Administration of Melatonin After Onset of Ischemia Reduces the Volume of Cerebral Infarction in a Rat Middle Cerebral Artery Occlusion Stroke Model

Zhong Pei, MBBS, MMed; Shiu Fun Pang, PhD; Raymond Tak Fai Cheung, MBBS, PhD

Background and Purpose—In both permanent and transient 3-hour middle cerebral artery occlusion rat stroke models, a single intraperitoneal injection of melatonin at 5 or 15 mg/kg given before ischemia was shown to reduce infarct volume at 72 hours. The present study was conducted to examine the treatment time window when melatonin was commenced after onset of ischemia.

Methods—Adult male Sprague-Dawley rats were anesthetized to undergo right-sided middle cerebral artery occlusion for 3 hours. A single intraperitoneal injection of vehicle or melatonin at 5 mg/kg was given at 0, 1, or 3 hours after onset of ischemia. Other groups received multiple injections of vehicle or melatonin at 5 mg/kg with the first injection given at 1, 2, or 3 hours after onset of ischemia and the second and third injections at 24 and 48 hours, respectively. Multiple injections of melatonin at 15 mg/kg with the first injection given at 3 hours were also made. The infarct volume was determined at 72 hours.

Results—A single dose of melatonin at 5 mg/kg given at 0 or 1 but not 3 hours after onset of ischemia reduced the infarct volume. Multiple doses of melatonin at 5 mg/kg also reduced the infarct volume when the first dose was given at 1 or 2 but not 3 hours after onset. Significant hemodynamic effects were not observed.

Conclusions—Our results indicate that melatonin at 5 mg/kg given as a single injection or multiple injections protects against focal cerebral ischemia when commenced within 2 hours of onset. (Stroke. 2003;34:770-775.)

Key Words: melatonin ■ middle cerebral artery occlusion ■ neuroprotection ■ stroke, experimental ■ rats

Most stroke patients are ineligible for recombinant tissue plasminogen activator (rtPA),1–3 and the risk of hemorrhagic transformation becomes unacceptable if rtPA is given to ineligible patients.4 Although remarkable insight into the pathogenic mechanisms of ischemic brain damage has been achieved in the last decade,5–8 no cerebroprotective agent is proven to be efficacious in controlled clinical trials.1 In addition, cerebroprotective treatment can potentially extend the therapeutic time window and/or enhance the efficacy of rtPA.

Oxidative damage due to overproduction of reactive free radicals plays a key role in the pathogenesis of ischemic brain damage, and free radicals are generated during both ischemia and reperfusion.5,7 Accumulating evidence suggests that oxidative damage does not occur in isolation but has complex interactions with excitotoxicity, apoptosis, and inflammation.6 Pharmacological modification of oxidative damage may protect against ischemia. Melatonin (N-acetyl-5-methoxytryptamine) is a potent free radical scavenger as well as indirect antioxidant.7 It is produced from L-tryptophan in a limited number of mammalian organs such as the pineal gland, retina, and gastrointestinal tract.9 Melatonin has been shown to be protective in gastrointestinal, myocardial, and cerebral models of ischemia/reperfusion injury when melatonin is given before ischemia10–14 and/or repeated doses are started before reperfusion.11–16

In both permanent and transient 3-hour middle cerebral artery occlusion (MCAO) rat stroke models, a single intraperitoneal injection of melatonin at 5 or 15 mg/kg given at 30 minutes before MCAO significantly reduces infarct volume at 72 hours.17,18 The present study was conducted to document the benefit of melatonin given after onset of ischemia and to identify the therapeutic time window.

Materials and Methods

Animal Preparation
All experiments were conducted according to the institutional guidelines with the protocol approved by the Committee on the Use of Live Animals in Teaching and Research, the University of Hong Kong. Adult male Sprague-Dawley rats, weighing between 280 and 360 g, were purchased from the Laboratory Animal Unit, the University of Hong Kong. The rats were maintained under a diurnal
lighting condition (12 hours of light beginning at 6 AM) with free access to food and water for a minimum of 4 days before experimentation.

