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Review

Oxidative Stress and Antioxidant Biomarkers in Clinical and Experimental Models of Non-Alcoholic Fatty Liver Disease

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Abstract: Non-Alcoholic Fatty Liver Disease (NAFLD) is a term that covers a range of hepatic disorders involving fat deposits in the liver. NAFLD begins with simple steatosis and progresses into non-alcoholic steatohepatitis (NASH) characterised by inflammation, fibrosis, apoptosis, oxidative stress, lipid peroxidation, mitochondrial dysfunction and release of adipokines and pro-inflammatory cytokines. Oxidative stress and antioxidants are known to play a vital role in the pathogenesis and severity of NAFLD/NASH. A number of oxidative stress and antioxidant markers are employed in the assessment of the pathological state and progression of the disease. In this article, we review several biomarkers of oxidative stress and antioxidants that have been measured at clinical and experimental levels. The levels/ activity in various models reviewed are also included. Also included is a comprehensive description of oxidative stress, sources and contribution to the pathogenesis of NAFLD/NASH.

Keywords: Liver; NAFLD; NASH; Biomarkers; Reactive Species; Oxidative Stress; Lipid Peroxidation; Antioxidants.

1. Introduction

Non-Alcoholic Fatty Liver Disease (NAFLD) is a range of hepatic disorders associated with fatty deposits in liver, and which occur in the absence of alcohol consumption or alcohol abuse [1]. NAFLD begins with an initial stage of fatty liver also known as hepatic steatosis (excessive fat loading in the hepatocytes). The progression from steatosis into cirrhosis of the liver due to inflammation and fibrosis results in irreversible damage to the liver [2]. This condition is called non-alcoholic steatohepatitis (NASH) – a term first introduced by Ludwig *et al.* [3] in clinical subjects with no history of alcohol consumption.

NAFLD is one of the most common chronic hepatic pathology. It has a worldwide distribution with an estimated worldwide prevalence of 25% for NAFLD and about 5 % for NASH [4]. The highest prevalence of NAFLD is observed in Western countries (17% to 46%) where it is the most common chronic liver disease (CLD) in adults with a high prevalence of NASH in the United States (16%). The World Gastroenterology Organisation, suggest that the prevalence of NAFLD had doubled over the last 20 years. NAFLD and NASH are closely associated with diabetes and obesity, and together are considered one of the major cause of liver disease in Western countries [5].

The pathophysiology of NASH was originally explained by the ''two-hit'' hypothesis [6]. NASH is currently described by the "multiple hit" hypothesis [7] which involve the first hit: increase in susceptibility of the liver to injury, leading to hepatic steatosis. The subsequent hit is responsible for the inflammation, fibrosis, apoptosis, oxidative stress (OS), and hepatic lipid peroxidation, release of adipokines, pro-inflammatory cytokines and mitochondrial dysfunction.

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An understanding of the mechanism of OS, its regulation as well as its role in NAFLD is vital. This will provide researchers in the area of NAFLD/ NASH with the best choice of OS/antioxidant (AO) biomarkers useful in pre-clinical investigations as well as in clinical diagnosis of NAFLD. Development of potent drugs in the treatment of NAFLD will also take into cognisance, the AO action required to counteract the OS associated with NAFLD. In this article, we review the role of oxidative stress in NAFLD and several AO and OS biomarkers that have been measured in pre-clinical and clinical evaluations.

2. Oxidative Stress

OS refers to an imbalance between the production of reactive species (RS) and AO defenses [8]. A more encompassing definition described it as "an imbalance between oxidants and AOs in favour of the oxidants, leading to a disruption of redox signalling and control and/or molecular damage" [9, 10]. RS are chemically reactive species containing oxygen (reactive oxygen species, ROS), nitrogen (reactive nitrogen species, RNS) etc. (Table 1) ROSs (which are the most extensively studied RS) are oxygen-containing molecules exhibiting higher chemical reactivity than oxygen (O_2). It has been classified according to severity as "eustress" (physiological oxidative stress) and "distress" (toxic oxidative burden which damages biomolecules) [10, 11]. In other words, low exposure to OS is useful for redox signalling, whereas high exposure results in disruption of redox signalling and causes damage important biomolecules.

