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# LABELING OF BRAIN MICROCIRCULATION AFTER SYSTEMIC ADMINISTRATION OF MELATONIN-FLUORESCEIN CONJUGATE IN HEALTHY MICE

Eliezer MASLIAH[1] and Paul TONE[2]

[1] Department of Neurosciences, University of California San Diego, La Jolla, CA, USA; Department of Pathology, University of California San Diego, La Jolla, CA, USA. E-mail: emasliah@ucsd.edu
[2] Department of Medicine, Richmond University Medical Center, Staten Island, New York. E-mail: paultonemd@yahoo.com

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We hypothesized that melatonin could be a carrier for small cargo of therapeutic molecule/s that can cross the BBB into the brain. To demonstrate the proof of concept, custom synthesized Melatonin-Fluorescein conjugate was administered intraperitoneally in healthy adult C57BL/6 mice. Confocal laser imaging showed that mice that received pretreatment with Colchicine and then Melatonin-Fluorescein conjugate, displayed intense cerebral microvascular labeling throughout the brain; the labeling was more intense in the vascular wall, endothelial cells and intravascular trafficking leukocytes, only a limited amount of the Melatonin-Fluorescein was seen in the adjacent neuropil.

*Keywords*: Melatonin, blood-brain-barrier (BBB), colchicine, melatonin-fluorescein conjugate, cerebral microvascular labeling, vascular wall, endothelial cells, intravascular trafficking of leukocytes, neuropil, confocal laser imaging.

## INTRODUCTION

### SYSTEMIC ADMINISTRATION OF EXOGENOUS MELATONIN *IN VIVO*

Melatonin crosses all morphophysiological barriers and enters equally well all cells in the organism[1].

A human PET study performed with Carbon-11 labeled melatonin in a healthy volunteer confirmed that melatonin readily crosses the blood-brain barrier (BBB) to brain without specific binding[2].

The mechanism by which melatonin enters into cells remains unknown, but its amphiphatic nature (amphiphilicity) seems to play an important role[3–4].

The ability of melatonin to cross the BBB has been attributed to its amphiphilic nature and octanol-water partition coefficient (logPoct = 1.2)[5].

5-metoxy group is responsible for lipophilicity of melatonin. If the 5-metoxy group is replaced by a hydroxyl group, under some in vitro conditions, the antioxidant capacity of this molecule may be enhanced. However, the costs of this change are decreased lipophilicity and increased prooxidative potential[6].

C3 side chain of melatonin is an amide, more precisely a secondary amide that contains a carbonyl group. It likely that C3 side chain of melatonin is responsible for hydrophillicity of melatonin taking into account our knowledge regarding the solubility of amides (Fig. 1).

Melatonin's O-methyl and N-acetyl residues are not only the basis of its amphiphilicity enabling the molecule to enter all organs and all subcellular compartments, but are also decisive for its antioxidant properties[7].

Melatonin can cross multilamellar lipid vesicles, which are used as model systems for the lipid phase of biological membranes, proving that melatonin can easily pass through the cell membrane and bath every part of the cell[8].

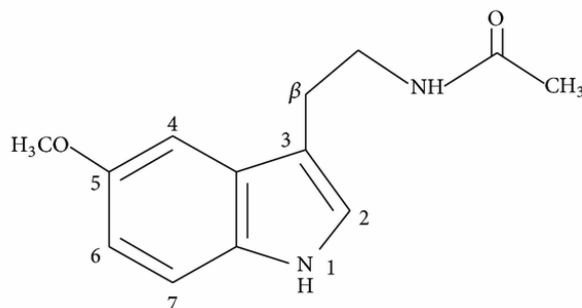


Figure 1. Melatonin structural formula [25].

Using small-angle neutron diffraction (SAND) from oriented lipid multi-layers, small-angle neutron scattering (SANS) from unilamellar vesicles experiments and Molecular Dynamics (MD) simulations to elucidate non-specific interactions of melatonin and cholesterol with 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) model membranes showed that melatonin decreases the thickness of both model membranes by disordering the lipid hydrocarbon chains, thus increasing membrane fluidity[9]. The interaction of melatonin with model membranes made of dimyristoylphosphatidylcholine (DMPC) at melatonin concentrations ranging from 0.5 mol% to 30 mol% showed that at low concentrations, melatonin molecules were found to align parallel to the lipid tails, and at high concentrations melatonin molecules align parallel to the bilayers, one melatonin molecule associates with 2 lipid molecules[10].