Experiments were performed during the light period. Rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (Rhone Merieux, Pinkenba) at a dose of 60 mg/kg. Most rats required an additional bolus of 20 mg/kg to ensure stable anesthesia. Rectal temperature was monitored continuously and maintained between 36.5°C and 37.5°C throughout the anesthesia via a feedback-regulated heating pad (FHC) placed underneath the rat. The right femoral artery was cannulated with PE-50 polyethylene tubing for monitoring of arterial blood pressure (BP) and heart rate (HR) (Powerlab/16 Data Acquisition System, AD Instruments Pty Ltd). These parameters were monitored continuously before and during the 3-hour MCAO as well as in the first 30 minutes of reperfusion.

### Determination of Infarct Volume

Seventy-two hours after onset of ischemia, the rats were deeply reanesthetized with sodium pentobarbital at a dose of 100 mg/kg. Their brains were collected after decapitation. Brains were cut into 2-mm-thick coronal slices with a rodent brain matrix. Extent of ischemic infarction was revealed by reaction with a 2% solution of 2,3,5-triphenyltetrazolium chloride (TTC; Sigma Chemical) for 20 to 30 minutes. After TTC reaction, the brain slices were fixed in 10% formalin at pH 7.4. The hemispheric volumes and volume of infarction between the bregma levels of +4 (anterior) and −6 mm (posterior) were integrated from the respective calibrated area measurements made on the digitalized images of the TTC-reacted brain slices with a computer-assisted image analysis system (SigmaScan Pro 4.0, SPSS Inc.). To correct for swelling due to cerebral edema, the infarct volume was normalized and expressed as a percentage of the ipsilateral hemispheric volume. A cerebral edema index was generated from the ratio of the right to left hemispheric volume.

### Statistical Analysis

There was no significant difference in the hemodynamic parameters and brain infarct volume between the control groups injected with vehicle at 30 minutes before or at the onset of MCAO. Thus, results of the control group (n=6) receiving an intraperitoneal injection at the onset of MCAO were combined with those of a previous control group (n=14) in which an intraperitoneal injection was made at 30 minutes before 3-hour MCAO. Data on regional cerebral perfusion were available from 5 rats of the previous control group. The data were analyzed with SPSS (Windows version 10.0) with the general linear model for univariate or repeated-measures 1-way or 2-way ANOVA because of the unequal number of rats in each group. For the groups receiving a single injection, the relative infarct volume and cerebral edema index were compared with univariate 1-way ANOVA, and the mean BP, HR, and normalized regional cerebral perfusion data were compared with repeated-measures 1-way ANOVA. The Dunnett post test was used to reveal any significant difference between any of the melatonin-treated groups and the combined vehicle-treated control group. For the groups receiving multiple injections at 1, 2, or 3 hours (including the groups treated with melatonin at 5 or 15 mg/kg) of ischemia, the relative infarct volume and cerebral edema index were compared with univariate 2-way ANOVA, and the mean BP, HR, and normalized regional cerebral perfusion data were compared with repeated-measures 2-way ANOVA. Additional comparisons were made for generating hypotheses only; Student’s t test was applied to reveal any significant difference in the relative infarct volume between each of the melatonin-treated groups and the respective vehicle-treated control group of part 2 of this study as well as between single and multiple injections of melatonin at 5 mg/kg with the first injection made at 1 or 3 hours of ischemia, and univariate 1-way ANOVA with the Dunnett post test was used to reveal any difference in the relative infarct volume among 0 (ie, vehicle only), 5, and 15 mg/kg of melatonin in the groups receiving multiple injections, with the first injection made at 3 hours of ischemia. A 2-tailed probability value of ≤0.05 was taken to indicate statistical significance.