Table 1: Major ROS and RNS of Physiological Importance

MAJOR ROS		**SOURCES**
Free Radicals	Hydroxyl radical ($OH^{\bullet}$);	Decomposition of $ONOO^-$ or , HOCl
	Superoxide radical ($O_2^{\bullet-}$)	Electron transport systems, and one-electron reduction of O_2 by respiratory burst via the action of membrane bound NADPH oxidase
	Peroxyl radical ($ROO^{\bullet}$)	Produced in the Fenton reaction
Non-Radicals	Hydrogen peroxide (H_2O_2)	Activated macrophages during inflammation
	Hypochlorous acid (HClO)	Combined activities of NADPH oxidase and myeloperoxidase (MPO) in phagocytes
	Lipid peroxides (ROOH)	Formed from oxidation of polyunsaturated fatty acid via lipid-peroxyl radical reaction
MAJOR RNS		**SOURCES**
Free Radicals	Nitric oxide ($NO^{\bullet}$)	Nitric Oxide Synthase (NOS)
	Nitrogen dioxide ($NO_2^{\bullet}$)	Activated neutrophils
Non-radicals	Dinitrogen trioxide (N_2O_3)	Produced in pathological conditions where (inducible nitric oxide (iNOS) is upregulated
	Peroxinitrite ($ONOO^-$),	Produced in pathological conditions where iNOS is upregulated
	Nitrite (NO_2^-)	Oxidation product from NO, formed during NOS activation in inflammatory diseases
	Nitryl ion (NO_2^+)	Activated neutrophils

3. Oxidative Damage to Macromolecule

OS is associated with many diseases, especially those with an inflammatory mechanism [12]. OS is known with several hepatic diseases with high levels of ROS and RNS which is an important description of the severity and disease progression [13, 14]. ROS is constantly generated in the cell

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due to partial reduction of O_2 or as a result of transfer of energy to O_2. ROS can attack vital cell components like polyunsaturated fatty acids, proteins, and nucleic acids and carbohydrates in few cases [15-17]. They can disrupt membrane properties like fluidity and ion transport, cause loss of enzyme activity, disruption of protein synthesis mechanism and induction of DNA damage, ultimately leading to cell death. Damages resulting from OS is often called 'oxidative damage'. Oxidative damage to macromolecules (lipids, proteins, DNA etc.) results in formation of oxidative damage products (Table 2) which are often measured as biomarkers of OS [18-21].

Table 2: Some Important Oxidative Damage Products

MACROMOLECULE	OXIDATIVE DAMAGE	DAMAGE PRODUCTS
Lipids	Lipid oxidation/ peroxidation	Malondialdehyde (MDA) Lipid peroxide, (lipid endoperoxides and lipid hydroperoxides) 8-Isoprostane 4-hydroxy-2-nonenal (4-HNE)
Proteins	Protein Oxidation, protein crosslinkage, Oxidative modification of amino acids	Protein carbonyl compounds, 3-Nitrotyrosine (product of ROS-mediated nitration of tyrosine), 2-oxohistidine, hydroxyproline etc.
DNA	RNA/ DNA fragmentation (single and double-strand breaks) Modification of base, sugar	8-hydroxy-2'-deoxyguanosine (8-OH-dG), 8-hydroxyguanine (8-OH-G)

4. Regulation of OS

There are several mechanisms for the cellular regulation of OS, which are extremely important to the cell homeostasis. This is achieved through the antioxidants system which can control the formation of ROS or RNS and also repair oxidative damage to cells. An antioxidant is any substance that can inhibit the oxidation of the cell components such as DNA, proteins and lipids. Several levels of antioxidative defense mechanism are used to prevent oxidative damages [22, 23]. The antioxidants can be derived from the diet or endogenously. Endogenously derived antioxidants are classified as enzymic or non-enzymic antioxidants (Table 3).

Table 3: Physiologically important Antioxidants and their functions

ANTIOXIDANT TYPE	ANTIOXIDANT NAME	FUNCTION(S)
Enzymic antioxidants	Superoxide dismutase (SOD)	Converts $O_2^{\bullet-}$ to H_2O_2 and O_2
	Catalase (CAT)	Converts H_2O_2 to $2H_2O$ and O_2
	Glutathione peroxidase (GPx)	Detoxifies H_2O_2 and Lipid peroxides using reduced glutathione (GSH) producing the oxidised form of glutathione (GSSG)
	Glutathione reductase (GR)	Reduces GSSG to GSH
Non-enzymic antioxidants	Ascorbic Acid (AA):	Detoxifies Superoxide radical, Hydroxyl radical, and H_2O_2
	Reduced Glutathione (GSH):	Neutralizes Superoxide radical, Hydroxyl radical, and H_2O_2; co-substrate for glutathione peroxidase

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α-Tocopherol:	Detoxifies H_2O_2; protects against membrane lipid peroxidation (LPO)
Ubiquinone	Detoxifies Lipid peroxides
Thioredoxin (TRX)	General thiol redox control of protein activity via reversible disulfide formation
Bilirubin	Effective in quenching/ scavenging secondary oxidants produced during OS

5. Sources and Role of OS in NAFLD/NASH

The sources of OS as well as its role in NASH has been extensively reviewed by Koek et al. [24] and Tariq et al. [25]. OS results from excessive generation of reactive species (RS) or depletion of physiological redox homeostasis. RS (ROS or RNS) from inflammatory response. The mitochondria, endoplasmic reticulum and peroxisomes also contribute to OS associated with NAFLD/NASH. Their sources and role in NAFLD/ NASH are summarized in table 4.

