Title:

Influence of Melatonin Pre-treatment and Preconditioning by Hypobaric Hypoxia on the Development of Cortical Photothrombotic Ischemic Lesion.

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Short title:

Melatonin, Hypoxic Preconditioning and Photothrombosis.

Summary: Photothrombotic model of ischemia (PT) is based on free radical-mediated endothelial dysfunction followed by thrombosis. Free radicals are also involved in hypoxic preconditioning. We tested the sensitivity of PT to preconditioning with hypobaric hypoxia and to pre-treatment with melatonin. In adult Wistar rats, after intravenous application of Rose Bengal, a stereo-tactically defined spot on denuded skull was irradiated by a laser for 9 minutes. The first experimental group underwent hypobaric hypoxia 3 days before irradiation. In the second experimental group, melatonin was applied intraperitoneally 1 hour before irradiation. Three days after irradiation, animals were sacrificed, the brains perfused, and stained with TTC. Ischemic lesions were divided into grades (I, II, III). In the control group (where no manipulation preceded photothrombosis), most animals displayed deep damage involving the striatum (grade III). The group pre-exposed to hypoxia showed similar results. Only 28.57% of the melatonin pre-treated animals exhibited grade III lesions, and in 57.14% no signs of lesions were detected.

Pre-exposure to hypoxia was not protective in our model. Pre-treatment with melatonin lead to a significant reduction of the number of large ischemic lesions. This result is probably caused by protection of endothelial cells by melatonin.

Key words:

Melatonin - Hypoxia – Ischemia - Free Radicals - Models, Animal

Introduction

Several experimental models have been devised for the study of the pathophysiology of focal ischemia of the central nervous system. Among these, the model of photothrombosis (PT) (Watson *et al.* 1985) is frequently used, because it is non-invasive and reliable. It involves photodynamic generation of free radicals (mainly singlet oxygen - $^1O_2$) triggered by the exposition of intravenously injected Rose Bengal (disodium tetraiodo-tetrachlorofluorescein) to green light. By mechanisms not completely understood, newly generated $^1O_2$ mediates endothelial dysfunction, which leads to thrombotization and vascular occlusion (Dietrich *et al.* 1987, Ishikawa *et al.* 2002). PT is pathophysiologically similar to naturally occurring ischemic stroke in man - not only due to common pathological mechanisms (including free radical mediated damage), but also due to the common possibility of spontaneous reperfusion.

Free radicals lead to cellular injury when generated in excess. However, their physiological role as signal molecules is now increasingly recognized (Kamsler and Segal 2004), (Brune 2005). Importantly, hypoxic preconditioning (i.e. tolerance to severe hypoxia/ischemia induced by previous exposure to mild hypoxia) is considered to be a free radical mediated phenomenon (Liu *et al.* 2005), at least because it is effectively abolished by pre-treatment with reactive oxygen species (ROS) scavengers (such as dimethylthiourea or ebselen) (Puisieux *et al.* 2004). It was shown that hypoxic preconditioning is most pronounced when the time between the first (mild) and the second (severe) hypoxia is approx. 24-72 hours. Based on these (and other) findings, the phenomenon was attributed to changes in the expression of immediate early genes (e.g. heat-shock proteins). Although theoretical background exists for possible redox regulation of heat-shock protein expression, the exact mechanism taking place in vivo was not yet fully elucidated (for review see Sharp 2004). The antioxidant abilities of melatonin (N-acetyl-5-methoxy tryptamine) were discovered in the

1990s. Its high antioxidant efficiency, both water and lipid solubility and lack of toxicity makes it a good candidate for experiments with free radical induced damage, such as hypoxia/ischemia. Melatonin metabolites, such as cyclic 3-hydroxy melatonin and N-acetyl-formyl kynurenine, also possess antioxidant abilities (Tan *et al.* 2002). Melatonin and its metabolites scavenge a wide range of free radical molecules including hydroxyl radical, hydrogen peroxide, singlet oxygen, nitric oxide and peroxynitrite (Reiter *et al.* 2003). By its effect on gene transcription, melatonin may also influence natural antioxidant systems (Rodriguez *et al.* 2004).

Because PT ischemia is essentially based on free radical generation, we were interested in comparing the effect of pre-exposition to hypobaric hypoxia (hypoxic preconditioning) and the effect of pre-treatment with melatonin on the extent of photothrombotic ischemic lesion in the rat.

Methods

A. Animals

All experiments were performed in accordance with the Ministry of Health of the Czech Republic guidelines and animal protocols were approved by the Ethics committee of the 3rd Medical School, Charles University, Prague.

