Published in final edited form as:

Am J Med Sci. 2011 October ; 342(4): 314–317. doi:10.1097/MAJ.0b013e31821d9905.

Oxidative Stress in Chronic Liver Disease: Relationship Between Peripheral and Hepatic Measurements

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Abstract

Introduction—Oxidative stress plays an important role in the pathogenesis of many liver diseases. Investigators often measure markers of oxidative stress in peripheral veins as a reflection of hepatic oxidative stress as it is not always feasible to measure oxidative stress in liver tissue. However, it is unknown whether markers of oxidative stress measured from peripheral sites accurately reflect hepatic tissue oxidative stress. The aim of this study is to examine the relationship of oxidative stress marker among hepatic tissue, hepatic and peripheral veins and urine.

Methods—Malondialdehyde (MDA), a marker of oxidative stress was measured in hepatic vein, peripheral vein and urine samples from 26 consecutive patients undergoing transjugular liver procedures. In 19 patients undergoing liver biopsies, we measured MDA by immunohistochemical staining of paraffin-embedded liver tissue.

Results—Peripheral venous MDA levels showed significant correlation with hepatic venous MDA levels ($r = 0.62$, $P = 0.02$), but they did not correlate with hepatic tissue MDA content ($r = 0.22$, $P = 0.4$). Hepatic venous MDA levels did not correlate with hepatic tissue MDA content ($r = -0.01$, $P = 0.9$). Subgroup analysis of patients without portal hypertension showed a positive correlation between hepatic venous and hepatic tissue MDA levels, but this was not statistically significant ($r = 0.45$, $P = 0.22$). Urinary MDA did not correlate with MDA from any other sampling location.

Conclusion—Oxidative stress measured from the peripheral venous samples is poorly reflective of hepatic tissue oxidative stress. Hepatic venous sampling might be suitable for assessing hepatic tissue oxidative stress in patients without portal hypertension, but a larger study is needed to examine this possibility.

Key Indexing Terms

Oxidative stress marker; Malondialdehyde; Cirrhosis

Oxidative stress is a prominent feature in the pathophysiology of both acute and chronic liver disease.¹⁻⁷ Oxidative stress occurs when reactive oxygen species generated from either

exogenous sources (eg, alcohol and acetaminophen) or endogenous sources (eg, liver cytochrome P-450 enzyme system) and cellular functions (mitochondrial metabolism) are not balanced by a similar rate of consumption by antioxidants (glutathione, superoxide dismutase, etc).⁸ Consequences of oxidative stress include oxidation of various cellular components such as DNA, proteins, lipids/fatty acids (lipid peroxidation) leading to damage and the potential for cell death (apoptosis).⁹ Once this process is initiated, there is a continuous cycle of cellular damage and release of proinflammatory cytokines resulting in hepatic inflammation, fibrosis and cirrhosis.⁴ Several end products of lipid peroxidation have been used as biomarkers of oxidative stress, including α - β unsaturated reactive aldehydes, such as malondialdehyde (MDA), 4-hydroxy-2-nonenal, 2-propenal (acrolein) and isoprostanes.⁹ These compounds are relatively stable and easily quantified in serum/plasma and urine as an indirect measure of oxidative stress.^{10,11}

Oxidative stress has also been implicated in a variety of conditions such as aging, neurodegenerative diseases, chronic inflammatory diseases and cancer.⁹ In general, investigators conducting human studies have measured lipid peroxidation in urine or blood from a peripheral vein under the assumption that it accurately reflects lipid peroxidation in the organ of interest. Rahman et al¹² reported that MDA levels in both bronchoalveolar lavage fluid and plasma were increased in patients with idiopathic pulmonary fibrosis. However, the degree of correlation was not examined in this study. Prior studies evaluating MDA as a marker for lipid peroxidation in the liver have only examined levels in either the peripheral blood or hepatic tissue.^{3,13,14} To our knowledge, a simultaneous assessment of systemic (peripheral venous blood, hepatic venous blood and urine) and liver tissue markers of lipid peroxidation has never been performed. Therefore, it is currently unknown whether peripheral markers of oxidative stress are an accurate reflection of hepatic oxidative stress. We conducted a study to examine the relationship of measures of oxidative stress among various sampling sites including peripheral and hepatic veins, urine and hepatic tissue.

