Neuroprotective effects of melatonin on kainic acid-induced oxidative stress in organotypic hippocampal slice cultures

Hyung A Kim

Department of Medical Science

The Graduate School, Yonsei University

Neuroprotective effects of melatonin on kainic acid-induced oxidative stress in organotypic hippocampal slice cultures

Directed by Professor Bae Hwan Lee

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This certifies that the Master's Thesis
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Thesis Supervisor : Bae Hwan Lee

Thesis committee Member : Dong Goo Kim

Thesis committee Member : Insop Shim

The Graduate School
Yonsei University

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Acknowledgements

고민과 시련의 먼 길을 돌아 이제 시작점으로 돌아온 것만 같습니다. 많았던 선택의 순간에 방황하고 가족과 친구들에게 기대며 울고 웃었던 기억이 부끄럽지만 아름다운 기억으로 자리잡은 날인 것도 같습니다. 처음 잡았던 맘이 이제 와서야 아쉬운 기억으로 남을 줄 알았더라면 조금 더 노력해 볼 것 을… 이란 안타까운 마음이 생깁니다.

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실험실에 들어와 아무것도 모르던 저에게 가장 중요한 기초를 잡아주셨던 선생님이시며 선배님이신 이경희선생님, 고민을 받아주시고 챙겨주신 김은정선생님, 멋쟁이 사영희 선생님, 걱정해주신 세정언니, 겁먹었던 시작에 울음을 받아주고 감싸 안아주신 경하언니, 세상 가장 착한, 말 안 듣는 후배 다은이, 때론 언니 같고 동생 같은 착한 우리 가민이, 마지막에 너무 고생시킨 준식이, 선지, 미란이에게 감사의 마음을 전합니다.

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대학원 생활의 정신적 지주셨던 포항공대의 우경철선생님. 대학원에 들어와 좌절하고 있었을 때 선뜻 도움을 주시고 인생의 선배님으로 마음을 열어 주신 것 항상 마음 깊이 감사 드립니다. 대학원 동기로 슬픈 일 기쁜 일을 함께 했던 노윤미양(62)과 나보다 내 논문에 신경을 써 주고, 도움을 준 멋쟁이 진형(59)군에게 외로울 뻔한 대학원의 추억을 함께 해준 것에 너무 고맙고 사랑한다고 말해주고 싶습니다.

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저자 씀

<ABSTRACT>

Neuroprotective effects of melatonin on kainic acid-induced oxidative stress in organotypic hippocampal slice cultures

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The Graduate School, Yonsei University

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The brain is organ with an high oxygen consumption where a lot of free radicals are present there. These free radicals attack proteins, lipids, and DNA that consist of intracellular elements to modify, damage and finally cause various diseases. In order to protect the cells and organs from diseases by this oxidative damage, the importance of antioxidant is getting increased. Melatonin, one of the free radical scavengers, is known as an antioxidant that removes the reactive oxygen species (ROS) effectively in various oxidative damage models. It is regarded as a terminal antioxidant because melatonin makes stabilized end product from melatonin by removing free radicals not through oxidation-reduction cycle.

The purpose of this study is to demonstrate the effectiveness of melatonin on kainic acid (KA)-induced oxidative stresses that affect various neurodegenerative diseases. The melatonin by different concentrations and secreted melatonin from cultured pineal gland were used in organotypic hippocampal slice cultures (OHSCs) model that causes oxidative damage by using KA. Postnatal 6~8 day rats were used for OHSCs model, and slices were

cultured for 3 weeks. 5 μM KA was treated to cultured tissues for 18 hours, and neuronal damage and ROS formation from KA by were observed measuring PI uptake and spectrum of 2',7'- dichlorohydrofluorescein diacetate (DCFH-DA) .

To observe protective effects of melatonin, two experiments were carried out. First, KA- injured OHSCs model was treated with 0.01, 0.1 and 1 mM of melatonin to measure the effects of melatonin by different concentrations,. Second, the co-culture method together with KA injured OHSCs was used to observe the effects of melatonin that was secreted from pineal cells.

Study Ⅰ demonstrated that 0.1 and 1 mM of melatonin protected neuronal cell death effectively at 24 and 48 hours after melatonin treatment after measuring the PI uptake. Besides, by measuring intact cells using cresyl violet staining, the measured data correlated with PI uptake at 48 hours after melatonin treatment. After measuring the DCFH-DA spectrum, ROS formation in 0.01, 0.1 and 1 mM melatonin treated groups after 24 hours were less than KA group. In order to detect the protein expression levels of 5-lipoxigenase (5-LO), caspase-3, calmodulin and superoxide dismutase-2 (SOD-2), western blot was carried out. 5-LO, caspase-3, calmodulin, and SOD-2 expression have similar tendency. All of the expressions increased in KA-induced injury group and melatonin treated groups had reduced expressions compared to the KA-injued group.

Study Ⅱ, neuronal cell death decreased at 48 hours after the treatment with melatonin co-culture using fetal and adult pineal cells. PI uptake and cresyl violet staining was carried out from this method to detect the neuronal cell death and intact neuronal cells,. Fetal and adult pineal cells co-cultured groups significantly protected neurons from neuronal cell death by KA-injury. For measuring DCFH-DA, the spectrum was decreased at 24 hours after co-culturing fetal and adult pineal cells. The expression of 5-LO, caspase-3,

calmodulin, and SOD-2 significantly decreased by fetal and adult cells compared with KA only-treated group. Therefore, these results demonstrated that melatonin that was secreted from pineal cells was effective for attenuating oxidative damage.

Based on the result, it is suggested that melatonin is effective on KA-induced oxidative stress in OHSCs model.

Key words: melatonin, kainic acid, organotypic hippocampal slice culture, reactive oxygen species

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Ⅰ. INTRODUCTION

The organisms are surrounded in high oxygen condition. Especially aerobe must consume the oxygen to make energy and metabolite. In this process, many byproducts such as nitric oxide, superoxide, hydrogen peroxide, and hydroxyl radical are formed. It is well known that the excessive production of oxygen free radicals has suggested that it generates many neuronal disorders. Especially, the brain is one of the most oxygen consuming organs in a human body.[1] Because of this oxygen consumption, reactive oxygen species (ROS) production in the brain is higher than ROS production in other body parts. ROS can be removed by various antioxidants and antioxidants do not have any harmful effects on other tissues or organs.[2,3] In general, antioxidant maintain and regulate the equilibrium between ROS formation and ROS extinction. However, if the equilibrium is distrupted, ROS acts as a stressor and damage intracellular elements and damage such as protein, lipid, and DNA. Because neuronal cells are especially sensitive and vulnerable to ROS, apoptosis or necrosis is easily occurred. Therefore oxidative stress produces neurotoxicity in the brain.

Specially, mitochondria oxidative stress deeply related with various neuronal disorders.[4]

The necessity of antioxidant is increased to reduced the oxidative stress,. Following this requirement, various antioxidants have been discovered. Melatonin is known as an antioxidant secreted from various organs such as pineal gland, retina, lens, and GI tract. Amongst, aforementioned organs pineal gland is the organ that release the majority of the melatonin compared to the others. Melatonin plays a variety of physiological roles such as adapting day and night cycle, adapting seasonal change, and participating in immune reaction, and it is known as a molecule has protect effects in many diseases such as Alzheimer, Parkinson disease, ischemia-reperfusion injury, mental disease, and cancer.[5,6,7,8] Because of melatonin's special characteristics, melatonin is able to pass morphophysiological barriers such as cellular membrane due to its high lipopilicity.[9] In addition, melatonin produces stabilized molecules by reacting with various ROS because it is an electron rich molecule. Particularly, melatonin produces stabilized molecules without producing any other ROS because there is no need to use redox cycling for melatonin, and it is titled suicidal or terminal antioxidant because the body excretes molecules produced by melatonin via urine without any secondary influence into a cell.[10] Furthermore the injection of melatonin from an outside the body does not affect the internal secretion of melatonin. Melatonin enter into a cell through binding with G-protein coupled melatonin receptor (MT) or directly permeable through a cell membrane. Melatonin binds with high affinity in the pico molar range to the membrane receptor, and nano molar range to the nuclear receptor as well as calmodulin.[11] At even higher concentration, melatonin has a free radical scavenging function. As a result, melatonin acts as an antioxidant.[12,13,14,15,16] The melatonin signaling pathway related with neuroprotective effects is presents in

figure 1.

