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Molecular, Biological, Physiological and Clinical Aspects of Melatonin

# Melatonin protects against transient focal cerebral ischemia in both reproductively active and estrogen-deficient female rats: the impact of circulating estrogen on its hormetic dose–response

Abstract: Melatonin (5–15 mg/kg) protects male animals against ischemic stroke. We explored the potential interactions and synergistic neuroprotection of melatonin and estrogen using a panel of lipid peroxidation and radical-scavenging assays, primary neuronal cultures subjected to oxygen–glucose deprivation (OGD), and lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. Neuroprotective efficacy of melatonin was also evaluated in both reproductively active and ovariectomized female rats subjected to transient focal cerebral ischemia. Relative to melatonin or estradiol (E2) alone, a combination of the two agents exhibited robust, synergistic antioxidant and radical-scavenging actions ($P < 0.05$, respectively). Additionally, the two agents, when combined at large doses, showed synergistic inhibition in the production of tumor necrosis factor alpha (TNF-$\alpha$) and interleukin-6 (IL-6) in the LPS-stimulated RAW 264.7 cells ($P < 0.05$, respectively). Alternatively, co-treatment with melatonin and E2 independently, but not combined, showed a U-shaped dose-responsive (hormetic) cytoprotection for neuronal cultures subjected to OGD. When combined at a dosage either positively or negatively skewed from each optimal dosage, however, co-treatment caused synergistic neuroprotection. Relative to vehicle-injected controls, melatonin given intravenously at 1–5 mg/kg, but not 0.1 or 15 mg/kg, significantly reduced brain infarction and improved neurobehavioral outcomes ($P < 0.05$, respectively) in reproductively active female rats. In ovariectomized stroke rats, melatonin was only effective at a large dosage (15–50 mg/kg). These results demonstrate complex interactions and synergistic antioxidant, radical-scavenging, and anti-inflammatory actions between estradiol and melatonin, and highlight the potential need to rectify the melatonin's hormetic dose–response by the level of circulating estradiol in the treatment of female stroke patients.

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## Introduction

Cerebral stroke affects both genders of patients and is a leading cause of mortality and morbidity in the United States. Several reports have demonstrated a gender-related difference in morbidity and mortality from patients with heart diseases [1, 2]. Experimental cerebral stroke research, however, has been performed almost exclusively on young male animals with results being translated to females [3, 4]. It is, however, known that the incidence of stroke occurrence in premenopausal women is lower than that in the same age group of male patients and that the incidence of cerebrovascular diseases increases rapidly in women after menopause [4, 5]. There also are sex differences with respect to ischemic vulnerability to neuronal death, with females being less sensitive. Thus, it is quite apparent that decreased incidence and severity for stroke development observed in young and premenopausal females may relate to their higher levels of circulating estrogens, principally, 17$\beta$-estradiol (E2). Thus, E2, similar to melatonin, is a well-known endogenous agent that protects the brain from various modes of ischemic brain injury through the up- and down-modulation of specific proteins [6, 7].

Melatonin (*N*-aceyl-5-methoxytryptamine) has a variety of actions that may be beneficial in the treatment of acute stoke [8, 9]. This natural neuroprotectant and its metabolites are well known to be potent free radical scavengers and antioxidants [10–14]. We have previously demonstrated

that melatonin reduced oxidative damage, protected against gray, white matter and dendritic pathology, and improved neurobehavioral and electrophysiological outcomes following transient focal cerebral ischemia in male mice and rats, respectively [15–17]. We have also documented that melatonin improved the preservation of early and late increases in the blood-brain barrier permeability and reduced the risk of hemorrhagic transformation following ischemic stoke in male mice [18, 19]. More recently, we have shown that melatonin effectively modulates MMP-9 activation and, therefore, inhibits systemic leukocyte transmigration and local microglial activation following transient focal cerebral ischemia in male mice and rats, respectively [20–22]. It is, however, not certain whether melatonin can protect female animals against ischemic stroke, as already demonstrated in various species of male animals.

Reproductive hormones generally inhibit the binding of melatonin receptor in a variety of cells, including cerebral vasculatures [4, 23–25]. Even though there is little definitive evidence to suggest that neuroprotection observed with melatonin against ischemic-reperfusion brain injury is receptor dependent, a reciprocal inhibitory action may exist between melatonin and estrogen in the regulation of vascular tone and receptor expression. In contrast, melatonin, combined with circulating levels of estrogen, may actually exhibit synergistic antioxidant, free radical scavenging and neuroprotective actions against the ischemic-reperfusion insults [26]. The hypothesis was further supported by the fact that concurrent decreases in the circulating levels of melatonin and estrogen and their modulatory effects on vascular biology and function have been linked with the increased risk of cerebrovascular disease and incidence of stroke observed in menopausal women [4, 5]. Accordingly, although melatonin is well known to protect against ischemic brain damage in various male subjects, there is a need to examine its potential gender-specific changes in the dosing regimen and efficacy both in reproductively active, with the presence of circulating estrogen, and senescent (ovariectomized) female subjects.

In attempt to extend our work and also to simulate the possible influence of circulating estrogen on melatonin's neuroprotection in female patients suffering from an ischemic stroke, we, herein, examine the potential interactions and synergistic neuroprotection between melatonin and E2 using a panel of lipid peroxidation and radical-scavenging assays, primary neuronal culture subjected to oxygen–glucose deprivation (OGD) as well as the lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. We also examined the neuroprotective dosing regimen of melatonin treatment and its effects on the functional outcomes following transient focal cerebral ischemia in both the reproductively active and estrogen-deficient (ovariectomized) female rats.

## Materials and methods

### Chemicals and reagents

Melatonin and E2 were dissolved in dimethylsulfoxide (DMSO). All chemicals were purchased from Sigma-Aldrich Co. (St Louis, MO, USA) unless otherwise indicated. Compounds were ordered in the highest grade available. Hank's balanced salt solution (HBSS 10×; GIBCO, Grand Island, NY, USA) was composed of (mM): glucose 55.56, KCl 53.33, NaCl 1379.31, $KH_2PO_4$ 0.44 and $Na_2PO4$ 3.36; pH 7.1. We first performed a preliminary dose–response of melatonin and E2 in each in vitro assay tested and then explored their potential interactions by using various combination formulae of the two agents at appropriate concentrations.

### Lipid peroxidation assay in vitro

The levels of malondialdehyde in rat brain homogenate induced by $Fe^{3+}$ were measured by the method described previously [27–29]. The reactions were initiated by adding 10 $\mu$L of vehicle (0.1% DMSO), melatonin (0.5–5 mM), E2 (10–100 $\mu$M), or a combination of various concentrations of melatonin and E2 to 30-$\mu$L brain homogenate supernatant and 5-$\mu$L freshly prepared ferric chloride hexahydrate ($Fe^{3+}$). Final iron concentration was 10 mM. The absorbance was measured at 532 nm using a plate reader (Stat Fax 2100; Awareness Technology, Inc., Palm City, FL, USA).