### Results

#### Groups With Single Injection

Table 1 summarizes the relative infarct volume and cerebral edema index after 3 hours of focal ischemia and 69 hours of reperfusion plus single or multiple injections of melatonin or its vehicle. Compared with the relative infarct volume of 32.7±2.7% in the combined control group, a single intraperitoneal injection of melatonin at 5 mg/kg at 0, 1, or 3 hours of ischemia reduced the relative infarct volume by 37.6%, 38.8%, and 17.4%, respectively (P<0.05 with a significant difference between the melatonin-treated groups at 0 or 1 hour of ischemia and the combined control group). There was no significant difference in the cerebral edema index (Table 1).

#### Determination of Infarct Volume

Seventy-two hours after onset of ischemia, the rats were deeply reanesthetized with sodium pentobarbital at a dose of 100 mg/kg.
bral perfusion (Table 2) among the different groups. In addition, the normalized regional cerebral perfusion ($P<0.001$) but not the mean BP or HR showed significant change at different time points; the mean regional cerebral perfusion was suppressed to $<30\%$ during MCAO and rapidly restored on reperfusion (Table 2).

### Groups With Multiple Injections

Compared with the respective relative infarct volume of $30.4\%\pm 7.4\%$, $31.3\%\pm 5.6\%$, and $31.2\%\pm 5.4\%$ in the vehicle groups when the first injection was made at 1, 2, and 3 hours, respectively (Table 1), multiple intraperitoneal injections of melatonin with the first injection given at 1, 2, or 3 hours of ischemia reduced the relative infarct volume by $54.5\%$, $42.9\%$, and $22.7\%$, respectively ($P<0.001$ for treatment with melatonin and $P=0.362$ for timing of injections). There was no significant difference in the cerebral edema index (Table 1).

There was no significant difference in the mean BP (data not shown), HR (data not shown), or normalized regional cerebral perfusion (Table 2) among the different groups. In addition, the normalized regional cerebral perfusion

### Table 1. Relative Infarct Volume (%) and Cerebral Edema Index

<table>
<thead>
<tr>
<th>Group</th>
<th>0 h of Ischemia</th>
<th>1 h of Ischemia</th>
<th>3 h of Ischemia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle only</td>
<td>32.7±2.7% (20)</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td></td>
<td>1.073±0.025</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Melatonin</td>
<td>20.4±3.6%* (8)</td>
<td>20.0±2.8%* (9)</td>
<td>27.0±5.3% (9)</td>
</tr>
<tr>
<td></td>
<td>1.014±0.014</td>
<td>1.042±0.010</td>
<td>1.042±0.009</td>
</tr>
<tr>
<td>Multiple IP Injections</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1, 24, 48 h of</td>
<td>2, 24, 48 h of</td>
<td>3, 24, 48 h of</td>
</tr>
<tr>
<td></td>
<td>Ischemia</td>
<td>Ischemia</td>
<td>Ischemia</td>
</tr>
<tr>
<td>Vehicle only</td>
<td>30.4±7.4% (6)</td>
<td>31.3±5.6% (8)</td>
<td>31.2±5.4% (10)</td>
</tr>
<tr>
<td></td>
<td>1.050±0.032</td>
<td>1.070±0.019</td>
<td>1.042±0.013</td>
</tr>
<tr>
<td>Melatonin</td>
<td>13.8±3.4%† (8)</td>
<td>13.4±1.5%† (9)</td>
<td>24.1±3.1%‡ (14)</td>
</tr>
<tr>
<td></td>
<td>1.033±0.007</td>
<td>1.023±0.007</td>
<td>1.072±0.016</td>
</tr>
</tbody>
</table>

Relative infarct volume (%) and cerebral edema index in different groups of rats following the middle cerebral artery occlusion (MCAO) for 180 minutes plus a single intraperitoneal (IP) injection of vehicle given at 0.5 hours before or at the onset of ischemia or of melatonin given at 0, 1, or 3 hours of ischemia or plus multiple IP injection of vehicle or melatonin with the first dose given at 1, 2, or 3 hours of ischemia in the rat. Data are expressed in mean±SEM. Number of rats in each group is indicated inside parentheses.