Especially, researchers have noticed that many MT1A and MT1B receptors are abundant in the CA1 and CA3 region of the hippocampus that regulate the activation of melatonin.[17]

KA was used to oxidative stress generator to make a oxidative stress model. Kainic acid (KA) is an agonist of ionotrophic glutamate receptor. It acts as an excitotoxin that leads to neuronal excitotoxcity and oxidative damage in the central nerve system.[18] KA binds to kainite receptor and produces neuronal death.[19] KA-induced ROS are generated in following fashion. First, endogenous glutamate is released by ionotrophic glutamate receptor activation in pre-synaptic neuron. After that, post-synaptic voltage dependent channels activated. It generates calcium entry in neuron cells that activates the ROS related enzymes such as a nitric oxide synthase (NOS). Those enzymes for the generate ROS in a cell region. As a result, cell compounds such as lipid, protein and DNA are damaged by the ROS.[20] Related diagram represents in figure 2. The hippocampus contains a large number of kainite receptor. Particularly, Cornu Ammoni 3 (CA3) region has more kainite receptors than Cornu Ammonis 1 (CA1) or dentate gyrus.[21] Thus, CA3 region in the hippocampus is well known region that is susceptible to KA-induced neurotoxicity. Several previous studies have demonstrated that antioxidants can attenuate KA-induced neuronal damage.[22,23,24] Especially, mitochondria superoxide radical production generates ROS and destroys hippocampus.[25]

Organotypic hippocampal slice cultures (OHSCs) model has been widely used for studies related in neurodegeneration widely, and it is known to be an effective test method to examine the effect of various substances to the neuron and tissue. Besides, it is proper model to preserve anatomical structure such as neuron, synapse and processes for long time that is almost same environment

with *in vivo*, and this model also has another advantage that can treat determine concentration of drug and treat drug in particular tie that is similar with *in vitro*. This could be an appropriate method to demonstrate and to study the neuroprotective effects of melatonin that acts as an antioxidant in the KA-induced OHSCs model. Therefore, the effects of melatonin in the hippocampus demonstrate that the oxidative damage by KA treatment using OHSCs model in this study.

In this study, antioxidant effects of melatonin was found via study I using melatonin from commercial product and via study Ⅱ using melatonin which is secreted from pineal gland. Further more, proper concentration of commercially useful melatonin was established from observing protective effects of melatonin from pineal gland. The comparison of the effect of each types of melatonin is the main point of this study.

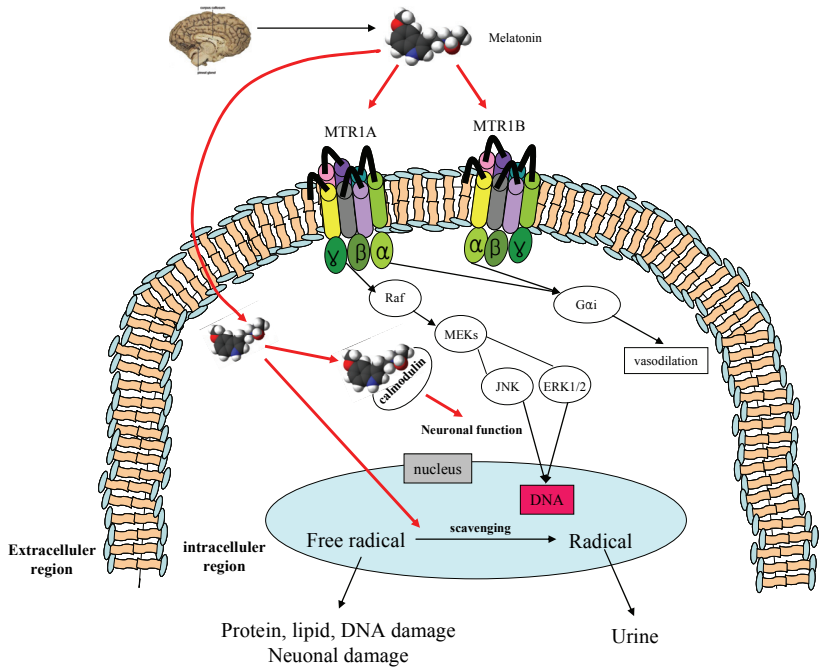


Figure 1. Melatonin pathway

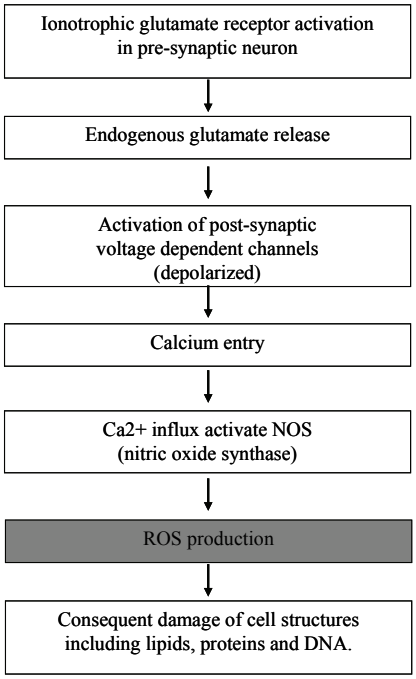


Figure 2. Kainic acid-induced ROS generation

Ⅱ. MATERIALS AND METHODS

1. Study Ⅰ: Protective effects of melatonin

A. Animals

Female (300 g) and her offspring (after birth of 1 day) Sprague-Dawley rats were purchased from KOATECH (Gyeonggi-do, Korea). They were feeding in 5 to 6 days in 37 ℃, and housed under 12 hr light and 12 hr dark condition with free access to food and water. All animal experiments were approved by Institutional Animal Care and Use Committee of Yonsei University College of Medicine.

B. Organotypic hippocampal slice cultures (OHSCs)

Sprague-Dawley rats brain aged 6 to 8 days postnatally were removed and transfer to Gey's Balanced Salt Solution (GBSS, Sigma, Saint Louis, MO, USA) containing 0.5 % glucose and 3 mM KCl, 1 N HCl. Rat hippocamppi were separated from whole brain and than dissected 350 ㎛ with McIlwain tissue chopper (Vibratome, O'Fallon, MO, USA) and choose the dissected tissues which have no damaged and cleared layer. Six to eight tissues were transferred onto Millicell-CM membrane insert (Millipore, Billerica, MA, USA) in 6-well plate containing 1 ㎖ culture media included 50 % Opti-MEM, 25 % Hank's Balanced salt solution (HBSS), 25 % heat inactivated horse serum (all from GIBCO BRL, Grand Island, NY, USA), 6.5 ㎎/㎖ D-glucose (AMRESCO Inc, Solon, Ohio, USA), pH 7.2. Cultured slices were incubated at

36℃ in a humidified atmosphere of 5 % CO_2. Culture media was changed three times a week. Slices were grown for 3 weeks in culture medium.

C. Drug prepation and treatment

Kainic acid (KA, sigma, Saint Louis, MO, USA) was dissolved in DW and applied for 18 h in 3 weeks cultured slices. Melatonin (Sigma, Saint Louis, MO, USA) was dissolved in 99 % ethanol (Merk, Darmstadt, Germany). 0.01, 0.1, 1 mM melatonin was diluted and treated with culture medium. Afterward, KA injured hippocampus kept on melatonin during 24 and 48 hr.

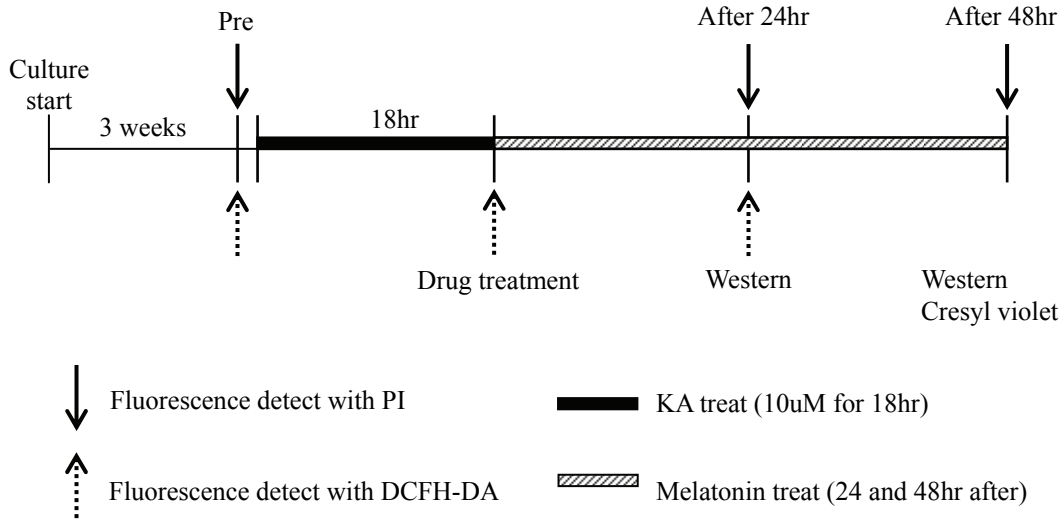


Figure 3. Diagram of experimental procedure. OHSCs perform for 3 weeks and 5 ㎍/㎖ PI was treated in the culture medium (pre phase). After 1 hr, pre phase cultured hippocampus pictures achieved. After that 10 μM DCFH-DA was treated for 30 min DCFH-DA picture achieved also. KA was diluted and treated with fresh culture medium for 18 hr. DCFH-DA picture was took in this phase but PI uptake was not. Subsequently, melatonin treated hippocampus picture of 24 and 48 hr for PI and 24 hr DCFH-DA were gathered.