Hevia *et al.*[3] demonstrated recently, that members of the SLC2/GLUT family glucose transporters have a central role in melatonin uptake. When studied by docking simulation, it was found that melatonin interacts at the same location in GLUT1 where glucose does. This is the first time that a facilitated transport of melatonin is suggested.

## MELATONIN HYBRID DRUGS

The idea that melatonin can be a carrier for small therapeutic molecules capable to cross the BBB into the brain was translated in reality by synthesis of melatonin hybrid ligands for the treatment of Alzheimer's disease.

Melatonin-Tacrine is a hybrid dual-acting drug for Alzheimer's disease, with improved acetylcholinesterase inhibitory and antioxidant properties. Tacrine, a lipophilic molecule, was linked to melatonin using an unsubstituted $C_2$-$C_6$ alkyl linker between the tacrine amine and the carbonyl of melatonin[10].

Melatonin –Tacrine hybrids were tested in vitro using parallel artificial membrane permeation assay for blood-brain barrier (PAMPA-BBB). It was suggested that these hybrids could cross the BBB and reach their biological targets located in the CNS[10].

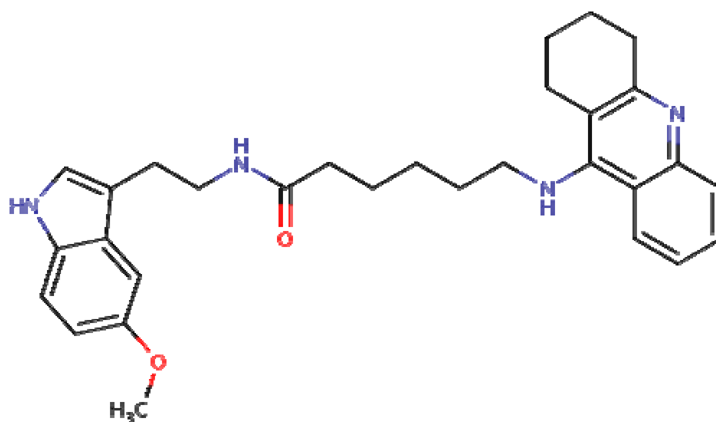


Figure 2. Structural formula of Melatonin-Tacrine hybrid [10].

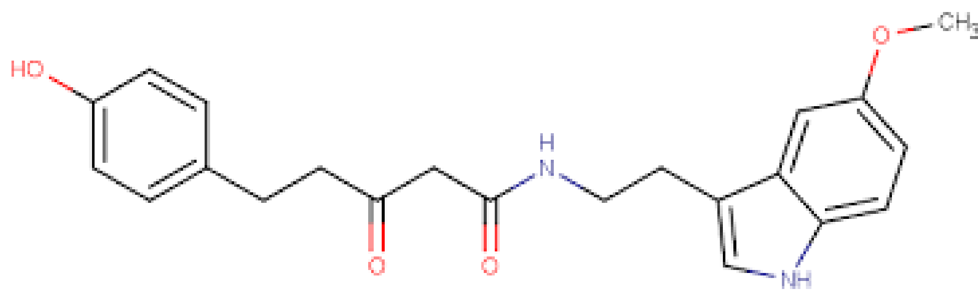


Figure 3. Melatonin-Curcumin hybrid structural formula [12].

Melatonin-Tacrine hybrid was tested in vivo by direct intracerebral administration in APP/Ps1 mice[11]. However, the efficacy of this hybrid drug in mice model of Alzheimer's disease was not verified after its systemic administration.

Melatonin-Curcumin hybrid (Z-CM-I-1) has been recently synthesized and tested on APP/PS1 transgenic AD model; a significant decrease in the accumulation of Aβ in the hippocampus and cortex regions of the brain and reduced inflammatory responses and oxidative stress were reported after treatment for 12 weeks at 50 mg/kg per dose via oral administration[12].

The ligand moiety of Melatonin hybrids is a small lipophilic therapeutic molecule (tacrine, curcumin, etc.).

## MATERIALS AND METHODS

### MATERIALS

Melatonin, 6-aminofluorescein, Formaldehyde, Trehalose, Protran Nitrocellulose Membrane, Triton X, Colchicine, were obtained from Sigma-Aldrich, Saint Louis, MO. Spectra Por MWCO CE dialysis tubing 500 MWCO was obtained from Spectrum Laboratories Inc., Ranch Dominguez, CA. Sheep anti-melatonin antibodies were obtained from Cortex Biochem Inc., San Leandro, CA. BSA was obtained from Equitech Bio, Inc., Kerrville, TX. Horseradish peroxidase labeled rabbit anti-sheep IgG was obtained from Jackson ImmunoResearch Laboratories, Inc., West Grove, PA. NFDM and TMB (BioFX) were obtained from SurModics Inc., Eden Prairie, MN. Wild-type (WT) C57BL/6 mice were purchased from Charles River laboratories (California, San Diego).

