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The Effects of Papaverine and Melatonin on the Biosynthesis of Reduced Glutathione in Selected Organs in Mice

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Papaverine – (3,4-dimethoxybenzyl)-6,7-dimethoxyisoquinoline, PAP) is a member of the benzylisoquinone subgroup of the opium alkaloids. It has been widely used for treating such diseases as pulmonary arterial embolism or renal and biliary colic. The aim of the research was to evaluate the influence of papaverine and melatonin on GSH metabolism in blood, brain, liver and kidney of mice. The distinct decrease in the GSH concentration was found after injection of papaverine. This decrease was reversed by earlier injection of melatonin. The obtained results suggest that the inhibiting effect of papaverine on the GSH level is connected with disturbances in energy mechanisms in cells, mainly inhibition of aerobic energy metabolism.

Key words: blood, brain, kidneys, liver, melatonin, mouse

INTRODUCTION

Reduced glutathione (GSH) participates in many physiologically important cell functions, partly because of its wide spread inside cells, but also because of its high concentration in cells and tissues. Due to its reduction properties it plays particularly important role in protection of cells against toxic effects of endogenous substances and xenobiotics. Interestingly, GSH is not an enzyme but it may participate as a co-substrate in some enzymatic reactions (Keller et al., 1990), e.g. it participates in phase II (conjugation) reactions of xenobiotic biotransformation (Sipes and Gandolfi, 1991). GSH also takes part in many other important cellular functions, such as DNA and protein synthesis regulation, apoptosis control and regulation of the cell cycle (Vina, 1990).

Similarly to GSH, melatonin (Mel) brings many benefits as regards preventive treatment, it delays the aging process, helps with the treatment of cancer, heart disease, Alzheimer's disease, cataracts, Parkinson's disease, multiple sclerosis, AIDS, it raises the body's resistance to many diseases, lowers cholesterol levels and normalizes blood pressure, reduces susceptibility to stress

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and provides a calm, physiological sleep, reduces the side effects of radiation and surgery, and neutralizes free radicals (Reiter et al., 2010; Bubenik and Konturek, 2011).

In animal cells, the level of GSH and its cellular functions depend not only on environmental factors and body condition but often also on the administered drugs which in addition to a therapeutic effect can also have an adverse influence. The question is whether papaverine as a substance with negligible toxicity possesses such features. Therefore, the aim of the study was to determine the concentration of GSH in the blood, brain, liver and kidneys of mice after a single administration of papaverine hydrochloride, melatonin alone, and melatonin followed by the injection of papaverine.

MATERIALS AND METHOD

The experiment was carried out on 72 four-month-old Swiss male mice with the average body weight of 27 g. The animals were kept in cages with full access to standard food and water throughout the experiment. Lighting was regulated in a cycle of LD 12:12 (light phase from 8 a.m. to 8 p.m.). The breeding room was soundproof; the average temperature was $20^0C \pm 2^0C$ and relative humidity was 55% ± 5%.

The animals were divided into 4 main groups: control and three experimental groups. The control animals received an intraperitoneal injection of 0.3 ml of 0.9% NaCl, the experimental groups were treated with 40 mg/kg b.w. of papaverine hydrochloride (Polfa, Poland), 10mg/kg b.w. of melatonin (Sigma, St. Louis, USA), and melatonin followed by papaverine hydrochloride one hour later, respectively. All the drugs were administered at 8 a.m. Each main group was divided into 3 subgroups which were decapitated at 3, 6 and 24 hours after drug injection. Before decapitation the animals were anesthetized by i.m. injection of 35 mg/kg b.w. of Vetbutal (Biowet, Poland).

Blood was collected from the carotid artery and deproteinized with 10% trichloroacetic acid (TCA) and EDTA (1:1 v/v). The mixture was placed in a refrigerator at 4^0C for 10 min, then centrifuged for 5 min at 5 000 rpm at 4^0C in a MPW-365 centrifuge.

The brain, liver and kidney were homogenized in a homogenizer with a Teflon plunger in 6 ml of cold 0.1 M phosphate buffer, pH=7.4, containing 10 mM EDTA. Homogenates were centrifuged in a MPW-365 centrifuge at 4^0C for 15 min at 15000rpm. The obtained supernatants were deproteinized in the same manner as the blood was.

GSH concentrations were determined in the blood and tissues by the colorimetric Ellman method (1959). Briefly, the samples of supernatants (200 µl) were added to a mixture containing 2.3 ml of distilled H_2O, 300 µl 3.2 M Tris-HCl, pH 8.1, and 100 µl 10 mM EDTA. Then 100 µl of 5,5'- dithiobis –(2-nitrobenzoic acid) (DTNB) dissolved in 0.05 mM acetate buffer, pH=5.0 were added. After 10 minutes the extinction was read out using a MARCEL S 330 spectrophotometer, at a wave length of 412 nm.