### 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging assay

DPPH radical-scavenging assay was carried out according to a published procedure [28–31]. The scavenging reactions were initiated by adding 100 $\mu$L of freshly prepared DPPH radical solution to 100 $\mu$L of vehicle (0.1% DMSO), melatonin (0.03–3 mM), E2 (0.03–3 mM), or a combination of various concentrations of melatonin and E2. The absorbance was measured at 517 nm using a plate reader (Stat Fax 2100).

### 2, 2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radical-scavenging assay

ABTS radical-scavenging activity was determined by the method described previously [13, 29, 32]. Briefly, ABTS radical cation was prepared by reacting ABTS (7 mM) with potassium persulfate (2.5 mM) in water in the dark overnight. Reactions were initiated by adding 100 $\mu$L of ABTS radical cation solution to 100 $\mu$L of vehicle (0.1% DMSO), melatonin (1–30 $\mu$M), E2 (1–30 $\mu$M), or a combination of various concentrations of melatonin and E2. The absorbance was measured at 734 nm using a plate reader (Stat Fax 2100).

### Primary cortical neuronal culture

Cultured cortical cells were obtained from the cerebral cortices of 1-day-old Sprague-Dawley rats under pentobarbital anesthesia, according to the method described previously [33, 34]. The cortices were placed in ice-cold Dulbecco's modified Eagle's medium (DMEM; GIBCO) and minced. Tissue chunks were then incubated in a papain solution (0.6 mg/ml papain and DNase I in HBSS) at 37°C for 30 min to dissociate the cells, and the reaction was terminated by adding heat-inactivated horse serum. After

the cell suspensions were centrifuged at 800 g, pellets were plated onto poly-D-lysine-coated petri dishes. Dissociated cells were suspended in DMEM with 10% horse serum and incubated at 37°C in a humidified incubator with 5% $CO_2$. Three hours after plating, the culture medium was replaced by a serum-free neurobasal medium containing 25 $\mu$M glutamate, 0.5 mM L-glutamine, and 2% $B_{27}$ supplement (17504-044; Invitrogen Corp., Carisbad, CA, USA). Cultured cells were allowed to grow for approximately 7–14 days. These cultures contained 80% neurons and 20% astrocytes, as determined by immunohistochemistry using monoclonal antibodies against microtubule-associated protein-2 (MAP-2; Boehringer Mannheim, Mannheim, Germany) and glial fibrillary acidic protein (GFAP, Sigma-Aldrich).

### Oxygen and glucose deprivation

At the 7–14th day in vitro, OGD was achieved by combining hypoxia with aglycemia [29]. The neuronal cultures were incubated with OGD medium consisting of ice-cold HBSS lacking glucose and previously bubbled with $N_2$ for 30 min to deplete glucose and oxygen from intracellular stores and extracellular space. Various concentrations of melatonin (1, 5, or 10 $\mu$M), E2 (0.01, 0.03, 0.01, or 0.1 $\mu$M) or a combination of different components of melatonin and E2, or vehicle were introduced into the medium. The neuronal cultures were then transferred to an anaerobic chamber at 37°C with $N_2$-enriched atmosphere for 2 hr. After the deprivation period, neuronal culture was incubated in culture medium under normal conditions (a humidified incubator with 5% $CO_2$ at 37°C), corresponding to the recovery periods. Each experiment consisted of triplicate samples. Four independent experiments were carried out for each experiment.

### Lactate dehydrogenase (LDH) assay

After a recovery period of 24 hr, neuronal cell injury was quantified by the measurement of the LDH levels in the extracellular medium using a LDH assay kit (Promega, Madison, WI, USA). Following the procedures provided by the vendor, the values of LDH were measured by the optical density (OD) taken at 490 nm in a plate reader (Stat Fax 2100; Awareness Technology, Inc.). Data are expressed as a value of the response % (i.e., a percentage relative to the values obtained from the medium of the OGD-treated control neuronal cultures).

### Cell cultures for RAW 264.7 cells

The immortalized murine macrophage RAW 264.7 cell line was maintained at a density of $1 \times 10^6$ cells/mL in DMEM supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT, USA) and incubated at 37°C in a humidified atmosphere containing 5% $CO_2$ and 95% air [29]. On the following day, the medium was replaced with fresh DMEM, and the cells were then stimulated with LPS (100 ng/mL) and co-treated with vehicle (0.1% DMSO), melatonin (1–100 $\mu$M), E2 (0.01–10 $\mu$M), or a combination of various concentrations of melatonin and E2. Culture supernatants were collected for tumor necrosis factor alpha (TNF-$\alpha$) and interleukin-6 (IL-6) measurements after incubation for 6 hr and for the measurements for nitrite/nitrate levels after incubation for 16 hr. All the experiments were conducted in duplicate with assays in triplicate [29].

### IL-6, TNF-$\alpha$, and nitrate/nitrite assay

The levels of IL-6 and TNF-$\alpha$ in culture supernatants were estimated by using enzyme-linked immunosorbent assay (ELISA) method [29]. ELISA was performed using a DuoSet ELISA development system (R&D Systems, Minneapolis, MN, USA) for mouse IL-6 and TNF-$\alpha$. The production of nitric oxide (NO) was assessed as the accumulation of nitrate/nitrite in the culture medium using a nitrate/nitrite fluorometric assay kit (Cayman Inc., Ann Arbor, MI, USA) [29]. Following the procedures provided by the vendor, the values of TNF-$\alpha$ and IL-6 were measured by the OD taken at 450 nm in a plate reader (Stat Fax 2100). Nitrate/nitrite assay was taken by Fluoroskan Ascent FL microplate reader (Thermo Electron Co., Milford, MA, USA) at an excitation wavelength of 360–365 nm and an emission wavelength of 430 nm.

### Animal preparation, anesthesia, ovariectomy, and monitoring

All procedures performed were approved by the Subcommittee on Research Animal Care of the University Medical Center, whose standards meet the guidelines of the National Institutes of Health (Guide for the Care and Use of Laboratory Animals). Adult young female Sprague-Dawley rats, weighing 210–250 g, were supplied by the University Laboratory Animal Center and were allowed free access to food and water before and after surgery. Animals were anesthetized with 1–2% halothane in 70% $N_2O$/30% $O_2$. During surgery, body temperature was maintained at 37.0 ± 0.5°C using a thermostatically controlled heating blanket and rectal probe (Harvard Apparatus, South Natick, MA, USA). For estrogen-deficient studies, age-matched, sexually mature female rats were ovariectomized and received ischemic experiments at approximately 1 month after ovariectomy to ensure estrogen depletion [7]. The right femoral artery was cannulated for measuring arterial blood gases, glucose, hematocrit, and blood pressure. Serum was separated from blood by centrifugation for 800 g and stored frozen (−20°C). Serum estradiol concentrations were determined with the use of 17$\beta$-Estradiol EIA kit (Cayman Inc.), according to the procedures provided by the vendor.