Table 4: Sources of the oxidative stress in NAFLD/NASH

SOURCE	CONTRIBUTION TO OS IN NAFLD/NASH	REFERENCE(S)
Mitochondrial metabolism (β-oxidation)	Production of ROS as a result of electron leakage during mitochondrial β-oxidation;	[26-30]
Peroxisomal β-oxidation	Generation of H_2O_2 during peroxisomal β-oxidation which is converted into hydroxyl radical contributing to OS	[31]
Mitochondrial electron transport chain	inhibition of electron transport chain by TNF-α and lipid peroxidation products;	[32, 33]
Microsomal Cytochrome P450 enzymes	ROS generation due to Increase in activity of cytochrome P4502E1 (CYP2E1) involved in lipooxygenation of longchain fatty acids	[34-37]
Endoplasmic reticulum (ER) stress	Endoplasmic reticulum stress response, promotes OS via increased expression of CHOP (also called DDIT –DNA Damage Inducible Transcript-3 protein)	[38]
Xanthine Oxidase (XO)	Increase in XO activity generates superoxide anions, due to induction by 4-HNE (a product of lipid peroxidation)	[39, 40]
Inflammatory Response	Abnormal inflammatory response mediated by gut microflora resulting in increase in pro-oxidants	[41-43]

OS has been reported to play a significant role in the pathophysiological mechanism of NAFLD and NASH [44, 45]. Studies conducted in humans and animal models showed a strong association between the level of OS and the severity of NASH [26, 46]. Although clinical and experimental studies have reported higher levels of lipid peroxidation in NASH patients, however, the level of circulating antioxidants are less reported.

6. Antioxidant and OS markers measured in NAFLD/ NASH

Several biomarkers of oxidative stress and antioxidants have been detected in clinical and experimental models of NAFLD and NASH. Most of these are assayed predominantly in the liver, serum, plasma, and in few cases in whole blood samples. Major assay procedures for the detection of these markers include Colorimetry, ELISA, and Immunohistochemistry. These biomarkers as

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reported in clinical and experimental models have been extensively reviewed and are presented in Tables 5, 6, 7 and 8.

Table 5: Antioxidant Markers Measured in Clinical NAFLD/ NASH

ANTIOXIDANT MARKER	SAMPLE	LEVEL/ ACTIVITY/ EXPRESSION IN SAMPLE	CLINICAL CASE	REFERENCE(S)
SOD	Serum	Decreased, Increased*	NASH	[47, 48*, 49]
	Serum/Liver	Decreased	NAFLD	[50]
	Plasma	Decreased	NAFLD	[51]
	Blood	Increased	NAFLD	[52]
	Liver	Decreased	NAFLD	[53]
CAT	Serum	Decreased, Increased*	NASH	[47, 48*]
	Plasma	Decreased	NAFLD	[51]
	Blood	Increased	NAFLD	[52]
	Liver	Decreased	NAFLD	[53]
GPx	Serum	Decreased, Increased*	NASH	[48, 49*]
	Plasma	Decreased	NASH	[54]
	Liver	Decreased	NAFLD	[53]
GR	Serum	Increased	NASH	[49]
Ascorbic Acid	Serum	nsc	NASH	[48]
	Serum	Decreased	NAFLD	[50]
GSH	Serum	Increased	NASH	[49]
	Blood	Increased	NAFLD	[52]
	Liver	Decreased	NAFLD	[50, 53]
α-Tocopherol	Serum	Increased	NASH	[48]
	Serum	Decreased	NAFLD	[50]
Ubiquinone	Serum	Decreased	NASH	[51]
Thioredoxin (TRX)	Serum	Increased	NAFLD	[55]
Bilirubin	Serum	Decreased	NASH	[56, 57]
	Serum	Decreased	NAFLD	[58]

nsc: no significant change

Table 6: Oxidative Stress Markers Measured in Clinical NAFLD/ NASH

OS MARKER	SAMPLE	LEVEL/ ACTIVITY/ EXPRESSION IN SAMPLE	CLINICAL CASE	REFERENCE(S)
Lipid peroxides	Plasma	Increased	NASH	[59]
NO$^{\bullet}$	Serum	Increased	NASH	[47, 49]
	Serum	Increased	NAFLD	[50, 60]

