Investigation of Dynamic Thiol/Disulfide Homeostasis and Nitrosative Stress in Patients with Wilson Disease

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Cite this article as: Yücel EM, Konduk BT, Saracaloglu A, *et al.* Investigation of dynamic thiol/disulfide homeostasis and nitrosative stress in patients with wilson disease. *Turk J Gastroenterol.* 2021; 32(9): 765-773.

ABSTRACT

Background: *Wilson disease (WD) is an autosomal recessive inherited disorder of copper (Cu^{2+}) metabolism, resulting in Cu^{2+} accumulation and liver and central nervous system toxicity. Oxidative stress may have a role in the pathogenesis of Wilson disease, but the roles of thiol/disulfide homeostasis and nitrosative stress have not been examined. The purpose of this study was to evaluate whether there is a modification in thiol/disulfide homeostasis and nitrosative stress in patients with Wilson disease.*

Methods: *A total of 50 patients with Wilson disease (42 under drug treatment and 8 newly diagnosed patients with no drug treatment) and 50 healthy gender- and age-matched controls were enrolled for this study. Serum native thiol and total thiol levels were measured with a spectrophotometric method. The number of disulfide bonds and the related ratios were determined from these measurements. Serum nitric oxide (NO) and 3-nitrotyrosine (3-NT) levels were analyzed using chemiluminescence and ELISA assays, respectively.*

Results: *The average native thiol levels of the patient group under drug treatment were found to be markedly higher than the levels of controls ($P < .05$). We detected no marked changes in total thiol and disulfide levels, and disulfide/total thiol, disulfide/native thiol, or native thiol/total thiol ratios between groups. We found significant elevations in NO levels in Wilson disease group before drug treatment, and the 3-NT levels in the Wilson disease groups prior to ($P < .05$) and under drug treatment ($P < .01$), when compared to controls.*

Conclusion: *Our data are the first to show that nitrosative stress and thiol/disulfide homeostasis can contribute to the pathogenesis of Wilson disease.*

Keywords: *Disulfide, nitric oxide, nitrotyrosine, thiol, Wilson disease*

INTRODUCTION

Wilson disease (WD) is a rare autosomal recessively inherited metabolic disorder of impaired copper (Cu^{2+}) transport caused by mutations of the *ATP7B* gene on chromosome 13q14.3.[1] A loss of function of this Cu^{2+} transporter (P-type ATPase) leads to a chronic Cu^{2+} accumulation in the liver and subsequently in the brain.[2,3] The disease has a worldwide frequency of 1/30 000-1/50 000 live births, but in some population-based studies, the genetic prevalence is 3-4 times higher than clinically based estimates.[4] Wilson disease can be successfully managed if diagnosed early and correctly treated; however, if untreated, WD is progressive and fatal.[2,4] Currently, the effective treatment for WD is life-long pharmacologic therapy. This can be achieved either by blocking intestinal uptake of Cu^{2+} with zinc or Cu^{2+} chelation with D-penicillamine and trientine.[3] Non-adherence to therapy can lead to worsening neuropsychiatric symptoms and irreversible hepatic injury.[2,3]

The increase in reactive nitrogen species is termed nitrosative stress. Nitrosative stress usually follows the production of nitric oxide (NO), which is formed by the decomposition of arginine catalyzed by the enzyme nitric oxide synthase (NOS) (Figure 1).[5] Reactive nitrogen species are formed when there is an abnormal increase in the level of NO produced by the inducible NOS (iNOS) and/or by the uncoupled endothelial NOS (eNOS). NO, a free radical, reacts with superoxide anion by radical–radical recombination and forms peroxynitrite ($ONOO^-$). At physiological pH, the protonation of peroxynitrite forms peroxynitrous acid (ONOOH), which decomposes into nitrogen dioxide (NO_2) and hydroxyl (OH) free radicals. Physiologically,

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Received: ***July 8, 2020*** *Accepted:* ***February 12, 2021*** *Available Online Date:* ***September 20, 2021***