PATIENTS AND METHODS

The study was approved by Institutional Review Board of Indiana University (IRB study protocol no. 0805-29). All patients gave a written informed consent before their participation in the study.

Patients

A total of 26 consecutive patients undergoing transjugular procedures as part of their clinical care participated in this study. Their indications included transjugular liver biopsy (n = 17), placement of TIPS (n = 7) and portal pressure measurements (n = 2). The presence of portal hypertension was established based on clinical, histological (when available) and radiological criteria. The medication lists were reviewed to identify patients receiving any anti-oxidant supplements. On the day of the procedure, 15 mL of peripheral venous blood and a urine sample were obtained in the preoperative suite, and the hepatic vein blood sample (15 mL) was obtained during the procedure after confirmation of hepatic vein cannulation under fluoroscopy. In the subgroup undergoing liver biopsy, we obtained paraffin-embedded liver tissue for assessing lipid peroxidation. All blood (centrifuged and protected from light) and urine samples were stored at -20°C until further analysis.

METHODS

MDA in Serum

MDA levels in serum samples were measured using high performance liquid chromatography with ultra violet detection as described previously with modification.^{15,16}

Briefly, 0.2 mL serum was mixed with 40 μ L of 6 M NaOH. The mixture was incubated at 60°C for 30 minutes to hydrolyze the protein-bound MDA. Protein was precipitated with 100 μ L of 35% (v/v) perchloric acid and centrifuged at $10,000 \times g$ for 10 minutes. Supernatant was transferred to a clean glass tube and 25 μ L of 2,4-Dinitrophenylhydrazine (5 mM solution in 2 M hydrochloric acid) was added. After incubation for 15 minutes at room temperature, the mixture was extracted with 1 mL of n-hexane 2 times. The organic phases were combined and dried under nitrogen flow. The extract was reconstituted in 200 μ L of 20% acetonitrile. A 50 μ L aliquot of reconstituted sample was injected onto a Waters Alliance 2690 high performance liquid chromatography system with a Waters 996 Photodiode Array detector (Waters, Milford, MA). MDA was separated on a reverse phase LC-18-DB column (3 μ M, 150 $\times$ 4.6 mm; Supelco, Bellefonte, PA) and detected with a ultra violet detector at a wavelength of 310 nm.

MDA in Urine

MDA levels in urine samples were measured as described previously with modification.¹⁷ Briefly, 2 mL urine was mixed with 0.2 mL of 35% (v/v) perchloric acid and 50 μ L 2,4-Dinitrophenylhydrazine (5 mM solution in 2 M hydrochloric acid). After incubation for 15 minutes at room temperature, the mixture was extracted with 1 mL of n-hexane 3 times. The organic phases were combined and dried under nitrogen flow. The extract was reconstituted in 200 μ L of 20% acetonitrile and analyzed as described previously for the serum analyses. Creatinine levels in urine were measured using a commercially available assay kit from Cayman Chemical Company (Ann Arbor, MI) following the manufacturer's instructions. MDA levels in urine were standardized against creatinine concentration.

Hepatic MDA Content in Paraffin-Embedded Liver Tissue

Immunohistochemistry was performed on 4- μ m thick sections cut from formalin-fixed paraffin-embedded tissue. Briefly, the sections were deparaffinized, and heat-induced antigen retrieval was carried out with ethylenediaminetetraacetic acid in a pressure cooker. Endogenous peroxidase was quenched by incubating with hydrogen peroxidase. Sections were incubated with anti-MDA antibody (Abcam, Cambridge, MA) and subsequently with a secondary antibody (EnVision+ from DAKO, Carpinteria, CA). The reaction was developed using streptavidin labeled with horse radish peroxidase and 3,3'-Diaminobenzidine as the chromogen. Immunohistochemical staining was digitally quantitated, and hepatic MDA content was expressed as a percent of total liver biopsy area using SPSS Sigma Scan Pro 5.0 software (SPSS, Chicago, IL).

