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Protective effect of melatonin on injuried cerebral neurons is associated with *bcl*-2 protein over-expression[1]

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KEY WORDS melatonin; free radicals; cerebral ischemia; proto-oncogene proteins c-*bcl*-2; *bax*; immunohistochemistry; cerebral arteries

ABSTRACT

AIM: To study the protective effect of melatonin against neuronal injury and the possible roles of alteration in the expression of *bcl*-2 and *bax* following brain ischemia. **METHODS**: Brain ischemia was induced by left middle cerebral artery occlusion (MCAO) for 60 min in rats. Brain damage was evaluated by the infarct area and the neuronal cell counting. The expression of *bcl*-2 and *bax* was analyzed by immunohistochemical method. **RESULTS**: Melatonin decreased the infarct area and prevented the neuronal death after 24-h reperfusion following 1-h MCAO. Melatonin given before the ischemia enhanced the expression of *bcl*-2 in the penumbra area and had no significant effect on the expression of *bax*. **CONCLUSION**: Melatonin effectively attenuated ischemic brain injury and increased the expression of neuronal *bcl*-2 in the ischemic brain, indicating that the protective effect of melatonin was associated with up-regulation of *bcl*-2 in ischemia-induced neuronal death.

INTRODUCTION

Oxygen free radicals were involved in acute and chronic neuronal damage. However, most scavengers of free radicals were not so effective as we expected because of their hemodynamics, pharmacokinetics, toxicity, blood-brain barrier permeability[1]. Melatonin was a potent scavenger of free radicals[2]. Because melatonin had high lipophilicity and diffusibility, it easily entered neuronal cells through blood-brain barrier and gained access to every subcellular compartment where it might exert local protective effect.

Cerebral ischemia resulted in neuronal damage which involved necrosis and apoptosis. Oxygen free radicals were participated in apoptotic cell death. The *bcl*-2 proto-oncogene had emerged as common regulator for multiple apoptotic pathway. The active form of *bcl*-2 protein, which promoted cell survival, was a heterodimer with *bax*, which accelerated cell death.[3] Expression of *bcl*-2 was observed in the survival neurons following cerebral ischemia and prevented programmed cell death *in vitro*[4]. Our previous study revealed that melatonin decreased production of hydroxyl radical during cerebral ischemia-reperfusion[5]. This led us to investigate whether melatonin could produce neuroprotection in the rat brains following ischemia-reperfusion and whether alterations in the expression of *bcl*-2 and *bax* proteins were associated with neuronal protection following subcutanous injection of melatonin prior to cerebral ischemic injury.

MATERIALS AND METHODS

Chemicals Melatonin was kindly gifted by Prof XIA Qi-Geng (Shanghai Chemical Reagent Factory), and freshly prepared with physiologic saline containing 3 % Tween 20. The *bax* and *bcl*-2 antibodies were purchased from Oncogene Science, USA. ABC kit was purchased from Vector Laboratories, USA.

Treatment of rats Sprague-Dawley rats (♂, $n = 49$) weighing 210 - 250 g (Experiment Animal

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Center of Shanghai Medical University, Grade II, Certificate № 02-22-2) were housed under a 12-h light/12-h dark cycle with free access to food and water. Rats were anesthetized with ip chloral hydrate 300 mg·kg^{-1}. Their arterial bloods were collected to measure pH, pO_2 and pCO_2. Heating lamp and pad were used to maintain brain temperature which was estimated using a 23-gauge stainless thermocouple probe (Barnhart Co, IL) inserted into the temporalis muscle[6]. Rats within normal range of physiologic variables were subjected to transient focal cerebral ischemia induced by left middle cerebral artery occlusion (MCAO). Briefly, a 4-0 nylon suture with a expanded tip was advanced from the external carotid artery into the lumen of the internal carotid artery until it blocked the origin of the left-lateral middle cerebral artery. Rats were randomly injected sc with vehicle or melatonin at doses of 2.5, 5, or 10 mg·kg^{-1}. Injection was given at 15 min before and at 6 h as well as 12 h after ischemia. Sham-operated rats were subjected to the same procedures without nylon suture inserted into the lumen of the carotid artery. All experiment procedures and injections were conducted in a blinded fashion.

