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# *EX VIVO* EPR EVALUATION OF OXIDATIVE STRESS BIOMARKERS IN DIABETES INDUCED WISTER RATS

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**ABSTRACT**

PURPOSE: By the present study using Electron Paramagnetic Resonance (EPR) spectroscopy method we report our investigations on some biomarkers of oxidative stress like as ascorbate radicals ($Asc^{\cdot}$) and ROS products in liver, kidneys and pancreas tissues isolated from streptozotocin (STZ) induced diabetes Male Wistar rats. Further, the effect of the melatonin (an antioxidant) on the levels of the same biomarkers was also studied.

METHODS: Male Wistar rats, separated into two groups, were used. First group was treated by a single injection of STZ at a dose of 60 mg/ kg and the second group - with the same dose of STZ and 7 consecutive days with melatonin (MLN) at a dose of 10 mg/ kg. The experiment lasted on 42-nd day, and livers, kidneys and pancreas homogenates isolated from tested rats was studied for the levels of $Asc^{\cdot}$ and ROS production by EPR spectroscopy methods.

RESULTS: Increased levels for the $Asc^{\cdot}$ and ROS products were established in the pancreatic and kidneys homogenates isolated from the rats treated by STZ and MLN comparing to those of the rats treated by STZ only. In the liver homogenates of the STZ and MLN treated group of animals were registered almost the same levels of $Asc^{\cdot}$ as those of rats treated with STZ, alone. At the same time ROS production in liver homogenates of STZ and MLN treated animals was statistical significant higher than that of the STZ treated rats.

CONCLUSION: These preliminary results demonstrate that the applied dose of melatonine was insufficient to overcome completely oxidative stress provoked by the STZ in the studied rats tissuses.

**Key words:** Diabetes; EPR spectroscopy, Antioxidants, Oxidative biomarkers.

## INTRODUCTION

Diabetes mellitus is a metabolic disorder, associated with many complications, which have been investigated in the experimental models of this disease (1). STZ a member of the glucosamine nitrosoureas class, exhibits strong selectivity and toxicity to pancreatic beta cells and is suitable for the induction of experimental diabetes in animals. STZ enters the target cells by GLUT-2 transporter and causes DNA alkylation and cell death (2-4).

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The literature contains many confusing and different data showing marked effects of diabetes on the antioxidant status of the organism (5). Increased oxidative stress is considered to play an important role in the pathogenesis of diabetes mellitus. Although the mechanisms underlying the diabetic changes are not currently understood, it is known that hyperglycemia leads to increased oxidative stress, since the production of several reducing sugars was improved. These reducing sugars can easily react with various biomolecules like functional proteins, enzymes, membrane lipids (a nonenzymatic glycation reaction), leading to increased production of oxygen-derived free radicals species, particularly superoxide ($O_2^{-}$), hydroxyl ($OH^{\cdot}$), hydroperoxyl ($HOO^{\cdot}$), peroxyl ($ROO^{\cdot}$), radicals, etc., by extracting an electron

from them to become stable structures (6). Thus, the chronic high glucose levels and the oxidative stress have detrimental effects on the structure and function of various organs (7).

The combination of diabetes - inducing drugs such as SZT with antioxidants, suggests a change in the levels of free radical formation in biological systems, in particular $Asc^{\cdot}$ , ROS and reactive nitrogen species. MLN- (N-acetyl-5 methoxy tryptamine), is an endogenous neurohormone produced by the pineal gland. It possesses antioxidant effect, does not undergo reduction and plays a role as a direct free radical scavenger (8, 9). Therefore, in studies of many researchers has been reported a reduction of oxidative stress by MLN application in experimentally induced diabetes (7, 10- 14).

The aim of the present research was using the unique technique for detection and characterization of free radical structures and antioxidant activity such as EPR spectroscopy to study the levels of oxidative biomarkers ($Asc^{\cdot}$ and ROS production) in STZ - diabetes induced rats with and without MLN treatment.

## MATERIALS AND METHODS

### *Chemicals*

STZ, MLN, Dimethyl sylfoxide (DMSO), N-tert-butyl-alpha-phenylnitrone (PBN) were purchased from Sigma Chemical Co, St. Louis, USA. All the other chemicals used in this study were with analytical grade.

### *Animals*

Male mature Wistar rats, weighting 300-360 g, were used. The animals were separated into 2 groups (8 rats per group). The first group was influenced by a single i.p. injection of STZ at a dose of 60 mg/ kg. The second group was influenced by a single i.p. injection of STZ at a dose of 60 mg/ kg and 7 consecutive days was treated with MLN at a dose 10 mg/ kg i.p. Only the rats with blood glucose levels more than 16 mmol/l, measured on the $72^{nd}$ hour after the STZ application, were considered to be diabetic. The study lasted at $42^{nd}$ days. Experiments were carried out in accordance with national regulations and the European directive 210/63/EU from 22.09.2010, concerning the protection of animals used for scientific and experimental purposes. The liver, kidneys and pancreas were immediately collected, washed in cool saline and tissues homogenates were prepared.

