The effects of melatonin on glutathione peroxidase activity in serum and erythrocytes after adriamycin in normal and pinealectomised rats

Wpływ melatoniny na aktywność peroksydazy glutationu w surowicy i erytrocytach po iniekcji adriamycyny u szczurów nietkniętych i poddanych pinealektomii

Katarzyna Dąbrowska[1, 2], Michał Stuss[1, 2], Jolanta Gromadzińska[3], Wojciech Wąsowicz[3], Ewa Sewerynek[1, 2]

[1]Department of Bone Metabolism, Medical University of Lodz, Poland
[2]Hospital of the Polish Mother Research Institute, Lodz, Poland
[3]Department of Toxicology and Carcinogenesis, the Nofer Institute of Occupational Medicine, Poland

Abstract

Introduction: Adriamycin (ADR) is a potent chemotherapeutic agent, effective in the treatment of leukaemias, lymphomas and many solid tumours. However, its clinical usage is often limited by cardiotoxicity, induced by oxygen radical damage of the membrane lipids. Melatonin (MEL) is a well-known antioxidant. It has been shown that MEL can scavenge free radicals, both directly and indirectly, stimulating the activity of antioxidative enzymes such as glutathione peroxidase (GSH-Px).

The aim of the study: The aim of the study was to examine the effect of MEL on serum and erythrocyte GSH-Px activity after ADR in normal and pinealectomised rats.

Material and methods: Wistar rats were divided into the three groups: control animals (Intact), sham-operated (Sham-PX) and pinealectomised (Px). Each of the groups was divided into four subgroups, injected with: 1 — saline, 2 — MEL, 3 — ADR and 4 —ADR + MEL. ADR was administered 2 months after Px as a single dose (15 mg/kg, *i.p.*) 1 hour after the fourth melatonin injection. Melatonin (5 mg/kg, *i.p.*) was administered for 4 days before and 2 days after ADR. After 6 days of treatment, the rats were killed by decapitation. Their blood was collected for measurements.

Results: In serum GSH-Px activity decreased in all the groups after ADR. Pinealectomy decreased the activity of the enzyme in all the groups of animals examined. In erythrocytes GSH-Px decreased after ADR in the Px-animals. The effect of pinealectomy on erythrocyte GSH-Px activity was not as strongly expressed as serum GSH-Px activity. MEL did not change GSH-Px activity after ADR.

Conclusion: Melatonin, in pharmacological concentrations, did not influence the activity of GSH-Px, either in normal or in pinealectomised rats after ADR. A deficiency of endogenous melatonin production may inhibit GSH-Px activity.

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Key words: glutathione peroxidase, adriamycin, melatonin, pinealectomy

Streszczenie

Wstęp: Adriamycyna (ADR, *adriamycin*) jest lekiem przeciwnowotworowym wykorzystywanym w leczeniu białaczek, chłoniaków czy guzów litych. Jej efekt leczenia jest ograniczony ze względu na kardiotoksyczność indukowaną produkcją wolnych rodników, które uszkadzają błony lipidowe.

Melatonina (MEL, *melatonin*) jest znanym antyoksydantem. Wykazano, że neutralizuje wolne rodniki w sposób bezpośredni lub pośredni, stymulując enzymy antyoksydacyjne, w tym peroksydazę glutationu (GSH-Px, *glutathione peroxidase*).

Cel: Celem badania było zbadanie efektu melatoniny na aktywność GSH-Px w surowicy i erytrocytach po adriamycynie u zwierząt nietkniętych i po usunięciu szyszynki.

Materiał i metody: Szczury samce szczepu Wistar podzielono na 3 grupy: grupę kontrolną (Intact — szczury nietknięte), po operacji pozornej (Sham-Px) i po usunięciu szyszynki (Px). Każdą grupę podzielono na 4 podgrupy, w których podano injekcje: 1 — soli fizjologicznej, 2 — MEL, 3 — ADR i 4 — ADR + MEL. Adriamycynę podano 2 miesiące po Px w dawce jednorazowej (15 mg/kg mc.), 1 godzinę przed czwartym podaniem MEL. Melatoninę podawano przez 4 dni przed Px i przez 2 dni po ADR w dawce 5 mg/kg mc. Po 6 dniach leczenia zwierzęta zabito przez dekapitację, a krew zamrożono do momentu pomiarów.