*Significant difference among the groups with Dunnett’s post test showing significant difference between the vehicle-treated group and these melatonin-treated groups.

†Significant treatment effect due to melatonin but no effect due to the different timing of injections with Student’s $t$ test showing possible difference between the vehicle-treated groups and the respective melatonin-treated groups.

‡This includes 8 rats receiving 3 injections of melatonin at 5 mg/kg (23.3±4.8%) and 6 rats given melatonin at 15 mg/kg (25.2±5.9%).

### Table 2. Normalized Regional Cerebral Perfusion (%)

<table>
<thead>
<tr>
<th>Group (No. of Rats)</th>
<th>Before MCAO</th>
<th>5 min of MCAO</th>
<th>60 min of MCAO</th>
<th>120 min of MCAO</th>
<th>5 min of Reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle at −0.5 or 0 h (11)</td>
<td>100.0</td>
<td>29.3±5.3</td>
<td>28.3±3.8</td>
<td>28.3±3.6</td>
<td>113.2±12.8</td>
</tr>
<tr>
<td>Melatonin at 0 h (8)</td>
<td>100.0</td>
<td>22.8±2.9</td>
<td>25.2±3.5</td>
<td>21.8±2.9</td>
<td>101.4±11.3</td>
</tr>
<tr>
<td>Melatonin at 1 h (9)</td>
<td>100.0</td>
<td>29.9±2.4</td>
<td>30.1±4.0</td>
<td>29.9±5.6</td>
<td>89.7±13.3</td>
</tr>
<tr>
<td>Melatonin at 3 h (9)</td>
<td>100.0</td>
<td>26.9±2.9</td>
<td>24.1±2.8</td>
<td>26.8±2.9</td>
<td>86.1±19.4</td>
</tr>
<tr>
<td>Vehicle at 1, 24, 48 h (6)</td>
<td>100.0</td>
<td>26.3±4.9</td>
<td>22.5±4.8</td>
<td>22.7±5.1</td>
<td>90.4±10.3</td>
</tr>
<tr>
<td>Melatonin at 1, 24, 48 h (8)</td>
<td>100.0</td>
<td>21.9±5.2</td>
<td>19.6±3.5</td>
<td>21.3±2.9</td>
<td>81.9±13.5</td>
</tr>
<tr>
<td>Vehicle at 2, 24, 48 h (8)</td>
<td>100.0</td>
<td>23.2±4.6</td>
<td>22.9±6.1</td>
<td>21.1±5.2</td>
<td>81.6±10.7</td>
</tr>
<tr>
<td>Melatonin at 2, 24, 48 h (9)</td>
<td>100.0</td>
<td>24.7±2.9</td>
<td>26.9±2.9</td>
<td>17.8±2.6</td>
<td>78.3±10.7</td>
</tr>
<tr>
<td>Vehicle at 3, 24, 48 h (10)</td>
<td>100.0</td>
<td>26.9±5.2</td>
<td>14.7±2.9</td>
<td>17.6±2.8</td>
<td>84.2±8.5</td>
</tr>
<tr>
<td>Melatonin at 3, 24, 48 h (14)*</td>
<td>100.0</td>
<td>23.5±2.6</td>
<td>20.0±2.3</td>
<td>17.1±2.1</td>
<td>83.3±9.6</td>
</tr>
</tbody>
</table>

Normalized regional cerebral perfusion (%) at time points in different groups of rats in relation to the middle cerebral artery occlusion (MCAO) for 180 minutes plus a single intraperitoneal (IP) injection of vehicle given at 0.5 hours before or at the onset of ischemia or of melatonin given at 0, 1, or 3 hours of ischemia or plus multiple IP injection of vehicle or melatonin with the first dose given at 1, 2, or 3 hours of ischemia in the rat. Data are expressed in mean±SEM.