Descriptive statistics, including means, standard deviations and percentages, were used to characterize the study patients and MDA data. Pearson's correlation coefficients were used to determine the degree of concordance between any 2 variables. Correlations were considered significant at the 0.05 level (2 tailed).

RESULTS

The mean $\pm$ standard deviation age of the study population (n = 26) was 52 ± 12 years (Table 1). The majority of the patients were men (61%) and white (85%). Sampling from all 3 sites (hepatic vein, peripheral vein and urine) was performed in all except in 3 patients in whom hepatic venous sample could not be obtained due to procedure-related issues. Paraffin-embedded liver tissue was available from all 17 (9 noncirrhotic and 8 with portal hypertension) patients who underwent liver biopsy (Table 1).

First, the relationship between MDA levels in the peripheral vein and other sampling sites was examined (Figure 1 and Table 2). Peripheral venous MDA levels exhibited a

statistically significant correlation with hepatic venous MDA content ($r = 0.62$, $P = 0.02$) (Figure 2). However, there was no relationship between peripheral venous and urinary MDA levels ($r = 0.19$, $P = 0.36$) or hepatic tissue MDA content ($r = 0.22$, $P = 0.4$).

Second, the relationship between MDA levels in the hepatic vein and other sampling sites was examined (Table 2). As shown earlier, they had significant correlation with MDA levels in the peripheral venous samples, but there was no correlation with either urinary MDA ($r = 0.11$, $P = 0.6$) or hepatic tissue MDA content ($r = -0.01$, $P = 0.9$).

Third, in patients undergoing liver biopsy, there was no correlation between hepatic tissue MDA content and peripheral venous ($r = 0.22$, $P = 0.4$), hepatic venous ($r = -0.01$, $P = 0.9$) or urinary MDA level ($r = -0.10$, $P = 0.7$). There was no relationship between hepatic tissue MDA content and peripheral venous MDA levels in patients with and without portal hypertension ($r = 0.19$, $P = 0.65$ and $r = 0.26$, $P = 0.5$, respectively) (Figure 3). Interestingly, in patients without portal hypertension, there was a positive correlation between hepatic MDA content and hepatic venous MDA levels ($r = 0.45$), but this did not reach statistical significance ($P = 0.22$). However, in patients with portal hypertension, there was no correlation between hepatic tissue MDA content and hepatic venous MDA levels ($r = -0.32$, $P = 0.44$).

DISCUSSION

The role of oxidative stress and resulting therapeutic potential for antioxidant supplements for liver disease has been a subject of interest for several decades.^{18,19} There is renewed interest in this area of research due to positive results from the PIVENS clinical trial that were reported recently by the NASH Clinical Research Network.²⁰ Importantly, mechanistic studies evaluating the role of oxidative stress in the pathogenesis of liver diseases human liver disease pathogenesis will require measurement of markers of oxidative stress, including end products of lipid peroxidation. Because repeated liver sampling is not ethical and/or feasible in human studies, a better understanding of the limitations of extrapolating peripheral markers of oxidative stress and lipid peroxidation to hepatic tissue is crucial to our study of liver diseases.

This study showed significant correlation between peripheral venous and hepatic venous MDA. However, MDA values from both these sites correlated poorly with hepatic tissue MDA content suggesting that peripheral venous measures of oxidative stress are not a valid reflection of hepatic tissue oxidative stress. Lack of significant correlation between hepatic venous MDA levels and hepatic tissue MDA content also excluded the possibility of hepatic venous sampling for a more satisfactory reflection of hepatic tissue oxidative stress. However, subgroup analysis suggested that in individuals without portal hypertension, there may be some relationship between hepatic venous MDA levels and hepatic tissue MDA content, but this did not reach statistical significance, perhaps due to our modest sample size.

