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6-METHOXYTRYPTOPHOL REDUCES LIPOPOLYSACCHARIDE-INDUCED LIPID PEROXIDATION *IN VITRO* MORE EFFECTIVELY THAN MELATONIN

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Bacterial lipopolysaccharide (LPS) causes lipid peroxidation (LPO). We have found that LPS induces LPO in tissue homogenates *in vitro* in a concentration-dependent manner, the concentration of 400 μg/ml demonstrating the most efficient lipid damaging effect. Antioxidant properties of melatonin are unquestionable and have been proved both *in vivo* and *in vitro*. It has been demonstrated that also melatonin metabolites and derivatives inhibit oxidative stress. The aim of our study was to compare the effects of melatonin (MEL) and indole compound: 6-methoxytryptophol, on LPS-induced LPO *in vitro*. Malondialdehyde (MDA) plus 4-hydroxyalkenal (4-HDA) concentrations were measured as the indicators of induced membrane peroxidative damage in brain, liver and kidney homogenates. Both melatonin and 6-methoxytryptophol were used at increasing concentrations, starting from 0.01-5 mM, together with LPS at one concentration of 400 μg/ml. In all the examined tissues, LPS stimulated LPO, while both melatonin and 6-methoxytryptophol released LPS-stimulated LPO. Furthermore, the capacity of 6-methoxytryptophol reducing LPO was higher than that of melatonin. The results of the reported study clearly indicate that 6-methoxytryptophol is a much stronger antioxidant *in vitro* than melatonin in terms of reducing oxidative damage to lipid membranes. However, it remains still unclear how the features relate to *in vivo* circumstances.

Key words: *6-methoxytryptophol, free radicals, indole derivatives, lipopolysaccharide, lipid peroxidation, melatonin, nitric oxide*

INTRODUCTION

Lipopolysaccharide (LPS) is an endotoxin produced by Gram-negative bacteria. It has been demonstrated that an addition of LPS impairs the structure and function of tissues and organs, a.o., lungs, kidneys, heart and brain. Oxygen is essential for life of organisms but its 5% is metabolized to free radicals and, due to oxygen ubiquity, this type of reactive species seems to be both the most important and common. LPS, used in large doses, stimulates the production of reactive oxygen and nitrogen species, especially of all superoxide anions (O_2^-), hydroxyl radicals (OH) and nitric oxide (NO) (1-3). According to some models, all these substances mediate tissue damage (1, 4, 5). The above-mentioned free radicals are also well-known promoters of lipid peroxidation, the severity of which can be assessed by measurements of malonaldehyde (MDA) or conjugated diene concentrations (6-8). An administration of compounds with antioxidant properties inhibits destructive lipopolysaccharide action. Melatonin (MEL), which is the main hormone produced by the pineal gland, strongly inhibits the action of many toxic substances, some of which exert their effects by generating free radicals (9, 10). Due to good solubility in fats and hydrophilic properties, high concentrations of melatonin may be present both in body fluids and in intracellular cavity (11). Melatonin inactivates primarily: hydroxyl (12, 13) and peroxyl radicals, hydrogen peroxide, hypochlorus acid, singlet oxygen, peroxynitrite and superoxide anion, as well as many others (8, 14-17).

It has been proved that melatonin protects tissues from damage, caused by many harmful compounds and in various conditions (2, 18-25). Melatonin also exerts its antioxidant activity against LPS (3, 26-29). Research data from recent years show that also melatonin intermediates possess antioxidant properties, a.o. tryptophan and serotonin (30, 31). It was shown in our previous paper, that N-acetylserotonin, the precursor of MEL reduced LPS-induced lipid peroxidation *in vitro* much better that melatonin (26). Several indole derivatives, similar to melatonin, a.o., 5-hydroxytryptophol, 5 hydroxytryptophan, 5-methoxytryptophol, 5-methoxytryptamine, pinoline (6-methoxy-1,2,3,4-tetrahydro-beta-carboline) and tryptoline (1,2,3,4-tetrahydro-beta-carboline) also exert their antioxidant activity in different experimental conditions (32-36). 6-methoxytrytophol (6MTX) (*Fig. 1*) is an indole compound, which is neither melatonin metabolite nor its precursor. However, due to its similar structure, it can also possess antioxidant properties.