Male Wistar albino rats (weight 200-220 g; ANLAB, Czech Republic, age postnatal day 60 - 67) were used. The animals were acclimated at least 3 days prior to the study and were maintained on a normal light/dark cycle (12 hours light/12 hours dark), with food and water available ad libitum. Experiments were performed in the morning, i.e. during the light cycle. All experimental and control animals were housed in standardized cages, four rats together prior to the experiment and later every animal alone.

B. Experimental groups and procedures

The rats were randomly assigned to the following four groups.

The first experimental (hypoxia) l group

The first experimental (hypoxia) group (N=11) was exposed to hypobaric hypoxia 72 hours before the induction of photothrombotic cortical lesion.

The hypobaric chamber allowed the use of two boxes with three animals in each one, (no more than six animals at the same time). The starting partial oxygen pressure (pO_2) value was approximately 150 mmHg (normal atmospheric pressure). During 15 minutes, the pO_2 was lowered to a value equal to 9 000 m above sea level (38 mmHg). This degree of hypoxia was maintained for 30 minutes. During the next 15 minutes, the pO_2 was returned to initial level. Throughout the experiment, ambient air was allowed to circulate through the system to prevent CO_2 accumulation. All animals were then returned to their cages.

After a 72 hour period of rest, the animals were anesthesized with ketamine-xylazine (ketamine 80 mg/kg; xylazine 7 mg/kg; Sigma, Czech Republic). During the anesthesia, the animals showed no deficits in vital signs. The scalp of the head was incised (2 cm length in midline) and the skull overlaying the left sensorimotor cortex was cleaned from soft tissues. The tail vein was cannulated and the photosensitive dye Rose Bengal (20 mg/2 ml/kg, dissolved in 0.9 % NaCl) was applied slowly into the systemic circulation.

A diode laser with the wavelength of 532 nm was used as a light source for photothrombosis (power density 50mW/mm^2, illuminated area <1 mm^2). The use of a laser is advantageous, because it produces strictly monochromatic green light without an infrared component. It is therefore cold and heat filtering is not necessary (in comparison with arc lamps). For a comparison of different methods of photothrombosis see (Pevsner *et al.* 2001), Laser-mediated photothrombosis was successfully used before (Sugimori *et al.* 2004, Watson *et al.* 1987, Yao *et al.* 2003), etc. The parameters of the laser used in our study were consulted

with and approved by the original author of the photothrombotic model (B.D. Watson, personal communication).

The laser irradiation started 5 minutes after the administration of Rose Bengal. Shortly before irradiation, each animal was placed in a stereotaxic frame to ensure precise positioning of the laser beam. The denuded skull on the left side (coordinates: posterior 2 mm, lateral 2 mm from bregma) was irradiated by a laser beam for 9 minutes. After irradiation, the scalp was sutured and the animals left to recover for three days.

The second experimental (melatonin) group

In the second experimental group (N=14) , melatonin (Sigma, Czech Republic), freshly suspended in 2% solution of Tween 80 in saline, was administered intraperitoneally 1 hour before the induction of photothrombosis, at the dose of 100 mg/2 ml/kg. The dose was chosen based on our previous experiments with epileptic seizures.

Photothrombosis was then performed as described above.

The control group

Prior to photothrombosis the animals in this group (N=16) were not exposed to any experimental manipulation. The photothrombosis was carried out in the same manner as in the previous two experimental groups.

The "test" group

The test group of animals (N=8) served to evaluate the efficacy of our model. In these animals, normal saline was applied instead of Rose Bengal. Otherwise, the experimental protocol was the same as in the control group.

C. Morphology

Brain slices preparation and staining

Three days after induction of photothrombotic lesion, in deep anesthesia, the animals were transcardially perfused with 0.9 % NaCl solution (37° C) and decapitated. Immediately after the perfusion, the brains were removed and cut into coronal slices 500 μm thick on the level of expected phototrombotic lesion. 2,3,5-triphenyltetrazolium chloride (TTC) reduction test to detect survived mitochondria was used (Khan *et al.* 2000). Slice preparations were submerged completely in the staining solution (2% TTC in 0.1 M phosphate buffer warmed to 37°C) and maintained at 37°C in an incubation box with no access of light for 45 minutes. At the end of the staining, the slices were washed in saline. Digital photographs of the slices were taken and saved for subsequent evaluation of morphologic changes.

Evaluation of the irradiated area

Tetrazolium salts are colorless and in metabolically active tissues, they are reduced to a colored form. Thus, the infarction zone appeared paler than the surrounding tissue.

Lesions detected on the digital photographs were evaluated by three independent raters and divided into three grades (I, II, III) according to their severity (I – no lesion, II - mild lesion, III – deep transcortical defect in some cases including the striatum) - see Figure 1. The grade of lesion was accepted when minimally two of three raters reported the same findings. Generally, the raters were in full agreement. The situation when each rater would report different finding never occurred. Inconsistent finding by one of the raters appeared twice in control group (once between grade I and II and once between grade II and III), in hypoxia group also two times (both between grade I and II) and in melatonin group four times (two times between grade I and II and two times between grade II and III).