### CUSTOM SYNTHESIS OF MELATONIN-FLUORESCEIN CONJUGATE

Custom synthesis of Melatonin-Fluorescein was done by Roveko Ltd (Gaithersburg, MD).

The Mannich reaction consists of condensation of formaldehyde (HCHO/CH2O) with ammonia, primary amine, secondary amine or even amides, and another compound containing active hydrogen. Reaction of melatonin (which has at C2 the active hydrogen) with free amino groups of glycine, piperidine, and proteins (BSA) occurs initially at the N-indole, and then a stable bond is formed at C2-indole position[13].

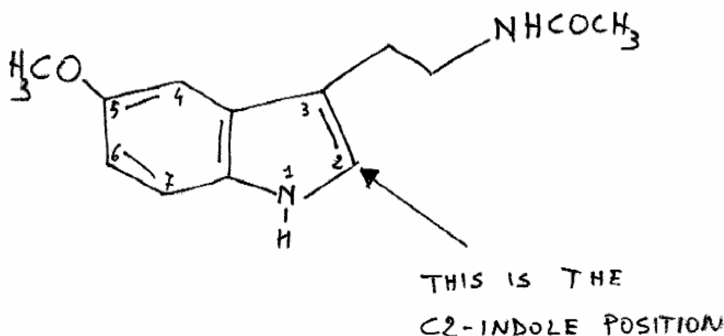


Figure 4. Melatonin with C2-indole position involved in Mannich reaction.

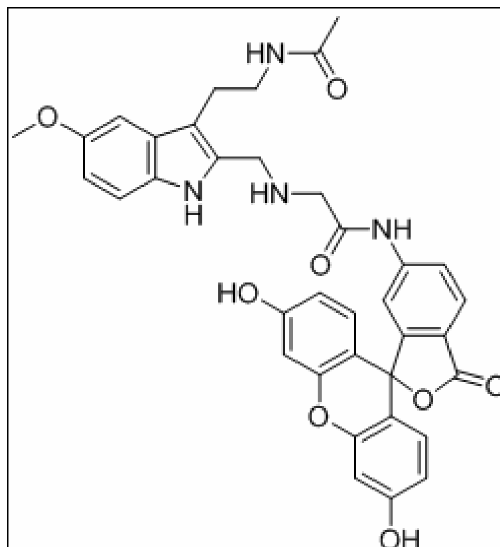


Figure 5. Melatonin-Fluorescein conjugate, chemical structure, molecular formula C33H30O7N3, MW (molar mass) 580.6103 ± 0.0005 g/mol.

50 mg of 6-aminofluorescein were dissolved in 5 ml of DMSO and added to 50 mg of melatonin dissolved in 5 ml of double distilled water: ethanol mixture (2:1, v/v). The following were then successively added: 3 ml of 8 M sodium acetate and 5 ml of 7.5% formaldehyde. The final volume was brought to 14 ml with double distilled water. The reaction mixture was then placed in the dark with constant magnetic stirring until a stable opalescent compound appeared at 24 h. Reaction was completed after an incubation time of 4 days in the dark. The conjugate was then dialyzed exhaustively in Spectra Por MWCO CE dialysis tubing 500 MWCO vs. borate buffer. 80 uL of 25% trehalose were added to the post dialysis conjugate, which was then aliquoted and freeze dried for later use.

To confirm the conjugation, the Melatonin-Fluorescein conjugate was tested by immunoblots. Sheep anti-melatonin antibody was purified with a Protein G column. The anti-melatonin IgG fraction was dissolved in PBS at a concentration of 10 ug/mL, and 1 uL aliquots were pipetted onto nitrocellulose membrane. The membrane was allowed to dry for 30 minutes then blocked with 5% NFDM (Non-Fat Dry Milk) for another 30 minutes. Melatonin, 6-AF, and melatonin-6AF were dissolved, respectively, in ethanol, DMSO, and DI water all at a concentration of 10 ug/mL. Separately, each of these three solutions were incubated and rocked for 30 minutes with the anti-melatonin antibody blots dried on membranes. After washing and drying, the membranes were analyzed using a dual wavelength fluorescent lamp. Fluorescence was detected only on membrane reacted with the Melatonin-Fluoresceine conjugate. In parallel, Protein G purified sheep anti-melatonin antibody at 10 ug/mL in 0.2% Triton X-100, 1% BSA, in PBS was incubated with membranes that have been blotted with serial dilutions of the Melatonin-Fluoresceine conjugate. After reaction with the antibody,horseradish peroxidase labeled rabbit anti-sheep IgG (H+L) at 1 ug/mL in Triton/BSA/PBS buffer was reacted for 30 minutes with the membrane. After a finalwashing and drying period the blot was visualized with TMB (BioFX) for the prescence of melatonin. The presence of 6-aminofluorescein in these reactive blots has been confirmed with the fluorescent lamp, prior to the application of TMB (tetra methyl benzidine).