### Experimental model, drug administration, and grouping of animal

Focal cerebral ischemia was employed by intra-arterial 4-0-nylon suture occlusion of the proximal right middle cerebral artery (MCA) for 90 min using the method described previously [15, 20, 21, 35–37]. Reperfusion was ensured by an improvement in the ipsilateral local cortical blood flow (LCBF) at a defined area of the ischemic core cortex (0.5 mm posterior and 7 mm lateral to the Bregma)

to about 50% of baseline following an initial decrease to about 20% of baseline caused by MCA occlusion as determined by Laser-Doppler flowmetry (Laserflo BMP$^2$; Vasamedics, St. Paul, MN, USA). LCBF was serially measured prior to and during the MCA occlusion, upon a brief period (10 min) of reperfusion and at 40 min after the onset of reperfusion.

Melatonin (Sigma-Aldrich Co.) was dissolved in a mixture of polyethylene glycol 400 (PEG 400; Sigma-Aldrich) and 0.9% normal saline (3:7, vol./vol.). For reproductively active rats, animals were intravenously administrated with melatonin at 0.1 mg/kg (n = 9), 1 mg/kg (n = 10), 5 mg/kg (n = 11), or 15 mg/kg (n = 11) or vehicle (PEG-saline, n = 21) at the initiation time of reperfusion (i.e., 90 min after the onset of ischemia). In ovariectomized rats, animals were intravenously administrated either with melatonin at 1 mg/kg (n = 10), 5 mg/kg (n = 11), 15 mg/kg (n = 10), or 50 mg/kg (n = 8) or vehicle (PEG-saline, n = 13) at the reperfusion onset. The dose of melatonin was adjusted based on the neuroprotective dose-responsive studies of melatonin in male rodent models of transient focal cerebral ischemia [15–21, 38, 39].

### Animal sacrifice and quantification of ischemic brain damage

Following 7 days of survival, sacrifice was performed under anesthesia by transcardiac perfusion accomplished with 200 mL of 0.1 M phosphate-buffered saline (PBS) and 200 mL of 3.7% formaldehyde in PBS. After postfixation overnight, the brains were embedded in optimal cutting temperature compound (OCT; Sakura Finetek USA, Inc., Torrance, CA, USA) and frozen in liquid nitrogen. The brains were sectioned in 40 $\mu$m thickness on a cryostat (HM-500O; Microm International GmbH, Walldorf, Germany). Serial sections of 40 $\mu$m, with 1-mm intervals from the stereotaxic coordinates of the Bregma anteroposterior (AP) +4.22 to −6.78 mm, were mounted on poly-L-lysine-coated Sigma-Aldrich Co., slides and dried at 37°C overnight [20–22, 40].

Sections were stained with 0.5% cresyl violet. Under light microscopy, the areas of neuronal perikarya displaying typical morphological features of ischemic damage were delineated. Brain infarction was measured using a computerized image analyzer MCID Elite; Imaging Research Inc., St. Catherines, ON, Canada and was expressed as a percentage of the contralateral hemisphere volume [15, 16, 20, 21, 35, 36]. The ipsilateral brain edema was determined by the summation of volume increment of the right hemisphere on each brain section, relative to the corresponding volume of the left hemisphere, and was expressed as a percentage index relative to the volume of the left hemisphere. In addition, individual cortical and subcortical (caudoputaminal and hippocampal) infarct sizes were separately calculated.

### Cell counting of surviving neurons

Coronal sections obtained between the Bregma AP −0.22 and −0.78 mm were chosen. Six random and nonoverlapping regions (500 × 400 $\mu$m$^2$) were sampled for the ischemic core (the unstained area), the inner (right side to the margin between the stained and unstained area), and the outer (left side to the margin between the stained and unstained area) boundary zones of the infarct areas at the parietal cortex. Cell counts were expressed as the mean number of viable neurons per mm$^2$.

### Neurobehavioral testing and body weight measurements

Body weight measurement was employed daily. A battery of sensory-motor tests was conducted prior to and after the ischemia-reperfusion insult and on a daily basis up to 7 days after reperfusion by two observers unaware of treatment protocol. Briefly, two neurologic grading systems were used: (i) a sensorimotor grading scale modified from previously published methods [15, 20, 21, 35–37, 41] with five categories (0–4) for forward and sideways visual placing tests of the affected forelimb and five categories (0–4) for motor outcome and (ii) a grading scale of 0–28 developed by Clark et al. [42].

### Statistical analysis

Neurobehavioral scores were expressed as the median ± 95% confidence interval (CI) and were analyzed by a nonparametric test for independent groups, i.e., the Kruskal–Wallis/Mann–Whitney $U$ test. The other data were expressed as the mean ± standard error of the mean (S.E.M.). Paired Students' $t$ test was used to evaluate the response to a change in conditions, and one-way analysis of variance (one-way ANOVA) with Fisher's protected least significant difference *post hoc* comparison was used to evaluate differences between groups. The LCBF values in each group were analyzed among groups at each sampling time by repeated analysis of variance (ANOVA), followed by Dunnett's *post hoc* tests. $P < 0.05$ was selected for statistical significance.

## Results

Both melatonin and E2 effectively inhibited the $Fe^{3+}$-induced lipid peroxidation in the rat brain homogenate ($P < 0.001$, respectively), and, when melatonin at 1, 3, and 5 mM was combined with E2 at 30, 50, and 100 $\mu$M, respectively, showed synergistic antioxidant actions (Fig. 1A; $P < 0.05$, respectively), compared to the compatible concentrations of each agent in isolation. Additionally, a combination of melatonin and E2 either at 1 or 3 mM, respectively, was more active in a DPPH radical-scavenging assay ($P < 0.05$, respectively), compared to the compatible concentration of either melatonin or E2 alone, although each of the latter two was effective in the assay (Fig. 1B; $P < 0.05$, respectively). In the ABTS radical cation scavenging assay, melatonin and E2, when combined at 3, 10, or 30 uM, respectively, exhibited effectively synergistic inhibition, compared to the compatible concentrations of either melatonin or E2 in isolation (Fig. 1C; $P < 0.001$, respectively).

As shown in Fig. 2A, treatment with melatonin and E2 at 1–100 $\mu$M and 0.01–10 $\mu$M, respectively, significantly