DOI: 10.5152/tjg.2021.20549

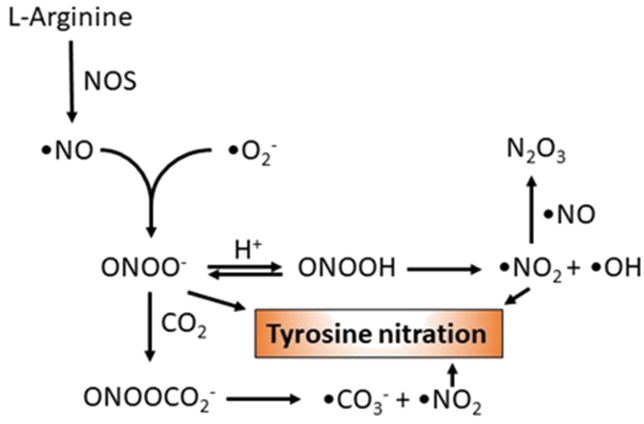

Figure 1. The synthesis of nitric oxide and some important nitrosative species. Tyrosine nitration, that is, 3-nitrotyrosine, is typically employed as a biomarker of peroxynitrite formation. NOS, nitric oxide synthase; NO, nitric oxide; O_2^-, superoxide radical; $ONOO^-$, peroxynitrite; ONOOH, peroxynitrous acid; NO_2, nitrogen dioxide; OH, hydroxyl radical; $ONOOCO_2^-$, nitrosoperoxocarbonate; CO_3^-, carbonate radical; N_2O_3, dinitrogen trioxide.

peroxynitrite also reacts with carbon dioxide (CO_2) to form nitrosoperoxocarbonate ($ONOOCO_2^-$), which can decompose into carbonate (CO_3^-) and nitrogen dioxide radicals (NO_2) (Figure 1).[5,6] Each of these radicals can induce lipid peroxidative cellular damage.[6] Recent studies identified mitochondria as a potential early target of Cu^{2+} toxicity.[7] NO alone causes only transient inhibition of mitochondrial respiration without mitochondrial uncoupling. Peroxynitrite can also be generated by increased leakage of complex I superoxide together with formation of NO by mitochondrial NO synthase (mtNOS) activation,[8] and cause mitochondrial permeability transition.[6] There is evidence that Cu^{2+}-induced oxidative stress has been shown to initiate mitochondrial permeability transition in cultured astrocytes.[7] Peroxynitrite nitrates tyrosine residues of cell proteins to form the stable product 3-nitrotyrosine (3-NT), which is accepted to be a very reliable footprint of peroxynitrite formation.[5,6]

MAIN POINTS

- *Determination of high native thiol levels in the patients suggests an involvement of dynamic thiol/disulfide homeostasis in Wilson disease (WD).*
- *Marked augmentation in 3-nitrotyrosine (3-NT) levels indicates the involvement of peroxynitrite formation in WD.*
- *Significant elevations in nitric oxide (NO) and 3-NT levels showed that nitrosative stress also contributes to the pathogenesis of WD.*

Free Cu^{2+} is normally incorporated in ceruloplasmin, cytochrome oxidase, superoxide dismutase, and metallothionein. Excess free Cu^{2+} is generally neutralized by glutathione (GSH) and amino acids. Excess free Cu^{2+} is toxic, and may cause inflammation and cell injury via various mechanisms including oxidative stress, mitochondrial toxicity, ER stress, cell membrane damage, inhibition of enzymes, and crosslinking of DNA.[9,10] Cu^{2+}-induced free-radical generation, increased lipid peroxidation marker (malondialdehyde, MDA) levels, and subsequent oxidative injury of hepatocytes' mitochondria are all involved in WD pathogenesis.[11] Indeed, depressed levels of different antioxidants and elevated oxidative stress have been described in patients with the hepatic presentation of WD.[12-14] The central nervous system is more vulnerable to nitrosative and oxidative stress, resulting in deterioration of motor and cognitive functions. To the best of our knowledge, there is no study reporting the contribution of dynamic thiol/disulfide homeostasis and nitrosative stress in patients with WD. Therefore, the goals of this study were to elucidate the possible contributions of thiol/disulfide homeostasis and nitrosative stress to the pathophysiology of WD.

MATERIALS AND METHODS

Study Populations

A total of 50 consecutive patients with WD under drug treatment [n = 42, age median (range) = 27 (16-66) years old] or newly diagnosed WD patients [n = 8, age median (range) = 25 (17-50) years old] who were admitted to the Gastroenterology Department of the University Hospital between April 2019 and April 2020 were enrolled prospectively to this study. The control group consisted of 50 age- and gender-matched healthy volunteers who did not have a family history of WD or a diagnosis of genetic, neurologic, liver, psychiatric, infectious, or chronic inflammatory disease. The healthy control individuals were selected from hospital staff and their families. The diagnosis of WD was based on laboratory findings and clinical signs. All patients met the diagnostic criteria of WD, were symptomatic, and were classified as having either the neurological or the hepatic form of WD, which was determined using widely accepted criteria.[15,16] Briefly, the neuropsychiatric form was defined by the presence of neurological and/or psychiatric symptoms at the time of diagnosis. The hepatic form was determined as the absence of any neurological symptoms and the presence of liver disease upon a detailed neurological examination at the time of diagnosis. All WD patients with the neuropsychiatric