In presented study, we evaluated the abilities of melatonin and 6MTX to prevent LPS-induced tissue damage. The antioxidative properties of 6MTX had never been studied before. Compound efficacy was based on the measurements of malondialdehyde and 4-hydroxynonenal concentrations as the indicators of cell membrane lipid peroxidation severity.

MATERIAL AND METHODS

Reagents

LPS (from *Escherichia coli* serotype 0111:B4), melatonin, 6-methoxytryptophol and ferrous sulphate were purchased from Sigma-Aldrich (St. Louis, USA). A Bioxytech LPO-586 kit was used to measure lipid peroxidation products; the kit was obtained from Cayman Chemical (Ann Arbor, MI).

Methods

Six adult Wistar male rats (body weight = 200±40 g) were kept in two plexi cages (3 animals per one cage). The room, where the animals were kept, was equipped with temperature and lighting control (light was on at 07.00 a.m. and off at 9.00 p.m.; 14 h light/10 h dark). Constant temperature of 22±1°C was maintained. The rats received food (standard laboratory chow) and water *ad libitum*. Melatonin and 6-methoxytryptophol were dissolved in absolute ethanol (when the homogenates were added, the final concentration of alcohol in solution was 1%). Lipopolysaccharide was dissolved in 20 mM Tris-HCl buffer, pH 7.4.

Sample preparation and assays

The animals were anaesthetized with ether and then decapitated. The organs, needed for the experiment, including livers, kidneys and brains, were collected and frozen in dry ice. Tissues were kept frozen at -80°C until assayed. On the day of assay, the organs were homogenized in a 20 mM Tris-HCl buffer, pH 7.4 with a Polytron-like stirrer. Homogenates were prepared in concentrations of 1 to 10. LPS (400 μg/mL) was added to each sample (3). For purpose of the experiment, six different doses of MEL and 6MTX (0.01–5 mM) were used. According to our previous studies, 10 mM of $FeSO_4$ was added to liver homogenates as an additional enhancer of lipid peroxidation (3, 26). The prepared samples were incubated at 37°C for 30 minutes. After incubation, the samples were centrifuged at 2500 g for 5 minutes at 4°C and supernatants were collected and promptly assayed for lipid peroxidation products: malodialdehyde (MDA) + 4-hydroxynonenal (4-HDA) (6). The Bioxytech LPO-586 kit was used for that purpose (37). One of the advantages of this kit is the possibility to obtain reaction products (MDA and 4-HDA) at relatively low temperature of 45°C and the selected wave length of maximum absorbance (586 nM) allows to avoid interference and artifacts. In order to assess protein concentrations, the samples were assessed by the Bradford`s method, using bovine serum albumin as standard (38).

Statistical analysis

The data were analyzed, using the one-way analysis of variance (ANOVA) and Student's t-test. If F values were significant, the Student-Newman-Keuls t-test was used. All the calculations were performed by the Statistica '99 computer software. The level of significance was accepted at $p<0.05$.

RESULTS

According to our assumptions, lipopolysaccharide, in concentration of 400 μg/mL, caused lipid peroxidation in all the examined organs (*Fig. 2-4*). Both melatonin and 6MTX inhibited that process in all of the examined tissue homogenates (*Fig. 2-4*). A statistically significant effect of lipid peroxidation inhibition in brain homogenates was obtained for melatonin at concentrations of 5 and 2.5 mM (*Fig. 2*), while 6MTX showed significant effectiveness at concentrations from 0.01 to 5 mM (*Fig. 2*). In liver homogenates, both indole derivatives exerted beneficial inhibitory effects for all the six concentrations (*Fig. 3*). In the third examined organ - the kidneys, the concentration of lipid peroxidation products was reduced in a statistically significant manner for melatonin concentrations of 0.5 to 5 mM (*Fig. 4*), and from 0.1 to 5 mM for 6-methoxytryptophol concentrations (*Fig. 4*). IC_{50} values for melatonin were higher than for 6MTX in homogenates of all the examined organ, especially in the brain homogenates (IC_{50}=204.5 mM).