## EXPERIMENTAL DESIGN

The study was approved by the University of California San Diego Institutional Animal Care and Use Committee (IACUC) and performed according to the National Research Council's Guide for the Care and Use of Laboratory Animals ($8^{th}$ ed., 2011).

Mice were injected intraperitoneally (IP) with 100 µL of colchicine 2 mg/kg (Colchicine mixed in 2% DMSO).

Two hours later mice were injected intraperitoneally (IP) with 250 mg/kg of the

Melatonin-Fluoresceine solution (5 mg diluted in 100 μL of DMSO + 150 μL of saline).

We administered IP the Melatonin-Fluorescein conjugate 2-hour after pretreatment with colchicine taking into account the optimum time for the action of colchicine (Morris, 1967).

Control animals were treated similarly but instead received 6AF alone in DMSO solution.

For each experiment a total 2 animals per group were used. Mice were euthanized by cervical dislocation 5 hrs later, following NIH guidelines for humane treatment of animals. Fresh brains (no perfusion) were carefully dissected with spatula and placed in 4% PFA for 4 days.

Vibratome sections (40 um thickness) were done from the whole hemibrain oriented in a longitudinal axis, each section includes most brain areas, the imaging and figures are from the frontal cortex but similar results are observed in the hippocampus.

Vibratome sections of the brain were analyzed with laser scanning confocal microscope (Biorad 1024), 40× objective, 1.2 NA, final Mag 630×, optical section thick 1um.

## RESULTS

Animals that received Colchicine and then Melatonin-Fluorescein displayed intense cerebrovascular labeling throughout the brain (Figs. 6, 7, 8); the labeling was more intense in the vascular wall, endothelial cells and intravascular trafficking leukocytes (Fig. 9), only a limited amount of the Melatonin-Fluorescein was seen in the adjacent neuropil (Fig. 10). No labeling was observed in any of the control groups.

The Melatonin-Fluorescein conjugate displayed excellent vascular labeling but limited intracerebral penetration; we might need to allow the conjugate to diffuse for a longer period of time.

The colchicine treatment appears to have dramatic effects at increasing the vascular labeling, but limited effect in terms of neuropil penetration.

Neuropil is a broad term defined as any area in the central nervous system gray matter composed of mostly unmyelinated axons, dendrites and glial cell processes that forms a synaptically dense region containing a relatively low number of cell bodies[15].