manifestation had undergone neurological and cranial magnetic resonance imaging (MRI) examinations. Cranial MRI was done using the 1.5-TMR GE scanner (Philips, Gyroscan Intera, Best, The Netherlands), and T1, T2, and FLAIR images were obtained. The abnormal signal intensities and their locations were identified. All subjects underwent a slit lamp examination for the presence of Kayser–Fleischer rings. Patients with a history of genetic or metabolic disease, congenital anomalies, infection, intake of drugs including vitamins, and smoking were excluded from the study. Our study was approved by the Institutional Ethics Committee (Reference No. 2019/121), and it was performed in accordance with the guidelines stipulated in the Declaration of Helsinki. Written informed consent was obtained from adult patients and volunteers. For children, consent was obtained from their parents.

Our WD patients received 3 different Cu^{2+}-reducing therapies: D-penicillamine, zinc, and trientine. Patients were treated with D-penicillamine at 250 mg daily, which was elevated every 2 weeks by 250 mg, up to 1000 mg daily. Zinc was prescribed in the form of zinc sulfate 50 mg, 3 times a day (150 mg/day). Trientine was administered as 250 mg 3 times a day (750 mg/day).

Blood Samples

Venous blood samples were obtained from patients and controls after overnight fasting. Laboratory biochemical analyses were carried out within 30 minutes after blood collection. For dynamic thiol/disulfide, NO, and 3-NT analyses, venous blood samples were withdrawn into tubes containing EDTA, stored for 20 minutes for clotting, and the sera were separated by cold (at 4°C) centrifugation at 1500 *g* for 10 minutes, transferred into plain tubes, and then stored at –80°C until analysis. The samples were assayed immediately after thawing to avoid decline in enzyme activity.

Thiol/Disulfide Detection

Commercially available kits (Rel Assay Diagnostics, Mega Tip Ltd, Gaziantep, Turkey) were used for analyzing the serum native thiol (–SH) and total thiol (–SH + –S–S–) levels. Erel and Neselioglu[17] developed these spectrophotometric methods, and we used them in our previous study.[18] A microplate reader (Epoch Microplate Spectrophotometer, BioTek Instruments, Winooski, VT, USA) was used to measure the total and native thiol levels. All the thiol groups, including the native and reduced thiols, were detected after reacting with 5,5′-dithiobis-(2-nitrobenzoic) acid. Half of the difference between the total and native thiols generated the dynamic disulfide (–S–S) content.

Nitric Oxide Analysis

Serum NO was measured as described previously.[18] The NO/ozone chemiluminescence technique used in this study converts nitrite (NO_2) or nitrate (NO_3) back to NO, and measures the gaseous form of NO. The serum samples were deproteinized with absolute ethanol at 0°C in a 1 : 2 v/v mix, incubated for 30 min at 0°C followed by centrifugation at 20800 g for 5 minutes. The supernatant was used to determine NO levels by the NO analyzer (Model 280i NOA, Sievers Instruments, Boulder, CO, USA). Standards and samples were injected into the purge vessel to react with the reducing agent, vanadium III chloride (dissolved in 1 M HCl at 95°C), and the resultant NO from the reaction vessel was analyzed under pure nitrogen. Sodium nitrate was used to obtain a standard curve, and the NO concentration of the sample was calculated from this curve. The NOAnalysis software (version 3.21, Sievers, Boulder, CO, USA) was used for data collection and analysis.

3-Nitrotyrosine Measurement

A commercially available ELISA kit (Cat.No. CK-bio-10045, Coon Koon Biotech Co. Ltd., Shanghai, China) was used for measuring serum 3-NT levels according to the procedure indicated by the manufacturer. The plate was read at an absorbance of 450 nm using a microplate reader (Epoch Microplate Spectrophotometer, BioTek Instruments, Winooski, VT, USA).

Other Biochemical Analyses

All other biochemical tests were performed in an autoanalyzer (Roche Hitachi Modular DP Systems, Mannheim, Germany) using commercially available kits. An automated hematology analyzer system (Beckman Coulter LH 780 Hematology Analyzer, Beckman Coulter, Brea, CA, USA) was used for determination of hematologic parameters. The measurements of Cu^{2+} were performed using the atomic absorption spectrophotometer (Shimadzu AA-680, Kyoto, Japan).

Statistical Analysis

Data were presented as mean ± standard error of mean (SEM) if normally distributed, or median and interquartile range if the variables were not normally distributed. Qualitative data were given as ratios with percentages.