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	Blood	Increased	NAFLD	[52]
TBARS	Serum	Increased	NAFLD	[47];
MDA	Serum	Increased	NASH	[61, 49, 51]
	Serum	Increased	NAFLD	[50]
Hydroperoxides	liver	Increased	NASH	[62]
8-Isoprostane	Plasma	Increased	NASH	[48]
4-HNE	Serum	Increased	NAFLD	[61]
	Liver	Increased	NASH	[63]
Protein carbonyl	Liver	Increased	NAFLD	[53]
Nitrotyrosine	Blood	nsc	NAFLD	[52]
8-OH-dG	Liver	Increased	NASH	[63, 64]
	Liver	Increased	NAFLD	[65]
	Plasma	Increased	NASH	[54]
CYP2E1	Liver	Increased	NASH	[66, 67, 68]
	Liver	Increased	NAFLD	[53]

nsc: no significant change

Table 7: Antioxidant Markers Measured in Experimental NAFLD/ NASH

ANTIOXIDANT MARKER	SAMPLE	LEVEL/ ACTIVITY/ EXPRESSION IN SAMPLE	EXPERIMENTAL MODEL	EXPERIMENTAL SPECIE	REFERENCE(S)
SOD	Liver	Decreased	NASH (MCD)	Wistar Rats	[69]
	Liver	Increased	NASH (MCD)	C57BL/6 Mice	[70]
	Liver	Increased	NASH (MCD)	C57BL/6 mice	[71]
	Liver	Decreased	NASH (MCD)	C57BL6/J mice	[72]
	Liver	Decreased	NASH (MCD)	N-Mary rats	[73]
	Liver	Decreased	NAFLD (HFD)	Mice	[74]
	Liver	Decreased	NASH (HF)	Kunming mice	[75]
	Liver	Increased	NAFLD (HFD)	Rat	[76]
CAT	Liver	Decreased	NASH (MCD)	Wistar Rats	[69]
	Liver	Decreased	NASH (MCD)	C57BL/6 Mice	[70]
	Liver	Increased	NASH (MCD)	C57BL/6 mice	[71]
	Liver	Decreased	NASH (HCD)	Wistar Rats	[77]
	Liver	Decreased	NAFLD (HFD)	Sprague-Dawley rats	[76]
GPx	Liver	Decreased	NASH (MCD)	Wistar Rats	[69]
	Liver	Increase	NASH (MCD)	N-Mary rats	[73]
	Liver	Decreased	NAFLD (HFD)	Mice	[74]
	Liver	Decreased	NASH (HF)	Kunming mice	[75]
	Liver	nsc	NAFLD (HFD)	Sprague-Dawley rats	[76]

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GR	Liver	Decreased	NASH (MCD)	N-Mary rats	[73]
GSH	Liver	Decreased	NASH (MCD)	Wistar Rat	[69]
	Liver	Decreased	NASH (MCD)	N-Mary rats	[73]
	Liver	Decreased	NAFLD (HFD)	Wistar Rats	[78]
	Liver	Decreased	NAFLD (HCD)	Wistar Rats	[77]
	Liver/RBC	Increased	NASH (HFMCD)	Sprague-Dawley rats	[79]
	Liver	Decreased	NASH (MCD)	Mice	[80]

MCD: Methionine/ Choline Deficient Diet;
HF: High fructose Diet
HFD: High Fat Diet
HCD: High Cholesterol diet
HF-MCD: High fat- methionine choline deficient diet
nsc: no significant change