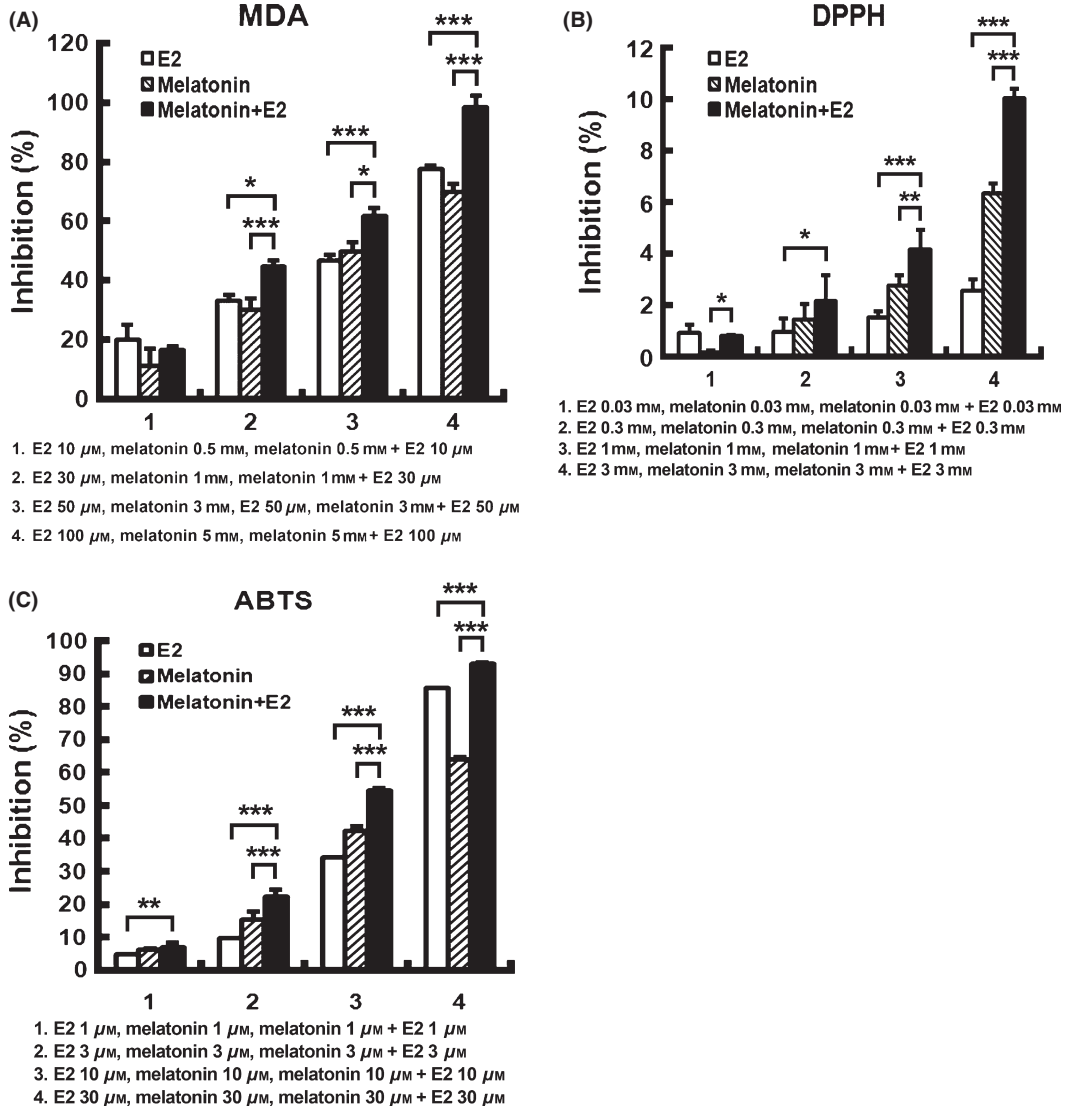


*Fig. 1.* Combined treatment with melatonin and estradiol (E2) exhibits synergistic antioxidant and radical-scavenging actions. (A) Inhibition of lipid peroxidation was induced by $Fe^{3+}$ (10 mM) in rat brain homogenate. Co-treatment with melatonin (1–5 mM) and E2 (30–100 $\mu$M) showed an additional synergistic action ($P < 0.05$, respectively), compared to either melatonin or E2 alone. In 2,2-diphenyl-1-picrylhydrazyl radical-scavenging assay (B), a combination of melatonin and E2 either at 1 or 3 mM, respectively, was more active ($P < 0.05$, respectively), compared to the compatible concentration of either melatonin or E2 alone. In the ABTS radical cation scavenging assay (C), melatonin and E2, when combined at 3, 10, or 30 uM, respectively, exhibited effectively synergistic inhibition ($P < 0.001$, respectively), compared to the compatible concentrations of either melatonin or E2 in isolation. Data are expressed by the mean ± S.E.M. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ calculated according to one-way ANOVA, followed by Fisher's protected least significant difference post hoc comparison.

reduced the TNF-$\alpha$ production in LPS-stimulated RAW 264.7 cells ($P < 0.05$, respectively), and melatonin at 50–100 $\mu$M, combined with E2 at 0.1–10 $\mu$M, showed additionally synergistic inhibition. Similarly, treatment with melatonin and E2 at 10–100 $\mu$M and 0.01–10 $\mu$M, respectively, significantly reduced the IL-6 production in the LPS-stimulated RAW 264.7 ($P < 0.05$, respectively; Fig. 2B). Melatonin at 50–100 $\mu$M, combined with E2 at 0.01–10 $\mu$M, also had additionally synergistic inhibition. However, melatonin and E2 at each testing dose failed to effectively inhibit the NO production in the LPS-stimulated RAW 264.7 cells ($P > 0.05$; Fig. 2C).

Compared to normal control cultures, the release of LDH was not significantly changed in nonlesioned cultures treated with melatonin or E2 at each testing dose (data not shown). In OGD-lesioned cultures co-treated either with melatonin at 1–10 $\mu$M or E2 at 0.01–0.1 $\mu$M, the LDH release taken at 24 hr was significantly reduced, compared to those in the OGD-lesioned control cultures (Fig. 2D; $P < 0.01$, respectively). Interestingly, melatonin and E2 independently, but not combined, showed a U-shaped (hormetic), dose-specific cytoprotection in neuronal cultures subjected to OGD. Curiously, a combination of melatonin and E2 at their optimal dose (5 and 0.03 $\mu$M,