Bartlett's test was used to analyze whether the standard deviations of the groups were equal. Additionally, the normality of the analyzed continuous variables was identified using the Kolmogorov–Smirnov test. For data with abnormal distribution, or if Bartlett's assumption test was significant, the Mann–Whitney *U*-test was used. Otherwise, an unpaired Student's *t*-test was used to compare 2 groups of normally distributed data. ANOVA was utilized to compare more than 2 groups when assumptions of normality and variance homogeneity were met. Then, a post hoc Student–Newman–Keuls test was applied for multiple comparisons. When these assumptions were not fulfilled, Kruskal–Wallis test (with Dunn's multiple comparisons post-test) was applied for comparison of more than 2 groups. Categorical data were analyzed using Fisher's exact or chi-square tests. Pearson's test was used to carry out correlation analysis. GraphPad Instat (version 3.05, GraphPad Software Inc., San Diego, CA, USA) statistical software was used. The level of significance was set at $P < .05$.

RESULTS

The duration of follow-up under treatment was 69.8 ± 5.1 months in patients with WD. Forty patients received D-penicillamine treatment, while 1 patient was on trientine, and another patient was on zinc therapy. Our neurological examination revealed that movement disorders including dystonia, tremor, ataxia, and parkinsonism, which were frequently associated with dysarthria, dysphagia, and slurred speech with drooling, were the most common neurologic symptoms among the patients in the neuropsychiatric WD group. Psychiatric manifestations such as depression, personality changes, and psychosis were observed in 7 patients (14%). Increased T2 signal intensity in subcortical structures such as the brainstem, thalamus, and basal ganglia was observed in all neuropsychiatric WD patients following MRI examination. We also noted asymptomatic hepatomegaly (2 patients), isolated splenomegaly (1 patient), fatty liver (3 patients), acute hepatitis (2 patients), resemblance to autoimmune hepatitis (1 patient), and acute liver failure (1 patient) among the hepatic WD group. Kayser–Fleischer rings were found in 26 patients (52%) with WD.

The demographic, laboratory, and clinical features of both patient and control groups are presented in Table 1. Compared with the control group, the average age, gender, albumin, hemoglobin, white blood cells, platelet count, alanine aminotransferase level, aspartate aminotransferase/alanine aminotransferase ratio, and fasting plasma glucose level in the patient group were similar ($P > .05$ for all). The clinical form of WD, the presence of cirrhosis and Kayser–Fleischer rings, and Cu^{2+} levels in urine were not significantly different between the groups with WD before drug treatment and under drug treatment. Serum gamma glutamyl transferase and aspartate aminotransferase enzyme levels were markedly elevated in the WD group before drug treatment, when compared to the controls and the WD group under drug treatment. Serum alkaline phosphatase level was markedly depressed in the WD group under drug treatment when compared to controls. Baseline 24-hour urinary Cu^{2+} excretion was found to be markedly elevated in patients with WD. There were low levels of serum ceruloplasmin in the WD groups when compared to controls (Table 1).

Figures 2 and 3 illustrate the distribution of the native thiol, total thiol, and disulfide levels between the study groups. These data are also summarized in Table 2. There was a significant increase in native thiol levels the WD group under drug treatment ($P < .05$) when compared to controls (Figure 2). No significant changes were observed in total thiol, disulfide, disulfide/total thiol ratio, disulfide/native thiol ratio, or native thiol/total thiol ratio between the groups (Figures 2 and 3).

Figure 4 shows serum NO and 3-NT levels in the study groups. We observed marked increase in NO levels in the WD group before drug treatment, when compared to the controls ($P < .05$) and the WD group under drug treatment ($P < .001$). Furthermore, NO levels were markedly reduced in the WD group under drug treatment when compared to controls ($P < .01$). We noted that serum 3-NT levels were significantly elevated in the WD groups before ($P < .05$) and under drug treatment ($P < .01$) when compared to controls.

We found no marked changes in NO levels between hepatic (206.65 ± 26.78 μM, n = 31) and neuropsychiatric (146.27 ± 13.34 μM, n = 19, $P > .05$) forms of WD. However, the NO levels in the neuropsychiatric form of WD (but not the hepatic form) were significantly low when compared to the control group (183.68 ± 6.38 μM, $P < .05$). There were also no significant differences in 3-NT levels between hepatic (0.94 ± 0.05 mmol/L, n = 31) and neuropsychiatric (1.03 ± 0.10 mmol/L, n = 19, $P > .05$) forms of WD. However, these values were markedly high when compared to the control group (0.72 ± 0.04 mmol/L, $P < .05$).