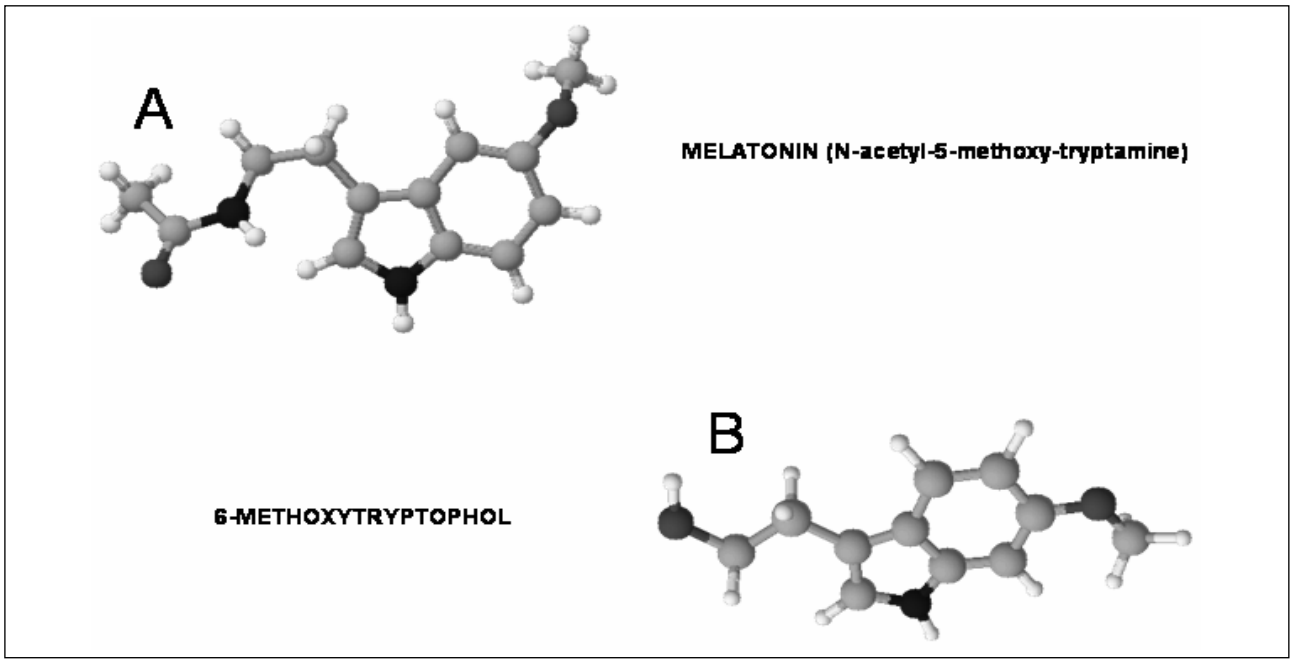


Fig. 1. Chemical sketches of melatonin (A) and 6-MTX (B) molecules.

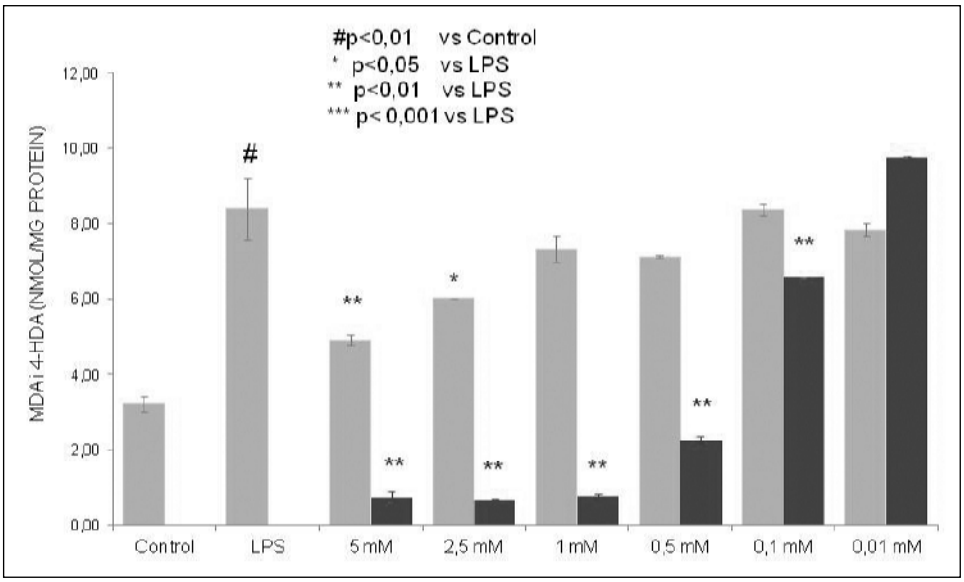


Fig. 2. Lipid peroxidation (MDA+4-HDA) induced by lipopolysaccharide (LPS) in brain homogenates, in the presence of melatonin (MEL) and 6-methoxytryptophol (6MTX).