Wyniki: Aktywność PSH-Px w surowicy obniżyła się po ADR we wszystkich badanych grupach. Po usunięciu szyszynki aktywność GSH-Px w surowicy zmniejszyła się w grupach poddanych injekcjom. Aktywność GSH-Px w erytrocytach obniżyła się po ADR u zwierząt poddanych Px. Zmiany aktywności GSH-Px w eytrocytach po usunięciu szyszynki były słabiej wyrażone w porównaniu z aktywnością GSH-Px w surowicy. Melatonina nie zmieniła aktywności peroksydaz po ADR.

Ewa Sewerynek, M.D., Ph.D., Professor of Endocrinology, Medical University of Lodz, Chair of General Endocrinology Head of Department of Bone Metabolism, ul. Zeligowskiego 7/9, 90–752 Łódź, tel./fax: +48 (042) 639 31 27; mobile +48 601 952 747, e-mail: ewa.sewerynek@wp.pl

Wnioski: Melatonina w dawce farmakologicznej nie wpłynęła na aktywność peroksydaz zarówno u zwierząt nietkniętych, jak i po usunięciu szyszynki. Deficyt endogennej produkcji melatoniny może odgrywać rolę w hamowaniu aktywności peroksydaz.
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Słowa kluczowe: peroksydaza glutationu, adriamycyna, melatonina, pinealektomia

Introduction

Antracycline antibiotics are widely used in the antineoplastic treatment of haemopoietic or solid tumours. Cardiotoxicity is one of the most serious side effects of these drugs [1]. Evidence collected for over 15 years indicates that melatonin (MEL) influences the cardiovascular system [2–4].

Morishima et al. [5] (1999) reported that MEL protected against adriamycin (doxorubicin hydrochloride)-induced cardiomyopathy, the pathogenesis of which may involve free radical and lipid peroxidation. In that study MEL was shown to affect zinc turnover, which acts as an antioxidant. Similar results were obtained by others; MEL was an effective antioxidant against adriamycin-induced cardiotoxicity of the myocardium [6–10]. The protective effect of MEL can partly depend on catalase activity stimulation in cardiomyocytes subjected to doxorubicin action [11]. Idarubicin is an antracycline antibiotic used in the treatment of acute leukaemia and other malignancies. Amifostine is a well-known cell protector and, like MEL, involves free radical scavenging. It has been shown that amifostine reduces apoptosis and DNA damage in normal (lymphocytes) and cancer cells (leukaemic K562 and HeLa cells). Melatonin protected both cell types against genotoxic effect and idarubicin-induced apoptosis. The authors concluded that, despite its recognised potential as an antioxidant, MEL should be administered with caution when used in combination with cancer chemotherapy agents, especially in leukaemias [12]. Additionally, the cytostatic effectiveness of daunorubicin was examined, when applied in parallel with MEL in rats with transplanted Morris hepatoma [13]. On the one hand, MEL protects cardiomyocytes by decreasing the intensity of daunorubicin-induced apoptosis but, on the other, it weakens the cytostatic activity of this drug, as demonstrated by less frequent necrosis and apoptosis in transplantable Morris hepatoma cells.

Glutathione peroxidase (GSH-Px) is one of the most important antioxidative enzymes. In some studies adriamycin (ADR) has been reported to have decreased the activity of this enzyme in the heart, and MEL did not restore the decreased activity [6], while in other reports ADR has been described as having no effect [11]. There have been some data to indicate that the effect of ADR can be dose- and time-dependent [6, 11, 14].

The aim of the study was to examine the effect of MEL on serum and erythrocyte GSH-Px activity in normal and pinealectomised rats after ADR.

Material and methods

Doxorubicin was donated by the Pfizer Company (Poland). Melatonin and other chemicals were purchased from Sigma. The experiment was performed in conformity with the principles of the Łódź Local Bioethics Commission for Experiments on Animals (L/BD/196/2004).

Male Wistar rats were used and these weighed approximately 200 g each at the onset of the study. There were 8 animals per cage and they were housed in a temperature-controlled and light-controlled room (the light was turned on at 6.0 a.m. and turned off at 6.0 p.m.) and had free access to food and water.

The groups of rats remained on a standard diet (Motycz, Poland) and water with *ad libitum* access to both. The Wistar rats were divided into the following three groups: control animals (Intact), sham-operated (Sham-Px), and pinealectomised (Px). Each group was divided into 4 subgroups, which were injected as follows: 1 — saline, 2 — MEL, 3 — ADR and 4 — ADR + MEL.