Table 1. Demographic and Laboratory Data of Patients With Wilson disease Before Drug Treatment and Under Drug Treatment, and in Healthy Controls

	Control, (n = 50)	WD – Before Drug Treatment (n = 8)	WD – Under Drug Treatment (n = 42)	*P*
Age (years)	28.9 ± 3.3	27.1 ± 3.8	30.8 ± 1.9	.8240
Gender				.5804
Male, n (%)	28 (56.0)	3 (37.5)	24 (57.1)	
Female, n (%)	22 (44.0)	5 (62.5)	18 (42.9)	
The clinical form of WD				.6933
Hepatic, n (%)	-	6 (75.0)	25 (59.5)	
Neuropsychiatric, n (%)	-	2 (25.0)	17 (40.5)	
Cirrhosis, n (%)	-	2 (25.0)	18 (42.9)	.4501
Kayser–Fleischer rings, positive, n (%)	-	3 (37.5)	23 (54.8)	.4561
Albumin (g/L)	45.1 ± 1.9	39.0 ± 2.1	40.0 ± 1.2	.0583
Hemoglobin (g/dL)	13.9 ± 0.7	12.7 ± 0.8	14.0 ± 0.3	.6674
White blood cells ($\times 10^3/mm^3$)	7.1 ± 0.9	6.6 ± 0.7	6.7 ± 0.3	.9064
Platelet count ($\times 10^3/mm^3$)	214.9 ± 15.4	224.3 ± 32.5	200.5 ± 13.9	.7200
AST (IU/L)	32.7 ± 4.2	102.3 ± 18.3	38.3 ± 7.7	<.001*+
ALT (IU/L)	30.1 ± 6.2	40.1 ± 6.5	31.1 ± 4.3	.7702
AST/ALT ratio	1.1 ± 0.7	2.8 ± 0.5	1.4 ± 0.2	.4717
Alkaline phosphatase (IU/L)	127.8 ± 4.1	109.6 ± 7.9	103.2 ± 6.0	<.01‡
Gamma glutamyl transferase (IU/L)	21.2 ± 3.7	156.5 ± 48.2	39.8 ± 6.1	<.001*+
Fasting plasma glucose (mg/dL)	88.2 ± 3.7	86.4 ± 2.3	86.8 ± 3.6	.9539
Copper in urine (μg/24 h)	26.8 ± 4.2	257.3 ± 102.3	362.8 ± 61.9	<.001*‡
Serum ceruloplasmin (mg/L)	213.8 ± 9.7	78.7 ± 13.6	106.3 ± 19.8	<.01*
				<.001‡

Data show mean ± SEM values.
*Control group versus WD group – before treatment.
+WD – before treatment versus WD group – under treatment.
‡Control group versus WD group – under treatment.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; WD, Wilson disease.

Correlation analysis revealed a positive correlation between duration of treatment and serum NO levels (*P* = .0035), but no marked correlation with 3-NT levels in the group of WD patients under drug treatment (Table 3).

DISCUSSION

The data of this study demonstrate for the first time the involvement of dynamic thiol/disulfide homeostasis and nitrosative stress in WD. Our data showed that serum levels of the native thiol were elevated in WD patients under drug treatment. We also observed an increased serum NO level in the WD patients before drug treatment, and augmented serum 3-NT levels in the WD groups before and under drug treatment.

Accumulated evidence suggests that reactive oxygen species (ROS) may play a significant role in the pathogenesis of tissue damage in WD.[13,19] As Cu^{2+} is a redox-active metal, it may create favorable conditions for superoxide-generating redox cycling and oxidative injury. Haber–Weiss or Fenton chemistry and lipid peroxidation are the important pathways through which Fe^{2+} and Cu^{2+} catalyze the formation of reactive hydroxyl and lipid radicals.[20] The ROS and the metal ions can cause a number of modifications in cellular macromolecular targets. Excess cellular Cu^{2+} can oxidize protein cysteine residues causing disulfide bond formation, which ultimately leads to altered protein activity.[21] Cu^{2+}-induced free radicals generate oxidative changes in hepatocyte organellar lipids