D. Measurement of neuronal injury

Those concentrations did not have negative effects that the drugs did not cause the neuronal death in non-KA treat group. To observe the neuronal cell death used propidium iodide (PI, Sigma, Saint Louis, MO, USA). After 3 weeks cultured normal hippocampus added 5 μM PI in culture medium and detected the neuronal death in pre phase which have no PI uptake in the hippocampus layers. Degenerated tissues were removed from membrane insert and 5 μM KA was treated for 18 hr. After all 0.01, 0.1, 1 mM melatonin was treated with PI during the 24 and 48 hr. Full kill was performed with 10 mM NMDA (Sigma, Saint Louis, MO, USA)) for generating the neuronal death in all tissue. Finally, pre, 24 hr, 48 hr and full kill phase result was detected fluorescence microscope analyzed with metamorph program. All value of fluorescence were calculated by using following formula; % of PI uptake = $100(F_t - F_{pre})/(F_{fk}-F_{pre})$. Each represents F_t = 24 or 48 hr after melatonin treatment, F_{pre} = pre, F_{fk} = Full kill.

E. Evaluation of intracellular ROS formation

The DCFH-DA method described by Yang et al.[26] 10 μM 2',7'-dichlorohydrofluorescein diacetate (DCFH-DA, sigma, Saint Louis, MO, USA) was treated 30 min before detect by using microscope. Cells incubated with DCFH-DA in the 36℃ humidified incubator for 30 min and washed twice with phosphate- buffered saline, pH 7.4 (1X dPBS, GIBCO, Grand Island, NY), after 30 min and detected the intra-cellular ROS formation at 10 sec after exposure time using a fluorescence microscope (IX-71, Olympus, Tokyo, Japan). DCFH-DA expression level was detected at pre, 18 hr after KA injury and 24 hr after melatonin treated phase. The DCFH-DA fluorescence intensity was measured

by metamorph program. And quantifying the intensity was measured the eluminated area ratio of whole tissue.

F. Cresyl violet staining

To detect the survival cells used the cresyl violet staining. Cultured hippocampus slice was take out carefully and frozen with OCT compound (Sakura, Torrance, CA, USA). After that each slice was sectioned 10㎛ thickness and transfer to micro slide. Each slides was soaked in 4 % paraformaldehyde (Duksan, Incheon, Korea) for 20 min, wash out twice with 1X dPBS, soaked into cresyl violet solution (Sigma, Saint Louis, MO, USA), wash with flowing tap water, dehyderated the tissue sections with 70, 90, 100 % EtOH (Ducksan, Incheon, Korea), soaked into the 50 %: 50 % (EtOH and xylene) mixture, and stored the 100 % xylene (Ducksan, Incheon, Korea). Finally the slides were mounted with permount. To detect the survived neuron cells in CA3 using MetaMorph program, which were detected the cells size and appeared nuclear.

G. Western blot analysis

For 24 and 48 hr melatonin treated samples were collected and homogenized with lysis buffer containing 10 % SDS, 1 M Tris-HCl (pH 7.4), 5 % Triton x-100, 50 % Sodium Deoxycholate, 1 M DTT, 0.5 M soduium orthovanadate, 2 ㎎/㎖ PMSF, 10X protease inhibitor (all from Sigma, Saint Louis, MO, USA). Freeze and thaw the homogenized solution in -80℃ three times. All mixtures were centrifuged at 15000 rpm during 20 min and supernatant were transfer to new tube. All procedure kept on ice. Protein concentrations in the sample were

measured with Bradford assay. Proteins were separated by SDS-PAGE, and transferred to a nitrocellulose membrane (Millipore, Billerica, MA, USA). The membrane blocked by 5 % skim milk in Tris-buffered saline added 0.5% tween-20, and incubated with primary anti bodies (5-LO, cayman, Ann Arbor, MI, USA; caspase-3, Santacruz biotecnology, Santacruz, CA; calmodulin, SOD-2, B-actin, Abcam, Cambridge, UK) for overnight at 4℃. That was developed with a peroxidase-conjugated secondary anti body, and protein detected by enhanced chemiluminescence (ECL) procedure (Amersham, Arlington, IL, USA).

H. Statistical analysis

All data was statistically evaluated to determine group difference between KA only treated group and comparison melatonin treated groups or pineal cells co-cultured groups. For all the results have been expressed as mean ± S.E.M.. Differences among groups were analyzed by LSD post-hoc test. The level of significance was accepted at *P< 0.05, **P<0.01 and ***P<0.001.

2. Study Ⅱ: Protective effects of pineal gland cells

A. Pineal gland cell culture

Sprague-Dawley rat brain aged 6 to 8 days postnatal and averaged 300 g female were removed and transfer to GBSS. Pineal gland was separated from brain region and than remove the extraneous tissue. The pineal gland was transferred to culture dish which contained 1ml 0.25 % trypsin-EDTA (GIBCO BRL, Grand Island, NY, USA). That tissue dissected trypsin-EDTA and and added Dulbecco's modified Eagle's medium (DMEM, GIBCO BRL, Grand Island, NY) include 5 % FBS (Standard FBS, Hyclone, USA) 5 % horse serum, 1 % penicillin/streptomycin sulfate (all from GIBCO BRL, Grand Island, NY, USA). The mixtures were transferred to 6-well culture plate, each well was coated with poly-L-Ornithine (Sigma, Saint Louis, MO, USA). The well contained mixture shaking gently and incubated at 36 ℃ in a humidified atmosphere of 5 % CO_2. Culture media was changed three times every 2 weeks.

B. Co-culture of hippocampus and pineal gland

Semiporous membrane which included 3 weeks cultured and KA treated hippocampal slices were transferred to 2 weeks pineal cells cultured well. Afterward, KA injured hippocampus kept on pineal cells during 24 and 48 hr.

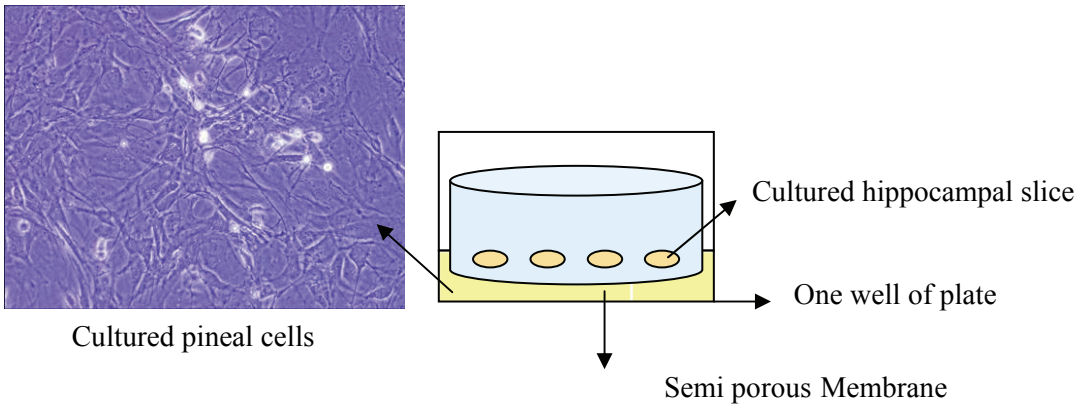
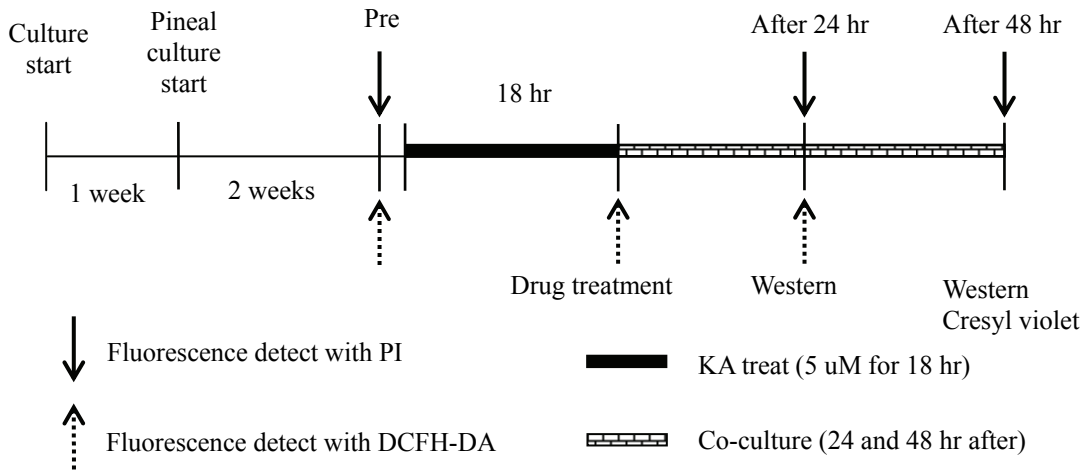


Figure 4. Pineal cells morphology and co-culture method. The picture represents cultured pineal cells and the method for co-culture of hippocampus and pineal cell.



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dichlorohydrofluorescein diacetate (DCFH-DA, sigma, Saint Louis, MO, USA) was treated 30 min before detect by using microscope. Cells incubated with DCFH-DA in the 36 ℃ humidified incubator for 30 min and washed twice with phosphate- buffered saline, pH 7.4 (1 X dPBS, GIBCO, Grand Island, NY), after 30 min and detected the intra-cellular ROS formation at 10 sec after exposure time using a fluorescence microscope (IX-71, Olympus, Tokyo, Japan). DCFH-DA expression level was detected at pre, 18 hr after KA injury and 24 hr after melatonin treated phase. The DCFH-DA fluorescence intensity was measured by MetaMorph program. And to quantifying the intensity was measured the luminescence area ratio of whole tissue.

