Physiol. Res. 57: 283-288, 2008

Influence of Melatonin Pretreatment and Preconditioning by Hypobaric Hypoxia on the Development of Cortical Photothrombotic Ischemic Lesion

I. MATĚJOVSKÁ[1], K. BERNÁŠKOVÁ[1], D. KRÝSL[1,2], J. MAREŠ[1]

[1] Department of Normal, Pathological and Clinical Physiology, Third Faculty of Medicine, Charles University, Prague and [2] Department of Neurology, University Hospital Motol, Prague, Czech Republic

Received October 25, 2006
Accepted February 6, 2007
On-line available February 8, 2007

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 pretreatment with melatonin. In adult Wistar rats, after intravenous application of Rose Bengal, a stereo-tactically defined spot on the denuded skull was irradiated by a laser for 9 min. The first experimental group underwent hypobaric hypoxia three days before irradiation. In the second experimental group, melatonin was applied intraperitoneally one 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 pretreated 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. Pretreatment 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

Corresponding author

J. Mareš, Department of Normal, Pathological and Clinical Physiology, Third Faculty of Medicine, Charles University, Ke Karlovu 4, 120 00 Prague 2, Czech Republic. Fax: +420 224 923 827, + 420 224 916 896. E-mail: jan.mares@lf3.cuni.cz

Introduction

Several experimental models have been devised for the study of the pathophysiology of focal ischemia of the central nervous system (Xi *et al*. 2004, Johnston *et al.* 2007, Tejkalová *et al.* 2007). 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 tetraiodotetrachloro-fluorescein) to green light. By mechanisms not yet 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 subsequent 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, Mareš *et al.* 2006). 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 pretreatment 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 about 24-72 h. 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 a 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 *et al.* 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 pretreatment with melatonin on the extent of photothrombotic ischemic lesions in the rat.

Methods

Animals

All experiments were performed in accordance with the Principles of Laboraory Animal Care (NIH publication No. 86–23, revised 1985) and animal protocols were approved by the Ethics committee of the Third Faculty of Medicine, Charles University, Prague.

Male Wistar rats (weighing 200-220 g; Anlab, Czech Republic, aged 60-67 days) were used. The animals were acclimated for at least three days prior to the study and were maintained on a normal light/dark cycle (12 h light/12 h 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 each animal alone. The rats were randomly assigned to four groups.

The first experimental (hypoxia) group

The first experimental (hypoxia) group (n=11) was exposed to hypobaric hypoxia for one hour 72 h before induction of the photothrombotic cortical lesion. The hypobaric chamber allowed the use of two boxes with three animals in each, (no more than six animals at the same time). The starting partial oxygen pressure (pO_2) value was approximately 150 mm Hg (normal atmospheric pressure). During 15 min, the pO_2 was lowered to a value equal to 9 000 m above sea level (38 mm Hg). This degree of hypoxia was maintained for 30 min. During the next 15 min, the pO_2 was returned to the 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-h period of rest, the animals were anesthetized 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 the 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 50 mW/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 comparison of different methods of photothrombosis see Pevsner *et al.* (2001). Laser-mediated photothrombosis was successfully used before Watson *et al.* 1987, Yao *et al.* 2003 Sugimori *et al.* 2004). 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 min 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 min. 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.

Brain slice preparation and staining

Three days after induction of photothrombotic lesion, the animals in deep anesthesia 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 at 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 min. 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) (Fig. 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 a different finding never occurred. Inconsistent finding by one of the raters appeared twice in the control group (once between grade I and II and once between grade II and III), in the hypoxia group also two times (both between grade I and II) and four times in the melatonin group (two times between grade I and II and two times between grade II and III).

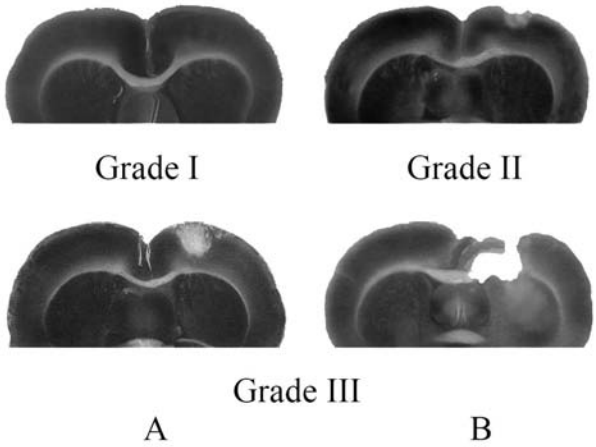

Fig. 1. Examples of lesion grades. Grade I = no lesion; Grade II = mild lesion, Grade III (A and B) = severe lesion. (Slices stained with triphenyltetrazolium chloride – pale regions represent metabolically inactive tissue, normal areas have a pink color. Pictures were transformed into a grayscale).

The number of animals with particular lesion grades 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, or both experimental groups with each other. The results were statistically evaluated by the 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 (Fig. 2).

