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PII: S0940-2993(09)00252-8
DOI: doi:10.1016/j.etp.2009.10.002
Reference: ETP 50415

To appear in: *Experimental and Toxicologic Pathology*

Received date: 19 July 2009
Accepted date: 22 October 2009

Cite this article as: Eugenia Murawska-Ciałowicz, Zbigniew Jethon, Jan Magdalan, Lidia Januszewska, Marzena Podhorska-Okoł ów, Marcin Zawadzki, Tomasz Sozański and Piotr Dzięgiel, Effects of melatonin on lipid peroxidation and antioxidative enzyme activities in the liver, kidneys and brain of rats administered with benzo(a)pyrene, *Experimental and Toxicologic Pathology*, doi:10.1016/j.etp.2009.10.002

Effects of melatonin on lipid peroxidation and antioxidative enzyme activities in the liver, kidneys and brain of rats administered with benzo(a)pyrene

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Keywords: Melatonin; Benzo(a)pyrene; Lipid peroxidation; Antioxidative enzymes; Superoxide dismutase; Glutathione reductase; Catalase

Abstract

Benzo(a)pyrene [B(a)P] is a widespread pollutant with a mutagenic, carcinogenic and strong prooxidative properties. The present study evaluated the melatonin effects on lipid peroxidation products levels and on activity of antioxidative enzymes in the course of B(a)P intoxication. Control rats were treated with 0.9% NaCl; another group was given 10 mg melatonin/kg b.w.; a third group was injected twice a week with B(a)P at the dose of 10 mg/kg b.w.; the fourth group received both B(a)P and melatonin at the dose as above. The experiment continued for 3 months. In homogenates of brain, liver and kidneys lipid peroxidation was appraised by evaluation of malonyldialdehyde and 4-hydroxyalkenal (MDA+4HDA) levels. Activities of glutathione peroxidase (GPx), superoxide dysmutase (SOD) and catalase (CAT) and concentration of reduced glutathione (GSH) were also estimated. In animals receiving both B(a)P and melatonin lower levels of MDA+4HDA were observed in all organs as compared to the group treated with B(a)P only. Following administration of B(a)P, GSH level decreased in brain and kidney. Melatonin in combination with B(a)P induced rises in the GSH level in liver and brain, as compared to the receiving B(a)P alone. The activity of SOD increased in the rats treated with melatonin alone but the highest activity was observed in rats treated with B(a)P plus melatonin. CAT activity in the melatonin-treated group increased in brain and liver. Similarly to SOD, activity of the enzyme significantly increased in the group treated in combination with B(a)P and melatonin, as compared to the remaining groups in all tested tissues. The results suggest that melatonin protects cells from the damaging action of B(a)P. According to our knowledge, there are no studies describing the effects of melatonin on lipid peroxidation markers and antioxidative enzymes during intoxication of B(a)P in the brain, liver and kidneys. The results of present

study gives a perspective for further studies of its free radical scavenger properties in prevention of oxidative stress dependent diseases, among others cancers caused by carcinogens such as B(a)P.

Introduction

Benzo(a)pyrene [B(a)P] is a polycyclic aromatic hydrocarbon (PAH), formed in processes of oxidation of organic matter (e.g., carbon and petrol) and in culinary processes such as roasting, curing and frying. The compounds enter human body from various sources, due to industrial emission, from a polluted environment and from contaminated food (Srivastava et al., 1997; Roos et al., 2002).

Previous studies have shown that B(a)P may induce cancer of lungs, stomach, breast, urinary bladder, prostate and skin (Sharma, 1997; Lee et al., 1998; Kumar and Das, 2000). The mechanism of carcinogenic activity of B(a)P is linked to formation of DNA adducts with metabolites of the compound (Kim and Lee, 1997; Lee et al., 1998). Following penetration into the organism, B(a)P undergoes turnover in processes of epoxidation and monoelectron oxidation. Both pathways are catalysed by cytochrome P-450 monoxgenases. The epoxidation yields highly reactive compound, 7,8-diol-9,10-epoxide-2 [BPDE], which manifests the most pronounced carcinogenic activity among all metabolites of B(a)P (Sullivan, 1985; Kim and Lee, 1997).