E. Cresyl violet staining

To detect the survival cells used the cresyl violet staining. Cultured hippocampus slice was take out carefully and frozen with OCT compound (Sakura, Torrance, CA, USA). After that each slice was sectioned 10um thickness and transfer to micro slide. Each slides was soaked in 4 % paraformaldehyde (Duksan, Incheon, Korea) for 20 min, wash out twice with 1 X dPBS, soaked into cresyl violet solution (Sigma, Saint Louis, MO, USA), wash with flowing tap water, dehyderated the tissue sections with 70, 90, 100 % EtOH (Ducksan, Incheon, Korea), soaked into the 50 %: 50 % (EtOH and xylene) mixture, and stored the 100 % xylene (Ducksan, Incheon, Korea). Finally the slides were mounted with permount. To detect the survived neuron cells in CA3 using metamorph program, which were detected the cells size and appeared nuclear.

F. Western blot analysis

For 24 and 48 h melatonin treated samples were collected and homogenized with lysis buffer containing 10 % SDS, 1 M Tris-HCl (pH 7.4), 5 % Triton x-100, 50 % Sodium Deoxycholate, 1 M DTT, 0.5 M soduium orthovanadate, 2 ㎎/㎖ PMSF, 10 X protease inhibitor (all from Sigma, Saint Louis, MO, USA). Freeze and thaw the homogenized solution in -80℃ three times. All mixtures were centrifuged at 15000 rpm during 20 min and supernatant were transfer to new tube. All procedure kept on ice. Protein concentrations in the sample were measured with Bradford assay. Proteins were separated by SDS-PAGE, and transferred to a nitrocellulose membrane (Millpore, Billerica, MA, USA). The membrane blocked by 5 % skim milk in Tris-buffered saline added 0.5% tween-20, and incubated with primary anti bodies (5-LO, cayman, Ann Arbor, MI, USA; caspase-3, Santacruz biotecnology, Santacruz, CA; calmodulin, SOD-2, B-actin, Abcam, Cambridge, UK) for overnight at 4℃. That was developed with a peroxidase-conjugated secondary anti body, and protein detected by enhanced chemiluminescence (ECL) procedure (Amersham, Arlington, IL, USA).

G. Statistical analysis

All data was statistically evaluated to determine group difference between KA only treated group and comparison melatonin treated groups or pineal cells co-cultured groups. For all the results have been expressed as mean ± S.E.M.. Differences among groups were analyzed by LSD post-hoc test. The level of significance was accepted at *P< 0.05, **P<0.01 and ***P<0.001.

Ⅲ. Results

1. Study Ⅰ: protective effects of melatonin

A. Effects of melatonin on KA-induced neuronal toxicity in OHSCs

PI uptake was used to investigate the effects of melatonin in KA-induced neuronal injury. In the pre phase, hippocampus tissues have almost no damage. 18 h after KA treatment increased the intensity of PI uptake was increased in the hippocampus CA1, CA3, and dentate gyrus layer. However, less intensity was shown in 0.01, 0.1, 1 mM melatonin treated group than that in KA only treated group (Fig. 6A). Difference fluorescence level each cell layer was used the method of Fig. 1. The CA1 and CA3 region of hippocampus layers value was presented the diagram (Fig. 6B). 0.01, 0.1, and 1 mM melatonin treated groups was shown reduced level of PI uptake in CA1 region. However, 0.01 mM had no significance in the CA3 region. On the other hands, CA3 region has more sensitivity and reduced range than CA1 region.

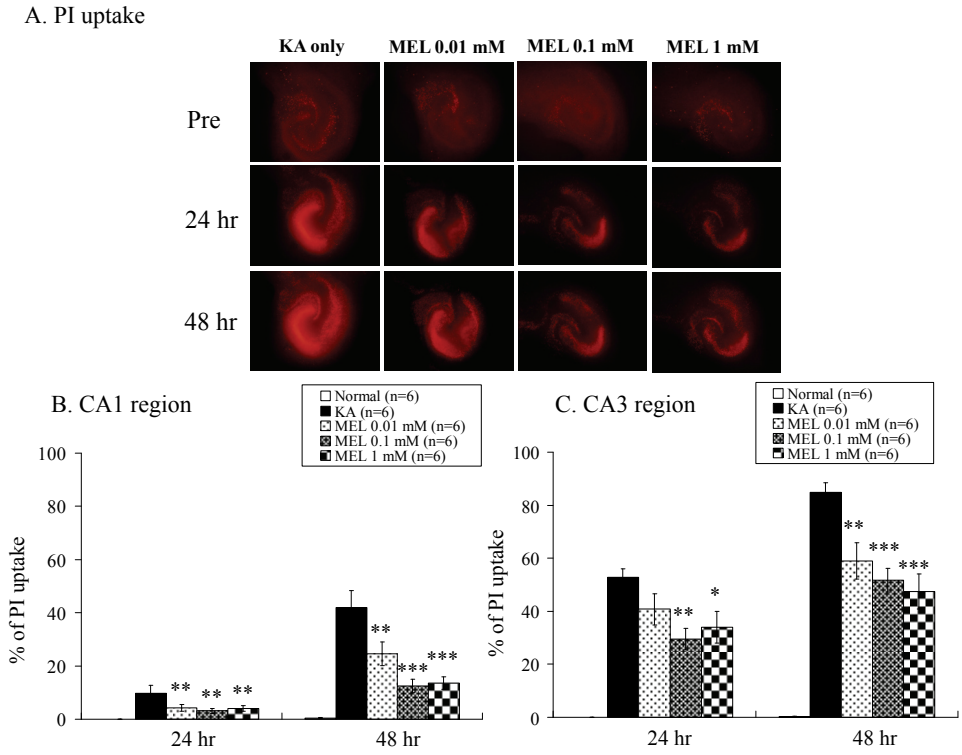


Figure 6. Melatonin reduces KA-induced neuronal injury in OHSCs. A: Difference PI uptake fluorescence intensities in KA only-treated group and melatonin treated groups using a fluorescence microscope. The horizontal axis indicates melatonin concentration and the vertical axis presents melatonin treated times. B: Quantification of PI uptake intensities using a MetaMorph program in CA1 region. C: CA3 region. The horizontal axis indicates after melatonin treated times and the vertical axis represents percent of PI uptake in each region. The value of fluorescence were calculated by using following formula; % of PI uptake = 100 $(F_t - F_{pre})/(F_{fk} - F_{pre})$. Data are mean ± SEM (n = 6). * < 0.05, ** <0.01 ***, and <0.001 one-way ANOVA followed by a LSD test for comparison with KA only-treated group.

B. Cresyl violet staining

Cresyl violet staining was used for detection survived neuronal cells. Normal tissues have many neuronal cells in the all of the cell layer. But KA only treated tissues have little cells in the layer, especially in the CA3 than CA1 region. However, melatonin treated tissues have more than KA only treated tissue but less than normal tissues (Fig. 7A). In the diagram of those pictures, melatonin treated groups have significance than KA only treated group.

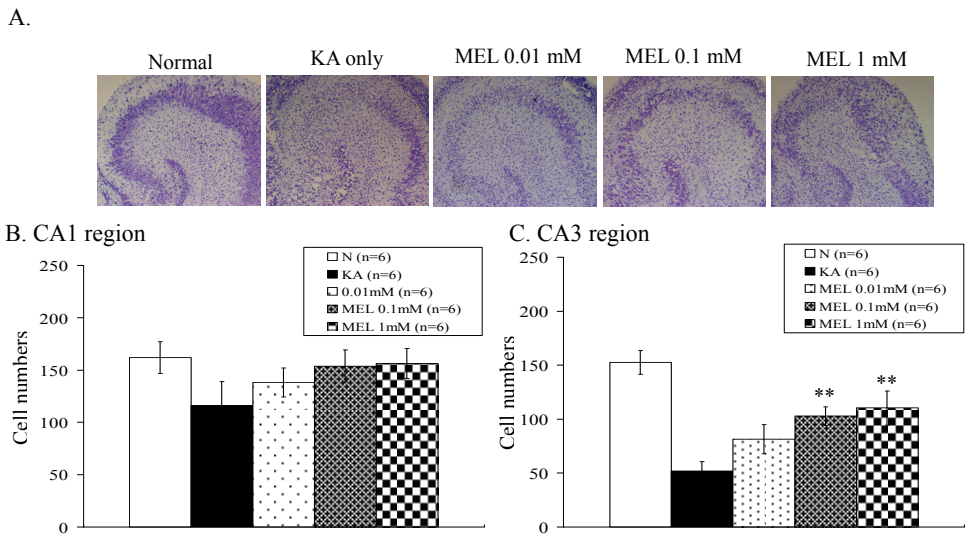
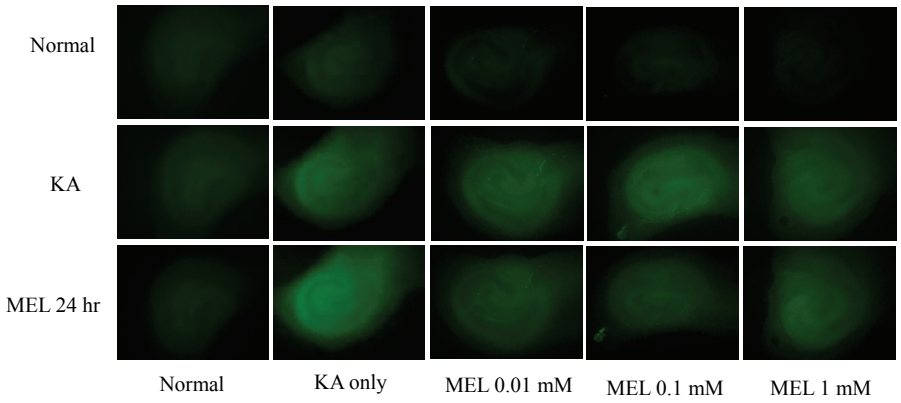