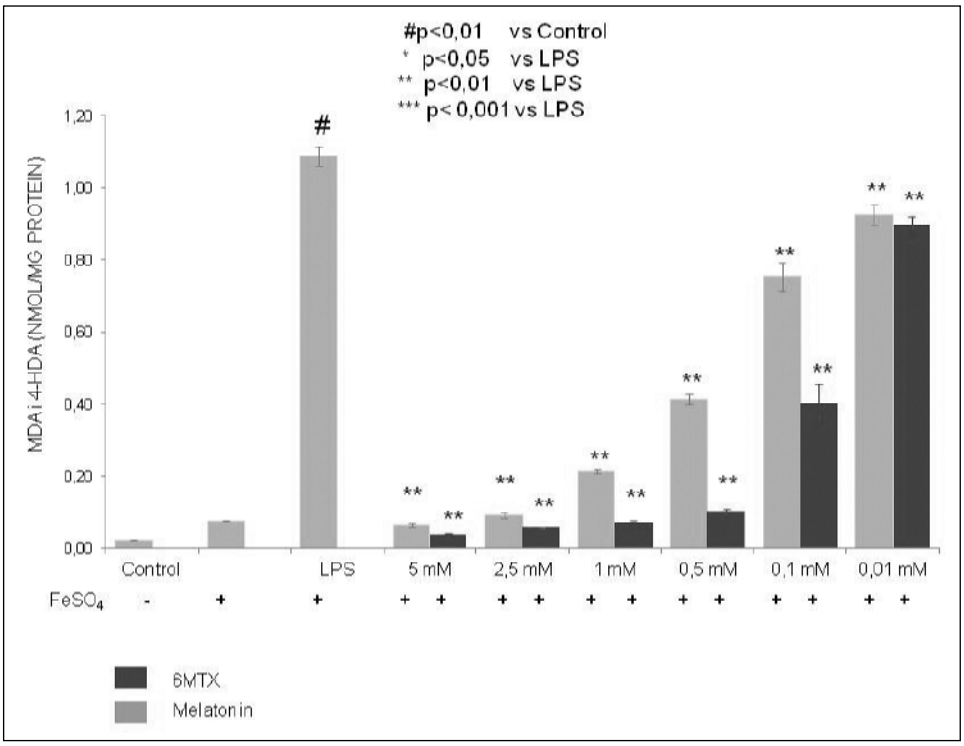


Fig. 3. Lipid peroxidation (MDA+4-HDA) induced by lipopolysaccharide (LPS) in liver homogenates, in the presence of melatonin (MEL) and 6-methoxytryptophol (6MTX).

The obtained results clearly show that both melatonin and 6-methoxytryptophol decreased lipid peroxidation, previously induced by LPS, in all the examined tissues. Additionally, 6MTX exerted stronger inhibitory effects (*Fig. 5*).

DISCUSSION

The presence and production of lipopolysaccharide are characteristic of Gram-negative bacteria. LPS is responsible for the pathogenicity of these microorganisms. Lipopolysaccharide is a well-known stimulator of lipid peroxidation. It induces the synthesis of nitric oxide by catalyzing L-arginine oxidation to citrulline and to the toxic form of nitric oxide (NO•) (39). The reaction of nitric oxide with superoxide anion (O_2•$^-$) leads to the formation of highly toxic peroxynitrite anion ($ONOO^-$), with successive derivatives, *e.g.*, NO_2•, CO_3•$^-$ are even more reactive. It has been shown that LPS is a stimulator of the production of many reactive oxygen species (1). The relationship between the overproduction of free radicals, including O_2•$^-$, H_2O_2, OH•, hypochlorus acid and damage of many tissues and organs, has been proven as well (4, 40). Fortunately, live organisms evolve numerous defense mechanisms. Many enzymes and compounds, *e.g.*, superoxide dysmutase (SOD), catalase, glutathione peroxidase, vitamin C and E, are used to protect tissues from damage caused by oxidative stress. LPS effects on activity changes of antioxidant enzymes have, so far, been analysed in several models, including our previous paper (26).