The other pathway of B(a)P turnover involves a monoelectron oxidation process, leading to formation of chionone derivatives, B(a)P-6,12-dione, B(a)P-3,6-dione and B(a)P-1,6-dione (Sullivan, 1985; Kim and Lee, 1997). These derivatives generate oxygen free radicals which cause to oxidative damage of macromolecules (DNA, RNA, protein, lipids and carbohydrates). This results in alterations in cellular structures and, hence, in organic structural and functional disturbances (Sharma, 1997; Kim and Lee, 1997; Lee and Lee, 1997).

Melatonin is present both in the plant world and in animals and it is a highly effective antioxidant. In its reaction with free radicals it donates electrons (Reiter et al., 1999; Reiter et al., 2001). Melatonin is a scavenger of both oxygen- and nitrogen-based reactive molecules, including peroxynitrite anion ($ONOO^-$) and its decomposition products, including hydroxyl radical ($OH^\bullet$), nitrogen dioxide (NO_2), and carbonate radical ($CO3^{\bullet-}$) (Korkmaz et al., 2009). Melatonin reacts rapidly with highly toxic $OH^\bullet$ resulting in several hydroxylated products formation. One of them, cyclic -3-hydroxymelatonin may serve as a biomarker of the amounts of $OH^\bullet$ scavenged (Tan et al., 2000; Reiter et al., 2001). Apart from $OH^\bullet$, melatonin directly inactivates NO_2, $CO3^{\bullet-}$, hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) (Gilad et al., 1997; Zang et al., 1998; Reiter et al., 2001; De Almeida et al., 2003; Fischer et al., 2004; Chuang and Chen, 2004, Korkmaz et al., 2009). The products of melatonin oxidation: N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK) and N(1)-acetyl-5-methoxykynuramine (AMK) have been also described as potent scavengers (Carampin et al., 2003; Tan et al., 2007; Manda et al., 2007). Interaction of melatonin with the lipid radical ($LOO^\bullet$) awaits clarification (Reiter et al., 1999; Reiter et al., 2001). Besides its ability to direct scavenge radicals and radical products, melatonin also augments the activities of antioxidative enzymes,

including glutathione peroxidase (GPx), superoxide dysmutase (SOD) and glutathione reductase (Longoni et al., 1998; Tan et al., 2000; Reiter et al., 2001; Reiter et al., 2005; Korkmaz et al., 2009). The findings relating to the stimulation of antioxidative enzymes by melatonin were reviewed by Rodriguez et al. (Rodriguez et al., 2004).

Present study demonstrated the protective activity of melatonin in the course of B(a)P intoxication through its effect on the antioxidative defense system.

Material and Methods

Chemicals

B(a)P and melatonin were obtained from Sigma Chemical Co. (St. Louis, USA) and reagent kits from Calbiochem (La Jolla, CA, USA) and OXIS (OXIS International Inc, USA).

Experimental protocol

The studies were performed on 40 male rats of the Buffalo strain, weighing 200 - 250g. The animals were kept in conditions consistent with requirements of the local commission for ethical matters of animal experimentation. They were maintained in controlled environmental conditions of ambient temperature (22 ± 2°C) and relative humidity

of 40 - 60%, in a 12:12 light / dark cycle. All animals were fed standard pelleted diet and water ad libitum.

The animals were placed into four groups, of 10 rats each: the control group received i.p. injections of 0.9% NaCl twice a week (NaCl); melatonin-treated rats, received s.c. injections of melatonin at the dose of 10mg/kg b.w., three times a week, rats intoxicated with B(a)P received an i.p. injection of 10mg B(a)P /kg b.w. twice a week; the final group was intoxicated with B(a)P in combination with melatonin. Melatonin dosage was decided according to previous studies of Dziegiel et al. (2002a, 2002b) and it was administered before each injection of a B(a)P and between them. A dosage of B(a)P was determined according to the study of Konstandi et al. (2007).

B(a)P was dissolved in a sunflower oil (Kim and Lee, 1997), while melatonin was dissolved in 10% C2H5OH in H2O at the ratio of 1:3. All the injections were administered between hours 13:00 and 14:00. The experiment Experimental protocol was approved by the Local Animal Ethics Committee of Wroclaw Immunology Institute.continued for 3 months. Forty-eight hours following the last injection of melatonin the animals were sacrificed by administration of bioketan at the dose of 100mg/kg body weight followed by cervical dislocation.