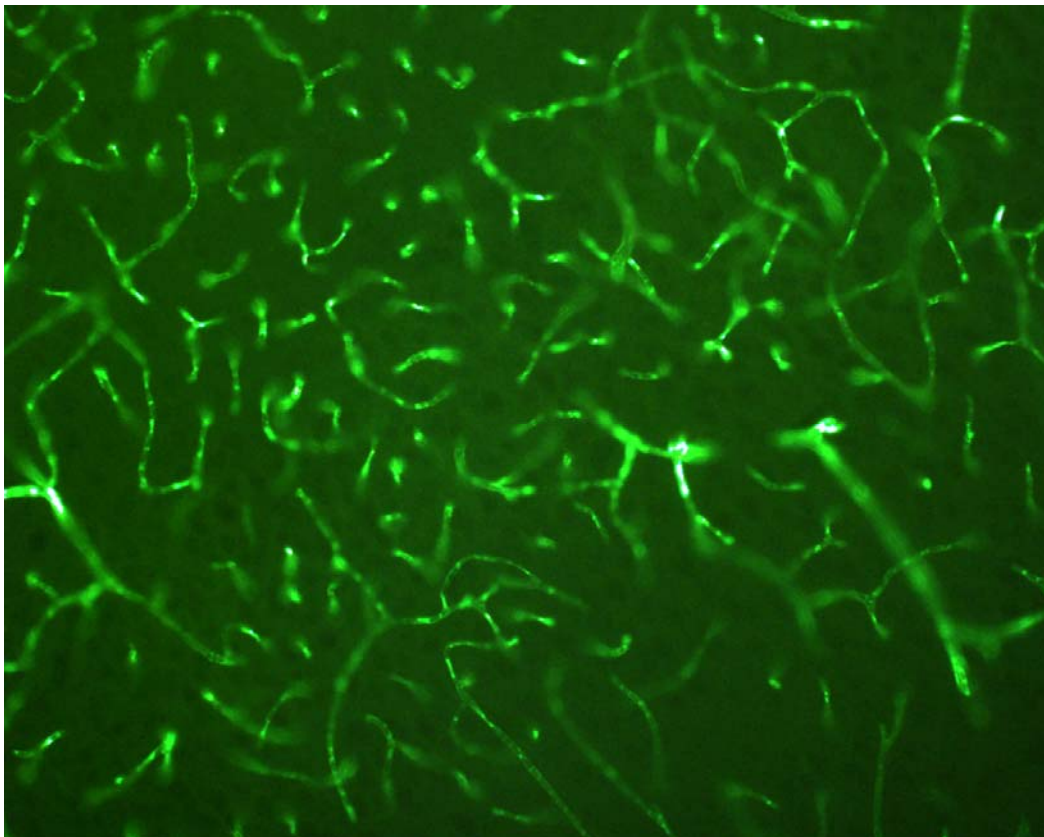

Figure 6. Confocal image showing labeling of mouse brain capillaries in the frontal cortex after systemic administration of Melatonin-Fluorescein conjugate.

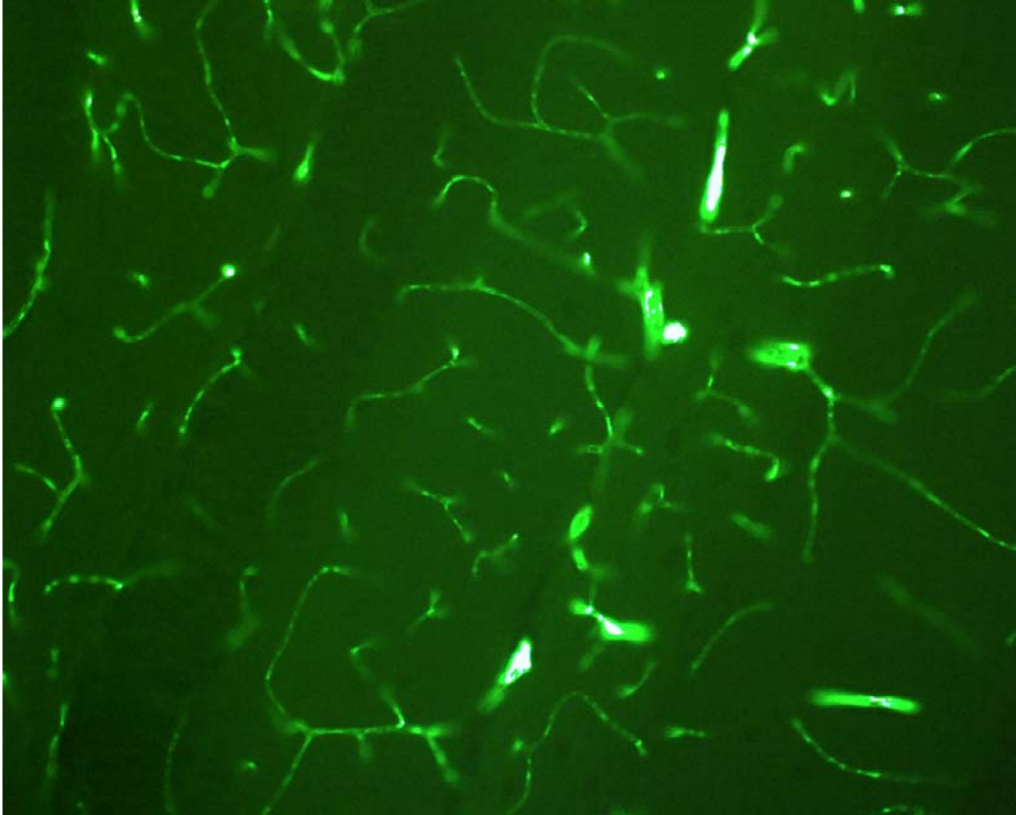

Figure 7. Confocal image showing labeling of mouse brain capillaries in the frontal cortex after systemic administration of Melatonin-Fluorescein conjugate.