### *Electron Paramagnetic Resonance measurements*

For all EPR measurements an X-band $EMX^{micro}$, EPR spectrometer (Bruker, Germany) equipped with standard Resonator was used. Quartz capillaries were used as sample tubes. The capillary tubes were sealed and placed inside a standard EPR quartz tube (i.d. 3 mm, length 150 mm, wall thickness 0.1 mm) that was placed in the EPR cavity. All EPR experiments were performed in triplicate and repeated at room temperature ($18^{o}$C-$23^{o}$C). Spectral processing was performed using Bruker WIN-EPR and SimFonia software. The levels of the Asc radicals and ROS products were calculated by double integration of the corresponding EPR spectrum and expressed in arbitrary units.

### *a) EPR investigations of ascorbate radical levels in organ homogenates of diabetes induced rats.*

The $Asc^{\cdot}$ levels in organ homogenates were studied according to Buettner et al., 1993 (15) with modifications by Zheleva et al., 2011 (Zheleva et al., 2011; 16 **).** Tissues from liver, kidneys and pancreas were collected in cold saline and processed immediately. Samples were weighed and homogenized in DMSO (10% w/v) and centrifuged at 4000 g, at $4^{0}$C for 10 min. Supernatants were collected and the concentration of radicals was evaluated by EPR spectroscopy. EPR settings were as follows: center field- 3505 G; sweep width- 30 G; microwave power- 20.37 mw; receiver gain 1 x $10^{4}$; mod. Amplitude 5.00 G; time constant 327.68 ms; sweep time 81.92 s; 5 scans per sample.

### *b) EPR study on the levels of PBN adduct registered in organ homogenates of diabetes induced rats.*

The levels of ROS production was studied according to Shi et al., 2005 (17) with some modifications by Zheleva et al., 2011 (18). Briefly, about 0.1 g of organs samples were homogenized after addition 1.0 ml of 50 mM solution of the spin-trapping agent PBN dissolved in DMSO. EPR settings were as follow: center field- 3503.74g; sweep width - 100 G; microwave power- 20.49 mw; receiver gain 1 x $10^{6}$; mod. amplitude 0.50 G; time constant 327.68 ms; sweep time 81.92 s, 1 scan.

## STATISTICAL ANALYSIS

Statistical analysis was performed with Statistica 8.0, StaSoft, Inc. and results were expressed as means ± standard error (SE). A value of $p < 0.05$ was considered statistically significant.

## RESULTS

The stable EPR signal with a g value of 2.0047 ± 0.0002 , typical for the $Asc^{\cdot}$ was recorded in all organ homogenates **(Figure 1)**.