*Each dose of melatonin is at 5 mg/kg except in this group: 6 rats received 3 doses of melatonin at 15 mg/kg.
The main findings of the present study are that (1) a single dose of melatonin at 5 mg/kg given at 0 or 1 hour but not 3 hours of ischemia significantly reduces infarct volume at 72 hours by approximately 40%; (2) multiple doses of melatonin at 5 mg/kg started after onset of ischemia significantly reduce the infarct volume by approximately 40%, and this benefit seems to disappear when the first dose is commenced at 3 hours of ischemia; (3) there may not be any additional benefit from giving the second and third doses of melatonin at 24 and 48 hours of ischemia; (4) treatment with melatonin does not significantly affect the cerebral edema index; and (5) treatment with melatonin does not produce significant hemodynamic effects during ischemia and the first 30 minutes of reperfusion.

In primary cultures, melatonin as a cotreatment was shown to protect rat cerebellar granule neurons against excitotoxicity due to kainate but not N-methyl-D-aspartate. Nevertheless, melatonin did not affect binding of glutamate to rat cerebellar membranes or kainate-stimulated inward currents or rise in free cytosolic calcium. Subsequently, 4 intraperitoneal injections of melatonin at 2.5 mg/kg, with the first injection made at 20 minutes before an intraperitoneal injection of kainate and other injections at 0, 1, and 2 hours after the injection of kainate, was found to ameliorate the kainate-induced neuronal death and apoptosis in the hippocampus, amygdala, and pyriform cortex as well as behavioral and biochemical disturbances in rats. Melatonin protects against kainate-induced excitotoxicity because of its direct and indirect antioxidant effects.

When melatonin deficiency was induced in rats by pinealectomy, melatonin-deficient rats were more susceptible to kainate-induced excitotoxicity (with rats killed at 72 hours), photothermonebotic brain injury (with rats killed at 24 hours), or focal ischemia (with rats killed at 4 or 6 hours) due to microsurgical clipping of both common carotid arteries and 1 MCA for 1 hour. In a follow-up study on the effects of pinealectomy and melatonin in the 3-vessel occlusion model of focal ischemia for 90 minutes, the extent of DNA damage and infarct volume at 6 or 24 hours after focal ischemia was increased in pinealectomized rats, and these effects of pinealectomy were reversed by 4 intraperitoneal doses of melatonin at 2.5 mg/kg (given at 30 minutes before as well as at 0, 1, and 2 hours after focal ischemia). The opposing effects of pinealectomy and melatonin on brain damage at 24 hours of ischemia in rats were confirmed in another study in which hemodynamic factors were also monitored and focal ischemia was induced by endovascular MCAO for 2 hours. In addition, melatonin at 4 mg/kg given before ischemia and reperfusion was shown to reduce infarct volume by 40% in both pinealectomized and sham-pinealectomized rats, suggesting a neuroprotective action of exogenous melatonin.

Three intraperitoneal injections of melatonin at 10 mg/kg in rats protected CA1 hippocampal neurons against forebrain ischemia for 10, 20, or 30 minutes only when the first injection was made immediately at reperfusion but not at 30 minutes before or 1 hour after reperfusion; the second and third injections were made at 2 and 6 hours of reperfusion. An intraperitoneal injection of melatonin at 10 mg/kg given at 30 minutes before ischemia was found to inhibit brain nitric oxide (NO) production and suppress NO synthase in a 10-minute gerbil model of forebrain ischemia and reperfusion. In another study, 4 intraperitoneal injections of melatonin at 10 mg/kg (given at 30 minutes before as well as at 1, 2, and 6 hours after reperfusion) were shown to reduce NO levels in the plasma at 4 hours, lipid peroxidation in the brain at 1 hour, cerebral edema at 48 hours, and CA1 neuronal loss at 96 hours after reperfusion in a 5-minute gerbil model of forebrain ischemia.