Pinealectomy was performed by the method of Kuszak and Rodin, a well-known model of endogenous deficiency of melatonin production [15, 16]. Adriamycin was administered 2 months after Px as a single dose (15 mg/kg, *i.p.*) 1 hour after the fourth MEL injection. MEL (5 mg/kg, *i.p.*) was administered for 4 days before and 2 days after ADR. After 6 days of treatment the rats were killed by decapitation.

Blood samples were taken into heparinised microtubes, free of any trace elements. After centrifugation, plasma was collected and red blood cells (RBC) were washed in 0.9% NaCl and centrifuged three times. Plasma and RBC samples were stored at –80°C for a maximum of 2 weeks until biochemical analysis.

The GSH-Px activities of RBC lysate plasma were assayed by the coupled methods of Paglia and Valentine with t-butyl hydroperoxide as substrate [17]. The reaction was carried out at 25°C in a spectrophotometer fitted with a constant-temperature cell housing. The method was based on the NADPH-coupled reaction, where oxidised glutathione, produced by GSH-Px and hydroperoxide, was reduced by exogenous glutathione reductase and NADPH. Enzymatic activities were
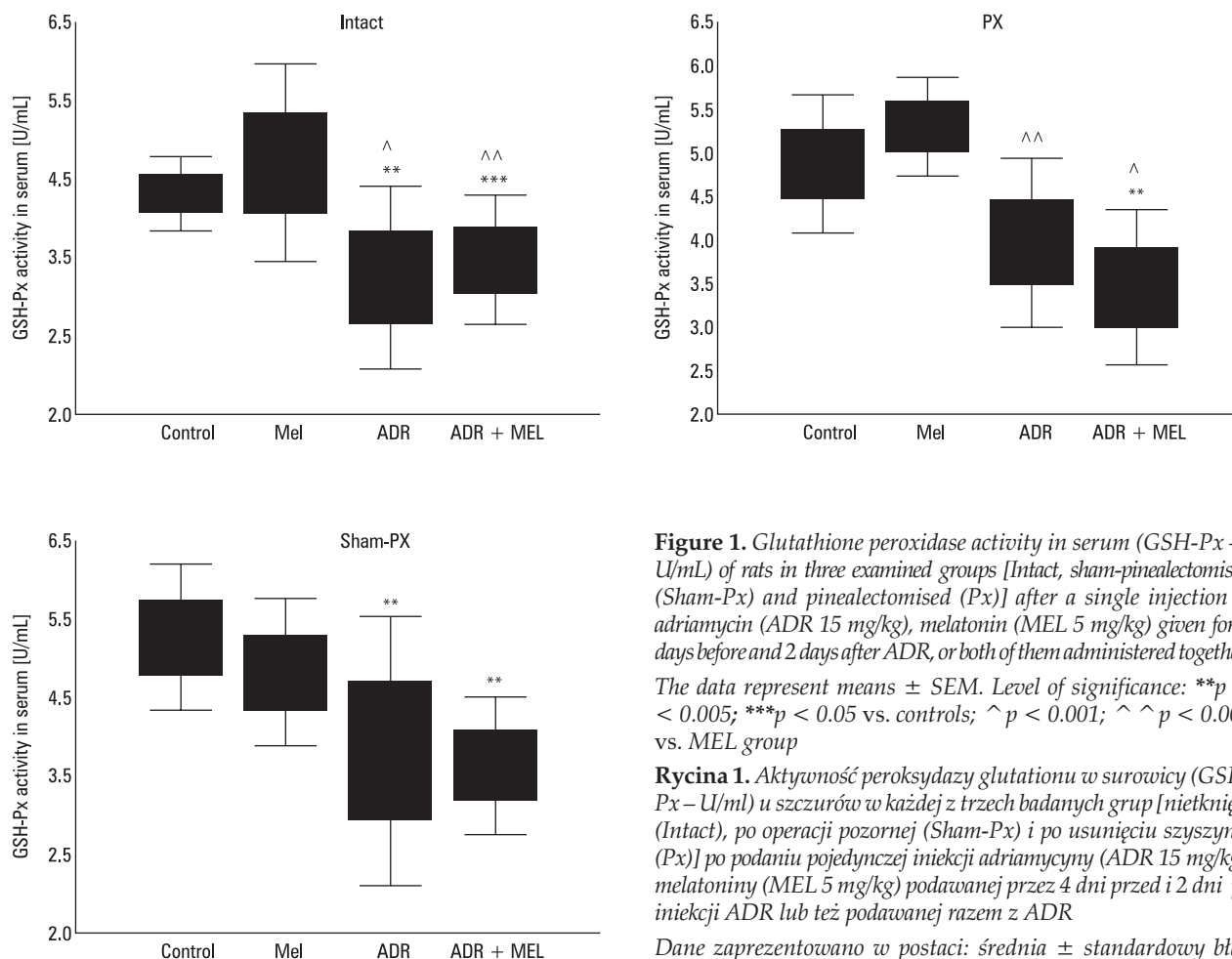
Figure 1. *Glutathione peroxidase activity in serum (GSH-Px — U/mL) of rats in three examined groups [Intact, sham-pinealectomised (Sham-Px) and pinealectomised (Px)] after a single injection of adriamycin (ADR 15 mg/kg), melatonin (MEL 5 mg/kg) given for 4 days before and 2 days after ADR, or both of them administered together.*