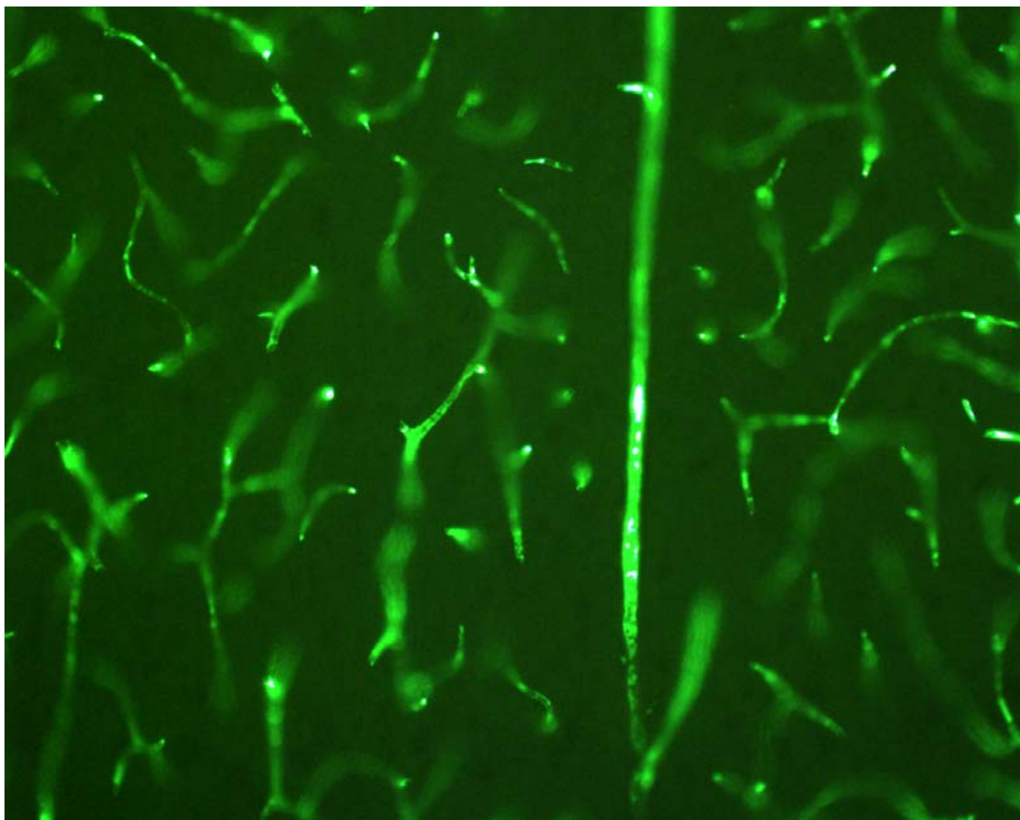

Figure 8. Confocal image showing labeling of brain capillaries in mouse hippocampus after systemic administration of Melatonin-Fluorescein conjugate.