In the melatonin group there was a highly significant reduction of the number of animals with

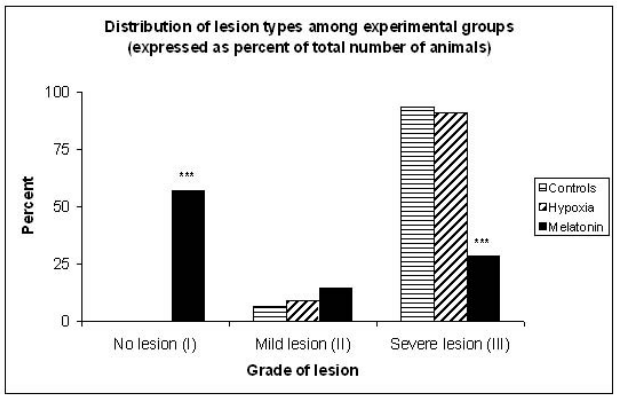

Fig. 2. Distribution of lesion types among experimental groups. Percent expression was used for better discrimination of inter-group differences (in each group, total number of animals was different). Note the highly significant increase in the percent of animals without signs of ischemic lesion (Grade I) and the significant decrease in the percentage of animals with severe lesions (Grade III) in the group pretreated with melatonin.

severe lesions (Grade III), when compared to the 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 pretreated with melatonin when compared to the 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) no ischemic lesion occurred.

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.

Pretreatment with melatonin significantly reduced the number of large lesions (grade III). We have not observed a reduction of lesion volume, i.e. a proportional increase in the number of mild lesions (Grade II). Instead, melatonin pretreatment resulted in a 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 because 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 also produced within the lesion. 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) or modification of intracellular signaling (Kilic *et al.* 2005). Although melatonin (given as a single injection pretreatment) 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 min (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, these authors do not consider the fact that free radical formation is the very basis of the above mentioned model and that melatonin and its metabolites, as potent free radical scavengers, act against it. Our study aimed to draw the attention to this point. In this context, it should be emphasized that still a significant number of melatonin pretreated animals develop severe lesions. 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 pretreatment and compare the results with melatonin alone. A 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 pretreatment 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-pretreated animals doubts a simple relation between antioxidant application and blocking of photothrombosis.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

This study was supported by the following grants: VZ 0021620816 and GAUK 104/2004/C/3LF.

References

ANDREADOU I, ILIODROMITIS EK, MIKROS E, BOFILIS E, ZOGA A, CONSTANTINOU M, TSANTILI-KAKOULIDOU A, KREMASTINOS DT: Melatonin does not prevent the protection of ischemic preconditioning in vivo despite its antioxidant effect against oxidative stress. *Free Radic Biol Med* **37**: 500-510, 2004.

BRUNE B: The intimate relation between nitric oxide and superoxide in apoptosis and cell survival. *Antioxid Redox Signal* **7**: 497-507, 2005.

CUZZOCREA S, COSTANTINO G, GITTO E, MAZZON E, FULIA F, SERRAINO I, CORDARO S, BARBERI I, DE SARRO A, CAPUTI A P: Protective effects of melatonin in ischemic brain injury. *J Pineal Res* **29**: 217-227, 2000.

DIETRICH W, WATSON B, BUSTO R, GINSBERG M, BETHEA J: Photochemically induced cerebral infarction. I. Early microvascular alterations. *Acta Neuropathol (Berl)* **72**: 315-325, 1987.

GUERRERO JM, REITER RJ, ORTIZ GG, PABLOS MI, SEWERYNEK E, CHUANG JI: Melatonin prevents increases in neural nitric oxide and cyclic GMP production after transient brain ischemia and reperfusion in the Mongolian gerbil (Meriones unguiculatus). *J Pineal Res* **23**: 24-31, 1997.

ISHIKAWA M, SEKIZUKA E, OSHIO C, SATO S, YAMAGUCHI N, TERAO S, TSUKADA K, MINAMITANI H, KAWASE T: Platelet adhesion and arteriolar dilation in the photothrombosis: observation with the rat closed cranial and spinal windows. *J Neurol Sci* **194**: 59-69, 2002.

JOHNSTON KC, WAGNER DP, WANG XQ, NEWMAN GC, THIJS V, SEN S, WARACH S: Validation of an acute ischemic stroke model: does diffusion-weighted imaging lesion volume offer a clinically significant improvement in prediction of outcome? *Stroke* **38**: 1820-1825, 2007.

KAMSLER A, SEGAL M: Hydrogen peroxide as a diffusible signal molecule in synaptic plasticity. *Mol Neurobiol* **29**: 167-178, 2004.

KHAN SH, BAZIANY A, BANIGESH A, HEMMINGS SJ, SHUAIB A: Evaluation of an optimal temperature for brain storage in delayed 2, 3,5-triphenyltetrazolium chloride staining. *J Neurosci Methods* **98**: 43-47, 2000.

KILIC U, KILIC E, REITER RJ, BASSETTI CL, HERMANN DM: Signal transduction pathways involved in melatonin-induced neuroprotection after focal cerebral ischemia in mice. *J Pineal Res* **38**: 67-71, 2005.