In the present study, LPS was used at concentration of 400 μg/ml and in conformity with our previous results (3, 26), causing significant lipid peroxidation in all the examined organs. Both indole compounds: melatonin and 6-methoxytryptophol,

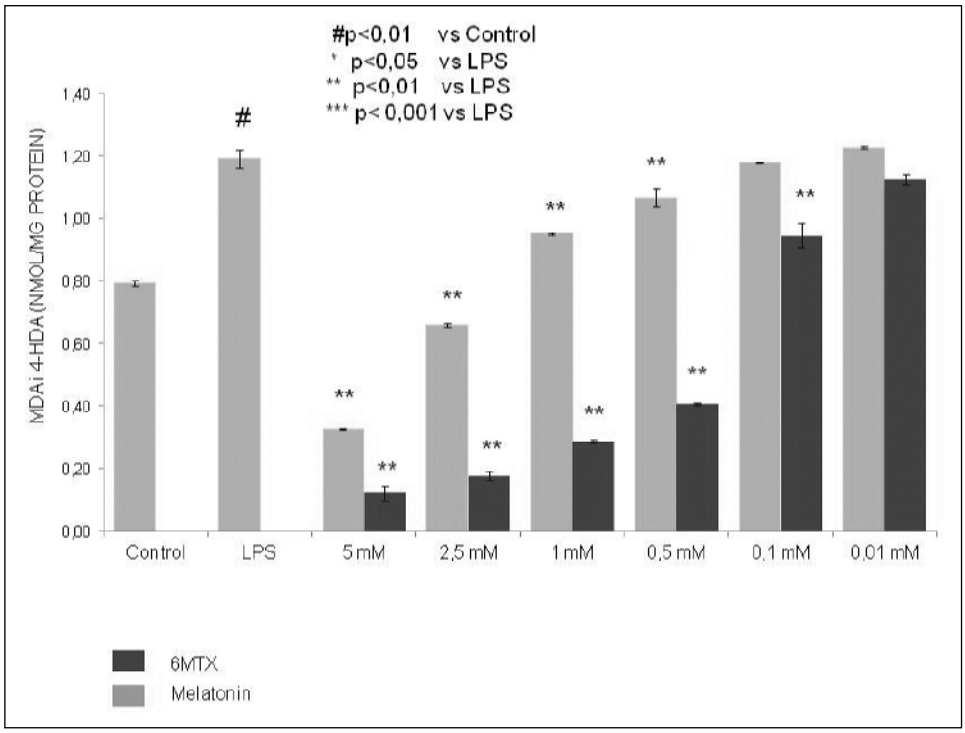
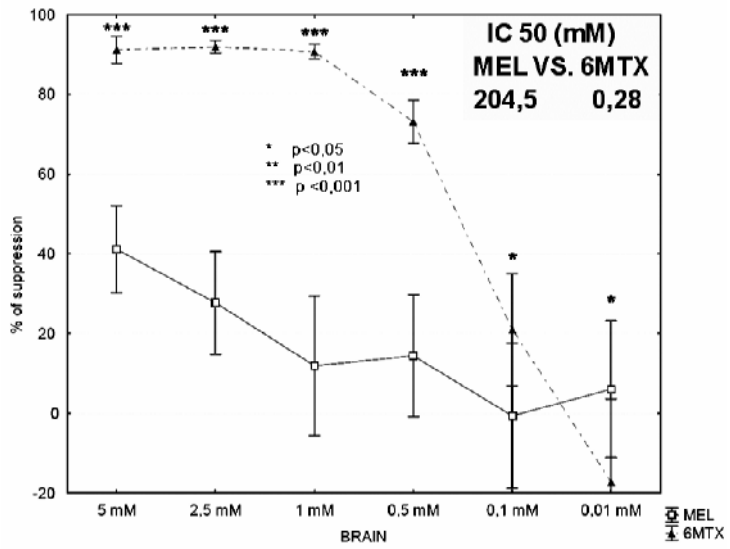