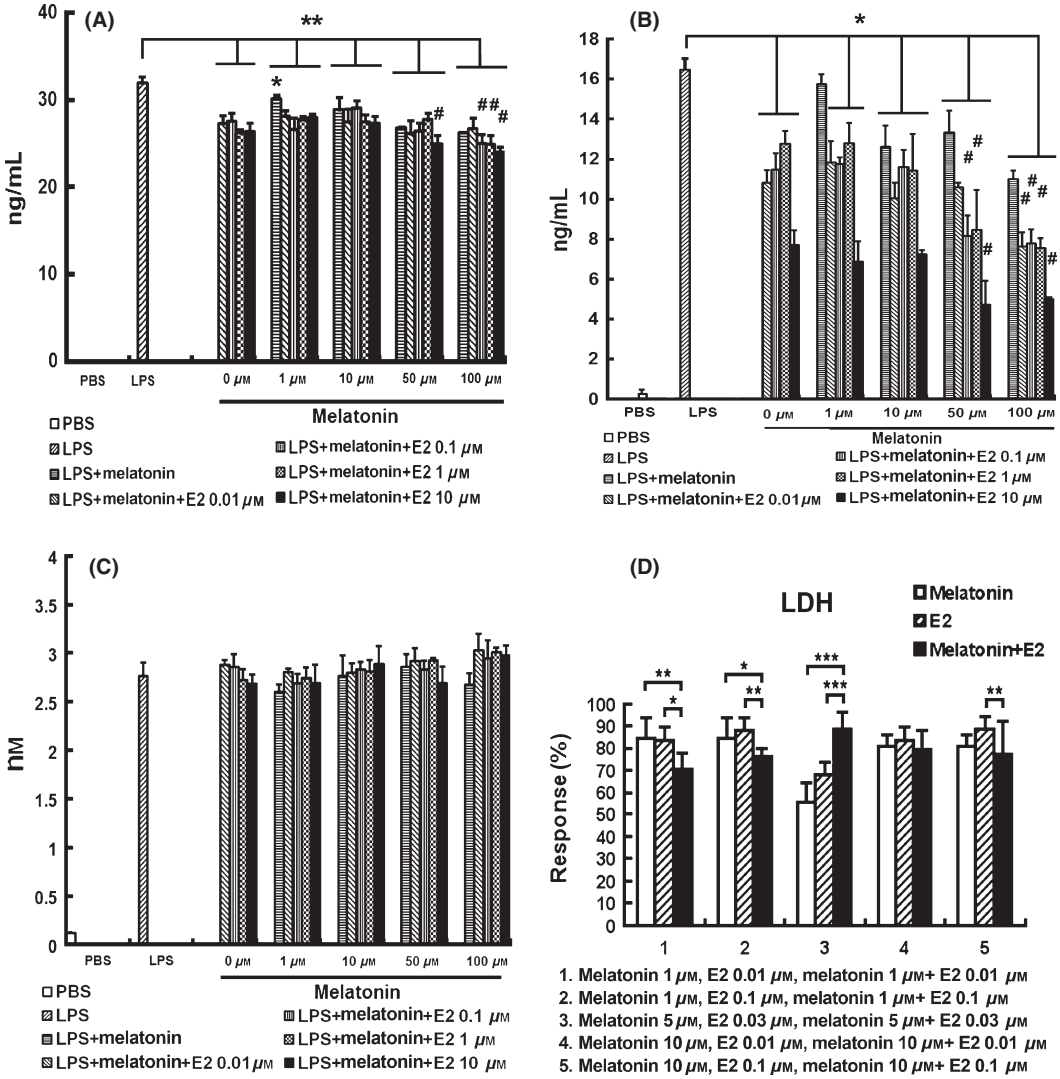


*Fig. 2.* Combined treatment with melatonin and estradiol (E2) showed synergistic inhibitions on (A) tumor necrosis factor alpha (TNF-α) and (B) interleukin-6 (IL-6), but not (C) nitric oxide (NO), production in LPS-treated RAW 264.7 cells and exerted a U-shaped cytoprotection in primary neuronal culture exposed to oxygen–glucose deprivation (OGD) (D). The cells were stimulated with LPS (100 ng/ml for RAW 264.7 cells) and co-treated with melatonin, E2, or a combination at various concentrations. The levels of IL-6, TNF-α, and NO in the supernatants were measured. The graphs are representative of two experiments with assays in triplicate. Data are expressed by the mean ± S.E.M. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ versus vehicle. $\#P < 0.05$ versus melatonin and E2 at compatible concentrations alone. (D) Reduced release of lactate dehydrogenase with melatonin and E2 in rat primary cortical neuronal cultures exposed to OGD. Co-treatment either with melatonin or E2, but not combined, showed a U-shaped dose-responsive cytoprotection in neuronal cultures subjected to 120 min OGD, when compared to control cultures. Notably, a combination of melatonin and E2 at their optimal dose (5 and 0.03 μM, respectively) substantially resulted in decreased neuroprotective efficacy ($P < 0.05$, respectively). $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ calculated according to one-way ANOVA, followed by Fisher's protected least significant difference post hoc comparison.

respectively) substantially resulted in nearly complete loss of their neuroprotective efficacy ($P < 0.05$, respectively), and when combined at a dosage either positively or negatively skewed from each optimal dosage, however, would exhibit synergistic neuroprotection ($P < 0.05$, respectively), compared to the compatible concentrations of melatonin and E2 alone.

Throughout the course of experiments, eight animals of 62 reproductively active female animals (12.9%) died prior to completing the recovery protocol and were excluded: three were in the vehicle-injected group, two were in melatonin (0.1 mg/kg)-treated group, and the other three were evenly distributed at other melatonin-treated groups at 1, 5, and 15 mg/kg, respectively. For the ovariectomized rats, four of 52 animals (7.7%) died. Two were in the vehicle-injected group, one was in melatonin (0.1 mg/kg)-treated group, and the other one was treated with melatonin at 50 mg/kg. Animals subjected to transient MCA occlusion invariably exhibited spontaneous hyperthermia in both the reproductively active and the estrogen-deficient groups of animals. The core temperature reached 37.6–39.2°C within 1 hr, remained high during the first 24 hr,

and then subsided. Relative to vehicle-injected controls, melatonin-treated animals, however, did not have significantly changed core temperatures at various dosing regimens, time intervals, or subgroups of experiments (data not shown).

For both groups of the vehicle-injected controls, the ipsilateral LCBF recorded at the ischemic core and the penumbral regions abruptly decreased to 11–16% and 35–40% of baselines, respectively, following the onset of MCA occlusion. The decreased LCBF at the ischemic core and the penumbral regions subsequently improved to 51–62% and 96–112% of baselines, respectively, after the initiation of reperfusion. In contrast, the contralateral LCBF did not significantly change over time during a course of experiments. Melatonin administered either at 0.1–15 mg/kg for the reproductively active or at 1–50 mg/kg for the estrogen-deficient female rats did not affect the LCBF recorded either at the ischemic core and the penumbral regions or at the contralateral cortical brain, as assessed within 40 min after treatment (data not shown). The other physiological parameters of the animals were kept within normal physiological limits during the course of experiments and did not differ significantly between melatonin-treated animals and vehicle-injected controls for both the reproductively active and the estrogen-deficient female rats (data not shown).

Transient (90 min) MCA occlusion typically produced a well-demarcated cortical and subcortical infarct in both the reproductively active and the estrogen-deficient female rats. Melatonin given at 1 and 5 mg/kg effectively reduced total cerebral infarct volumes by 36.4% and 27.1% ($P < 0.05$, respectively) in reproductive active female rats when compared with vehicle (PE400-saline)-injected controls (Fig. 3A,B). In particular, exogenous melatonin administrated at 1 mg/kg effectively decreased cortical and subcortical infarction by 37.9% and 35.8% ($P < 0.01$, respectively; Fig. 3C). Treatment with melatonin at 5 mg/kg also effectively reduced cortical brain infarct by 27.9% ($P < 0.05$; Fig. 3C), but showed marginal protection for reducing subcortical brain infarct ($P = 0.055$). Melatonin