Biochemical analysis

Brain, liver and kidneys were collected. The isolated organs were cut into smaller fragments, weighed and frozen at the temperature of -86°C. For biochemical tests the samples were thawed, washed with 0.9% NaCl plus EDTA, homogenized in ice-cold 20mM TRIS-HCL buffer, pH 7.4 and centrifuged for 10 min at the temperature of 4°C at 15,000 rpm. The supernatants were decanted and used to estimate lipid peroxidation (malondialdehyde + 4 hydroxynonenal, MDA+4HDA) levels using Lipid Peroxidation Assay Kit (Calbiochem) and of GSH using Bioxytech GSH-400 kit (OXIS). Activities of GPx and SOD were estimated using Bioxytech kit GPx-340 (OXIS) and Bioxytech kit SOD-525 (OXIS) while catalase activity was assayed by the technique of Aebi (Aebi, 1984) against H_2O_2. Total protein concentration was estimated by the technique of Lowry (Lowry et al., 1951), using the Protein Assay Kit (Sigma Diagnostics Co., ST. Louis, USA). All analyses were performed as recommended by manufacturers of the reagent kits.

Statistical analysis

Results were subjected to statistical analysis using the Statistica 5.1 PL software (StatSoft, Cracow, Poland). Significance of differences was tested employing the non-parametric Mann – Whitney *U*-test at the confidence level of $p<0.05$.

Results

In control group levels of lipid peroxidation markers varied with the type of tissue studied. The highest concentration of MDA+4HDA was noted in kidneys. Administration of melatonin induced a decrease of lipid peroxidation markers in homogenates of kidney and liver. In the group receiving B(a)P with melatonin, levels of MDA + 4HDA were significantly lower in all studied tissues, as compared to the group receiving B(a)P alone (Fig. 1).

Initial concentrations of GSH were the highest in liver homogenates. In the group treated with melatonin, GSH concentrations were significantly increased in all studied tissues as compared to the control group, especially in the liver. In the groups intoxicated with B(a)P concentration of GSH was significantly lower in the brain and kidney as compared to the control. In B(a)P plus melatonin group only GSH level in liver homogenates was significantly higher than in the group receiving B(a)P alone. (Fig. 2).

The highest control GPx activities were noted in liver. In the group receiving melatonin, activity of the enzyme was significantly higher than in the control in all studies tissues. Significantly augmented activities of GPx in the group of B(a)P-intoxicated animals as compared to the control group, were demonstrated in homogenates of liver and brain. As compared to the B(a)P group, rats treated with B(a)P plus melatonin demonstrated a significantly higher activity of the enzyme in liver and brain homogenates (Fig. 3).

In the control groups the highest activity of SOD was noted in the liver. Melatonin - treated rats demonstrated significantly augmented SOD activity in comparison to the control

in the brain. In rats treated with B(a)P alone activity of SOD was significantly higher in kidney and liver as compared to control and melatonin groups. In rats receiving B(a)P plus melatonin, activity of SOD was significantly augmented as compared to the control and melatonin treated groups in each experimental organ (Fig. 4).

In control the highest CAT activity was observed in liver. As compared to this the group treated with melatonin alone showed significantly higher CAT activity only in the liver. In rats intoxicated with B(a)P significantly lower CAT activity was observed in comparison to control rats in the kidney, and as compared to the group treated with melatonin alone the values were significantly lower in all organs. In the group treated with B(a)P plus melatonin activity of CAT augmented significantly in all studied tissues, as compared to any other group (Fig. 5).