The data represent means ± SEM. Level of significance: **p < < 0.005; ***p < 0.05 vs. *controls; ^p < 0.001; ^ ^p < 0.005* vs. *MEL group*

Rycina 1. *Aktywność peroksydazy glutationu w surowicy (GSP–Px – U/ml) u szczurów w każdej z trzech badanych grup [nietknięte (Intact), po operacji pozornej (Sham-Px) i po usunięciu szyszynki (Px)] po podaniu pojedynczej iniekcji adriamycyny (ADR 15 mg/kg), melatoniny (MEL 5 mg/kg) podawanej przez 4 dni przed i 2 dni po iniekcji ADR lub też podawanej razem z ADR*

Dane zaprezentowano w postaci: średnia ± standardowy błąd pomiaru (SEM, standard error of measure*). Poziom istotności: **p < 0,005; ***p < 0,05* vs. *kontrola; ^p < 0,001; ^ ^p < < 0,005* vs. *grupa MEL*

expressed as units (U) per gram of haemoglobin or units per ml of plasma. One unit of enzyme was defined as 1 mmol NADPH oxidised per minute per g of Hb (U/gHb) or per ml of plasma (U/ml). The intra-assay coefficient of variation for both materials (6–8 analyses) was below 3%.

The Kruskal-Wallis test was applied to evaluate the statistical significance of the results.

Results

GSH-Px activity in serum decreased 3 days after a single injection of ADR in a dose of 15 mg/kg in Intact, Sham-Px and Px rats (Fig. 1). MEL, given for 4 days before, in a dose of 5 mg/kg/day and continued for 2 days after ADR, did not change the activity of the enzyme. Pinealectomy decreased the activity of the enzyme in all the groups of animals examined (Fig. 2).

In erythrocytes GSH-Px activity decreased only after ADR in the Px animals (Fig. 3). This effect was not observed either in the Intact or in the Sham-Px rats. MEL

did not change GSH-Px activity after ADR in any of the groups examined (Fig. 3). The effect of pinealectomy on the activity of GSH-Px in erythrocytes was not as strongly expressed as that of serum GSH-Px (Fig. 4).