The number of animals with each lesion grade was noted in each group. Contingency tables were constructed (for the occurrence of each lesion grade) to compare either one of the experimental groups with the control group, and both experimental groups with each other. The results were statistically evaluated by Fisher exact test.

Results

In the hypoxia group, ischemic lesions developed in all animals (N=11). Ten animals showed severe lesions (grade III, two of them grade III B – see Fig, 1) and one animal displayed a surface defect (grade II).

In the melatonin group, four animals developed deep lesions (grade III A), while mild lesions (grade II) occurred in two animals. In the remaining eight animals, ischemic lesions were not detectable (see Figure 2).

In the melatonin group there was a highly significant reduction of the number of animals with severe lesions (Grade III), when compared to controls ($p < 0.0005$), and when compared to the hypoxia group ($p<0.005$). The number of animals with no detectable lesions (Grade I) was significantly higher in the group pre-treated with melatonin when compared to controls ($p<0.0005$), and when compared to animals pre-exposed to hypoxia ($p<0.0005$). The number of animals with mild lesions (Grade II) was small, and the difference was not significant among the groups.

In the test group of animals subjected to laser irradiation without Rose Bengal application (the test group), ischemic lesion did not occur.

In the control group (i.e. laser irradiation with Rose Bengal application), ischemic lesions developed in all animals. In 15 animals deep transcortical damage and in four cases also deep damage involving the striatum (grade III) was found. One animal exhibited a mild defect (grade II).

Discussion

The model of photothrombosis (PT) used in our study proved to be a reliable technique of producing focal ischemic lesions in the rat.

Pre-treatment with melatonin significantly reduced the number of large lesions (grade III). We have not observed a reduction of lesion volume, i.e. proportionate increase in the number of mild lesions (Grade II). Instead, melatonin pre-treatment resulted in significant increase in the number of animals without any signs of ischemia (Grade I). Apart from methodological limitations (semi-microscopic evaluation of lesions), this "all or nothing" effect may be partially explained by the pathophysiological mechanisms of PT.

The key event in PT is the generation of short-lived cytotoxic molecules of singlet oxygen ($^1O_2$) by photosensitization of Rose Bengal. In morphological (electron-microscopic) studies of PT, significant endothelial denudation was not observed (Watson *et al.* 1985). Despite this finding $^1O_2$ probably causes a functional disturbance at the endothelial plasma membrane, which secondarily leads to increased platelet adhesion to the vessel wall (Ishikawa *et al.* 2002) and thrombosis. It is plausible that melatonin causes a substantial reduction of $^1O_2$ also at this point, therefore thrombosis, and subsequent ischemia may not occur. In models with middle cerebral artery occlusion (MCAO), melatonin was also efficient. It rather reduced the infarct size (Pei *et al.* 2002). In this case, melatonin may have more affected free radicals generated within the lesion itself. In the photothrombotic model, as ischemia develops, secondary free radicals are produced within the lesion as well. However, the primary oxidative burst takes place at the vascular endothelium.

The points at which melatonin and its metabolites may interfere with free radical mediated events within the ischemic lesion are numerous, including attenuation of lipid peroxidation (Tutunculer *et al.* 2005), (Wakatsuki *et al.* 1999), prevention of the increase in

neuronal NO and protein nitration (Guerrero *et al.* 1997), (Cuzzocrea *et al.* 2000), modification of intracellular signaling (Kilic *et al.* 2005). Although melatonin (given as a single injection pre-treatment) possibly does not interfere with some of the listed events directly (its elimination half-life after intravenous application in the laboratory rat being approximately 20 minutes (Yeleswaram *et al.* 1997)), its indirect effects can be widespread and long lasting. In addition, melatonin metabolites may have different pharmacokinetics.

Papers examining the role of melatonin in cortical photothrombosis are scarce. Recently, one such study was published, stressing the neuroprotective potential of melatonin (Zou *et al.* 2006). However, the authors do not consider the fact that free radical formation is the very basis of the mentioned model and that melatonin and its metabolites, as potent free radical scavengers, act against it. Our study aims to draw attention to this point. In this context, the finding that still a significant number of melatonin pre-treated animals develop severe lesions should be emphasized. It may give a signal of more complicated relations between antioxidant application and blocking of photothrombosis.