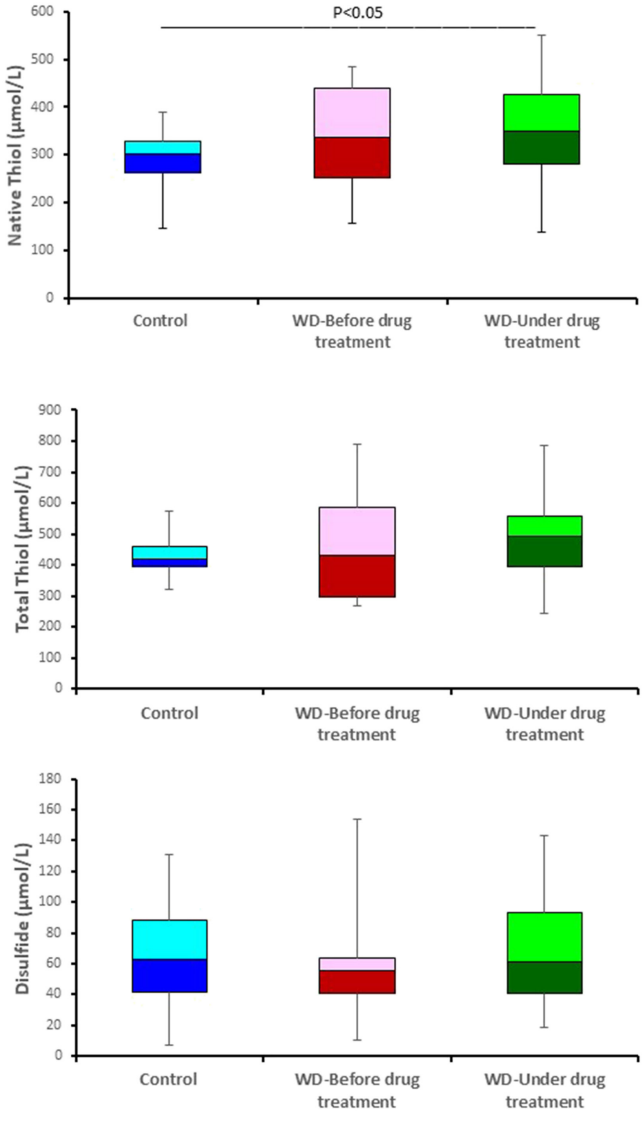

Figure 2. Box-plots of native thiol, total thiol, and disulfide levels between the study groups. The lower and upper margins of each box represent 25th and 75th percentiles, horizontal lines in the middle of the boxes represent median value, and whiskers represent the lowest and highest values. WD, Wilson disease.

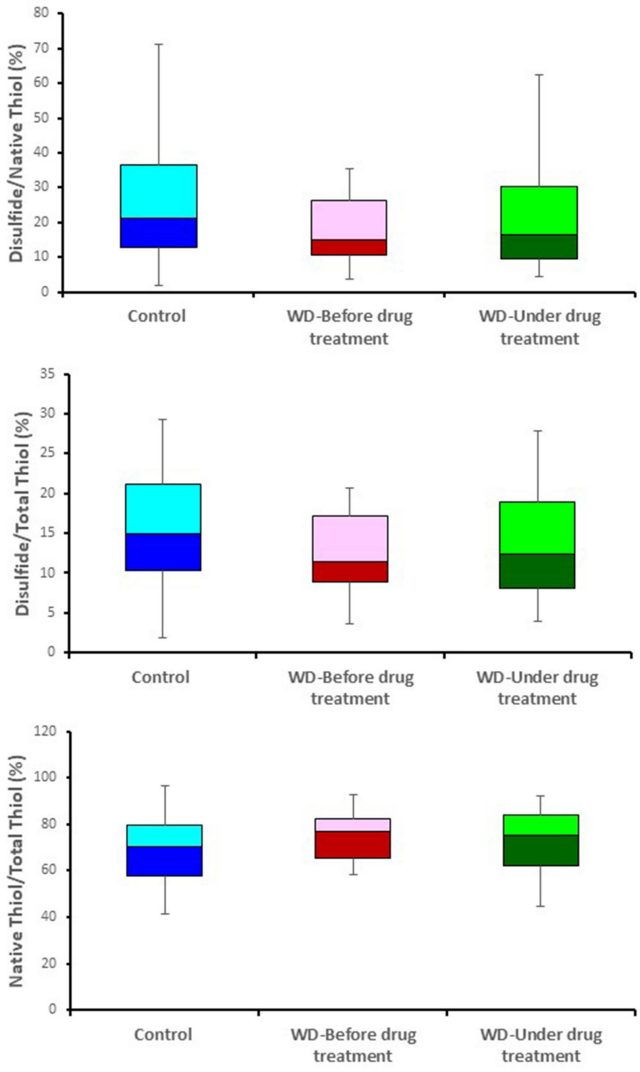

Figure 3. Box-plots of disulfide/native thiol, disulfide/total thiol, and native thiol/total thiol ratios between the study groups. The lower and upper margins of each box represent the 25th and 75th percentiles, horizontal lines in the middle of the boxes represent median value, and whiskers represent the lowest and highest values. WD, Wilson disease.

and thiol-containing proteins, causing hepatocyte injury or dysfunction.[22] Furthermore, it has been demonstrated that a sudden onset of fulminant hepatic failure in patients with WD results from the extensive apoptosis of hepatocytes attributable to extraordinarily intense oxidant stress loads.[22] The generation of free oxygen radicals from Cu^{2+} overload in WD causes mutations in the p53 tumor suppressor gene.[23] It has been shown that excess free Cu^{2+} induces not only oxidative stress but also endoplasmic reticulum (ER) stress via the accumulation of abnormal proteins in hepatocytes, contributing to disease progression in WD.[9] DNA damage and lipid peroxidation have been detected in livers of WD patients.[13,14] Collectively, these findings suggest that there is an involvement of oxidative stress in the etiology of WD.