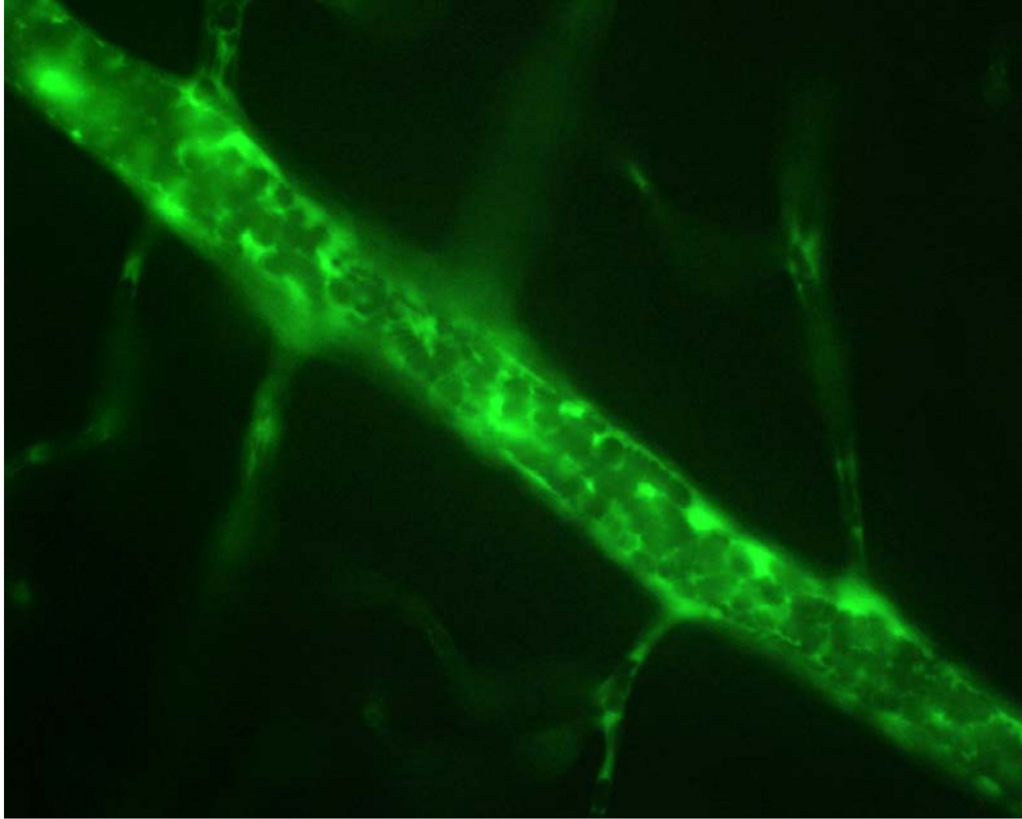

Figure 9. Confocal imageshowing labeling of a mouse brain arteriole in hippocampus with intense staining in the vascular wall, endothelial cells and intravascular trafficking leukocytes, after systemic administration of Melatonin-Fluorescein conjugate.

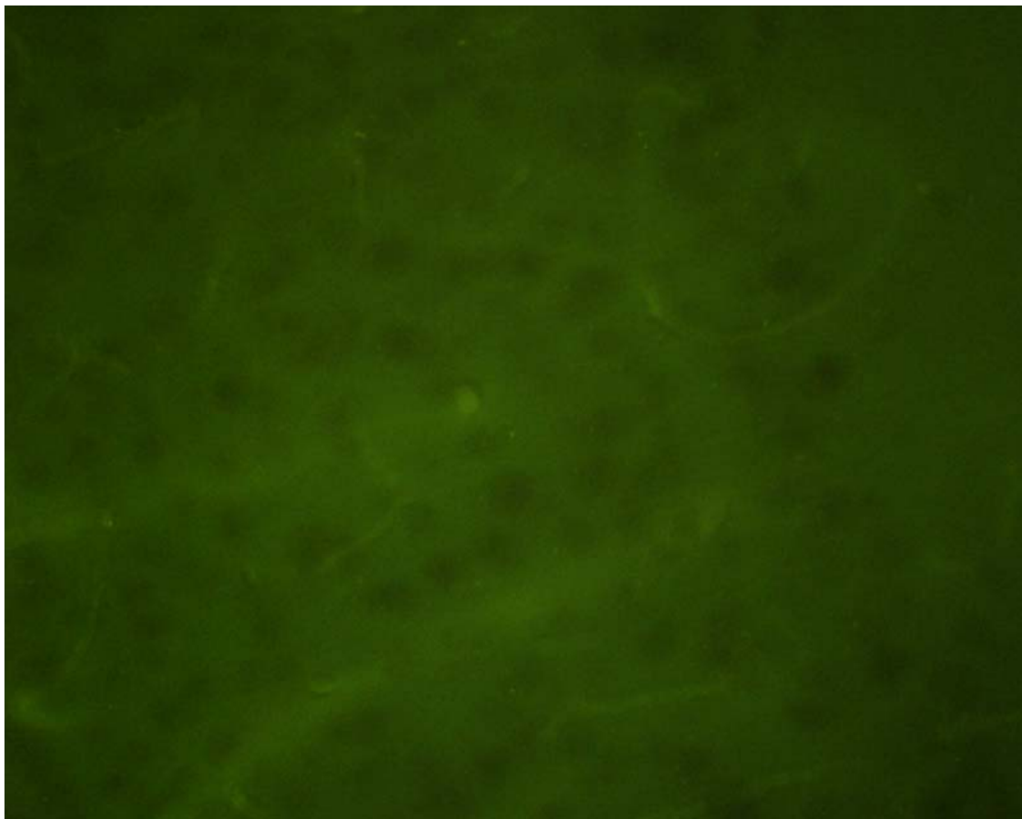

Figure 10. Confocal image showing that only a limited amount of Melatonin-6AF conjugate injected systemically in mouse was seen in the adjacent neuropil.