Figure 7. Melatonin increases neuronal survival on KA-induced neuronal injury in OHSCs. A: Survived cells morphology in 10㎛ dissected hippocampus tissues. Each picture represents normal, KA only-treated, 0.01, 0.1, and 1 mM melatonin treated groups at 48 hr after melatonin treatment. B: Quantify of cell survival using a MetaMorph program in CA1 region. C: CA3 region. The horizontal axis indicates 48 hr after melatonin treatment and a vertical axis represents survived cell numbers. Data are mean ± SEM (n = 6). ** < 0.01 one-way ANOVA followed by LSD test for comparison with KA only-treated group

C. Formation of ROS in KA-induced oxidative stress

DCFH-DA fluorescence dye was used to detect the ROS formation in the hippocampus tissues. The ROS formation level was detected in the total cell layer. 10 μM dye was represents a green fluorescence. Before the KA treatment fluorescence in the tissues was little but, 5 μM KA was generated the fluorescence more than normal. After melatonin treatment, the fluorescence was reduced compared with KA treated groups.

A.



B.

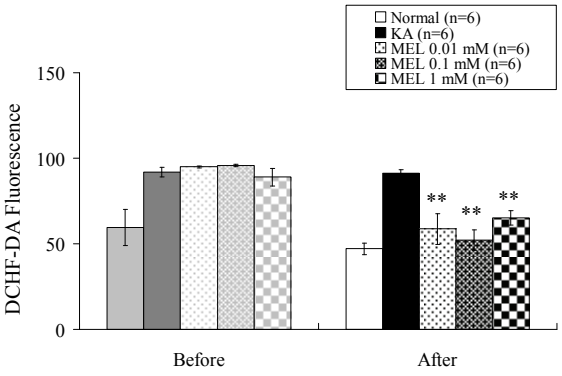


Figure 8. Relationships between melatonin treatment and ROS formation. A: Represents DCFH-DA intensities in each group. The bottom of the horizontal axis indicates melatonin concentration and the vertical axis represents after KA and melatonin treated. B: Quantification of DCFH-DA intensities using a MetaMorph program. The horizontal axis indicates before melatonin treatment (Before) and after melatonin treatment for 24 hr (After) and a vertical axis represents DCFH-DA intensity in the total tissues. Data are mean ± SEM (n = 6). ** < 0.01 one-way ANOVA followed by LSD for comparison with each group.

D. 5-lipoxigenase expression level on KA-induced neuronal injury

5-lipoxigenase (5-LO) expression is a marker of lipid peroxidation. 5-LO diluted 1:500 in 5 % skim milk than overnight incubated at 4℃. Quantification of 5-LO expression at 24 hr. increased melatonin concentration gradually attenuate 5-LO expression. KA-injury group has higher expression than other groups.

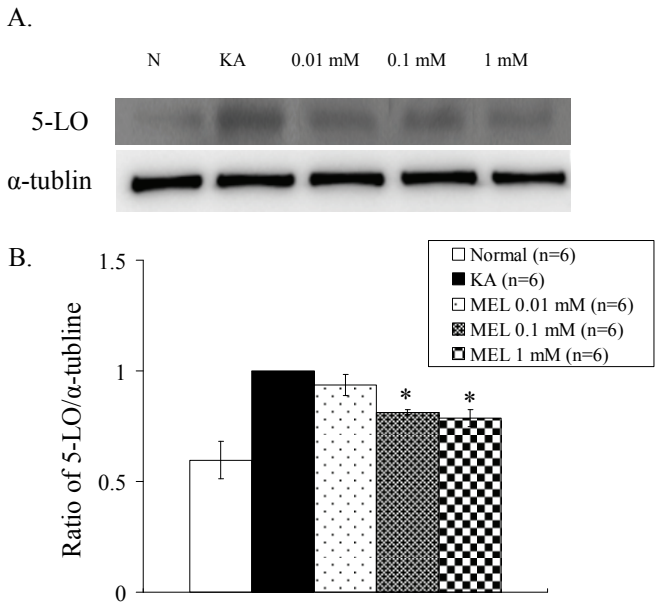


Figure 9. Melatonin changes the 5-LO expression level. A: representing western blotting of 5-LO expression at 24 hr after melatonin treat. B: Quantification of 5-LO at 24 hr after melatonin treat. The horizontal axis indicates concentration of melatonin and the vertical axis represents level of 5-LO expression ratio (5-LO expression/ α-tubline expression). Data are mean ± SEM (n = 6). * < 0.05 one-way ANOVA followed by a LSD test.

E. Caspase-3 expression level on KA-induced neuronal injury

Caspase-3 expression is a marker of apoptosis, also play a role in necrosis and inflammation. Increased caspase-3 expression is showing some possibility of triggering apoptosis. Caspase-3 diluted 1:500 in 5 % skim milk than overnight incubated at 4℃. In normal group presents lower expression than KA-treated group. 0.01 mM, 0.1 mM, 1 mM melatonin treated groups gradually reducing the caspase-3 expression.

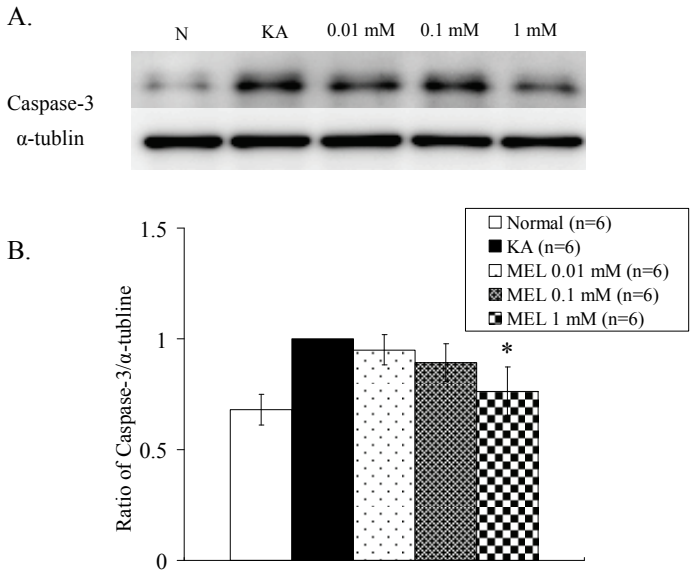


Figure 10. Melatonin changes the caspase-3 expression level. A: representing western blotting of caspase-3 at 24 hr after melatonin treat. B: Quantification of caspase-3 at 24 hr after melatonin treatment. The horizontal axis indicates concentration of melatonin and the vertical axis represents level of caspase-3 expression ratio (caspase-3 expression/ α-tubulin expression). Data are mean ± SEM (n = 6). * < 0.05 one-way ANOVA followed by a LSD test.

F. Calmodulin expression level on KA-induced oxidative stress

Calmodulin diluted 1:500 in 5 % skim milk than overnight incubated at 4℃. Quantification of calmodulin expression at 24 h and 48 hr. increased melatonin concentration reduced calmodulin expression. KA-injury group has higher expression than other groups.

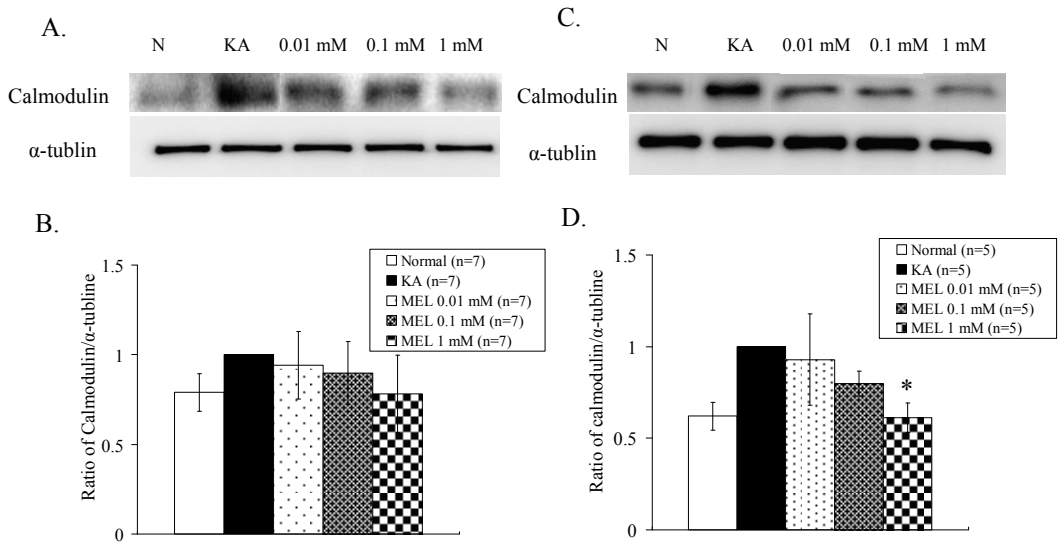


Figure 11. Melatonin changes the calmodulin expression level. A: Representing western blotting of calmodulin expression at 24 hr after melatonin treatment. B: Quantify of calmodulin at 24 hr after melatonin treatment. C: representing western blotting of calmodulin expression at 48 hr after melatonin treatment. D: Quantify of calmodulin at 48 hr after melatonin treatment. The horizontal axis indicates concentration of melatonin. And vertical axis represents level of calmodulin expression ratio (calmodulin expression/ α-tubline expression). Data are mean ± SEM (n = 5 to 7). * < 0.05 one-way ANOVA followed by a LSD test.