Discussion

This study aimed to investigate whether melatonin protects cells from oxidative injury, induced by B(a)P. Benzo(a)pyrene, a five-ring polycyclic aromatic hydrocarbon is a widespread pollutant with a well documented mutagenic and carcinogenic properties. It's action involves generation of free radicals which cause oxidative damage of macromolecules. Melatonin is a highly effective antioxidant. It was also shown that it inhibits mutagenesis and clastogenic effect of a number of chemical mutagens (Anisimov et al., 2006). Only a few papers described the effects of melatonin on B(a)P intoxication, showing the possible inhibitory effect of this pineal hormone on B(a)P induced carcinogenesis in rodents. In Vesnushkin's et al. surveys melatonin decreased the incidence of skin tumors and subcutaneous sarcomas after superficial and subcutaneous administration of B(a)P, respectively (Vesnushkin et al., 2006, Vesnushkin et al., 2007). Moreover, melatonin decreased, enhanced by B(a)P, lipid peroxidation markers both in the serum and tumor tissue. (Vesnushkin et al., 2006). According to our knowledge, there are no studies describing the effects of melatonin on lipid peroxidation markers and antioxidative enzymes during intoxication of B(a)P in the brain, liver and kidneys. The effects of melatonin on brain were examined since it is clearly dependent on rich oxygen supply and containing vast amounts of lipid. The brain is also characterized by relatively low levels of anti-oxidative enzymes (Reiter, 1995; Escames et al., 1997; Esparza et al., 2005). Moreover, both investigated substances: exogenous melatonin and B(a)P readily cross a blood-brain barrier and reach the brain in a clinical relevant concentrations (Menendez-Pelaez et al., 1993; Moir et al., 1998; Saunders et al., 2006; Reiter et al., 2008; Zhang et al., 2008). The liver was included because of its multiple detoxication pathway including during B(a)P intoxication. The kidney was studied because its excretes metabolites of B(a)P. Among the selected organs, the lowest level of enzymatic antioxidative protection was manifested by the brain while the highest enzyme

activities were found in the liver. However, the brain may be physiologically protected by many antioxidants, especially by melatonin, the receptors for which are present in various brain structures (Reiter, 1995; Sewerynek et al., 1995; Reiter, 1996; Uz et al., 2005).

B(a)P crosses a blood-brain barrier and induces acute neurobehavioral toxicity through oxidative stress due to inhibition of the brain antioxidant scavenging system (Moir et al. 1998; Saunders et al., 2006; Zhang et al., 2008). Generation of oxygen-based reactive molecules in brain can increase permeability of the blood-brain barrier and modify synaptic transmission (Evans, 1995). B(a)P can also reach the brain directly by passing through the olfactory nerve (Persson et al., 2002). In the present study B(a)P has stimulated lipid peroxidation and augmented activities of SOD and GPx in the brain. Administration of melatonin alone or in combination with B(a)P clearly stimulated CAT, SOD and GPx activities. Numerous authors (Tan et al., 1993; Acuna-Castroviejo et al., 1995; Escames et al., 1997; Bongiovanni et al., 2007; Hung et al., 2008; Bharti and Srivastava 2009) have shown melatonin to be an effective neuroprotective agent. Its actions involve not only stimulation of antioxidative enzymes but also direct scavenging of oxygen free radicals and prevention of free radical generation (Sewerynek et al., 1995; Escames et al., 1997; Hung et al., 2008; Reiter et al., 2008). Urata et al. (1999) found that the concentration of GSH increases following administration of melatonin similar to our results.

Among the examined organs, kidneys contained the highest physiological level of lipid peroxidation markers. Thus, they may be assumed to be the least protected from free radicals even if, compared to the brain, they have higher activities of antioxidative enzymes. Perhaps despite the high physiological activities of the enzymes, they are more sensitive to the

toxic effects of B(a)P metabolites. Similarly to other polycyclic aromatic hydrocarbons, B(a)P becomes mainly trapped in the kidneys, liver and adipose tissue (Collins et al., 1991). Urinary excretion includes one forth of the total B(a)P (Becher and Bjorseth, 1983). Moreover, various B(a)P turnover products are eliminated in urine mainly as conjugates with endogenous compounds, i.e., with glutathione (Yang et al., 1994). A small percent of unmetabolized B(a)P is also excreted in urine (Jongeneelen, 1990). In the groups receiving melatonin alone or in combination with B(a)P, MDA + 4HDA concentrations in kidney homogenates were significantly lower as compared to the control and B(a)P groups. Melatonin alone also stimulated renal GPx activity, The activity of SOD was significantly increased following injection of B(a)P. Lower levels of GSH both after administration of B(a)P alone and B(a)P plus melatonin as compared to the control group may have resulted from increased GSH usage by GPx and from it complex formation with B(a)P metabolites (Sharma, 1997). There was very pronounced reduction in concentration of MDA+4HDA in rats that received melatonin with or without B(a)P to values below those observed in the control rats. This may supports melatonin strong protective actions against lipid peroxidation as observed by others (Kim and Lee, 1997; Dziegiel et al., 2002a,b; Dziegiel et al., 2003; Buyukokuroglu, 2008).