Section preparation Rats were anesthetized by chloral hydrate and intracardially perfused with 0.9 % saline, followed by 300 mL fixative of 4 % paraformaldehyde in phosphate-buffer saline (PBS pH 7.2) 0.1 mol·L^{-1} at 24-h reperfusion after 1-h ischemia. Following the reperfusion, brains were postfixed in the same fixative overnight, then immersed in 20 % and 30 % sucrose solutions in PBS until sinking. Coronal brain sections (30 μm) were cut at 1.2 mm to −3.3 mm bregma level on freezing microtome and stored at −20 ℃ in cryo-protectant solution consisting of sucrose/ethylene glycol/PBS for histological study.

Cresyl violet staining Sections were stained with cresyl violet. Infarct areas were measured by computer image analyzer. Total and viable cell counting was performed on the microscopy in 3 different areas per section, the ischemic core (center part of caudate putamen), penumbra area 1 (lateral ventral part of caudate putamen), and penumbra area 2 (parietal cortex), which corresponded to the areas of the areas of A, B, and C, respectively (Fig 1).

The number of cells in a section was given by an average from 3 countings in each area per section.

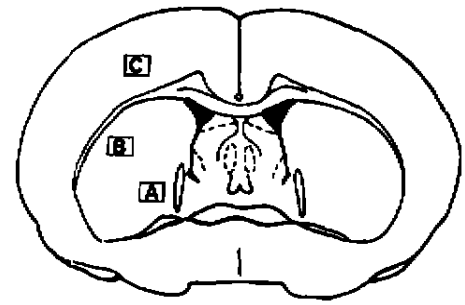

Fig 1. Cell counting area in brain slice. A) ischemic core, B) penumbra area 1, and C) penumbra area 2.

Percentage of viable cells was calculated: % of viable cells = (viable cells in ipsilateral)/(total cells in controlateral) × 100 %.

***Bax* and *bcl-2* immunohistochemical study** Sections adjacent to cresyl violet staining were subjected to immunohistochemical analysis. Sections were fixed in 4 % paraformaldelyde PBS for 15 min, incubated with 0.3 % H_2O_2 for 10 min, 10 % normal goat serum with 0.2 % Triton X-100 for 30 min, either *bax* or *bcl-2* polyclonal rabbit antibody (1:40, from Oncogene Science, USA) at 4 ℃ for 72 h, biotinylated goat anti-rabbit IgG (ABC kit from Vector Lab, USA) at 1:200 dilution for 30 min, avidin-biotin-peroxidase (from ABC kit) at 1:100 dilution for 45 min, stained with 0.05 % diaminobenzidine (Sigma, USA)/0.01 % H_2O_2.

Immunocytochemical control sections received no primary or secondary antibody and showed no specific staining. *Bax* and *bcl-2* positive cells were counted in the center part of caudate putament, lateral ventral part of caudate putamen and parietal cortex.

Statistical analysis Data were expressed as $\bar{x} \pm s$ and analyzed using a one-way ANOVA followed by *t*-test.

RESULTS

Melatonin decreased schemic infarct area of rat brain Melatonin was injected sc into rats with a transient cerebral ischemia and attenuated the infarct area at 24-h reperfusion after 1-h ischemia. Compared with vehicle injection [$n = 9$, infarct area (IA) = (9 ± 9) mm^2], melatonin 2.5 mg·kg^{-1} [$n = 11$, IA = (4 ±

5) mm^2], 5 $mg \cdot kg^{-1}$[$n = 10$, IA = (3 ± 4) mm^2], or 10 $mg \cdot kg^{-1}$[$n = 10$, IA = (2.3 ± 4.1) mm^2], showed respectively 55.3 %, 68.0 %, and 75.6 % decreases in the infarct area induced by ischemia.

Melatonin decreased the ischemic neuronal death in rats Histochemical analysis showed that cells in the ischemic penumbra areas 1 (lateral ventral part of caudate putament) appeared pyknosis, karyorrhexis, and karyolysis and dramatically lost in the ischemic core at 24-h reperfusion after 1-h focal ischemia. Melatonin improved ischemia-induced pathohistological change and attenuated cell loss in lateral ventral part of caudate putamen. Melatonin itself did not change the cell morphology in the sham-operated rat brains (Tab 1, Fig 2).

