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Application of stable nitroxide radicals and their non-contrast forms in diagnostics of oxidative stress in patients with diabetes mellitus type 2 and non-alcoholic fatty liver disease

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Non-alcoholic fatty liver disease has emerged as one of the main causes of chronic liver damage, which occurs as a result of a wide range of complications such as obesity, T2DM, inflammation, fibrosis, and the development of non-alcoholic steatohepatitis, and cirrhosis. Elevated serum-free fatty acid concentrations, hepatic triglyceride accumulation, cytotoxic reactive oxygen species, and increased levels of oxidative stress are believed to be major contributors to the development and progression of the disease. The present study highlights the application of the stable nitroxide radical TEMPOL as an effective redox sensor for redox changes monitoring in T2DM and NAFLD patients. The oxidative stress levels and antioxidant status were investigated in T2DM and NAFLD patients (group 2) and healthy volunteers (group 1) by conventional EPR spectroscopy. The obtained data show a statistically significant increase in ROS levels and EPR signal intensity of nitroxide and hydroxylamine in patients with NAFLD and T2DM compared to the control group - healthy volunteers without metabolic disorders (post hoc test; (*) $p < 0.05$ *vs.* control). The spectroscopic analysis allows the prediction of diabetic complications and will guide the scientific community and clinicians to conduct effective antioxidant therapy.

Keywords: oxidative stress, antioxidant enzymes, ROS, NAFLD, EPR, nitroxide radicals, TEMPOL

INTRODUCTION

Non-alcoholic fatty liver disease

Non-alcoholic fatty liver disease (NAFLD) is a common liver disorder characterized by fat accumulation in the liver cells. It is one of the most prevalent liver conditions in the world and can range from relatively benign to more severe forms that can lead to liver damage and other health complications. Non-alcoholic fatty liver is the milder form of NAFLD, where there is excessive fat accumulation in the liver, but little or no inflammation or liver cell damage [1]. The main risk factors of NAFLD include excess body weight, especially around the abdomen, insulin resistance often associated with type 2 diabetes mellitus (T2DM), high blood triglyceride and cholesterol levels (hyperlipidemia), metabolic syndrome presenting as high blood pressure, high blood sugar, excess body fat, and abnormal lipid profiles, family history of NAFLD, etc. It is known that there exists a close relationship between NAFLD and diabetes.

with diabetes are at an increased risk of developing NAFLD, and on the other, the presence of NAFLD can exacerbate metabolic disease since diabetes type 2 increases the risk of NAFLD, and having NAFLD can make it more challenging to manage blood sugar levels in the body [2].

Role of inflammation and oxidative stress in diabetes-related NAFLD

Non-alcoholic fatty liver disease is one of the most common chronic liver diseases in obese and diabetic patients, and its incidence continues to rise. The disease ranges from mild hepatic steatosis to liver fibrosis and cirrhosis, which increase overall mortality in elderly patients and those with chronic diseases [3]. Several studies have shown that insulin resistance (IR) plays a critical role in the pathophysiology of NAFLD and the natural history of the disease [4-6]. Accelerated lipolysis associated with IR increases hepatic glucose production in NAFLD patients, which up-regulates de novo fat synthesis, accelerating NAFLD progression [7].

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Other pathogenic pathways such as changes in lipid metabolism, mitochondrial leakage, and activation of pro-inflammatory cascades have been described in disease progression [8].

It is known that the reactive oxygen species (ROS) and inflammation play a significant role in the pathogenesis of diabetes and the progression of NAFLD, and that is accompanied by the development of oxidative stress (OS) in the body [9]. When diabetes and NAFLD coexist, these two processes can interact and exacerbate each other, leading to more severe liver damage. Inflammation is one of the most common players of liver damage in NAFLD, especially in the more severe form of NAFLD - non-alcoholic steatohepatitis. In individuals with diabetes, there is often a chronic low-grade inflammation throughout the body, known as systemic inflammation. This systemic inflammation can also affect the liver and contribute to the non-alcoholic steatohepatitis (NASH). In the liver, inflammation can lead to the recruitment of immune cells and activation of pathways that promote cell damage, fibrosis, and cirrhosis. Another factor in the NAFLD pathogenesis is OS, which is presented as abnormal or high levels of ROS and reactive nitrogen species (RNS) and reduced antioxidant defenses, or a combination of both [10]. At the rule, OS is a physiological condition that occurs in an imbalance between ROS or free radicals production, and the body's ability to neutralize or detoxify them with antioxidants. In NAFLD, the excess fat in the liver can lead to the production of free radicals, the process as slow as lipid peroxidation, which can cause cellular damage and inflammation. In NASH, the inflammation and immune response in the liver can further promote OS [11].