Results obtained here indicate that among examined organs the liver is least sensitive to damaging action of oxygen radicals in B(a)P treated rats. Administration of melatonin alone or with B(a)P reduced MDA +4HDA levels compared to the control rats and rats intoxicated with B(a)P. Among the antioxidative enzymes activity of which may be stimulated by melatonin, liver cells are protected by SOD, CAT and GPx and by the augmented levels of GSH (Ohta et al., 2000; Ohta et al., 2001; Leaden et al., 2002; Ohta et al.,

2003; Tùnez et al., 2003; Taysi et al., 2003; Mauriz et al., 2007). The least pronounced increase in lipid peroxidation markers in the liver of B(a)P-treated animals clearly proves that the organ is most effectively protected from oxidative stress.

Conclusion

On the basis of present results we conclude that melatonin may protect the brain, kidneys and liver from damaging effects of reactive oxygen species formed by B(a)P turnover. The mechanism of melatonin's protective effects involves most probably stimulation of activities of antioxidative enzymes. Melatonin is regarded as a safe substance with low risk of side effects after the treatment even in a large doses. It gives a perspective for further studies of its free radical scavenger properties in prevention of oxidative stress dependent diseases, among others cancers caused by carcinogens such as B(a)P.

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Fig. 1. Concentration of lipid peroxidation markers (MDA+4HDA) in homogenates of brain, kidney and liver, *p<0.05 as compared with control, $^{\#}p$<0.05 as compared with melatonin, $^{\$}p$<0.05 as compared with B(a)P in all organs.

Fig. 2. Concentration of reduced glutathione (GSH) in homogenates of brain, kidney and liver, *p<0.05 as compared with control, $^{\#}p$<0.05 as compared with melatonin, $^{\$}p$<0.05 as compared with B(a)P in all organs.

Fig. 3. Activity of glutathione peroxidase (GPx) in homogenates of brain, kidney and liver, *p<0.05 as compared with control, $^{\#}p$<0.05 as compared with melatonin, $^{\$}p$<0.05 as compared with B(a)P in all organs.

Fig. 4. Activity of superoxide dismutase (SOD) in homogenates of brain, kidney and liver, *p<0.05 as compared with control, $^{\#}p$<0.05 as compared with melatonin, $^{\$}p$<0.05 as compared with B(a)P in all organs.

Fig. 5. Activity of catalase (CAT) in homogenates of brain, kidney and liver, *$p<0.05$ as compared with control, $^{\#}p<0.05$ as compared with melatonin, $^{\$}p<0.05$ as compared with B(a)P in all organs.