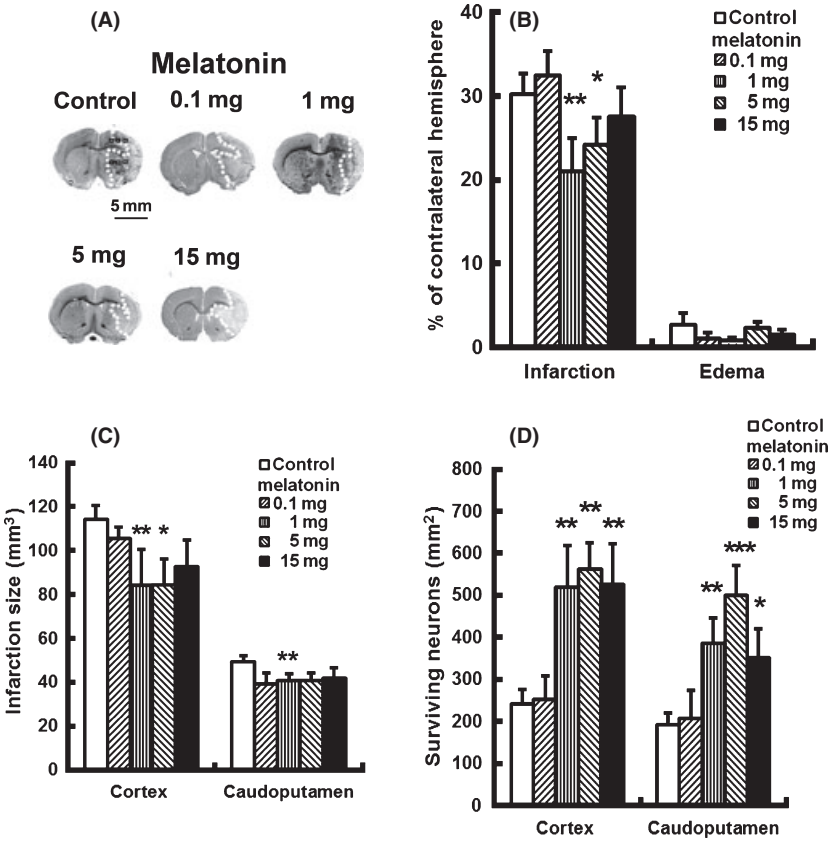


*Fig. 3.* Treatment with melatonin reduced brain infarction and increased the number of surviving neurons in reproductively active female rats subjected to middle cerebral artery (MCA) occlusion for 90 min. The cresyl violet–stained coronal sections (A) were from representative animals that received an intravenous injection of vehicle (polyethylene glycol-saline) or melatonin (0.1–15 mg/kg) at 90 min following the onset of ischemia. Brain infarction volumes were much smaller in animals treated with melatonin at 1 and 5 mg/kg, compared to controls. Scale bar = 5 mm. Percentage infarction volumes (B) and individual cortical and striatal lesion sizes (C), but not the ipsilateral brain edema (B), are much smaller in animals treated with melatonin at 1 mg/kg (n = 9) and 5 mg/kg (n = 10) 90 min after the onset of MCA occlusion, compared to control data (n = 18). Treatment with melatonin either at a dosage of 0.1 mg/kg (n = 7) or 15 mg/kg (n = 10), however, failed to offer effective neuroprotection in term of reducing brain infarct. The numbers of surviving neurons in the ipsilateral parietal cortex and caudoputamen (D) are, however, significantly increased in each melatonin-treated group, compared to control data. Percentage infarct volume and brain edema are expressed as a percentage of the contralateral (control) hemisphere. Data are represented as the mean ± S.E.M. $*P < 0.05$ versus vehicle data; $**P < 0.01$ versus vehicle data, and $***P < 0.001$ versus vehicle data.

given at 0.1 mg/kg or 15 mg/kg, however, did not show effective infarct volume reduction ($P > 0.05$, respectively). In contrast, animals treated with melatonin at 1–15 mg/kg invariably showed improved surviving neurons in penumbral cortical and striatal regions, respectively, compared to controls ($P < 0.05$, respectively; Fig. 3D). In estrogen-deficient female rats, the E2 level was at subnormal low limits (Fig. 4A). Exogenous administration with melatonin at 15 and 50 mg/kg significantly reduced brain infarction by 39.1% and 29.4%, compared to controls ($P < 0.05$, respectively; Fig. 4B). This reflected a melatonin-mediated reduction in cortical infarct sizes by 44.3% and 31.4% ($P < 0.05$, respectively; Fig. 4D) and improvements in the number of the cortical surviving neuron by 29.1% and 36.4% ($P < 0.05$, respectively; Fig. 4E). Melatonin given at 50 mg/kg also effectively decreased brain infarction and improved the surviving neuron in the caudoputamen regions ($P < 0.05$, respectively; Fig. 4D,E). In contrast, melatonin given at 1 or 5 mg/kg did not show effective infarct volume reduction ($P > 0.05$, respectively). Consequently, melatonin, when given at 1–15 and 5–50 mg/kg for the reproductively active and the estrogen-deficient female rats, respectively, significantly improved sensory, motor, and the 28-point neurologic scores taken 1, 3, and 7 days after the onset of reperfusion than did the vehicle-injected controls ($P < 0.05$; Tables 1 and 2).

## Discussion

This study has shown that a combination with melatonin and E2 exhibits potent and synergistic antioxidant, radical-scavenging, and anti-inflammatory actions. Additionally, we demonstrated that administration either with melatonin or E2 showed a U-shaped (dose-specific hormetic) neuroprotective response in primary neuronal cultures exposed to OGD, and the two agents, when combined at each optimal dosage, exhibited an opposite hormetic response [43]. Moreover, we showed that melatonin, when administered at a dosage ranged between 1–5 and 15–50 mg/kg, 90 min after the onset of transient MCA occlusion, was effective in