Inflammation in diabetes is driven by various factors, including the release of pro-inflammatory cytokines from fat tissue, activation of the immune system, and accumulation of advanced glycation end-products (AGEs) that can stimulate inflammation [12]. Elevated blood sugar levels can lead to the overproduction of reactive oxygen species and reduce the body's antioxidant defenses. Hyperglycemia can also lead to the formation of AGEs, which not only promote inflammation but also contribute to OS [13]. In individuals with diabetes-related NAFLD, there is a synergistic effect between inflammation and OS. Inflammation can promote OS, witch can further intensify inflammation [14]. This interplay can result in a vicious cycle where liver inflammation and OS can lead to NAFLD progression to more advanced stages, including fibrosis and cirrhosis [15].

Nitroxide radicals as a redox sensor in monitoring of diabetes and related complication

Nitroxides (aminoxyl radicals) are a class of paramagnetic heterocyclic nitroxide derivatives of piperidine, pyrroline, and pyrrolidine that possess an unpaired electron. The ability of nitroxides to interact with a wide range of free radicals determines their important biological significance [16]. They are characterized by unique antioxidant properties, the ability to modify OS and change the redox status of tissues by breaking down superoxide anion radicals and hydrogen peroxide, inhibiting Fenton reactions, and participating in radical-radical recombination reactions [17, 18]. The unpaired electron gives them paramagnetic properties, making them detectable by various spectroscopic techniques. By redox-transformation of one-electron transfer reaction, nitroxide becomes the reduced form hydroxylamine (>N-OH) and oxoammonium cation (>N=O+), and donates an electron, returning the non-contrast form to the contrast oxidized nitroxide radical (>N-O•) [17]. The involvement of nitroxide radicals in reversible redox reactions makes them valuable tools in studying redox processes at the cellular and molecular level in a wide range of fields, including chemistry, biochemistry, and biomedicine [19]. This is particularly important when studying the role of OS in the context of diseases such as cancer, neurodegenerative disorders, cardiovascular diseases, diabetes, etc [20]. As a redox-active species, nitroxide can provide information about the dynamic nature of redox processes in the body. This makes it useful tools in the development of diagnostic models, treatments, and interventions targeting diseases characterized by redox imbalance [21].

This study describes a new methodological protocol for the evaluation of redox imbalance in T2DM and non-alcoholic fatty liver disease and the application of EPR spectroscopy in monitoring the occurr redox changes.

EXPERIMENTAL

Sample preparation

The study used whole blood from T2DM and NAFLD patients (n=50) and clinically healthy volunteers (n=20) (age 45-65 years). The blood samples (900 μL) were mixed with 100 μL of nitroxide standards in DMSO (Sigma-Aldrich, Germany) and incubated for 30 min at room temperature. All reagents are of high purity ("HPLC-grade"). Quartz non-heparinized capillaries were used, which were placed in the EPR cuvette, and the measurement was started. EPR spectral analysis was

performed at room temperature. All EPR measurements were performed at the following parameters: microwave frequency – 9.4 GHz, magnetic field strength – 336 mT, microwave power – 2.0 mW, field modulation frequency – 100 kHz, field modulation amplitude – 0.063 mT, time constant – 0.01 s, sweep width – 10 mT, scan time (sweep time) – 2 min. Each sample was scanned in triplicate, and spectral processing was performed using Simfonia software, Win-EPR (Brucker, Germany). Results are presented as a percentage of the nitroxide radical/DMSO control and a.u. in hydroxyl amine.

RESULTS AND DISCUSSION

As a result of normal metabolic processes, ROS are constantly produced in the human body, as the main source of oxidants are mitochondria. On the one hand, low levels of ROS are involved in the immune response to deal with various pathogens, modulating and maintaining physiologically important redox reactions. However, higher concentrations of ROS lead to OS, which can lead to oxidative damage to cells and provoke metabolic collapse, compromising their normal functions [12].