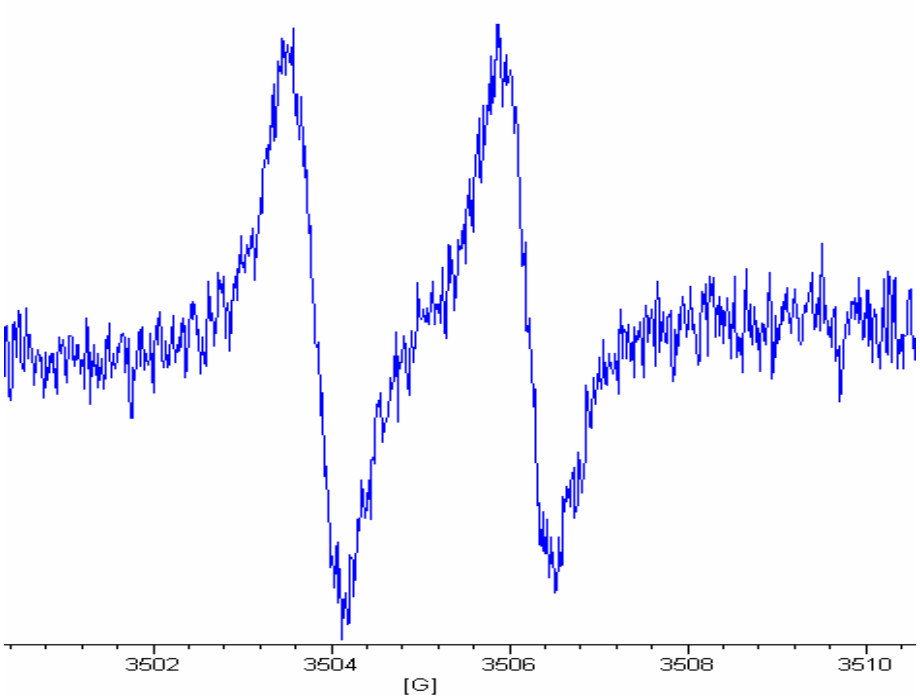


**Figure 1.** EPR spectrum of ascorbate radical registered in all rat tissues.

PBN six-line spin adducts were registered in all studied organ homogenates **(Figure 2).** Based on the calculated values of $a_N$ and $a_H$ slpitting constants of the registered PBN spin adducts (Takeshita et al., 2004, 19) the radicals trapped were recognized as high toxic $^{\cdot}OH$ radicals.

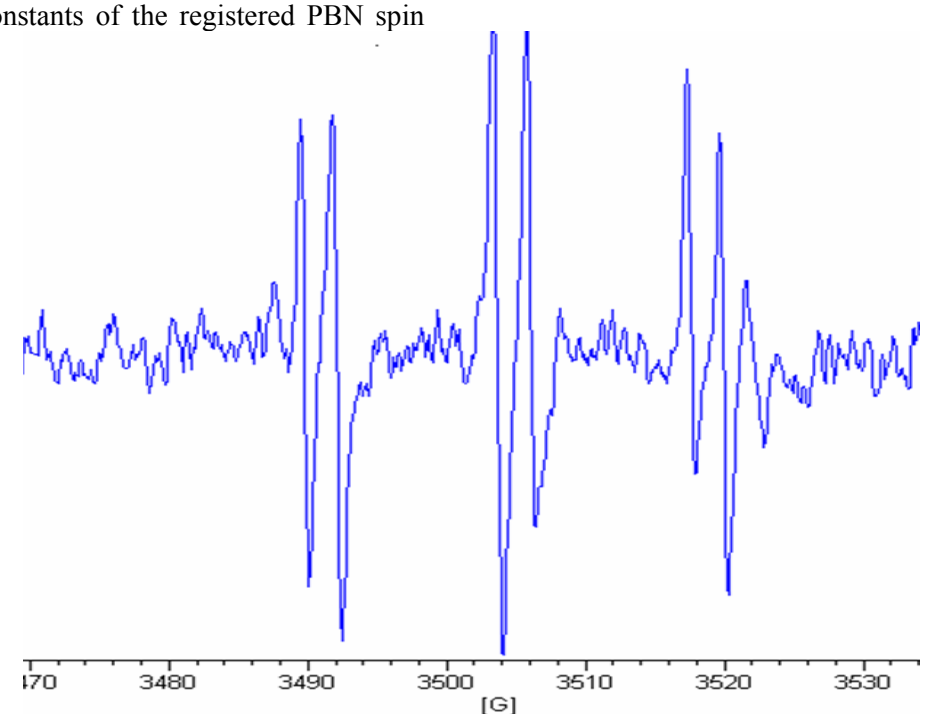


**Figure 2.** EPR spectrum of ROS products trapped as PBN spin adducts and registered in all rat tissues.

Results from the EPR *ex vivo* studies on the levels of oxidative stress biomarkers are shown on **Figure 3** and **Figure 4**.