Discussion

The antioxidative properties of MEL have already been demonstrated over a period of more than 15 years [18–20]. The results of previous studies have shown a protective effect of MEL against oxidative stress, as induced by many xenobiotics or carcinogens, such as lipopolysaccharide, hydrogen peroxide, iron, iodide, thyrotoxicosis, potassium bromate, delta-aminolevulinic acid, cadmium and liver ischaemia-reperfusion [21–33]. It has been suggested that MEL influences the cardiovascular system [2, 3]. It was shown in one of the initial studies that MEL protected against arrhythmia induced by ischaemia-reperfusion in isolated rat hearts, well-known as a model of the induction of free radicals [34]. Sahna et al. [35] suggested that MEL in physiological
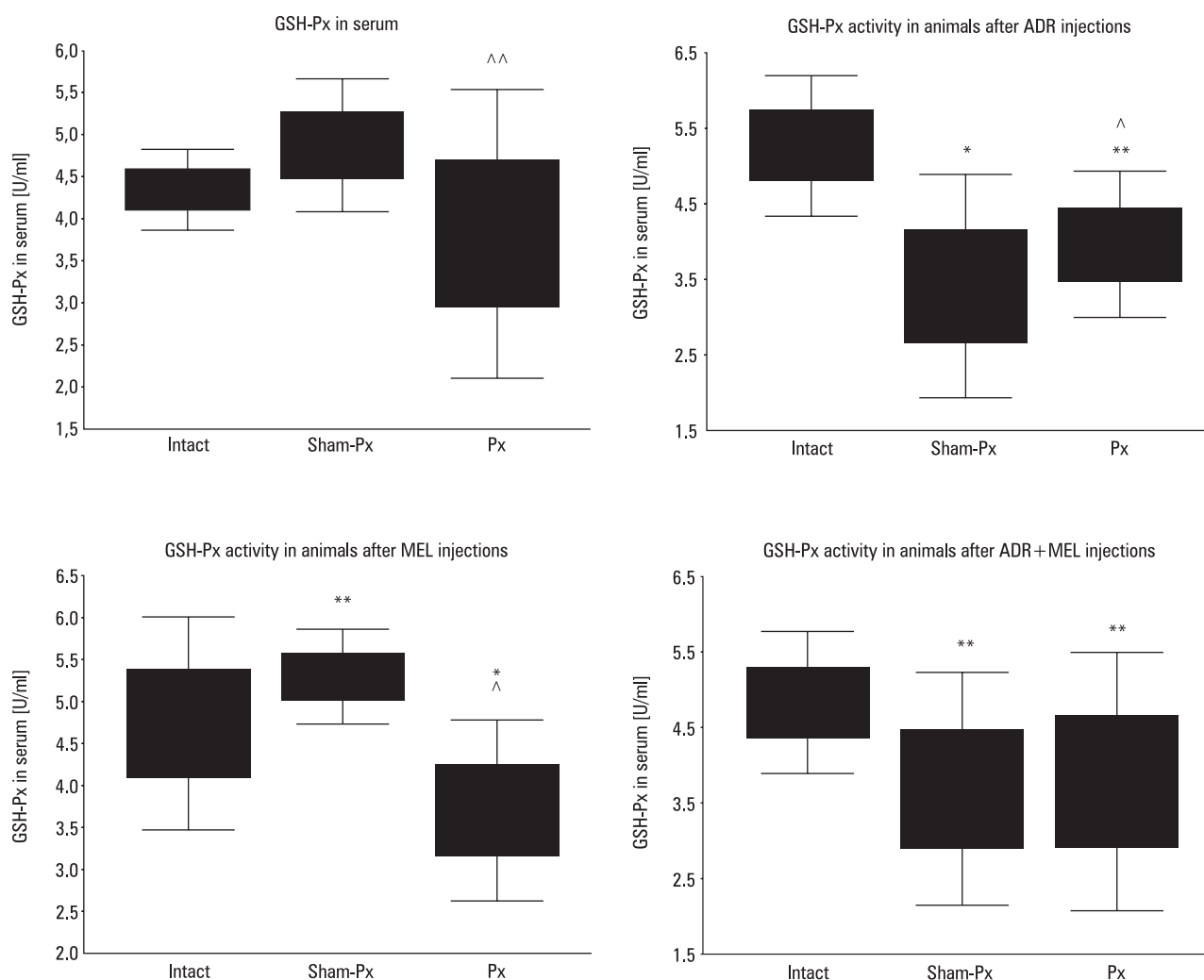
Figure 2. *The effect of pinealectomy on glutathione peroxidase activity in serum (GSH-Px — U/mL) after a single injection of adriamycin (ADR 15 mg/kg), melatonin (MEL 5 mg/kg) given for 4 days before and 2 days after ADR, or both of them administered together.*
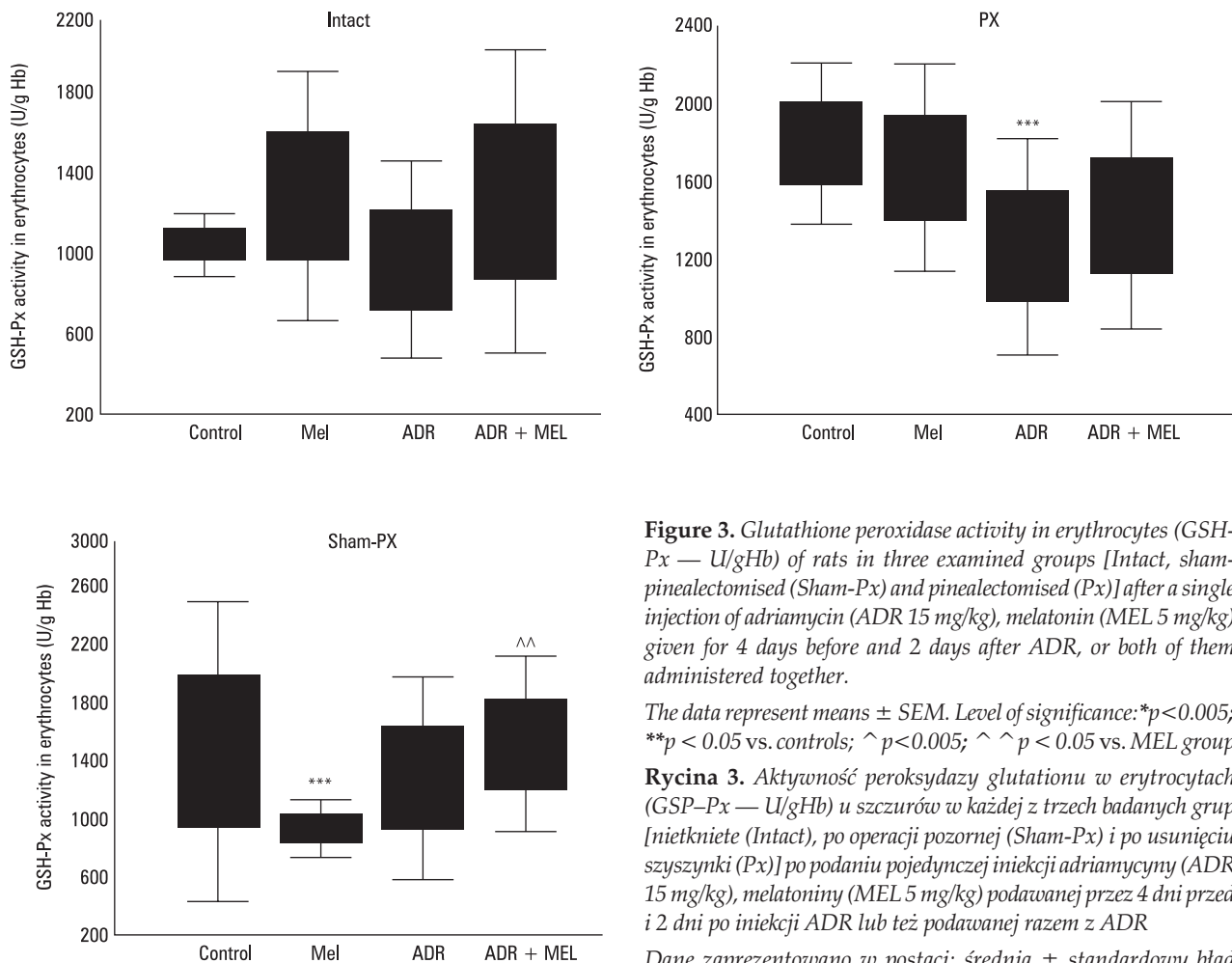
The data represent means ± SEM. Level of significance:***p<0.05 vs. controls; ^ ^ p < 0.05 vs. MEL group