Determination of total OS levels by electron paramagnetic spectroscopy (CW-EPR spectroscopy)

X-band EPR was used to determine the total OS levels. Solutions of the nitroxide radicals TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl, 4-hydroxy-TEMPO) (0.2 mM) and 3- carbamoyl-PROXYL (2,2,5,5-tetramethyl-1-pyrrolidinyloxy-3-carboxamide; 3-CP) (0.5 mM), which are characterized as suitable redox sensors for the determination of ROS levels ($O_2^{\bullet-}$, H_2O_2, etc.). A confirmatory assay was performed to determine superoxide radical levels using 0.5 mM hydroxyl amine 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetra-methylpyrrolidine (CMH) in DMSO. Pronounced changes in the EPR signal intensity of nitroxide radicals TEMPOL and 3-CP were observed in blood samples of patients with T2DM and NAFLD, compared to the control group (healthy volunteers, gray). The graph profile shows high levels of free radicals in patients with T2DM and NAFLD (Figure 1A and B), while normal redox status in healthy volunteers does not initiate a decrease in the signal intensity of the radical forms of nitroxide, as well as oxidation of the hydroxyl amine form (CMH). Values presented are mean ± SEM. *p < 0.05 compared to control group.

Oxidative stress occurs as a result of metabolic reactions with the participation of oxygen and is expressed in a violation of the pro-oxidant-antioxidant balance of cells and tissues. As a result, a redox imbalance in favor of pro-oxidants is observed, which is due to the excessive production of reactive oxygen and/or nitrogen species and the inability to overcome this superproduction of oxidants by the body's antioxidant defense. Various macromolecules such as lipids, nucleic acids, and proteins are major targets of oxidative damage involving ROS [22-25].

The high ROS concentrations can initiate lipid peroxidation, DNA oxidation, and irreversible oxidative modifications of redox-sensitive residues in proteins (including enzymes), leading to structural and functional changes in various macromolecules [26]. The control group included patients without diabetes, renal, respiratory, or cardiovascular diseases.

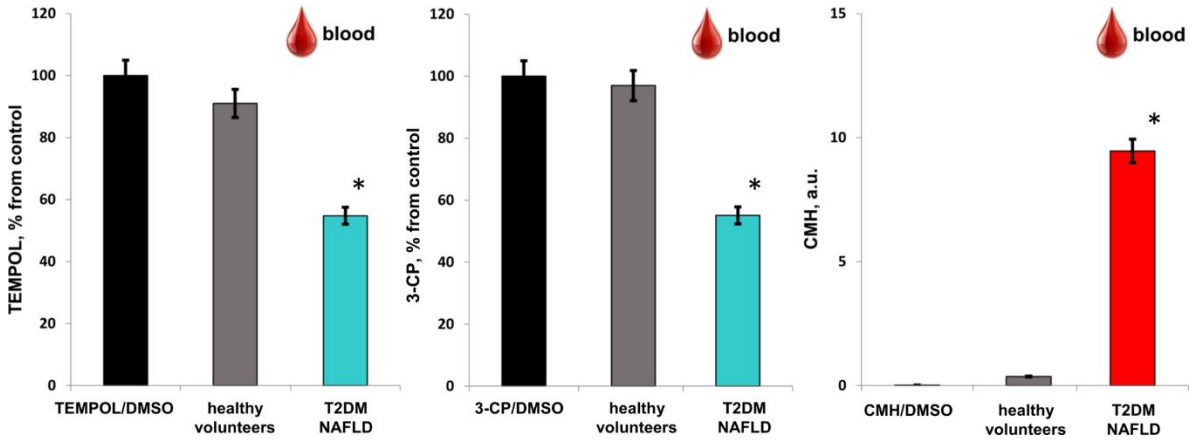

Figure 1. A, B, C. X-band EPR signal intensity in the T2DM and NAFLD patient blood samples, control nitroxide radical/DMSO (black) and healthy volunteers (in gray): (A) TEMPOL, (B) 3-Carbamoyl-PROXYL (3-CP), and (C) (CMH).