In our experiment, preconditioning by hypobaric hypoxia did not influence the severity of lesions. This result, implicating the lack of protection by preconditioning, could be influenced to some extent by methodological aspects. With our rating method we may not be able to detect possible small changes in lesion volume and penumbra in preconditioned animals. However, apart from this consideration, the protective mechanisms initiated in neural cells by hypoxic preconditioning may possibly not be as effective as melatonin in attenuating damage induced by the burst of free radicals at the endothelial surface.

In further studies, it would be interesting to combine hypoxic preconditioning and melatonin pre-treatment and compare the results with melatonin alone. To date, one similar study was performed in the myocardium, where melatonin was demonstrated not to inhibit beneficial effect of ischemic preconditioning (Andreadou *et al.* 2004). This finding also

illustrates the complexity of relations between oxidative stress mediated processes and antioxidant treatment.

To summarize, preconditioning by hypobaric hypoxia was not effective in our experiment. The changes may have been subtle and difficult to detect by our methods. Melatonin pre-treatment leads to a significant decrease in the number of severe lesions in the photothrombotic model of focal brain ischemia, with a proportionate increase in the number of semi-microscopically intact animals. This result is probably caused by diminished free radical mediated endothelial cell damage. However, the fact that severe lesions did occur in some melatonin pre-treated animals impugns a simple relation between antioxidant application and blocking of photothrombosis.

Acknowledgments

This study was supported by the following grants:

VZ 0021620816.

GAUK 104/2004/C/3LF.

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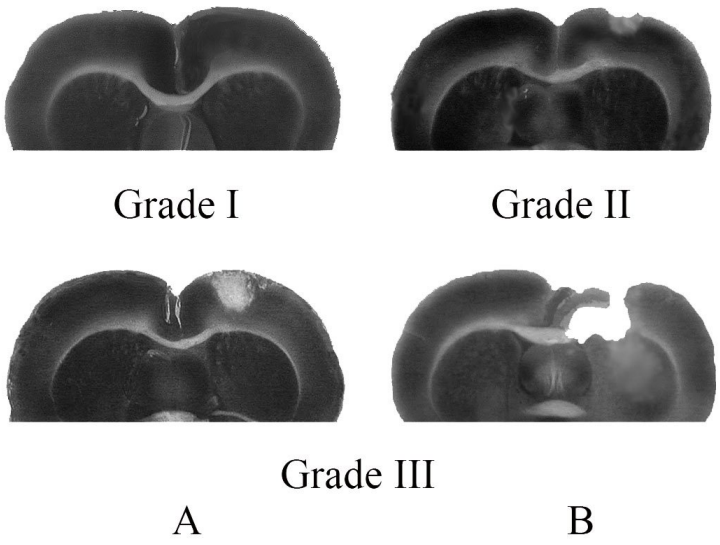

Figure 1: Examples of lesion grades. Grade I = no lesion; Grade II = mild lesion, Grade III (A and B) = severe lesion. (Slices stained with triphenyltetrazoliumchloride - pale regions represent metabolically inactive tissue, normal areas have a pink color. Pictures were transformed into grayscale).

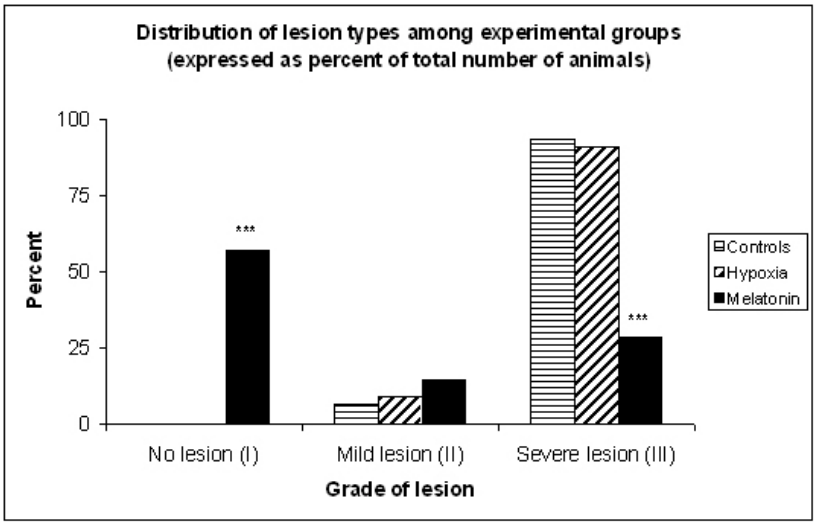

Figure 2: Distribution of lesion types among experimental groups. Percent expression was used for better discrimination of inter-group differences (in each group, total number of animals was different). The test group is not shown - it was used only to demonstrate the efficacy of our model. Note the highly significant increase in the percent of animals without signs of ischemic lesion (Grade I) and the significant decrease in the percent of animals with severe lesions (Grade III) in the group pre-treated with melatonin.