Tab 1. Increase of viable cells by melatonin following a transient cerebral ischemia. $\bar{x} \pm s$. $^bP < 0.05$ *vs* **vehicle group.**

	Percentage of viable cells			
	Sham-operated group		Ischemic group	
	Vehicle ($n = 4$)	Melatonin ($n = 5$)	Vehicle ($n = 9$)	Melatonin ($n = 10$)
Ischemic core	96 ± 5	99.3 ± 2.3	6 ± 10	9 ± 6
Penumbra area 1	96 ± 8	100 ± 12	15 ± 29	70 ± 37[b]
Penumbra area 2	98 ± 6	98 ± 5	74 ± 32	88 ± 20

Melatonin up-regulated ischemia-induced expression of *bcl*-2 in rat brains Immunohistochemical analysis was used to detect the expressions of *bcl*-2 and *bax* in the rat brain following a transient focal ischemia with or without melatonin treatment. A few of *bcl*-2 and *bax* immuno-positive cells were observed in the sham-operated rats. In the ischemic group, *bcl*-2 positive stained cell highly expressed in the survived neurons of penumbra areas and very few in the ischemic core (Tab 2).

In the ischemic rats treated with melatonin 10 $mg \cdot kg^{-1}$, the number of *bcl*-2 positive stained cell was further enhanced, especially in the penumbra area 1 ($P < 0.05$ compared with vehicle treatment). However, melatonin treatment did not change the expression of *bax* in rat brains (Tab 2, Fig 3). Melatonin increased the expression of *bcl*-2 in the lateral ventral part of caudate putamen.

Tab 2. Effect of melatonin on the expression of *bcl*-2 and *bax*. $\bar{x} \pm s$. $^bP < 0.05$ *vs* **vehicle.**

	Immuno-possitive stained cell (number/mm^2)			
	bcl-2		*bax*	
	Vehicle ($n = 9$)	Melatonin ($n = 9$)	Vehicle ($n = 9$)	Melatonin ($n = 10$)
Ischemic core	0 ± 0	0.5 ± 1.2	2 ± 5	4 ± 7
Penumbra area 1	328 ± 283	1005 ± 672[b]	36 ± 46	28 ± 54
Penumbra area 2	896 ± 363	1165 ± 391	202 ± 252	140 ± 129

DISCUSSION

In the present study, it was obviously found that melatonin could decrease in the infarct areas and protect neuronal death following a transient MCAO. Combined with the previous report that the levels of melatonin in the plasma and urine were lower in the stroke patients[7] and rats with melatonin deficiency by pinealectomy were more susceptibility to ischemia than normal rats[8], it indicates that melatonin in the brain may be involved in the physiological mechanisms of neuronal protection.

Several lines of evidence have shown that melatonin is a potent hydroxyl and peroxyl radical scavenger through different mechanisms. Our previous study found that melatonin could prevent the formation of hydroxyl radicals during the ischemia and reperfusion[5]. Putting together, all data suggest that the neuroprotective effect of melatonin may be related to its antioxidant effect. However, direct evidences are still needed to show the relationship between antioxidant and neuroprotection. Besides, other mechanisms might be involved in the neuroprotection such as progesterone and adrenal hormones[9].

Cerebral ischemia induced cell death in the ischemic territory and penumbra areas, which include necrosis and apoptosis. As we understood, apoptosis is an active cell death process regulated by endogenous specific enzymes[10] and genes[11]. Among them, *bcl*-2 family is considered as a group of important factors to determine cell death or survival. Physically, *bcl*-2 can interact with several of its homologous proteins in the form of heterotypic dimers[3]. One of the critical interactions appears to be *bcl*-2/*bax* dimerization[12]. Over-expressed *bax* accelerates cell apoptotic death.