The obtained results were calculated by Student's t-test and analysis of variance using STATISTICA 9 (StatSoft).

RESULTS

The results obtained for the concentration of GSH in the blood, brain, liver and kidneys of male mice of the control and experimental groups at 3, 6 and 24 hours after the drug treatment are summarized in Figures 1-4.

In comparison with the control values, the intraperitoneal injection of papaverine hydrochloride at a dose of 40 mg/kg b.w. resulted in a significant decrease in GSH concentration at 3 and 6 h after the injection in all the tested tissues (Figures 1-4). After 3 and 6 hours the GSH concentration in blood decreased by 30.80 % and 24.80 % , in the brain by 32.65 % and 49.62 %, in the liver by 27.31 % and 34.83% and in the kidney by 18.10 % and 15.19 %, respectively ($P<0.001$). After 24 hours the level of GSH was similar to the control values.

Intraperitoneal injection of melatonin caused a statistically significant increase in GSH concentration in blood after 3 hours ($P<0.001$), 6 hours ($P<0.001$) and 24 hours ($P<0.01$, Fig.1). As regards the brain, melatonin significantly increased the concentration of that tripeptide only after 3 h ($P<0.001$). After 6 h and 24 h the effect of melatonin on the GSH concentration was not statistically significant (Fig. 2). In the liver, a statistically significant increase in the GSH level also occurred only after 3 h ($P<0.001$, Fig.3). As regards the kidneys, the intraperitoneal administration of melatonin re-

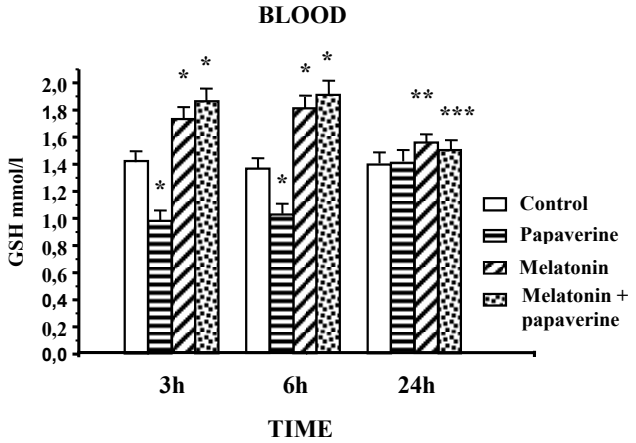

Fig. 1. GSH concentration in the blood of mice treated with a single injection of papaverine, melatonin, and melatonin followed by papaverine (X+SEM, *P<0.001, **P < 0.01; ***P <0.05). Each value represents 6 animals.

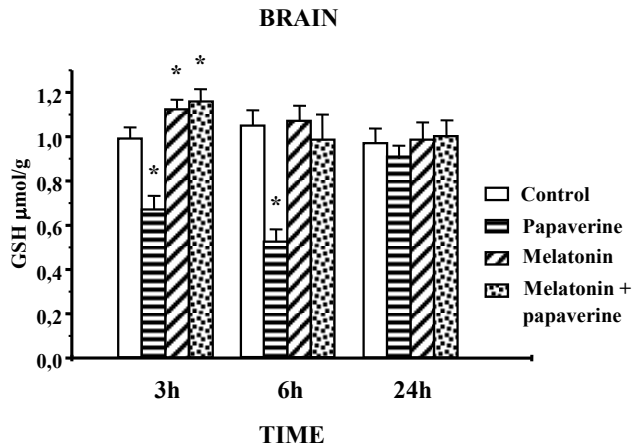

Fig. 2. GSH concentration in the brain of mice treated with a single injection of papaverine, melatonin, and melatonin followed by papaverine (X+SEM, *P<0.001). Each value represents 6 animals.

sulted in a statistically significant increase in GSH concentration after 3 h (P<0.01, 11.18 %) and 6 h (P<0.001; 15.67%). After 24 h melatonin did not have any effect on the GSH concentration (Fig. 4).

In comparison with the control values, the intraperitoneal administration of melatonin (10 mg/kg b.w.), followed by papaverine chloride (40 mg/kg b.w.) one hour later, resulted in a statistically significant increase in GSH concentration in the blood at 3 h (P<0.001; 25.92%), 6 h (P<0.001, 39.49%) and 24 h (P<0.05, 6.77%) after the injection of papaverine chloride (Fig.1).

These drugs caused a statistically significant increase in GSH concentration in the brain only after 3 h (P<0.001, 16.83%), while no significant reduction was found after 6 h (6.82%) (Fig.2).