G. SOD-2 expression level on KA-induced oxidative stress

SOD-2 is crucial for cell survival that reported antioxidant enzyme. SOD-2 diluted 1:10000 in 5 % skim milk than overnight incubated at 4℃. Quantification of SOD-2 expression at 24 hr and 48 hr. Fetal and adult cell co-cultured groups reduced SOD-2 expression. KA-injury group has higher expression than other groups.

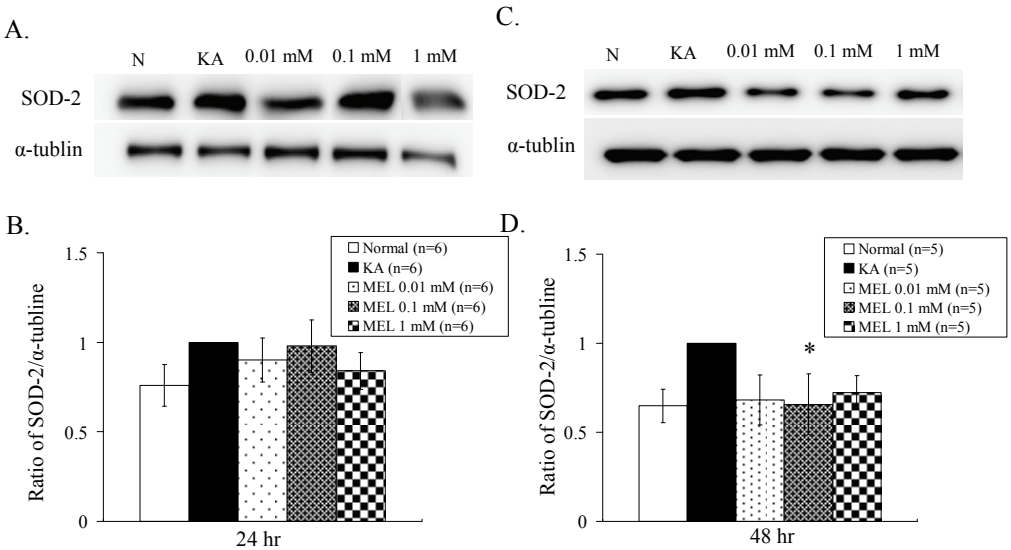


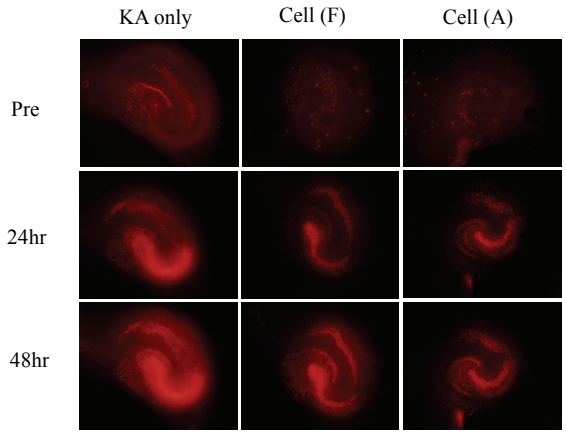
Figure 12. Melatonin changes the SOD-2 expression level. A: Representing western blotting of SOD-2 expression at 24 hr after melatonin treatment. B: Quantification of SOD-2 at 24 hr after melatonin treatment. C: representing western blotting of SOD-2 expression at 48 hr after melatonin treatment. D: Quantify of SOD-2 at 48 hr after melatonin treatment. The horizontal axis indicates concentration of melatonin. And vertical axis represents level of SOD-2 expression ratio (SOD-2 expression/ α-tubline expression). Data are mean ± SEM (n = 5 to 6). * < 0.05 one-way ANOVA followed by a LSD test.

2. Study Ⅱ: : Protective effects of pineal gland cells

A. Effects of pineal cells on KA-induced neuronal toxicity in OHSCs

PI uptake was used to investigate the effects of cell types in KA-induced neuronal injury. In the pre phase, hippocampus tissues have almost no damage. 18 hr after KA treatment increased the intensity of hippocampus CA1, CA3, and dentate gyrus layer. However, fetal and adult cell co-cultured groups were shown less intensity than KA only treated group (Fig. 13A). Difference fluorescence level each cell layer was used the method of Fig 3. The CA1 and CA3 region of hippocampus layers value was presented the diagram (Fig. 13B). Both of fetal and adult cell co-culture groups in the CA3 region has less % of sensitivity than KA only treated groups. However, the difference has no exist in cell types.

A. PI ultake



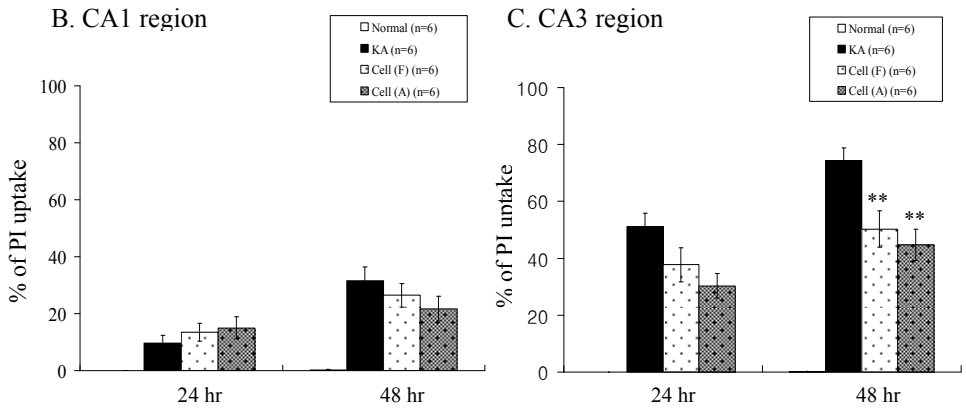


Figure 13. Co-culture with pineal cells reduces KA-induced neuronal injury in OHSCs. A: represents PI uptake intensities in KA only treated group and indicated cell co-culture groups with a fluorescence microscope. The (F) means pineal cells from fetal (after birth of 6 to 7 days) and (A) means pineal cells from adult (300g). The width indicates cell types and the vertical line presents co-cultured times. B: Quantify of PI uptake intensities with a MetaMorph programs in CA1 region. C: CA3 region. The horizontal axis indicates after melatonin treated times and a vertical axis represents % of PI uptake in each region. The value of fluorescence were calculated by using following formula; % of PI uptake = 100 $(F_t - F_{pre})/(F_{fk} - F_{pre})$. Data are mean ± SEM (n = 6). * < 0.05 and **< 0.01 one-way ANOVA followed by LSD test.

B. Cresyl violet straining

Cresyl violet staining was used detecting method for survived neuronal cells. In normal tissues have many neuronal cells in the all of the cell layer. But KA only treated tissues have little cells in the layer, especially in the CA3. However, melatonin treated tissues have more than KA only treated tissue but less than normal tissues (fig. 14A). In the diagram of those pictures, melatonin treated groups have significance than KA only treated group.