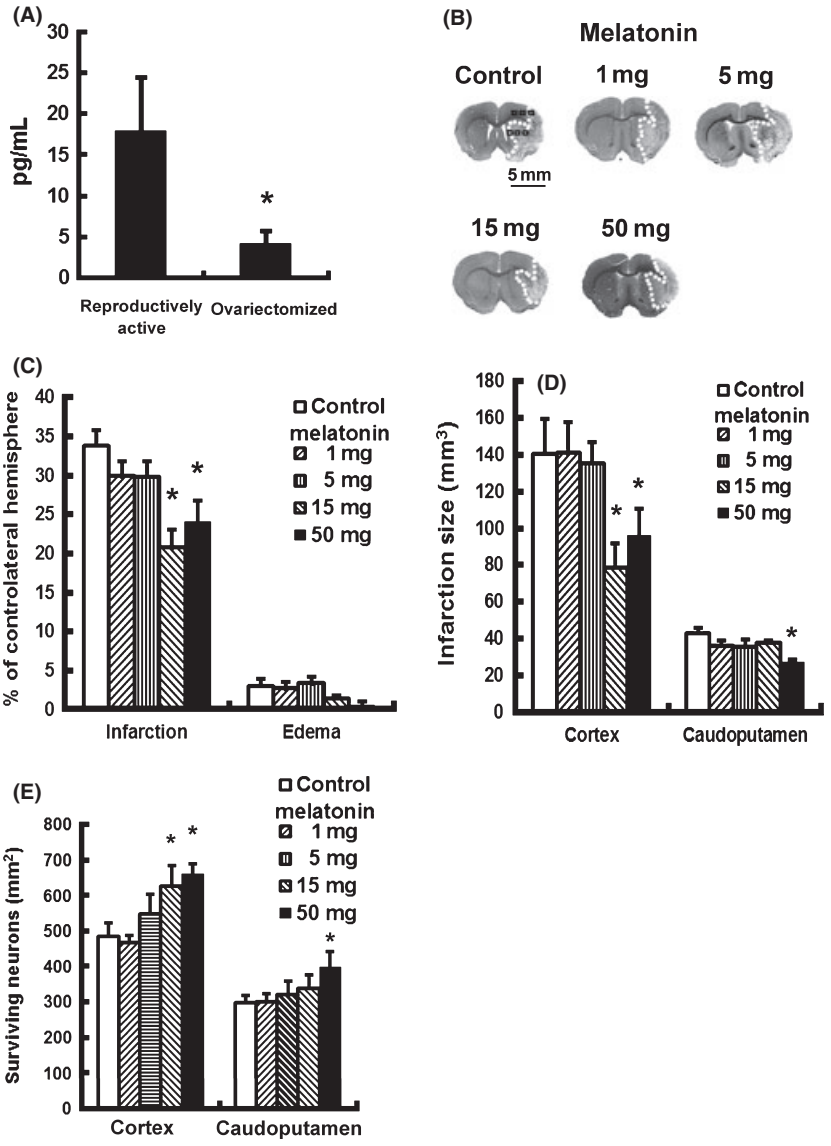


*Fig. 4.* Treatment with melatonin reduced brain infarction and increased the number of surviving neurons in estrogen-deficient (ovariectomized) female rats subjected to middle cerebral artery (MCA) occlusion for 90 min. (A) The estradiol levels in ovariectomized rats were kept in subnormal low limits, compared to reproductive active control rats. The cresyl violet–stained coronal sections (B) were from representative animals that received an intravenous injection of vehicle (polyethylene glycol-saline) or melatonin (1–50 mg/kg) at 90 min following the onset of ischemia. Brain infarction volumes were much smaller in animals treated with melatonin at 15 and 50 mg/kg, compared to controls. Scale bar = 5 mm. Percentage infarction volumes (C) and individual cortical lesion sizes (D), but not the ipsilateral brain edema (C), are much smaller in animals treated with melatonin at 15 mg/kg (n = 10) and 50 mg/kg (n = 7) 90 min after the onset of MCA occlusion, compared to control data (n = 11). Treatment with melatonin either at a dosage of 1 mg/kg (n = 9) or 5 mg/kg (n = 11), however, failed to offer effective neuroprotection in term of reducing brain infarct. The numbers of surviving neurons in the ipsilateral parietal cortex and caudoputamen (E) are, however, significantly increased only in the group treated with melatonin at 50 mg/kg, compared to control data. Data are represented as the mean ± S.E.M. *$P < 0.05$ versus vehicle data.

Table 1. Melatonin improves sensorimotor behavioral scores after cerebral ischemia-reperfusion in the reproductively active female rats

| Neurologic behavioral score | n | Day 1 | Day 3 | Day 7 |
|---|---|---|---|---|
| *28-Point clinical scale* | | | | |
| Control | 18 | 16.5 (15.6–18.4) | 17 (15.8–18.2) | 18 (17.2–18.8) |
| Melatonin (mg/kg) | | | | |
| 0.1 | 7 | 16 (13.7–18.3) | 15 (13.8–16.2) | 15 (13.1–15.9) |
| 1 | 9 | 9 (6.2–11.8)** | 8 (7.0–9.0)* | 7 (6.4–7.6)** |
| 5 | 10 | 9.5 (8.4–10.6)** | 9 (8.0–10.0)** | 8.5 (7.9–9.1)* |
| 15 | 10 | 12.5 (10.1–14.9) | 9.5 (7.1–11.9)** | 10 (8.3–11.7)** |
| *Sensory* | | | | |
| Control | 18 | 4 (3.9–4.1) | 4 (3.8–4.2) | 4 (3.8–4.2) |
| Melatonin (mg/kg) | | | | |
| 0.1 | 7 | 4 (3.4–4.6) | 4 (3.4–4.6) | 3 (3.0–4.0) |
| 1 | 9 | 2 (1.4–2.6)** | 2 (1.7–2.3)** | 1 (0.8–1.2)** |
| 5 | 10 | 2 (1.5–2.5)** | 2 (1.3–2.7)** | 2 (1.7–2.3)** |
| 15 | 10 | 2.5 (1.7–3.3)* | 2.5 (1.7–3.3)* | 2 (1.3–2.7)** |
| *Motor* | | | | |
| Control | 18 | 2 (1.8–2.2) | 2 (1.8–2.2) | 3 (2.8–3.2) |
| Melatonin (mg/kg) | | | | |
| 0.1 | 7 | 2 (1.6–2.4) | 2 (1.7–2.3) | 3 (2.6–3.4) |
| 1 | 9 | 1 (0.8–1.2)** | 1 (0.7–1.3)** | 1 (0.7–1.3)** |
| 5 | 10 | 1.5 (1.2–1.8)* | 1 (0.7–1.3)** | 1 (0.7–1.3)** |
| 15 | 10 | 2 (1.6–2.4) | 1.5 (1.1–1.9)* | 2 (1.6–2.4)* |

Neurologic behavioral scores are expressed by median (95%CI). Intravenous injection of melatonin (1–15 mg/kg) 90 min after the onset of middle cerebral artery occlusion (i.e., upon reperfusion) significantly improved sensorimotor neurologic scores compared to vehicle-injected control values.
n, number of animals.
*$P < 0.05$ and **$P < 0.01$ versus vehicle data, respectively.

Table 2. Melatonin improves sensorimotor behavioral scores after cerebral ischemia-reperfusion in the estrogen-deficient (ovariectomized) female rats

| Neurologic behavioral score | n | Day 1 | Day 3 | Day 7 |
|---|---|---|---|---|
| *28-Point clinical scale* | | | | |
| Control | 11 | 16 (14.7–17.3) | 15 (13.5–16.5) | 13 (15.6–10.4) |
| Melatonin (mg/kg) | | | | |
| 1 | 9 | 15 (13.0–17.0) | 15 (12.8–17.2) | 11.5 (10.1–12.9) |
| 5 | 11 | 13 (11.3–14.7) | 13 (11.4–14.5) | 9 (7.0–11.0)* |
| 15 | 10 | 13 (11.2–14.8) | 12 (9.7–14.2) | 9 (8.6–9.4)* |
| 50 | 7 | 13 (11.5–14.4)* | 9 (7.4–10.6)* | 9 (7.8–10.1)* |
| *Sensory* | | | | |
| Control | 11 | 3 (2.5–3.5) | 3 (2.4–3.6) | 2 (1.5–2.5) |
| Melatonin (mg/kg) | | | | |
| 1 | 9 | 3 (2.4–3.6) | 3 (2.3–3.6) | 2 (1.5–2.4) |
| 5 | 11 | 3 (2.4–3.6) | 2 (1.7–2.3) | 2 (1.6–2.4) |
| 15 | 10 | 3 (2.3–3.7) | 2.5 (1.8–3.1)* | 2 (1.5–2.5) |
| 50 | 7 | 3 (2.2–3.8) | 2 (1.5–2.5) | 1 (0.6–1.4)* |
| *Motor* | | | | |
| Control | 11 | 3 (2.7–3.3) | 3 (2.5–3.5) | 2 (1.5–2.5) |
| Melatonin (mg/kg) | | | | |
| 1 | 9 | 2 (1.5–2.5) | 2 (1.7–2.3) | 2 (1.6–2.4) |
| 5 | 11 | 2 (1.5–2.5) | 2 (1.5–2.5) | 1.5 (1.0–2.0) |
| 15 | 10 | 2 (1.5–2.5) | 2 (1.7–2.3) | 1 (0.7–1.3)* |
| 50 | 7 | 2 (1.7–2.3)* | 2 (1.6–2.3)* | 1 (0.8–1.2)* |