Table 8: Oxidative Stress Markers Measured in Experimental NAFLD/ NASH

ANTIOXIDANT MARKER	SAMPLE	LEVEL/ ACTIVITY/ EXPRESSION IN SAMPLE	EXPERIMENTAL MODEL	EXPERIMENTAL SPECIE	REFERENCE(S)
H_2O_2	Liver	Increased	NASH (MCD)	C57BL/6J-mt$^{FVB/N}$ mice	[81]
Nitrite/ nitrate	Liver	Nsc	NAFLD (HFD)	Wistar Rats	[78]
TBARS	Liver	Increased	NASH	Rat	[82]
	Liver	Increased	NASH (MCD)	C57BL/6 Mice	[70]
	Liver	Increased	Steatosis/ NASH (HFD)	Albino rats	[83]
	Liver	Increased	NASH (MCD)	C57BL/6 mice	[71]
	Liver	Decreased	NAFLD (HCD)	Wistar Rats	[77]
	Liver	Increased	Steatosis (HFD/HSD)	Wistar rats	[84]
	Liver	Increased	HF/HGD	Wistar rats	[85]
	Liver	Increased	NAFLD (HFD)	C57BL/6J mice	[86]
	Liver	Increased	NASH (MCD)	C57BL/6 mice	[87]
MDA	Liver	Increased	NAFLD/ NASH (CDAA diet)	Wistar Rats	[88]
	Liver	Increased	NAFLD (HFD)	Sprague-Dawley rats	[76]
	Liver	Increased	NASH (MCD)	C57BL6/J mice	[72]
	Liver	Increased	NASH	Rat	[82]
	Liver	Increased	NAFLD (HFD)	Wistar Rats	[78]
	Liver	Increased	NASH (CDHF diet)	Wistar Rats	[89]
	Liver	Increased	NASH (MCD)	N-Mary rats	[73]
	Liver	Increased	NAFLD (HFD)	Mice	[74]
	Liver	Increased	NASH (HF)	Kunming mice	[75]
	Liver	Nsc	NASH (MCD)	C57BL/6J-mt$^{FVB/N}$ mice	[81]
Lipid peroxide	Liver	Increased	NASH (MCD)	Wistar Rat	[69]
8-Isoprostanes	Liver	Increased	NASH (HFMCD)	Sprague Dawley Rat	[79]

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4-HNE	Liver	Increased	NASH (MCD)	Wistar Rat	[69]
	Liver	Increased	NASH	Rat	[82]
	Liver	Increased	NASH (HFD)	Sprague Dawley Rat	[90]
	Liver	Increased	NASH (CDHF diet)	Wistar Rats	[89]
	Liver	Increased	NASH	leptin-deficient (ob/ob) mice	[91]
	Liver	Increased	NASH (HF-HSD)	C57BL/6 J mice	[92]
	Liver	Increased	NASH (MCD)	C57BL6 mice	[54, 93, 94]
Protein carbonyl	Liver	Increased	NASH (MCD)	N-Mary rats	[95]
Dityrosine	Liver	Increased	NAFLD (HFD)	C57BL/6J mice	[86]
Hydroxyproline	Liver	Increased	NAFLD/ NASH (CDAA diet)	Wistar Rats	[88]
	Liver	Increased	NASH (MCD/ WD)	C57BL6 mice	[54]
8-OH-dG	Liver	Increased	NASH (MCD)	Wistar Rat	[69]
	Liver	Decreased	NASH (HF-MCD)	Sprague-Dawley rats	[79]
	Liver	Increased	NAFLD (HFD)	C57BL/6J mice	[86]
CYP2E1	Liver	Increased	NAFLD	CYP2E1 transgenic (Tg) mice	[94, 95]
	Liver	Increased	NASH (HFD)	Sprague Dawley Rat	[90]
	Liver	Increased	NASH (HFD)	Sprague Dawley Rat	[96]
	Liver	Increased	Steatosis (HFD/HSD)	Wistar rats	[84]
	Liver	Increased	NASH (HFD)	Sprague-Dawley rats	[97]
	Liver	Increased	NASH (CDHF diet)	Wistar rats	[98]
NADPH Oxidase	liver	Increased	NASH	ob/ob mice	[91]
Xanthine Oxidase	Liver	Increased	NAFLD (HFD)	Sprague-Dawley rats	[76]
Nitrotyrosine	Liver	Increased	NAFLD	CYP2E1 transgenic (Tg) mice	[95]

MCD: Methionine/ Choline Deficient Diet
HF: High fructose Diet
HFD: High Fat Diet
HCD: High Cholesterol diet
HSD: High Sucrose diet
CDAA: Choline Deficient L-Amino Acid-defined
CDHF: Choline Deficient High Fat diet
HFMCD: High fat methionine choline deficient diet
HF-HSD: High fat-high sucrose diet
WD: Western diet
HF/HGD: High-Fructose/High-Glucose Diet
nsc: no significant change

5. Conclusions

Oxidative stress play an important role in the pathophysiology of NAFLD/NASH. Several markers of oxidative stress and antioxidants have been shown to be very useful in assessing the redox state in NAFLD/NASH. Among the oxidative stress biomarkers reviewed, TBARS, MDA, CYP2E1 and 4-HNE are unique; they are represented in both clinical and experimental measurements and

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they consistently increase. Antioxidants of interest in clinical and pre-clinical assessment of NAFLD/NASH will include: GSH, SOD, CAT, GPx which appears most reliably detected in the liver samples.

Conflicts of Interest: The authors declare no conflict of interest.

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