In the case of liver and kidney, a statistically significant increase in GSH concentration was found after 3 and 6 h. After 3 and 6 h, the increase in the concentration of GSH in the liver was 15.14% (P<0.001) and 8.49 % (P<0.05), and in the kidney 21.74% (P<0.001) and 23.86 % (P<0.001), respectively. After 24 h the level of GSH was similar to the control values (Fig. 3, 4).

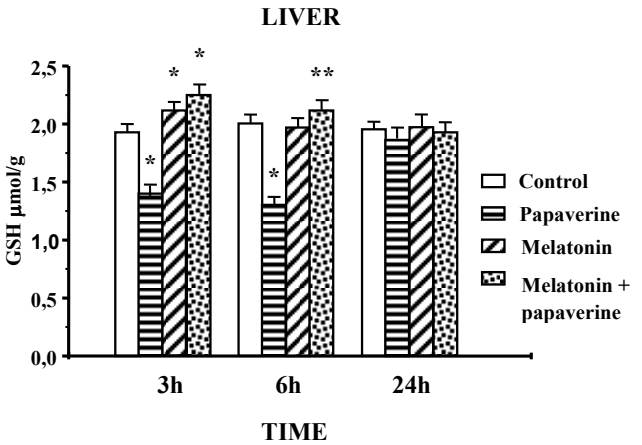

Fig. 3. GSH concentration in the liver of mice treated with a single injection of papaverine, melatonin, and melatonin followed by papaverine (X+SEM, *P<0.001, **P < 0.01). Each value represents 6 animals.

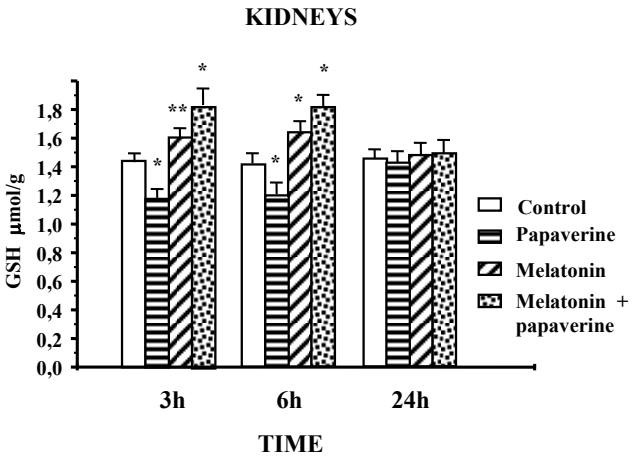

Fig. 4. GSH concentration in the kidney of mice treated with a single injection of papaverine, melatonin, and melatonin followed by papaverine (X+SEM, *P<0.001, **P < 0.01). Each value represents 6 animals.

DISCUSSION

Papaverine is one of the numerous alkaloids present in opium. It is a strong relaxant of smooth muscles of internal organs with a direct effect on the muscle cell. The antispasmodic action occurs in the biliary tract, gastrointestinal tract, blood vessels and bronchi, causing a slight decrease in blood pressure. Besides the beneficial effects, papaverine impairs ventricular heart rhythm and causes arrhythmias (Kim et al., 2008).

The mechanism of papaverine action is associated with inhibition of the enzyme phosphodiesterase (the enzyme that decomposes cAMP) and an increase in intracellular cAMP, which leads to inhibition of the influx of calcium ions into the cell and diastole. With the increase in cAMP, a reduction in cell energy metabolism and a decrease in ATP levels occur (Kaneda et al., 2010).

A decrease in cell energy metabolism and a decrease in the amount of ATP inhibit the rate of GSH synthesis in the cell. It is known that GSH synthesis is limited not only by the presence of substrates, cysteine in particular, but also depends on the level of ATP. In many experiments it has been shown that ATP affects the activity of an enzyme associated with the synthesis of this tripeptide (Meister and Anderson, 1983; Kirlin et al., 1999, McBean and Flynn, 2001; Chorostowska –Wynimko, 2007). Moreover, GSH transport by Mona cells through cell membranes to extracellular space also depends on the amount of ATP (Rebbeor et al., 2000). The observed decrease in the GSH level in the examined organs of mice after administration of papaverine confirms these data, suggesting that a decrease in the metabolic activity of cells determines the synthesis of GSH.

The results obtained in the present experiment confirm earlier findings reported by Davila et al., (1990, 1991) who in *in vitro* studies demonstrated hepatotoxicity of papaverine, manifesting itself in a decrease in the amount of GSH and a distinct decrease in cell viability. Moreover, those studies demonstrated that a decrease in the GSH level was positively correlated with a decrease in ATP.