Rycina 2. *Wpływ usunięcia szyszynki na aktywność peroksydazy glutationu w surowicy (GSP–Px — U/ml) po podaniu pojedynczej iniekcji adriamycyny (ADR 15 mg/kg), melatoniny (MEL 5 mg/kg) podawanej przez 4 dni przed i 2 dni po iniekcji ADR lub też podawanej razem z ADR*

Dane zaprezentowano w postaci: średnia ± standardowy błąd pomiaru (SEM, standard error of measure*). Poziom istotności: ***p < 0,05* vs. *kontrola; ^ ^ p < 0,05* vs. *grupa MEL*

concentrations is important in reducing ischaemia-reperfusion arrhythmias, myocyte damage and mortality, while pharmacological concentrations of this hormone do not increase its beneficial effect. In order to examine the role of physiological concentrations of pineal indoleamine, the animals were kept in constant light conditions [36]. It was shown in a subsequent study [37] that MEL administration exerted a mitigating effect on infarct extension. As suggested by Castagnino et al. [38], a significant cytoprotective effect of MEL is especially demonstrable in the early phases of myocardial infarction in rats.

Melatonina also suppresses iron-induced lipid peroxidation in many tissues, including the heart [39]. Arteaga et al. [40] compared the antioxidative effect of a few antioxidants in protecting against the oxidation of LDL-cholesterol from postmenopausal women. They showed that the antioxidant potency of oestradiol *in vitro* was 10–100 times higher than that of either α- and γ-tocopherol or of MEL. Benot et al. [41] suggest that the antioxidative mechanism of MEL also plays a very important role in blood pressure reduction and in protection against atherosclerosis.

Figure 3. *Glutathione peroxidase activity in erythrocytes (GSH-Px — U/gHb) of rats in three examined groups [Intact, sham-pinealectomised (Sham-Px) and pinealectomised (Px)] after a single injection of adriamycin (ADR 15 mg/kg), melatonin (MEL 5 mg/kg) given for 4 days before and 2 days after ADR, or both of them administered together.*

The data represent means ± SEM. Level of significance:*p<0.005; **p < 0.05 vs. controls; ^ p<0.005; ^ ^ p < 0.05 vs. MEL group

Rycina 3. *Aktywność peroksydazy glutationu w erytrocytach (GSP–Px — U/gHb) u szczurów w każdej z trzech badanych grup [nietknięte (Intact), po operacji pozornej (Sham-Px) i po usunięciu szyszynki (Px)] po podaniu pojedynczej iniekcji adriamycyny (ADR 15 mg/kg), melatoniny (MEL 5 mg/kg) podawanej przez 4 dni przed i 2 dni po iniekcji ADR lub też podawanej razem z ADR*

Dane zaprezentowano w postaci: średnia ± standardowy błąd pomiaru (SEM, standard error of measure*). Poziom istotności: *p < 0,005; **p < 0,05 vs. kontrola; ^ p < 0,005; ^ ^ p < 0,05 vs. Grupa MEL*

In our study ADR was used, its cardiotoxicity being a well-known feature. The mechanism of heart damage by ADR is complex, but oxidative stress is one of the most important effects [1]. Pinealectomy was done in order to examine the effect of MEL at physiological concentrations on one group of animals [15, 16].

As suggested by others, the effect of ADR could be dose- and time-dependent [6, 11, 14]. Li et al. [42] measured myocardial antioxidative enzyme activities, GSH-Px abundance and protein levels at 1, 2, 4 and 24 h after an ADR single injection at a dose of 2.5 mg/kg B.W. They found that, while manganese superoxide dismutase (MnSOD), GSH-Px and catalase activities were not significantly changed, copper-zinc superoxide dismutase (CuZnSOD) activity was reduced at all the time points. In earlier studies ADR, in a dose of 13.5 mg/kg B.W. in rabbits, decreased GSH-Px activity in the heart [43]. While a single dose of ADR (15 mg/kg) resulted in a 56% decrease in cardiac GSH-Px activity, 24 h after injection in mice, a lesser decrease was noted with

10 mg/kg and no changes at all were observed after 5 mg/kg [44].

In our experiment GSH-Px activity in serum decreased 3 days after the single injection of ADR at a dose of 15 mg/kg in Intact, Sham-Px and Px rats. MEL, given for 4 days before ADR administration, in a dose of 5 mg/kg/day and continued for 2 days after ADR, did not change the activity of the enzyme. Pinealectomy decreased the activity of serum GSH-Px activity in all the groups of animals examined, which suggests that endogenous MEL production deficiency can inhibit the activity of this enzyme.