We have measured dynamic serum thiol-disulfide homeostasis which allowed us to determine whole dynamic SH groups providing an advantage over measuring only glutathione (GSH) level or redox couples (such as reduced/oxidized glutathione and cysteine/cystine) separately. We

Table 2. Dynamic Thiol/Disulfide Parameters of the Patients With Wilson Disease and in Healthy Controls

	Control (n = 50)	WD – Before Drug Treatment (n = 8)	WD – Under Drug Treatment (n = 42)	*P*
Native thiol (μmol/L)	294.9 ± 7.9	339.7 ± 44.5	348.8 ± 16.7	<.05*
Total thiol (μmol/L)	426.8 ± 7.3	460.5 ± 66.8	486.4 ± 20.4	>.05
Disulfide (μmol/L)	66.0 ± 4.6	60.4 ± 15.4	68.8 ± 5.7	>.05
Disulfide/native thiol (%)	24.9 ± 2.3	18.3 ± 4.0	22.4 ± 2.4	>.05
Disulfide/total thiol (%)	15.2 ± 1.0	12.5 ± 2.1	14.1 ± 1.0	>.05
Native thiol/total thiol (%)	69.6 ± 2.0	75.0 ± 4.3	71.8 ± 2.1	>.05

Data show mean ± SEM values.
*Control group versus WD group – under treatment.
WD, Wilson disease.

detected a marked increase in native thiol levels of the WD group under drug treatment. The majority of our patients received D-penicillamine as the Cu^{2+} chelating agent, and this drug has a free thiol group. This may have contributed to the increased native thiol levels seen in our study. Our data agree with the findings of Gromadzka et al.,[24] who demonstrated that anti-Cu^{2+} drug (D-penicillamine or zinc sulfate)-treated WD patients had significantly higher GSH levels than non-drug-treated WD individuals. Gromadzka et al.[24] also demonstrated that anti-Cu^{2+} drug-treated WD patients had significantly diminished Cu^{2+} metabolism parameters and augmented total antioxidant potential than non-drug-treated WD individuals, but both groups had decreased serum catalase, manganese superoxide dismutase (Mn-SOD), glutathione S-transferase, and glutathione peroxidase (GPx) activities compared to controls. They concluded that anti-Cu^{2+} drugs affect Cu^{2+} metabolism as well as improve, but do not normalize, the natural antioxidant capacity in patients with WD.[24] In another study, activities and protein expressions of catalase, $Cu^{2+}Zn^{2+}$-dependent superoxide dismutase (CuZn-SOD), and Mn-SOD were found to be reduced in all WD patients.[13] In particular, a diminished Mn-SOD activity can contribute to mitochondrial damage, leading to cell apoptosis. It has been shown that GPx activity was reduced only in WD patients with fulminant hepatic failure.[13] In WD patients, the GSH and total serum antioxidant capacity (TAC) were reduced, and MDA was increased compared to controls.[19,25] It has also been reported that TAC levels are similar in controls and WD patients.[26] Patients with WD have low vitamin E levels in plasma compared to controls,