Certain limitations of our study deserve further discussion. Although several reactive aldehydes have been reported as biomarkers of oxidative stress, we chose MDA for our study as it has been used as a biomarkers of lipid oxidation for several decades. Although we do not have any data on other biomarkers, we have no reason to believe that use of any other biomarker would yield different results in terms of correlation. A study that evaluated serum levels of both MDA and nitric oxide as markers of oxidative stress in patients with liver diseases reported similar conclusion using MDA and nitric oxide.²¹ Similarly, investigators in another study evaluated various biomarkers of oxidative stress in patients with chronic hepatitis C and alcoholic liver disease reported similar conclusions with MDA, 4-hydroxynonenal and 8-isoprostane.²² Another limitation of the study was the inability to use

the sensitive high performance liquid chromatography-ultra violet methods to measure liver tissue MDA as was done in the blood samples. This was due to the fact that we were unable to obtain enough tissue for these types of measures, as only the formalin-fixed, paraffin-embedded tissue was available for immunohistochemical analysis. The results of our study would be much more robust with a larger sample size. However, the results of this study are unique because concordance was assessed in the same subject and not in an intersubject manner. Notwithstanding these limitations, we believe our study offers important information for investigators studying the role of oxidative stress in the pathogenesis of chronic liver disease.

In summary, this study shows that peripheral venous sampling is not a satisfactory reflection of hepatic tissue oxidative stress. It is possible that hepatic venous sampling might be suitable for assessing hepatic tissue oxidative stress in patients without portal hypertension, but a larger study is needed to examine that possibility.

Acknowledgments

This study was supported by K24DK069290 (to NC).

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FIGURE 1.
Box plots for malondialdehyde levels at various peripheral sampling sites.

FIGURE 2.
Significant correlation between peripheral vein malondialdehyde (MDA) and hepatic vein MDA levels.

FIGURE 3.
Lack of correlation between peripheral vein malondialdehyde (MDA) and hepatic tissue MDA by immunohistochemistry.

TABLE 1

Select demographics and biochemical parameters of study patients

Entire cohort (N = 26)		
Age, yr (mean ± SD)	51 ± 12	
Males (%)	62	
Whites (%)	85	
BMI (kg/m ²), (mean ± SD)	28 ± 5	
Diabetes (%)	27	
Smoking (%)	12	
Patients on antioxidant therapy (n)	1	
Liver biopsy subgroup		
	Portal hypertension present (n = 8)	Portal hypertension absent (n = 9)
Biochemical parameters (mean ± SD)		
Total bilirubin (mg/dL)	5.1 ± 7.0	2.7 ± 3.9
AST (IU/L)	109 ± 101	93 ± 96
ALT (IU/L)	67 ± 68	77 ± 68
Albumin (mg/dL)	2.8 ± 0.6	2.9 ± 1.0
Serum creatinine (mg/dL)	1.0 ± 0.5	1.2 ± 0.6
INR	1.5 ± 0.4	1.4 ± 0.6
MELD score	15 ± 8	N/A

SD, standard deviation; BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; INR, International Normalized Ratio; MELD, Model for End Stage Liver Disease; N/A, not applicable.

TABLE 2Correlation (*r* value) of malondialdehyde levels among various sampling sites

	Peripheral vein (n = 26)	Hepatic vein (n = 23)	Urine (n = 26)	Hepatic MDA (n = 17)
Peripheral vein	—	0.62 ^a	0.19	0.22
Hepatic vein	0.62 ^a	—	0.11	−0.01
Urine	0.19	0.11	—	−0.10
Hepatic MDA	0.22	−0.01	−0.10	—

^a*P* value attained statistical significance (*P* = 0.02).

MDA, malondialdehyde.