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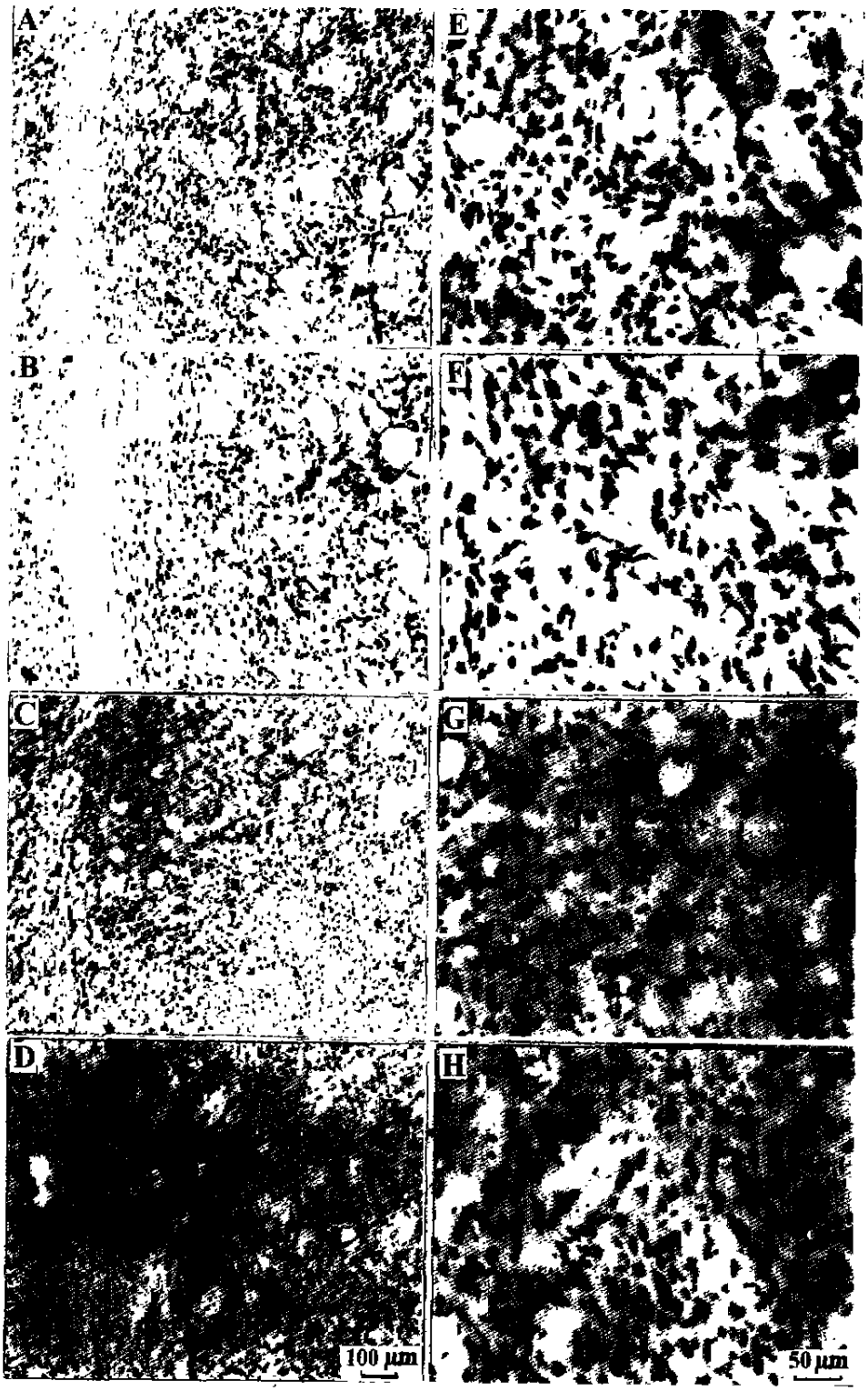

Fig 2. Effect of melatonin (10 mg·kg^{-1}) on the cell morphology in lateral ventral part of caudate putamen at 24-h reperfusion following 1-h MCAO. HE stain. A – D: × 40, E – H: × 100. Sham-operated (A, E) and sham-operated + melatonin (B, F) group showed normal morphology, ischemia group (C, G) appeared pyknosis, karyorrhexis, karyolysis and cell lost which was attenuated by melatonin (D, H).

