<0.05 was considered significant for statistical analyses (Spearman's correlation test) **p<0.01

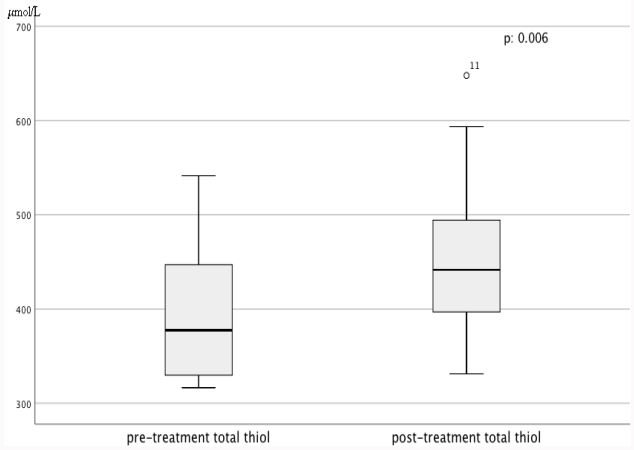

Figure 2: Pre-treatment and post-treatment total thiol levels.

treatment (Table 3). There was no correlation between age, gender, relative weight, hemoglobin, platelet, urea, creatinine, AST, ALT, bilirubin levels and thiol parameters before and after treatment. The toxicities were as follows: Hepatotoxicity in 5 patients (4 grade I and 1 grade II), mucositis in 2 patients (grade II), myelosuppression in 3 patients (1 grade IV and 2 grade II), and dermatitis in 1 patient. None of the patients developed nephrotoxicity. There were no deaths. There was no statistically significant difference in native thiol, total thiol and disulfide levels between patients with and without toxicity.

Discussion

Dynamic thiol/disulphide homeostasis takes part a critical role cell signaling mechanisms, intracellular enzymatic activity, antioxidant protection and apoptosis. Furthermore, abnormalities in dynamic thiol/disulphide homeostasis have been reported in numerous diseases [7-13]. This study is one of the first studies to investigate thiol and dynamic thiol/disulphide homeostasis changes after HDMTX treatment in the pediatric cancer patients. In our results, there were significantly higher post-treatment levels of native thiol, and total thiol compared to pre-treatment levels. This result supports the reduction of oxidant stress with HDMTX treatment. The best known cytotoxic mechanism of MTX is based on inhibition of DHFR. New studies have demonstrate that MTX is also able to target other metabolic pathways that are independent of DHFR inhibition, including the oxidative stress, cell differentiation, anti-inflammatory effect, demethylation, protein acetylation and effects on protein kinases and other signaling proteins [2-5,15,16]. The various effects of MTX are obviously concentration-dependent and some of the non-DHFR mediated effects of MTX can be relevant *in vivo* only when patients are treated with HDMTX [17-22]. Nowadays, a previously undescribed mechanism of MTX was reported. In the study by Zimmerman and et al. [15] it was indicated that MTX directly scavenges free radicals, specifically superoxide, inhibits the formation of Malondialdehyde-Acetaldehyde (MAA) adducts and decreases intracellular oxidative stress. Therefore, it is likely that the capability of MTX to scavenge frees radicals and reduces the activation of redox-sensitive intracellular signaling pathways and decreases oxidative stress. Interestingly, MTX has a structure similar to polyphenols. Polyphenols are a large family of compounds characterized by more than one phenol ring or hydroxylated benzene. Many polyphenols have been present to have antioxidant features [23]. There are parallel studies in the literature demonstrating the effect of low dose MTX on antioxidant system [24,25]. However, clinical studies evaluating oxidant status in children treated with HDMTX are rare. We think that HDMTX may have a positive effect on the oxidative system in pediatric cancer patients, because in our study, post-treatment thiol levels were significantly higher than pre-treatment levels. In contrast to our data, increased cellular oxidative stress during cancer treatment has been previously shown in pediatric patients [26,27]. Papageorgiou et al. [28] showed total antioxidant status of plasma

from children with different cancers was decreased during treatment compared to capability at diagnosis and after treatment. However, this study used total antioxidant capacity, a combination of endogenous and dietary anti-oxidants, as opposed to direct measures of oxidative stress. Raber and et al. [29] reported that chemotherapeutics increase direct measures of oxidative stress in pediatric acute leukemia patients. But, they could not determine if all chemotherapeutic drugs (vincristine, methotrexate, and dexamethasone) affect oxidative stress measurements or only certain medications. We think that our study is valuable since the plasma thiols are the biggest antioxidant components of serum. This is the first study that highlights an association between thiol/disulphide homeostasis and HDMTX treatment. We think that these findings enhance our understanding about the relationship between oxidative stress and MTX. Therefore, in the light of this new knowledge, a prospective study must be performed. There was a positive correlation between white blood cell, total neutrophil, and albumin and thiol levels. This result reveals the importance of antioxidant supplements for body defense. On the other hand, there was no statistically significant relationship between the development of toxicity and thiol/disulfide balance. There were some limitations in this study. First of all the sample size was small. This study should be interpreted as a preliminary study and its findings should be interpreted with caution. Further and more comprehensive studies are required on this matter. Second, our results were not compared with other oxidative stress indicators such as total antioxidant status, total oxidant status and oxidative stress index. Moreover, multiple factors may influence to the effects of MTX such as genetic polymorphisms, glutathione levels and specific dynamic processes. Nevertheless, the novelty of our study is that it is the first study to investigate the thiol/disulphide homeostasis in pediatric cancer patients treated with HDMTX.

Conclusion

Our study indicates that HDMTX may influence the thiol/disulphide homeostasis. HDMTX has antioxidant effects and decreases oxidative stress, which can be demonstrated through dynamic thiol/disulphide homeostasis. The newly spectrophotometric method used in this study helped providing more accurate and precise measurements of oxidative status. Identification of these new targets for MTX is especially important for a better understanding of MTX action in new protocols of combination therapy.

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