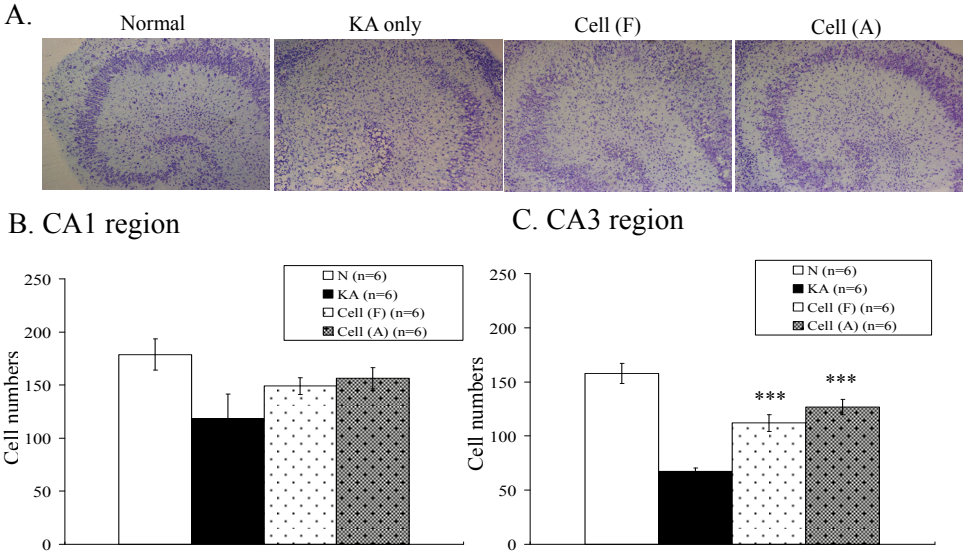
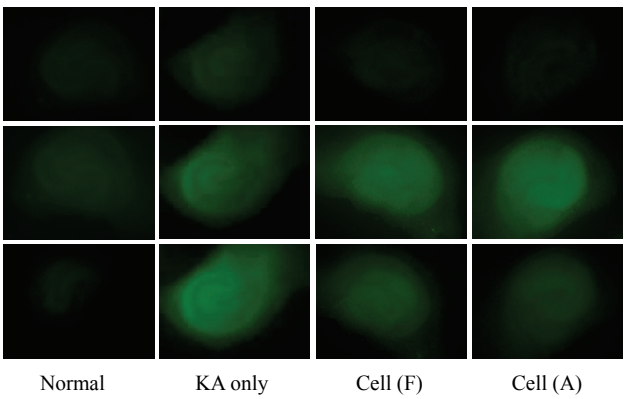


Figure 14. Co-culture with pineal cells increases neuronal survival against KA-induced neuronal injury in OHSCs A: Survived cells morphology in 10㎛ dissected hippocampus tissues. Each picture represents normal, KA only treat, fetal and adult pineal cells co-cultured groups for 48 h. B: Quantification of cell survival with a MetaMorph programs in B: CA1 and C: CA3 region. The horizontal axis indicates co-culture times and a vertical axis represents survived cell numbers. Data are mean ± SEM (n = 6). *** < 0.001 one-way ANOVA followed by LSD test.

C. ROS formation on KA-induced oxidative stress

DCFH-DA fluorescence dye was used to detect the ROS formation in the hippocampus tissues. The ROS formation level was measured in the total cell layer. 10 μM dye was represents a green fluorescence. Before the KA treatment fluorescence in the tissues was little but, 5 μM KA was generated the fluorescence more than normal. After co-culture, the fluorescence was reduced compared with KA treated groups.

A.



B.

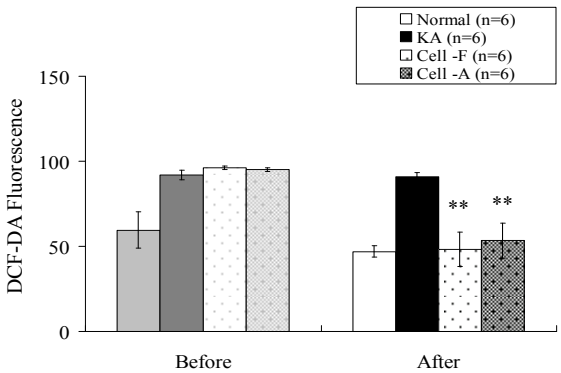


Figure 15. Relationship between ages of cells and ROS formation. A: Represents DCFH-DA intensities in each groups. In the middle of the width indicates cell types and the vertical line present after KA and co-culture time. B: Quantification of DCFH-DA intensities with a MetaMorph programs in total tissues region. The horizontal axis indicates before co-culture (Before) and after co-culture (After) and a vertical axis represents DCFH-DA intensity in the total tissues. Data are mean ± SEM (n = 6). ** < 0.01 one-way ANOVA followed by LSD test

D. 5-lipoxigenase expression level on KA-induced oxidative stress

5-lipoxigenase(5-LO) expression is a marker of lipid peroxidation. 5-LO diluted 1:500 in 5 % skim milk than overnight incubated at 4℃. Quantification of 5-LO expression at 24 hr. increased Fetal and adult cell co-cultured groups attenuate 5-LO expression. KA-injury group has higher expression than other groups.

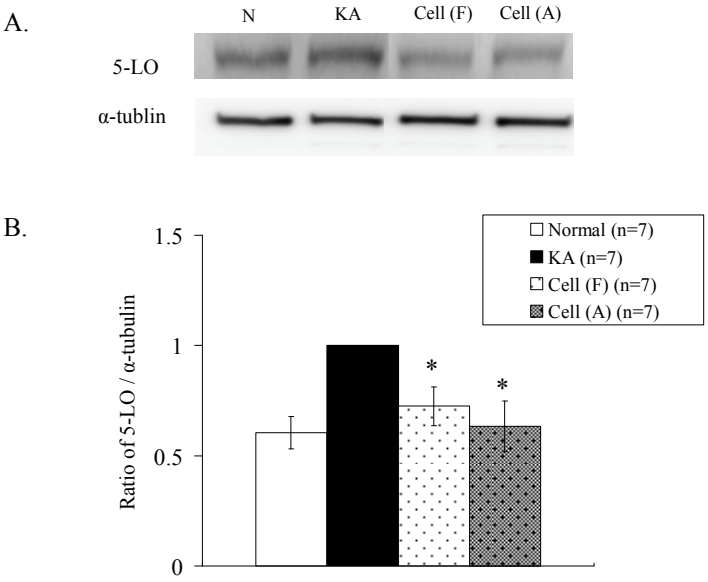


Figure 16. Pineal cells change the 5-LO expression level. A: Representing western blotting of 5-LO expression at 24 hr after co-culture with fetal and adult cells. B: Quantify of 5-LO at 24 hr. The horizontal axis indicates cell type. And vertical axis represents level of 5-LO expression ratio (5-LO expression/ α-tubline expression). Data are mean ± SEM (n = 7). * < 0.05 one-way ANOVA followed by a LSD test.

E. Caspase-3 expression level on KA-induced oxidative stress

Caspase-3 expression is a marker of apoptosis, also play a role in necrosis and inflammation. Increased caspase-3 expression is showing some possibility of triggering apoptosis. Caspase-3 diluted 1:500 in 5 % skim milk than overnight incubated at 4℃. In normal group presents lower expression than KA-treated group. Fetal and adult cell co-cultured groups reducing the caspase-3 expression.

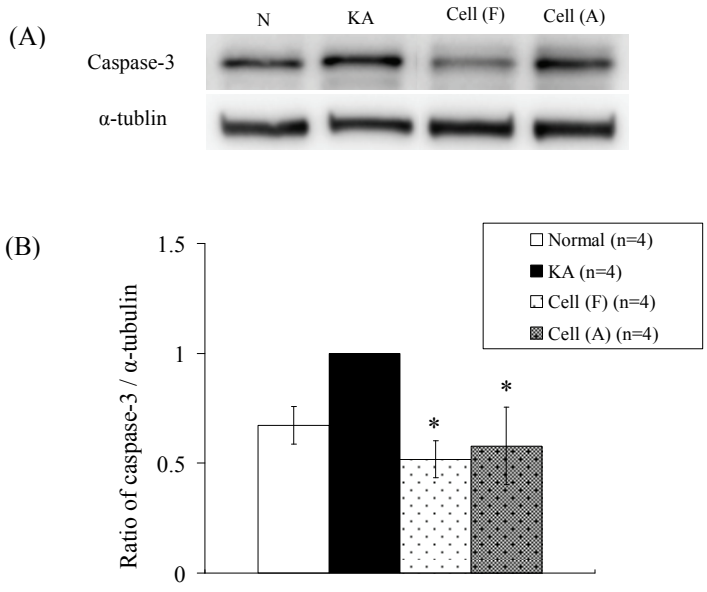


Figure 17. Pineal cells change the caspase-3 expression level. A: Representing western blotting of caspase-3 at 24 hr after co-culture with fetal and adult cells. B: Quantify of caspase-3 at 24 hr. The horizontal axis indicates co-cultured cells and the vertical axis represents level of caspase-3 expression ratio (caspase-3 expression/ α-tubulin expression). Data are mean ± SEM (n = 4). * < 0.05 one-way ANOVA followed by a LSD test.

F. Calmodulin expression level on KA-induced oxidative stress

Calmodulin diluted 1:500 in 5 % skim milk than overnight incubated at 4℃. Quantification of calmodulin expression at 24 and 48 hr. Fetal and adult cell co-cultured groups reduced calmodulin expression. KA-injury group has higher expression than other groups.