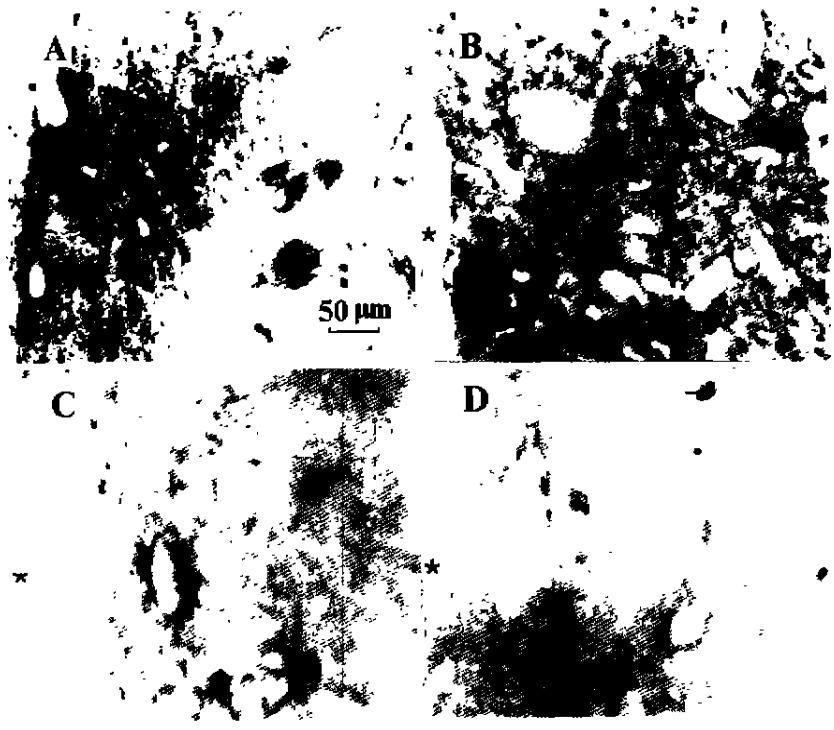

Fig 3. The *bcl*-2 (A, B) and *bax* (C, D) immunoreactive cells in lateral ventral part of caudate putamen (B area indicating in method) at 24 h reperfusion after 1 h MCAO. ＊: corpus callosum.

Therefore, the ratio of *bcl*-2 to *bax* could be used to reflect the direction of cell survival or death after an apoptotic stimulus[3,13]. It had been reported that *bcl*-2 over-expression could sufficiently suppress cell death induced by *L*-glutamate, free radicals and glutathione depletion[14]. Kane *et al* recently also found that *bcl*-2 could prevent cell death by decreasing the cellular generation of reactive oxygen species[15]. The present results showed that melatonin could increase the expression of *bcl*-2 in the survival neurons of ischemic penumbra areas, but not *bax*, showing the increase in the ratio of *bcl*-2 over *bax*. Therefore, it suggests that one of the mechanisms of the neuroprotective effect of melatonin in the neuronal cell death following cerebral ischemia probably by inhibiting apoptotic process.

In conclusion, our results suggest that melatonin can reduce the infarct area and decrease the neuronal death following cerebral ischemia and reperfusion. Its neuroprotective effect might be related to the increasing of the ratio of *bcl*-2/*bax*.

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褪黑激素对受损伤脑神经元的保护作用与 *bcl*-2 蛋白过度表达有关

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关键词 褪黑激素；自由基；脑缺血；原癌基因蛋白 c-*bcl*-2；*bax*；免疫组织化学；脑动脉

目的：研究褪黑激素对受损伤脑神经元的保护作用和对脑缺血后 *bcl*-2，*bax* 表达的影响。**方法**：用大鼠大脑中动脉梗死再灌注脑缺血模型，以脑梗死面积和存活细胞百分率作为脑损伤指标。用免疫组化的方法分析 *bcl*-2，*bax* 的表达。**结果**：缺血再灌后 24 h 褪黑激素呈剂量依赖性的减少脑梗死面积，减少缺血周边区细胞死亡，并显著增加该区 *bcl*-2 的表达，而对 *bax* 的表达则无明显影响。**结论**：褪黑激素具有明显减少缺血性神经细胞死亡的作用，该作用机制可能与神经细胞上 *bcl*-2 的表达有关。

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