## DISCUSSIONS

It is important to make the distinction between visualization of brain microcirculation using an intravascular fluorescent agent like Dextran-FITC[15'] and fluorescent labeling of the walls of brain capillaries and arterioles with Melatonin-Fluorescein conjugate.

The mechanism of enhancing effect of colchicine on increasing staining and residence of Melatonin-Fluorescein in the brain arteriolar and capillary walls of healthy adult mouse is unknown.

Colchicine is a highly lipophilic drug that is a substrate for P-glycoprotein (P-pg) ATP-dependent efflux pumps localized on the luminal plasmalemma of BBB endothelial cells and co-localize with caveolin-1[16,17]. Colchicine inhibits microtubule polymerization by binding to tubulin, one of the main constituents of microtubules[18,19].

The role of microfilaments and microtubules in cerebral endothelial permeability to the protein horseradish peroxidase (HRP) was studied in rats pretreated with agents known to disrupt the microfilaments and microtubules. Increased cerebrovascular permeability (capillaries, arterioles, and venules) consisted in accumulation and labeling of tracer in the intraendothelial spaces except tight junctions which were not disrupted[20].

We believe that by disrupting microtubules in mouse brain capillaries and arterioles, colchicine could explain the increase accumulation of Melatonin-Fluorescein into intraendothelial spaces of capillaries and arteriolar walls.

The microtubule cytoskeleton has been thought to play a role in the trafficking of vesicles containing reserves of transmembrane transporters to their docking sites in the plasma membrane[21].

Evidence has suggested that the integrity of microtubule cytoskeleton is a requirement for similar trafficking of Na-K pump[22].

Conditions inducing a reduction or dissipation of the ionic gradients caused a decrease in the rate of Fluorescein release from the living cells. In contrast, enhancement of the gradients increased the efflux rate. The release of Fluorescein from living cells is influenced by the membrane potential[23].

By disrupting the microtubules with colchicine, the transport of vesicles containing NA-K-ATPase pump to the docking sites in plasma membrane can be impaired, and the membrane ionic gradient can be reduced. This could explain the decrease in the rate of Melatonin-Fluorescein release from capillary endothelial cells and arteriolar wall.

Fluorescent proteins have been used to monitor the dynamics of secretory vesicle movements and their fusion with the plasma membrane of chromaffine cells[24].

If Melatonin-Fluorescein locates in the secretory vesicles of vascular endothelial cells, pretreatment with colchicine can destabilize the microtubule network that interconnects the Golgi apparatus with plasma membrane to guide secretory vesicles for export. In this way, pretreatment with colchicine could increase the residence time of Melatonin-Fluorescein conjugate into the brain capillaries and arteriolar walls and could explain the dramatic effect on labeling of brain microcirculation by the fluorescent conjugate.

Labeling intravascular trafficking leukocytes in arteriole demonstrates capacity of Melatonin-Fluorescein conjugate to cross the cellular membranes of these cells.

Melatonin-Fluorescein conjugate could be a valuable research tool for confocal studies of brain microcirculation in transgenic mice model of Alzheimer’s disease and other neurodegenerative diseases, ischemic stroke, and neuroinflammatory diseases particularly EAE (Experimental Autoimmune Encephalomyelitis) model of multiple sclerosis.

If the brain arteries can be also stained with Melatonin-Fluorescein conjugate, this will represent a breakthrough in the delivery of therapeutic molecules to the arterial wall in cerebral atherosclerosis. Melatonin could carry a low molecular weight therapeutic cargo load into the arterial wall which is an active metabolic participant in atherogenesis.

Perhaps the greatest application of Melatonin-Fluorescein conjugate will be the labeling of retinal microcirculation that is accessible to clinical examination with confocal laser fluorescent microscope.

## CONCLUSIONS

The study is preliminary and no definitive conclusion could be drawn regarding the ability of Melatonin-6AF to cross the BBB in mice. The poor penetration of Melatonin-Fluoresceine conju-

gate in neuropil does not mean total absence of crossing of BBB by the conjugate. We might need in the future work to allow the conjugate to diffuse for a longer period of time for crossing the BBB.

There were two unexpected new findings revealed by this study:

1. Labeling of mouse brain microcirculation after intraperitoneal (IP) administration of Melatonin-Fluorescein conjugate;

2. Pretreatment with colchicine (IP) 2 hours prior to IP administration of Melatonin-Fluorescein conjugate enhanced dramatically penetration of the conjugate which displayed five hour-residence time in the brain microvessels walls.

**Conflict of interests.** The authors declare that there is no conflict of interests regarding the publication of this paper.

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