Oxidative stress plays a major role in the initiation and development of various pathological conditions such as diabetes mellitus (DM), cancer, atherosclerosis, neurodegenerative diseases, ischemia/reperfusion injury, aging, etc., and their resulting complications [27-29]. According to the literature data known to date, metabolic disorders are characterized by an increased production of ROS compared to a normal healthy organism. Hypoglycemia is believed to be involved in the production of free radicals, the initiation of OS, inflammation, hypercoagulation, and endothelial dysfunction and may lead to increased levels of oxidative markers of DNA damage, lipid peroxidation, etc. [9]. Diabetes is associated with several complications that are due to a higher production of free radicals and a sharp drop in the body's antioxidant defense expressed in reduced superoxide dismutase (SOD) and catalase (CAT) activity and leads to low concentrations of GSH and Vit E [30]. These complications are characterized by well-expressed changes in the normal redox balance and impaired activity of antioxidant protective enzymes, in conditions of hyperglycemia [12]. Disturbances in the normal metabolism of the body lead to a pronounced redox imbalance, which allows the use of nitroxide-enhanced EPR for monitoring OS and the degree of free-radical damage in patients with NAFLD and type 2 diabetes mellitus.

Abnormal levels of oxidants in these diseases can lead to a loss of the EPR signal of the nitroxide radical form (R-O•) and an increase in the EPR-silent hydroxylamine (R-OH) signal (Figure 1 A-C). The methodological approach presented in this article applies to direct and non-invasive assessment of OS in patients with NAFLD and T2DM. Diabetic patients have an increased risk of micro-, macro-vascular and cardiovascular complications, and increased mortality, with OS and cytokine expression considered to be one of the key sources of diabetic complications [9].

The EPR analysis with the participation of oxidized and reduced forms of nitroxides aims to show the application of their radical and hydroxyl-amine forms in determining the levels of free radicals and OS in patients with endocrine diseases. Through various acid and nitrogen radical and non-radical forms, transition metal ions, NAD(P)H, ascorbate, etc. the nitroxide radical, which gives an EPR signal, can be rapidly converted to the non-contrast form hydroxylamine or oxoammonium [31]. The presented results suggest that the nitroxide radicals TEMPOL and 3-CP can be successfully used for *in vitro* assessment of OS and redox imbalance in patients with T2DM and NAFLD. As a result of the high levels of free radicals, the EPR signal intensity decreased after 30 minutes of incubation of the blood sample in T2DM and NAFLD patients relative to the nitroxide / DMSO control, while in healthy volunteers, the reduction in nitroxide signal intensity was about 10% (Figure 1A and B).

3-CP is a neutral membrane-permeable nitroxide that can be distributed in the intracellular and extracellular environment and can be used to measure the levels of oxygen radicals [32]. A high concentration of ROS leads to a downgraded EPR signal of paramagnetic 3-CPH due to the formation of non-contrast hydroxylamine (3-CPOH) or oxidation of protonated superoxide (•OOH) to oxoammonium cation [33]. The results demonstrated that the intensity of 5-membered ring nitroxide 3-carbamoyl-proxy markedly reduced in diabetic patient blood samples, which can be a result of high ROS production, such as superoxide anion radicals and hydroxide radicals [34, 35] (Figure 1B).

The hydroxyl amine 1-hydroxy-3-methoxy-carbonyl-2,2,5,5-tetramethylpyrrolidine (CMH) is the most effective centrifugation probe for measuring ROS production [31] and characterize as a neutral detector for intra- and extracellular production of superoxide radical levels [36]. The interaction of hydroxylamine with ROS (O_2•$^-$ and H_2O_2) and RNS (peroxyl radicals or peroxynitrite) leads to the appearance of an EPR signal [37]. Abnormal levels of ROS in patients with T2DM, and in particular in oxidative stress-related diabetes and NAFLD, lead to oxidation of the hydroxylamine form of CMH and its conversion to the radical form (CM•). The normal redox state of the organism in healthy controls did not initiate the appearance of a significant EPR signal (Figure 1C). This result can be associated with low levels of ROS, well-coordinated redox regulation, and immune responses in healthy volunteers [38].

The results showed that EPR spectroscopy could be a powerful tool for assessing OS in endocrine conditions and their distinction. Based on this, the *in vitro* EPR could be successfully applied for the assessment of redox imbalance in oxidative stress-related metabolic diseases. Patients with T2DM and NAFLD are characterized by a completely different redox status compared to healthy people, which can be a basis for introducing antioxidant therapy to the main treatment.

CONCLUSION

The present study proposes a new reading of the application of nitroxide radicals and hydroxyl amines in monitoring of the patients redox status, in the context of T2DM and NAFLD-related OS. The

developed methodological protocol will allow a more detailed study of the dynamic nature of the disease and will contribute to the development of treatments and interventions aimed at limiting the effects of oxidative damage in patients with diabetes and its complications.

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