Fig 1

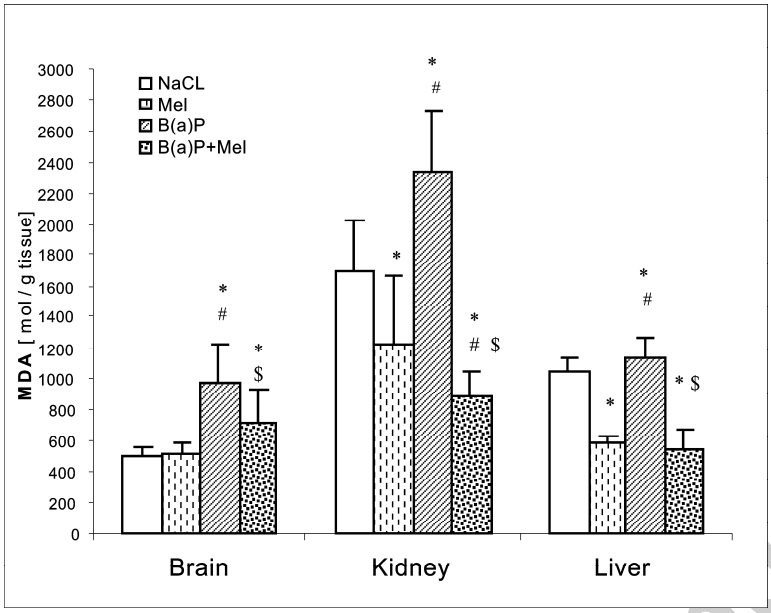

Fig 2

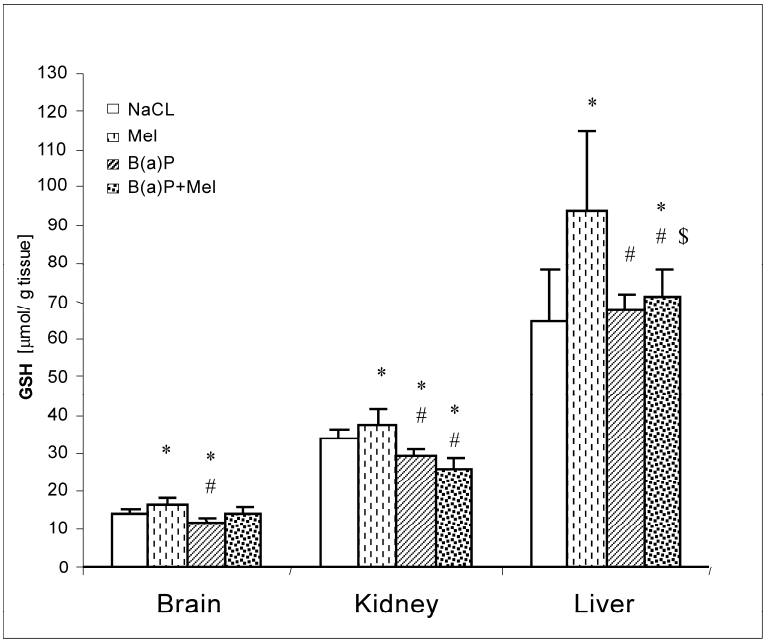

Fig 3

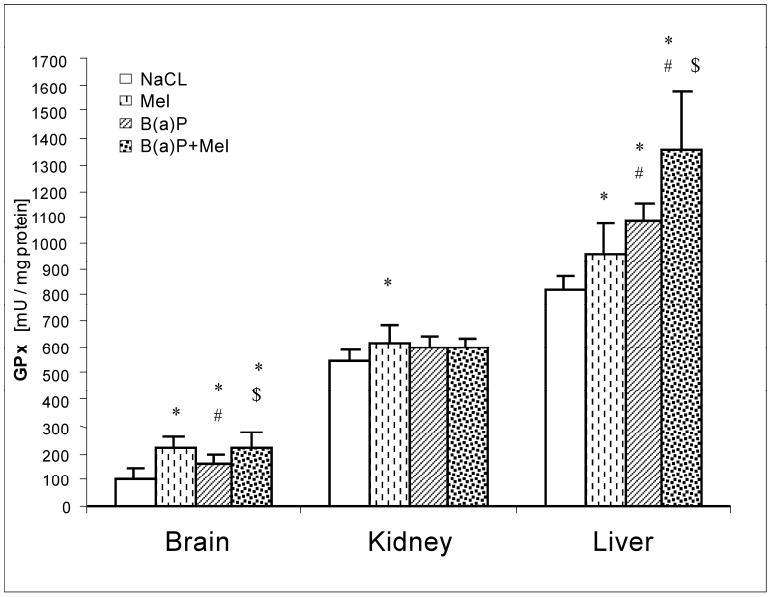

Fig 4

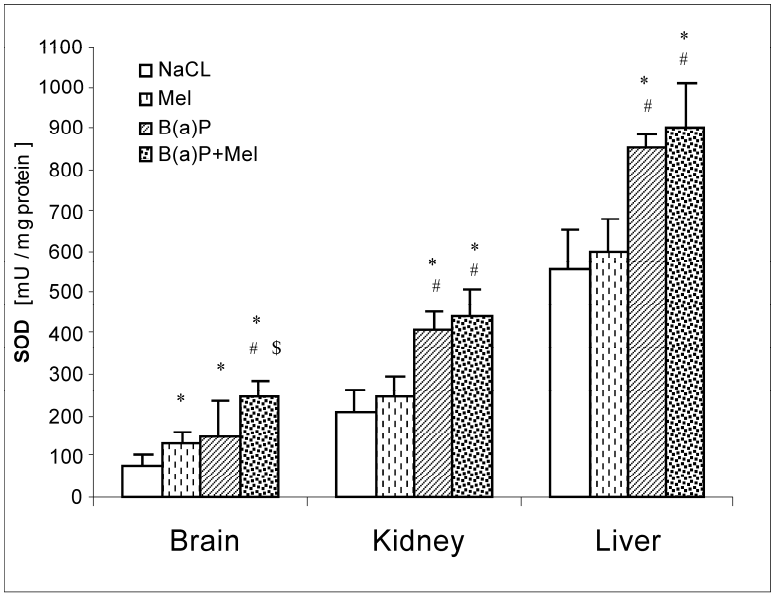

Fig 5