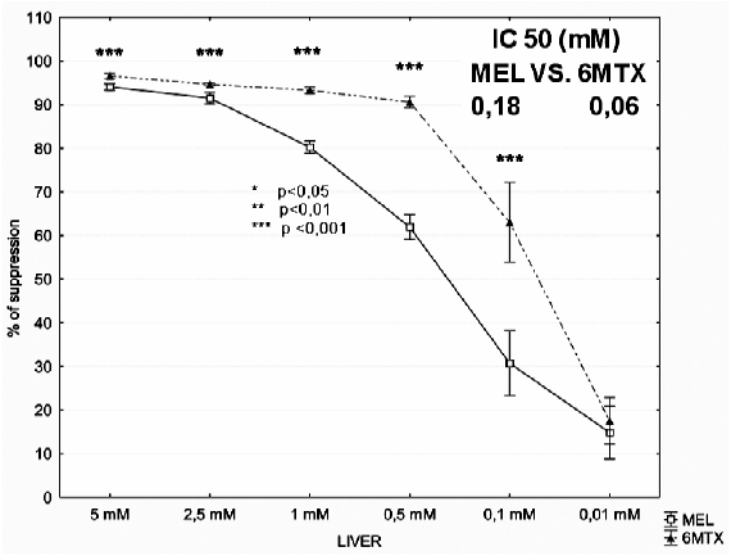
Fig. 4. Lipid peroxidation (MDA+4-HDA) induced by lipopolysaccharide (LPS) in kidney homogenates, in the presence of melatonin (MEL) and 6-methoxytryptophol (6MTX).

effectively inhibited that process in all the organ homogenates. The strongest effects of stimulation and inhibition of lipid peroxidation were observed in liver homogenates, in which $FeSO_4$ was used as an additional stimulator of peroxidation (8, 41). Ferrous sulfate stimulates hydroxyl radical production in Fenton`s reaction. Our present and previous experiments have proven high efficacy of indole compounds in their scavenging effects on hydroxyl radicals (3, 26). The role of melatonin in preventing tissue damage by reactive oxygen species is unquestionable. Melatonin may exert its effect directly, neutralizing primarily hydroxyl radicals (12, 13), hydrogen peroxide, hypochlorus acid, superoxide anion radical, singlet oxygen and many others (8, 14-17, 32). It has been demonstrated that melatonin stimulates antioxidant enzymes, such as superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and also glucoso-6-phosphate, dehydrogenase (42) the high activity of which is the source of NADPH, a substrate for glutathione reductase. Melatonin also inhibits the activity of prooxidant enzymes, a.o., nitric oxide and aminolevulinate synthases (42-45). An additional advantage of this indole compound is its hydro-and lipophilicity that greatly increases its bioavailability. Moreover, there is evidence that the precursors, derivatives and metabolites of melatonin are also able to scavenge free radicals. In our previous experiment, it was shown that N-acetylserotonin - a direct precursor of MEL - has got stronger antioxidant properties than melatonin (26). N-acetylserotonin effectively inhibited lipid peroxidation process that was induced by LPS in all the examined organs: the liver, the brain and the kidneys, while the eventual effect was concentration dependent (26). Our results are consistent with the findings of other authors (46-49) while, on the other hand, there are also some reports that call into question our data (50-52). Several indole derivatives, similar to melatonin, a.o., 5-hydroxytryptophol, 5 hydroxytryptophan, 5-methoxytryptophol, 5-methoxytryptamine, pinoline and tryptoline also exert their antioxidant activity in different experimental conditions (32-36). It has been demonstrated that 5-hydroxytryptophan is a stronger neutralizer of hydroxyl radicals than melatonin and vitamin C *in vitro* (34). It is suggested that pinoline is a more powerful antioxidant *vs*. melatonin, N-acetylserotonin, 5-hydroxytryptophan, 5-methoxytryptamine, 5-methoxytryptophol and tryptoline *in vitro* (32). It has also been proved that pinoline inhibits LPS activity 2.8 × more strongly than Trolox (water soluble vitamin E) in retina (35). In addition, Matuszak *et al*. (53) have proven that melatonin has a comparable efficacy to 5-hydroxytryptamine, 5-methoxytryptamine, 6-chloromelatonin and kynuramine in scavenging hydroxyl radicals *in vitro* (Fenton`s reaction with DPMO). In contrast to unhydroxylated indole derivatives (melatonin, 6-chloromelatonin, 5-methoxytryptophol), the hydroxylated ones (5-hydroxytryptophol, 6-hydroxymelatonin) can react both as promoters and neutralizers of hydroxyl radicals. It has also been demonstrated that melatonin neutralizes singlet oxygen equally effective as tryptophan, 5-methoxytryptamine, 5-hydroxytryptamine, 6-chloromelatonin or 6-hydroxymelatonin (16). Melatonin is most sensitive to O_2• bleaching, in comparison to other tested indoleamines, which could result in better neutralization of singlet oxygen. It has been proven that the presence of hydroxyl group in the molecule increases the sensitivity to photodegradation (16). 6-methoxy-2-benzoxazolinone (6-MBOA) is a plant origin compound, structurally similar to melatonin and 6-methoxytryptophol. Apart from its antioxidant properties, this substance exerts antimicrobial and antifungal effects (36). The indole compounds inhibit oxidative processes with variable effectiveness and potency. It seems that the presence of additional substituents and changes in their position can modify their solubility and affinity. In addition the kind of experimental model and the types of radicals produced are also very important. According to Poeggeler *et al*.' (54) conclusions, the biggest role is played by the presence and position of O-methyl and N-acetyl groups in the indole derivative molecules. In the reported experiment, 6MTX was a stronger antioxidant than melatonin. The IC_{50} values of the tested compounds in all the examined organs were significantly lower in case of 6MTX. In liver homogenates, where an additional production of hydroxyl radicals was evoked, both tested compounds showed potent, almost similar inhibitory effects. We suggest that both MEL and 6MTX exert strong inhibitory effects against hydroxyl radicals. In brain and kidney homogenates, the rate of lipid peroxidation inhibition could depend on the different