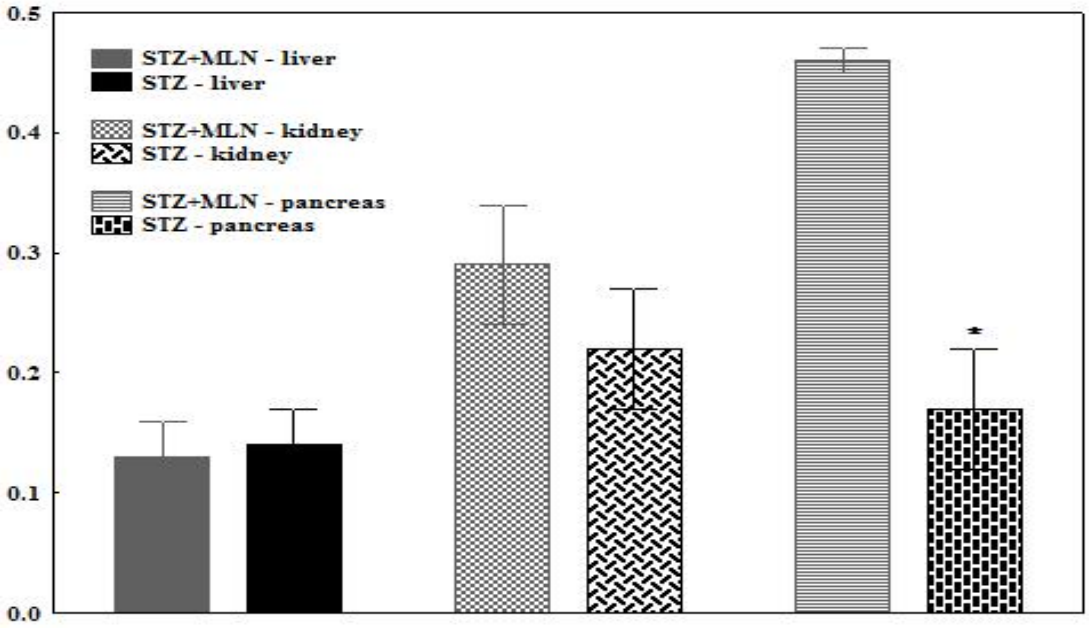


**Figure 3.** Levels of Ascorbate radicals in organ homogenates from Wistar STZ - induced rats and rats treated with combination of STZ + MLN.

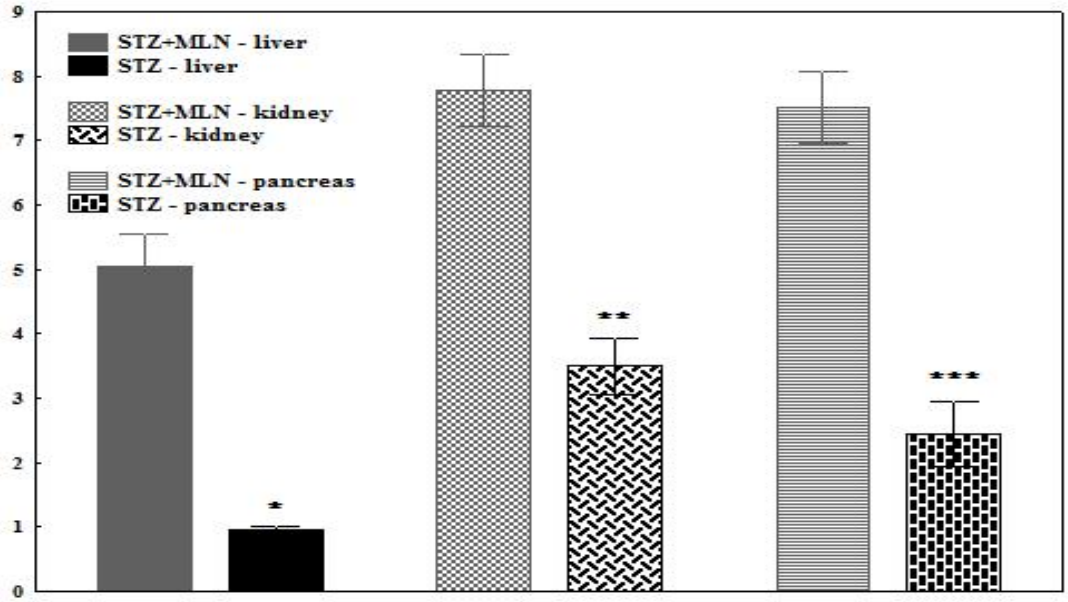


**Figure 4.** Levels of ROS products in organ homogenates from Wistar STZ - induced rats and rats treated with combination of STZ + MLN.

Statistical significant higher levels of Ascorbate radicals in pancreases and increased levels in kidneys (without statistical significance) of rats treated by STZ and MLN were found in comparison with those of rats treated by STZ, only **(Figure 3)**. In the livers of rats treated by STZ and MLN was found slight decrease in the levels of $Asc^{\cdot}$, comparing to the STR treated animals.

As is also seen on **Figure 4**, the levels of ROS products in liver, kidney and pancreas of rats treated with STZ and MLN were significantly higher than those of rats treated with STZ, only.

## DISCUSSION

Oxidative stress induced by drugs generates free radicals, that play a critical role in the initiation and progression of oxidative damage in the biological systems (20). The oxidative stress induced in rats treated with STZ was not decreased when MLN was applied at a dose of 10 mg/kg. Since, ascorbate radical is a real-time biomarker of the oxidative status in biological systems (14) and based on our present results it is obvious that in spite application of STR together with MLN, oxidative reactions are still going off in the tissues of the treated rats. Apparently, the first 7 days treatment by MLN was not enough to improve the oxidative status of the tested animals. Because diabetes is a chronic illness and complications occur in a long period of time, a long-term use of melatonin in diabetics is necessary to be investigated (21).

## CONCLUSION

We consider that 7-days period of MLN administration was insufficient to overcome completely the provoked oxidative stress in STZ – induced diabetic rats. Further studies with joint application of MLN with other proper antioxidants are in progress in our laboratory.

## ACKNOWLEDGEMENTS

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