Taken together, previous studies have shown the neuroprotective effects of exogenous melatonin in kainate-induced excitotoxicity in rats, focal cerebral ischemia in pinealectomized rats, and forebrain ischemia in rats and gerbils only when melatonin was given as multiple doses with the first one as a pretreatment or cotreatment. Two recent studies show that pretreatment with a single intraperitoneal injection of melatonin at doses between 5 and 15 mg/kg significantly reduces the infarct volume at 72 hours by approximately 40% without affecting the mean BP, HR, and regional cerebral perfusion in both permanent and 3-hour transient MCAO stroke models in rats. Compared with these studies, commencement of single melatonin injection at 1 hour or less after onset of ischemia produces a similar reduction in infarct volume. Addition of the second and third doses at 24 and 48 hours of ischemia may not achieve a larger reduction in infarct volume or an extension of the treatment time window beyond 2 hours of ischemia. Increasing the individual dose of melatonin from 5 to 15 mg/kg may not extend the treatment time window to 3 hours of ischemia. The relatively short time window of <3 hours for both single and multiple injections as well as the absence of significant effects on cerebral edema and hemodynamic parameters suggests that melatonin mediates its cerebroprotective actions against the early pathophysiological events of severe focal ischemia. On the other hand, the treatment regimen can be as simple as a single dose given within 2 hours of ischemia.
Melatonin probably ameliorates oxidative damage of reactive free radicals generated during both ischemia and reperfusion. In addition to being a direct free radical scavenger and an indirect antioxidant, melatonin may help to preserve mitochondrial function during ischemia and reperfusion. Mitochondrial dysfunction is an important mediator of ischemic cell death. When mitochondria generate ATP through the mitochondrial respiratory chain, reactive oxygen species are produced by complex I and complex III. When mitochondrial respiration is disturbed during ischemia, ATP synthesis is impaired, and excessive generation of reactive oxygen species occurs. Melatonin preserves mitochondrial function by improving the electron transport chain, ameliorating mitochondrial oxidative stress, maintaining ATP production, and restoring glutathione levels.

Melatonin possesses anti-inflammatory effects via indirect inhibition of NO synthase, suppressed induction of inducible NO synthase and cyclooxygenase-2, and inhibition of neutrophil infiltration, but the present results do not support an important role of its anti-inflammatory effects in protection against focal ischemia because inflammation is important in the delayed evolution of ischemic infarction.

Melatonin is a lipid soluble molecule and a relatively safe drug with excellent oral absorption and tissue bioavailability. It is hypothesized that melatonin can be used in stroke patients to alleviate the ischemic lesion. The possibility of high-risk patients, who are willing to keep melatonin pills or patches, being treated immediately with melatonin after any signs of stroke should be considered. A reduction of the infarct volume in stroke patients would lessen the brain damage and facilitate recovery after appropriate hospital care. Moreover, melatonin may be used in combination with other cerebroprotectors or with rtPA to improve the treatment effects as well as extend the treatment time window of rtPA. For example, melatonin can be offered as a prehospital treatment in combination with other strategies. Melatonin may be an important cerebroprotective agent against ischemic and reperfusion injury because of its excellent diffusion in brain tissue after either systemic injection or oral administration and lack of toxic effects in animals and humans. Both rtPA and melatonin have a short treatment time window, but the treatment with melatonin is very simple and convenient.

In conclusion, melatonin as single or multiple intraperitoneal injections of 5 mg/kg given as late as 2 hours after ischemia significantly reduces infarct volume without affecting cerebral edema and hemodynamic parameters. It appears that a single dose of melatonin at 5 mg/kg given as late as 2 hours after ischemia is sufficient and that subsequent dosing or higher doses of melatonin may be unnecessary. In vitro studies using neuronal cell culture are useful in unraveling the cerebroprotective mechanisms of melatonin in focal cerebral ischemia. Further studies should also be conducted to explore the benefit of combining melatonin with other cerebroprotectants or rtPA.

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References


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