A. brain homogenates



B. liver homogenates



C. kidney homogenates

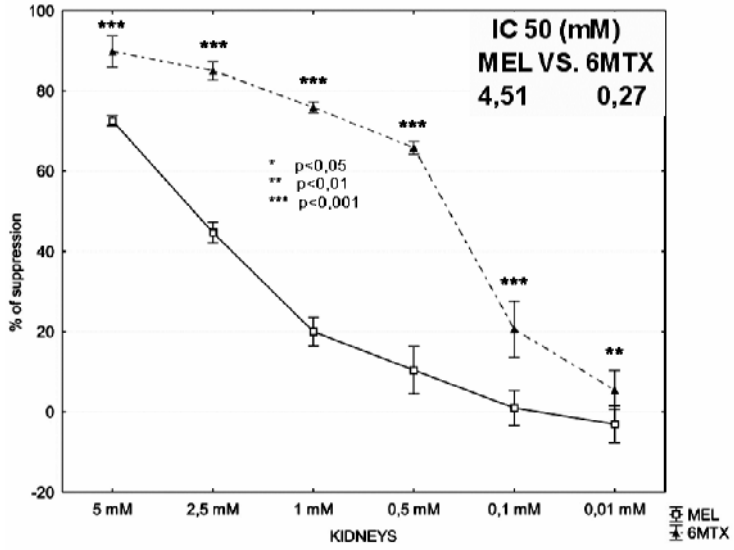


Fig. 5. Efficacy of lipid peroxidation inhibition by melatonin (MEL) and 6-methoxytryptophol (6MTX).

reaction of MEL and 6MTX on other than hydroxyl radicals. No data about antioxidative properties of 6MTX had been published before.

Despite the fact that nearly 20 years have passed since the discovery of antioxidative properties of melatonin, the knowledge has constantly been updated with new reports. In addition, numerous attempts of synthesis of new compounds, based on its structure, are undertaken to create molecules, whose medicinal properties (including antioxidative one) would be stronger and more durable (55).

In summary, our results indicate that 6-methoxytryptophol is a more effective inhibitor of lipid peroxidation *in vitro* than melatonin. Further *in vivo* studies are needed to show its antioxidative properties.

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Conflict of interests: None declared.

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