LIU J, NARASIMHAN P, YU F, CHAN PH: Neuroprotection by hypoxic preconditioning involves oxidative stress-mediated expression of hypoxia-inducible factor and erythropoietin. *Stroke* **36**: 1264-1269, 2005.

MAREŠ J, POMETLOVÁ M, KRÝSL D, ROKYTA R: Influence of scavengers of reactive oxygen species on learning impairment elicited by flurothyl epileptic seizure. *Physiol Res* **55**: 31P, 2006.

PEI Z, PANG SF, CHEUNG RT: Pretreatment with melatonin reduces volume of cerebral infarction in a rat middle cerebral artery occlusion stroke model. *J Pineal Res* **32**: 168-172, 2002.

PEVSNER PH, EICHENBAUM JW, MILLER DC, PIVAWER G, EICHENBAUM KD, STERN A, ZAKIAN KL, KOUTCHER JA: A photothrombotic model of small early ischemic infarcts in the rat brain with histologic and MRI correlation. *J Pharmacol Toxicol Methods* **45**: 227-233, 2001.

PUISIEUX F, DEPLANQUE D, BULCKAEN H, MABOUDOU P, GELE P, LHERMITTE M, LEBUFFE G, BORDET R: Brain ischemic preconditioning is abolished by antioxidant drugs but does not up-regulate superoxide dismutase and glutathione peroxidase. *Brain Res* **1027**: 30-37, 2004.

REITER RJ, TAN DX, MANCHESTER LC, LOPEZ-BURILLO S, SAINZ RM, MAYO JC: Melatonin: detoxification of oxygen and nitrogen-based toxic reactants. *Adv Exp Med Biol* **527**: 539-548, 2003.

RODRIGUEZ C, MAYO J C, SAINZ RM, ANTOLIN I, HERRERA F, MARTIN V, REITER RJ: Regulation of antioxidant enzymes: a significant role for melatonin. *J Pineal Res* **36**: 1-9, 2004.

SHARP F R, RAN R, LU A, TANG Y, STRAUSS K I, GLASS T, ARDIZZONE T, BERNAUDIN M: Hypoxic preconditioning protects against ischemic brain injury. *NeuroRx* **1**: 26-35, 2004.

SUGIMORI H, YAO H, OOBOSHI H, IBAYASHI S, IIDA M: Krypton laser-induced photothrombotic distal middle cerebral artery occlusion without craniectomy in mice. *Brain Res Brain Res Protoc* **13**: 189-196, 2004.

TAN DX, REITER R J, MANCHESTER LC, YAN MT, EL-SAWI M, SAINZ RM, MAYO JC, KOHEN R, ALLEGRA M, HARDELAND R: Chemical and physical properties and potential mechanisms: melatonin as a broad spectrum antioxidant and free radical scavenger. *Curr Top Med Chem* **2**: 181-197, 2002.

TEJKALOVÁ H, KAISER M, KLASCHKA J, ŠŤASTNÝ F: Does neonatal brain ischemia induce schizophrenia-like behavior in young adult rats? *Physiol Res* **56**: 815-823, 2007.

TUTUNCULER F, ESKIOCAK S, BASARAN U N, EKUKLU G, AYVAZ S, VATANSEVER U: The protective role of melatonin in experimental hypoxic brain damage. *Pediatr Int* **47**: 434-439, 2005.

WAKATSUKI A, OKATANI Y, IZUMIYA C, IKENOUE N: Melatonin protects against ischemia and reperfusion-induced oxidative lipid and DNA damage in fetal rat brain. *J Pineal Res* **26**: 147-152, 1999.

WATSON B, DIETRICH W, BUSTO R, WACHTEL M, GINSBERG M: Induction of reproducible brain infarction by photochemically initiated thrombosis. *Ann Neurol* **17**: 497-504, 1985.

WATSON B, DIETRICH W, PRADO R, GINSBERG M: Argon laser-induced arterial photothrombosis. Characterization and possible application to therapy of arteriovenous malformations. *J Neurosurg* **66**: 748-754, 1987.

XI GM, WANG HQ, HE GH, HUANG CF, WEI GY: Evaluation of murine models of permanent focal cerebral ischemia. *Chin Med J (Engl)* **117**: 389-394, 2004.

YAO H, SUGIMORI H, FUKUDA K, TAKADA J, OOBOSHI H, KITAZONO T, IBAYASHI S, IIDA M: Photothrombotic middle cerebral artery occlusion and reperfusion laser system in spontaneously hypertensive rats. *Stroke* **34**: 2716-2721, 2003.

YELESWARAM K, MCLAUGHLIN LG, KNIPE J O, SCHABDACH D: Pharmacokinetics and oral bioavailability of exogenous melatonin in preclinical animal models and clinical implications. *J Pineal Res* **22**: 45-51, 1997.

ZOU LY, CHEUNG RT, LIU S, LI G, HUANG L: Melatonin reduces infarction volume in a photothrombotic stroke model in the wild-type but not cyclooxygenase-1-gene knockout mice. *J Pineal Res* **41**: 150-156, 2006.