Papaverine belongs to a group of isoquinoline alkaloids; unlike phenantrene alkaloids, it is characterized by cytotoxicity manifesting itself in DNA damage, leading to the death of thymocytes by apoptosis (Kishko and Dmitrenko, 2000). This alkaloid also induces apoptosis in the vascular endothelium and smooth muscle cells (Gao et al., 2002, 2003). The induction of apoptosis observed in thymocytes or smooth muscle cells after administration of papaverine is probably due to a decline in the GSH level in these cells. It suggests, in fact, that the concentration of GSH in the cells plays an important antiapoptotic role (Schnelldorfer et al., 2000, Pollack and Leeuwenburgh, 2001, Tormos et al., 2004, Bilska et al., 2007).

In the present study a distinct increase in the GSH concentration after administration of melatonin only was demonstrated. This increase was observed in all the examined organs after 3 hours and, in addition, in blood and kidneys also after 6 hours.

Melatonin stimulates the activity of several antioxidant enzymes, such as superoxide dismutase (SOD), γ –glutamylcysteine synthetase (γ –GCS), glutathione peroxidase (GSH-Px), glutathione reductase (GSH-Rd), glucose -6-phosphate dehydrogenase (6-GPD) and catalase (CAT). It also inhibits the activity of pro-oxidative enzymes, such as nitric oxide synthase (NOS) (Reiter et al., 2000, Tan et al., 2000, Reiter et al., 2001).

It has been shown that melatonin is also a very effective scavenger of the highly toxic hydroxyl radical (OH), and indirectly or directly detoxifies free radicals or reactive oxygen species (ROS): peroxynitrite anion ($ONOO^-$), superoxide anion radical ($O_2^{\cdot -}$), nitric oxide ($NO\cdot$), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O2$) (Reiter et al., 2002a). Because of its antioxidant properties, stimulation of antioxidant enzyme activity, as well as direct scavenging of free radicals and ROS, melatonin creates appropriate conditions for an increase in GSH synthesis and inhibition of its degradation in mice. The studies carried out by Reiter et al., (2002) on the effects of melatonin on GSH synthesis have shown that it stimulates the synthesis of GSH in the cytoplasm of cells and stimulates its transport into mitochondria. It is vitally important because mitochondria do not synthesize GSH and are particularly vulnerable to ROS attack. It has been found that in the case of a weak antioxidant defense mitochondrial dysfunction occurs, resulting in energy deficits in cells. Such cells lose their ability to adapt to different physiological stresses. In addition, some observations indicate that melatonin also stimulates the transport of electrons in the respiratory chain and stimulates the synthesis of ATP. This is extremely important because the

rate of GSH biosynthesis depends on the amount of ATP (Reiter et al., 2002 b; Okatani et al., 2006).

In the group of animals treated with melatonin followed by papaverine one hour later, the GSH concentration in the examined organs of mice increased as compared with the animals which received only papaverine and, interestingly, with the control animals.

This increase seems entirely justified in the group of animals which are treated only with melatonin because melatonin has been experimentally shown to stimulate the transport of electrons in the mitochondrial respiratory chain, to increase the synthesis of ATP and GSH (Leon et al., 2004).

Melatonin stimulates oxidative phosphorylation, reduces the leakage of electrons from the respiratory chain and decreases the amount of generated ROS. With the decrease of the amount of ROS generated in mitochondria the utilization of GSH is reduced, which results in the increased amount of this tripeptide (Leon et al., 2005). The increased synthesis of GSH and an increase in its amount after melatonin injection is also caused by increased activity of the enzyme limiting GSH production, such as γ-glutamylcysteine synthetase (γ – GCS), and enzymes involved in the regeneration of the tripeptide – glutathione peroxidase (GSH-Px), glutathione reductase (GSH –Rd) and glucose -6-phosphate (G6PD) (McBean and Flynn, 2001).

The differences in the GSH concentration found between the animals which received melatonin only and those which received melatonin plus papaverine were similar though statistically insignificant. It seems to confirm the protective effect of melatonin on glutathione metabolism in the examined mice. The protective effect of melatonin on the level of GSH has been demonstrated in the brain and liver of rats after administration of tetra-butyl hydroperoxide causing severe oxidative stress. In this experiment a decrease in the activity of GSH-Px and GSH-Rd, and an increase in the activity of oxidized glutathione GSSG in the mitochondria of the examined organs were observed. After administration of melatonin these changes were reversed (Martin et al., 2000).

The results obtained in the present experiment showing an increase in the level of GSH in the group of experimental animals which were given melatonin only and melatonin plus papapverine suggest that melatonin not only stimulates the synthesis of GSH but also neutralizes the negative action of papaverine. With a view to the reduction of negative effects of papaverine on the synthesis of GSH the question arises whether besides melatonin other antioxidants have also a similar effect.

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