In erythrocytes GSH-Px decreased only after ADR in the Px animals. This effect was not observed either in the Intact or in the Sham-Px rats. Melatonin did not change GSH-Px activity after ADR in any of the groups examined. The effect of Px on the activity of GSH-Px was not as strongly expressed as that of serum GSH-Px activity. This reaction could have depended on the half-life of erythrocytes, which is about 120 days.
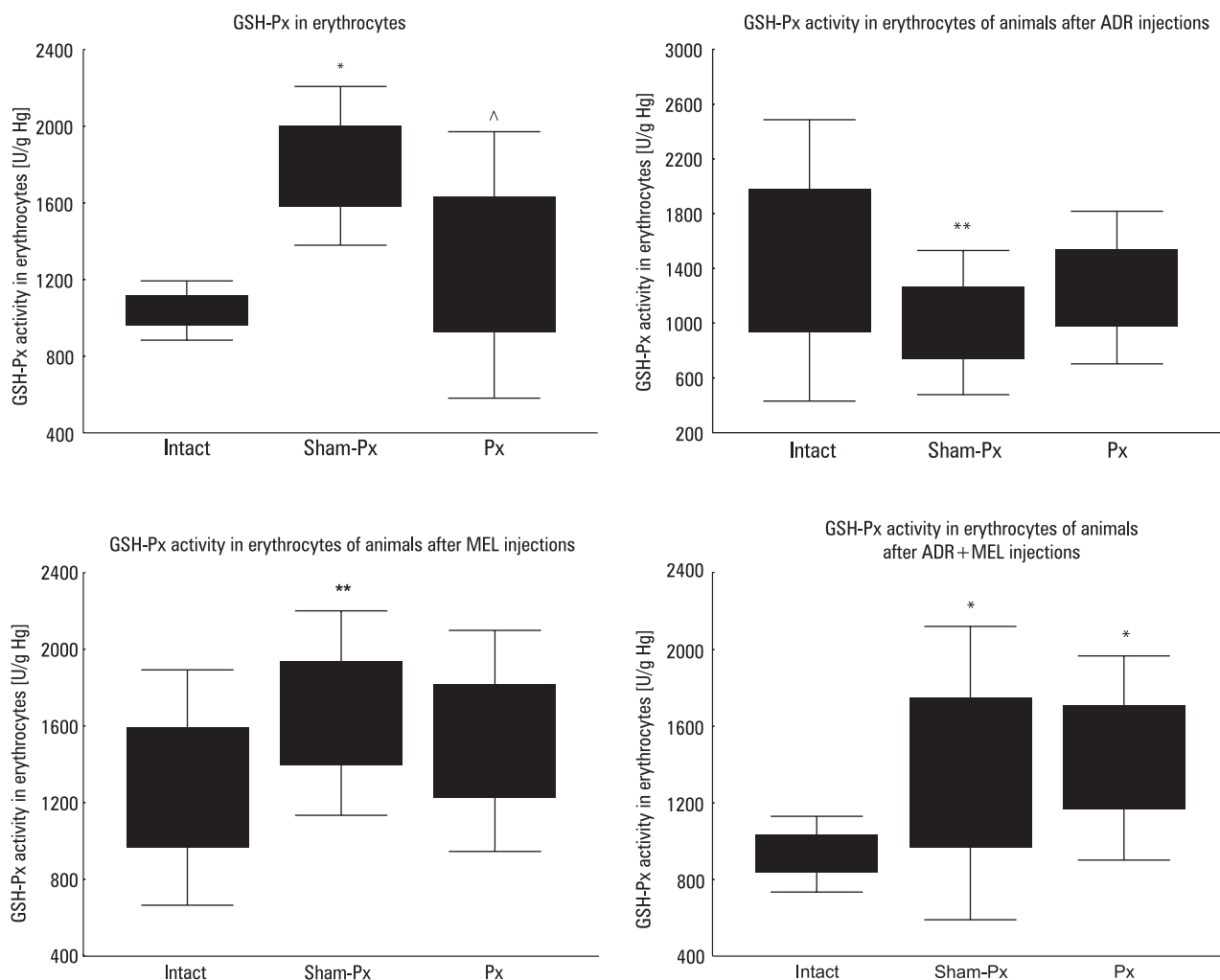
Figure 4. *The effect of pinealectomy on glutathione peroxidase activity in erythrocytes (GSH-Px — U/g Hb) after a single injection of adriamycin (ADR 15 mg/kg), melatonin (MEL 5 mg/kg) given for 4 days before and 2 days after ADR, or both of them administered together.*

The data represent means ± SEM. Level of significance:*p < 0.001; **p < 0.05 vs. controls; ^ p<0.001 vs. MEL group

Rycina 4. *Efekt usunięcia szyszynki na aktywność peroksydazy glutationu w surowicy (GSP-Px — U/g Hb) po podaniu pojedynczej iniekcji adriamycyny (ADR 15 mg/kg), melatoniny (MEL 5 mg/kg) podawanej przez 4 dni przed i 2 dni po iniekcji ADR lub też podawanej razem z ADR*

Dane zaprezentowano w postaci: średnia ± standardowy błąd pomiaru (SEM, standard error of measure). *Poziom istotności: *p < 0,001; **p < 0,05 vs. kontrola; ^ p < 0,001 vs. grupa MEL*

Conclusion

Melatonin, administered in pharmacological concentrations, did not influence serum or erythrocyte glutathione peroxidase activity, either in normal or in pinealectomised rats after adriamycin. The deficiency of endogenous melatonin production may inhibit serum glutathione peroxidase activity.

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PRACE ORYGINALNE