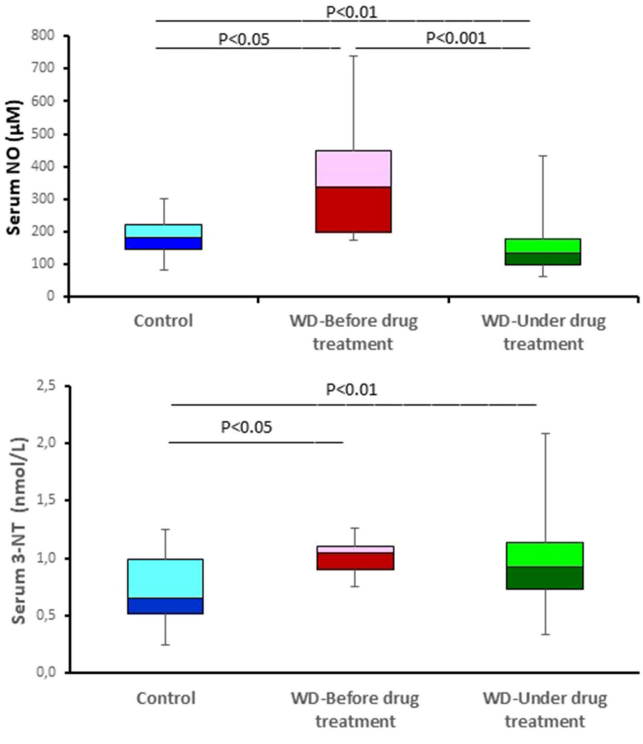

Figure 4. Box-plots of nitric oxide (NO) and 3-nitrotyrosine (3-NT) levels between the study groups. The lower and upper margins of each box represent the 25th and 75th percentiles, horizontal lines in the middle of the boxes represent median value, and whiskers represent lowest and highest values. WD, Wilson disease.

Table 3. Correlations Between Duration of Treatment and Nitrosative Stress Markers in Wilson disease Patients Under Drug Treatment

Parameters	Correlation Coefficient (r)	Coefficient of Determination (r^2)	*P*
Duration of treatment ⟷ NO	0.4403	0.1939	.0035
Duration of treatment ⟷ 3-NT	0.0469	0.0022	.7682

NO, nitric oxide; 3-NT, 3-nitrotyrosine.

presumably due to increased production of ROS.[27] This is in agreement with the suppressed levels of different antioxidants,[25] and elevated oxidative stress in WD.[12,14] It has been suggested that the augmented oxidative stress contributes markedly to the clinical manifestation of WD; as a diminished TAC is associated with the neurological symptoms in WD patients.[19] Kalita et al.[28] reported that addition of vitamins C and E to D-penicillamine or zinc treatments reduced oxidative stress and improved the clinical outcome in WD patients with neurological manifestation. Thus, decreased antioxidant enzyme activities and increased oxidative stress are believed to play a crucial role in the pathophysiology of WD.

We observed a marked increase in NO levels in non-drug-treated WD patients. There are only limited numbers of published studies related to nitrosative stress in WD. Hussain et al.[23] reported that 60% of the WD cases demonstrated a higher expression of inducible NO synthase in the liver, which suggests NO as a source of elevated nitrosative stress.[23] Increased NO levels were also found in one study performed in children with WD.[26] Collectively, these results imply that high levels of NO are generated in WD. We showed for the first time that serum 3-NT levels were significantly elevated in WD. Thus, the oxidative or nitrosative stress associated with Cu^{2+}accumulation can be considered as a central mechanism underlying the detrimental effects of WD.

There are also limitations of this study. One of them is that the number of study subjects in the non-drug-treated WD group was relatively small. Since WD is a rare inherited autosomal recessive disease, and it is difficult to diagnose, our group of WD patients before drug treatment was small. Therefore, further studies enrolling a larger cohort of patients are required. The other limitation of this study was related to genetic factors. We did not perform a genetic analysis in the present study. Since over 700 different mutations have been described in the *ATP7B* gene,[2] direct molecular/genetic diagnosis is difficult in WD. Moreover, attempts to link the *ATP7B* gene mutations to phenotypic presentation have mostly been unsuccessful, prompting researchers to evaluate other explanations for the heterogeneous phenotypes of WD.[29,30]

In conclusion, our results strongly suggest that augmented native thiols, NO, and 3-NT levels may have a role in the pathogenesis of WD. Our findings imply that increased oxidative and nitrosative stress play an important role in WD. An antioxidant that could suppress nitrosative stress in WD deserves further investigation.

Ethics Committee Approval: This study was approved by the Institutional Ethics Committee (Reference No. 2019/121).

Informed Consent: Written informed consent was obtained from patients who participated in this study.

Peer Review: Externally peer-reviewed.

Author Contributions: Concept – E.M.Y., B.T.K., S.B., F.K., B.D.G., A.T.D.; Design – B.T.K., A.T.D.; Supervision – B.T.K.; Resource – E.M.Y., B.T.K., A.S., A.T.D.; Materials – B.T.K., A.S., S.D., F.K., A.T.D.; Data Collection and/or Processing – E.M.Y., B.T.K., A.S., S.B., S.D., F.K., B.D.G., A.T.D.; Analysis and/or Interpretation – E.M.Y., B.T.K., A.S., S.B., S.D., B.D.G., A.T.D.; Literature Search – E.M.Y., B.T.K., S.D., F.K., A.T.D.; Writing – B.T.K., S.D., A.T.D.; Critical Reviews – E.M.Y., B.T.K., A.S., S.B., S.D., F.K., B.D.G., A.T.D.

Conflict of Interest: The authors have no conflicts of interest to declare.

Financial Disclosure: This study was supported by a research project (TF.UT.19.46) from the Gaziantep University.

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