Neurologic behavioral scores are expressed by median (95%CI). Intravenous injection of melatonin (15–50 mg/kg) 90 min after the onset of middle cerebral artery occlusion (i.e., upon reperfusion) significantly improved sensorimotor neurologic scores compared to vehicle-injected control values.
n, number of animals.
*$P < 0.05$ versus vehicle data.

infarct volume reductions and, thus, improved the functional neurobehavioral outcomes in the reproductively active and estrogen-deficient female Sprague–Dawley rats, respectively. Thus, melatonin had an apparently gender-specific and circulating estrogen-dependent difference in the dose–response curve in the treatment of ischemic stroke in the female animals [4, 6, 7]. This neuroprotection observed in female stroke rats cannot be accounted for by changes in

hemodilution (as measured by blood hematocrit), arterial blood pressure, heart rate, or differences in core temperature, because these were not significantly different when compared between vehicle-injected and melatonin-treated animals.

The U-shaped neuroprotective dose–response curve for melatonin has been observed in primary neuronal cultures exposed to OGD as well as both in female and male stroke rats, as described in the present study and well shown in previous studies [39]. Although the reasons underlying these U-shaped findings are not yet clear, a possible adverse effect counteracting the melatonin-mediated neuroprotection has been implicated, when melatonin was administrated at high doses [39]. This assumption, however, was contradicted by our finding that melatonin, even when it was administrated at large doses, actually did not exhibit any observable cytotoxicity in primary cortical neuronal cultures (data not shown).

Several experimental reports have documented the importance of circulating estrogen in attenuating ischemic brain damage in rats [4, 5]. We have observed that, in the reproductively active female rats, melatonin's hormetic dose–response for effectively reducing brain infarct volumes was negatively skewed from the optimal dosage (5–15 mg/kg) reported in male rats [39], whereas, in the estrogen-deficient stroke rats, there was a contrary (positive) shift of the hormetic dose–response [43]. This change was apparently induced by the presence of the circulating estrogen or not and might unlikely be attributed to a difference in the route of drug administration, as melatonin could well penetrate the blood–brain barrier by a regimen of either intraperitoneal, intravenous, or, even, oral administration route [8, 15–21, 44]. Thus, increasing the dosage of melatonin in the presence of the circulating estrogen may not necessarily lead to improved neuroprotection, as observed here in the reproductively active female ischemic rats. Given the differences described in the report in female and presently highlighted in the literature in male rats [39], it is important that putative neuroprotective agents for the treatment of stroke may need to be investigated both in male as well as in female animals.

In the present study, we observed that co-treatment with melatonin and E2 at each optimal dosage actually induced an opposite change in cytoprotection in primary cortical neuronal cultures exposed to OGD, compared to each agent in isolation, and when combined at a dosage either positively or negatively skewed from each optimal dosage, however, would exhibit synergistic neuroprotection. This finding further supported that an optimal regimen dosing reciprocally between melatonin and estrogen may be needed for exhibiting their optimal and synergistic neuroprotection in the neuronal cultures exposed to OGD, regardless of their synergistic antioxidant, radical-scavenging, and anti-inflammatory actions apparently observed in the acellular preparations and the LPS-stimulated RAW 264.7 cells in vitro. Consistently, an optimal regimen dosing reciprocally to the level of circulating estrogen might be needed to protect reproductively active and senescent female rats against ischemic stroke, as observed here. It is, therefore, very likely that melatonin and estrogen may share some common, but competitive, receptors or pathways in exhibiting neuroprotection for both primary neuronal cultures in vitro and female stroke rats in vivo and this, however, needs further evaluations [45].

The mechanism(s) underlying the gender-specific and circulating estrogen-dependent shift in melatonin dose–response curve for reducing brain infarct in female animals are not exactly known, but several possibilities are apparent. It is very likely that melatonin might exhibit synergistic neuroprotection with physiologically circulating estrogen for reducing brain infarct in reproductively active female stroke rats [26]. Thus, a decrease in the melatonin's dosing regimen in reproductively active female rats might, at least in part, be as a result of an add-on of antioxidant, free radical-scavenging, and anti-inflammatory actions. However, this gender-specific neuroprotection might also have been influenced by the estrogen-induced activation of the melatonin receptors. Specifically, estrogen differentially affects MT1 and MT2 melatonin receptor functions, attenuates melatonin responses through activation of MT1 receptors, and increases the MT2 receptors density [4, 23, 25, 45] and this could partly account for the finding with a negative and positive shift of the melatonin's hormetic dose–response in the reproductively active and senescent female rats, respectively.

Melatonin is already being used clinically in the treatment of disturbed sleep disorders, irritable bowel syndrome, and in the treatment of some solid tumors, as well as being used as anti-oxidative and anti-hypertensive agents [46–50]. Numerous studies in humans show a lack of toxicity for melatonin, even at high doses. Combined with the substantial reduction in infarct volume in male [15–21, 39, 44] and reproductively active and senescent (estrogen deficient) female rats (this study), melatonin is highly recommended as one drug of choice for clinical trial testing in the treatment of ischemic stroke. It is, however, essential to rectify melatonin's hormetic dose–response according to the level of circulating estradiol/the status of menstrual cycle, thereby, offering optimal neuroprotection in the treatment of female stroke patients [5, 43].

Our results demonstrate that intravenous administration of melatonin (1–5 and 15–50 mg/kg, respectively), 90 min after the onset of stroke, reduces brain infarct volumes and improves neurobehavioral outcomes in a model of transient focal cerebral ischemia in reproductively active and estrogen-deficient female rats. In addition, we showed complex interactions and synergistic antioxidant, radical-scavenging, and anti-inflammatory actions between estradiol and melatonin and highlighted the need to rectify the melatonin's hormetic dose–response by the status of menstrual cycle in the treatment of female stroke patients. This encouraging neuroprotective profile of melatonin in male and female experimental animals, as reported previously and observed here, and the safety established by numerous clinical studies in patients for other indications suggest that melatonin may be of use for both male and female stroke patients.

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