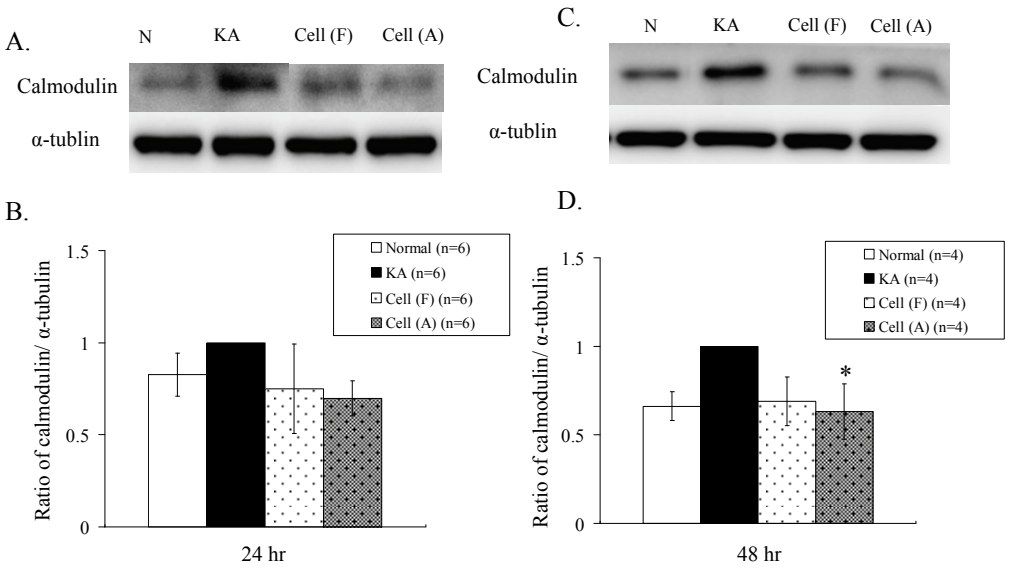


Figure 18. Melatonin changes the calmodulin expression level. A: Representing western blotting of SOD-2 expression at 24 hr after pineal cell co-culture. B: Quantify of calmodulin at 24 hr after melatonin treatment. C: representing western blotting of calmodulin expression at 48 hr after melatonin treatment. D: Quantify of calmodulin at 48 hr after melatonin treatment. The horizontal axis indicates concentration of melatonin and the vertical axis represents level of calmodulin expression ratio (calmodulin expression/ α-tubline expression). Data are mean ± SEM (n = 4 to 6). * < 0.05 one-way ANOVA followed by a LSD test.

G. SOD-2 expression level on KA-induced oxidative stress

SOD-2 is crucial for cell survival that reported antioxidant enzyme. SOD-2 diluted 1:10000 in 5 % skim milk than overnight incubated at 4℃. Quantification of SOD-2 expression at 24 and 48 hr. Fetal and adult cell co-cultured groups reduced SOD-2 expression. KA-injury group has higher expression level than other groups.

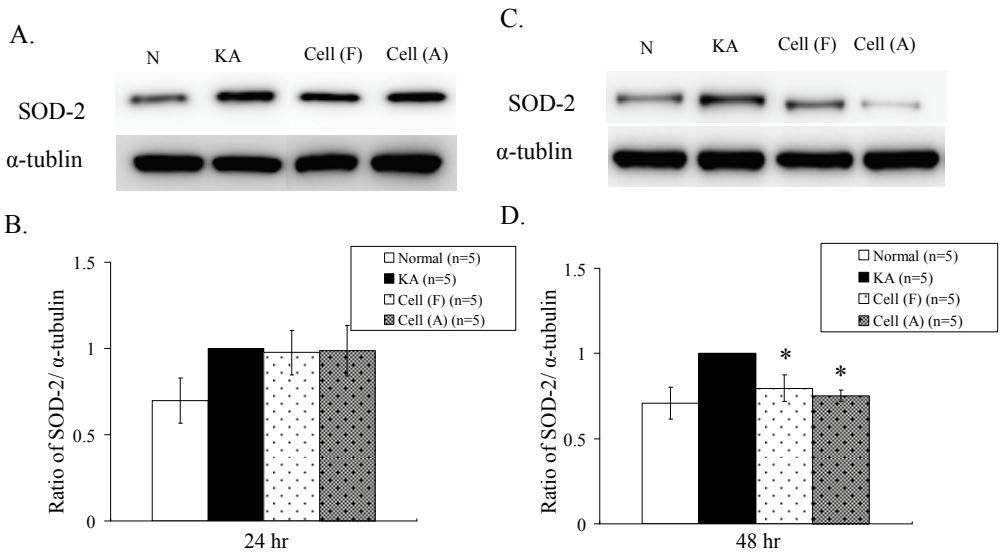


Figure 19. Melatonin changes the SOD-2 expression level. A: Representing western blotting of SOD-2 expression at 24 hr after pineal cell co-culture. B: Quantify of SOD-2 at 24 hr after melatonin treatment. C: Representing western blotting of SOD-2 expression at 48 hr after melatonin treatment. D: Quantify of SOD-2 at 48 hr after melatonin treatment. The horizontal axis indicates concentration of melatonin and the vertical axis represents level of SOD-2 expression ratio (SOD-2 expression/ α-tubline expression). Data are mean ± SEM (n = 5). * < 0.05 one-way ANOVA followed by a LSD test.

Ⅳ. DISSCUSSION

This study is to investigate the antioxidant effects of melatonin by drug and by secreted substance that is related to the neuroprotective effects whose mechanisms and relationship of melatonin had not been clearly demonstrated. Because oxidation generated in a body is one of the factors that induce cell death, it would be important to identify developing a novel and the most effective antioxidant against cell death. KA-induced oxidative stress model was used to generate cell death and ROS measurement was used to decide the relationship between cell death and antioxidant to demonstrate the effect from melatonin treatment.

In the previous study of KA, Zaja[17] demonstrated that oxygen free radical was generated in limbic structure by KA-induced seizures, and another study demonstrated that KA-induced damage in neuron generated a mitochondrial superoxide production.[27] ROS generated from KA is harmful to neurons. Because this ROS can remove by various antioxidants, the importance of studying about antioxidants is increased. Recently, some studies demonstrate a remarkable and likely possibility that melatonin plays a major role to attenuate a brain injury through its antioxidant function. Following a study related with melatonin, a broad spectrum antioxidant, is reduces amyloid-β induced neurotoxicity. According to the study, 0.05mM and 0.1mM melatonin were administered for its antioxidant effects and neuroinflammatory effects in hippocampal region.[28] in addition, the administration of melatonin in rat attenuated the oxidative stress-induced neurodegeneration and microglial generation.[29]

In our first experiment to demonstrate the antioxidant effects of melatonin as a drug, KA-induced oxidative stress established model was chosen because ATPA, AMPA, and NMDA are also neurotoxin like KA, but KA-induced injury

is more effective to the hippocampal CA3 region than CA1 which makes it easy to specify injury model.[30,31,32] The concentration of 0.01mM, 0.1mM, and 1mM of melatonin was treated to the KA-induced injury model to observe the extent of cell death by KA and cell survival by each melatonin. PI is an index of detecting death cells. PI-uptake method was used. PI is a dye that can stain DNA and in turn, it emits red fluorescence. PI generally penetrates to damaged membranes and then interacts with DNA, and it is not toxic to neurons.

PI-uptake intensity was higher in KA only-treated group than the rest of the group. In this manner, KA induced a noticeable neuronal cell death, and 0.1 and 1 mM melatonin treated groups demonstrated lower intensity and increment than that of KA only- group. The data suggested that the cell death was less than KA only treated group. Also, melatonin showed improve results concentration level increased and the intensity growth of PI-uptake in melatonin treated groups was slower than KA only-treated group.

Some previous *in vivo* experiments, injection of high concentration (200mg/kg) of melatonin did showed any harmful effects and little impacts on melatonin level. This study would be valuable support for previous studies related to antioxidant effects of melatonin by KA-induced oxidative stress. Because the hippocampal slice culture has both advantageous of *in vivo* and *in vitro* for KA-induced oxidative stress model, it is possible to determine the proper concentrations of melatonin. In this results demonstrated that melatonin effectively suppressed the neuronal cell death in CA1 region and CA3 region in hippocampus from measuring of PI uptake. The amount of PI uptake increased from 15 % to 50 % in whole tissue on KA-only treated group in CA1 region in hippocampus for 24 hr and 48 hr treatment, respectively, but increased amount was slightly down according to the dosage level. Further more PI uptake in melatonin treated groups were less than KA only treated group compare at each

time measurement. For standardizing the tissue damage, total amount of sensitivity from KA-induced injury in hippocampus was regarded as 100% of cell death in hippocamal CA1 and CA3 in full kill phase. In this analysis, the damage from KA only at 48 hr after treatment was increased about 30 % from that of 24 hr after. But the damage was decreased in melatonin treated groups by dose dependent manner, the increment was less than 20 % from that of KA only treated-group.

Previous reports demonstrated that melatonin is effective in numerous diseases such as Alzheimer disease's, Parkinson disease's, porphyric neuropathy, ischemia-reperfusion injury, traumatic brain injury and spinal cord injury.[33] It is, therefore, possible that melatonin can effectively suppress various neuronal disorders. KA, a ionotrophic gluatamate receptor agonist,[34] is known to cause limbic seizure and neuronal damage by systemical or intracerebral injection.

In immunohistochemistry using cresyl violet, which dyes nucleus in live cells on hippocampus tissues, the number of survived cells supported the tendency of PI uptake result. In normal group, 150 cells were counted in CA3 in hippocampus. The number of survived cells in KA only-treated group were decreased to one-third of normal group, a half in 0.01 mM melatonin treated group and one-tenth in 0.1 mM and 1 mM melatonin treated groups.

The result from fluorescence measurement using DCFH-DA demonstrated the relation between neuronal death and ROS and found a relation between PI and cresyl violet staining. Detection of DCF is a fluorescence-based method. It is widely used in ROS measurements in mitochondria. The DCFH-DA emits the green fluorescence after deacylated and then oxidized mainly by H_2O_2. It is a major advantage of DCFH-DA that detects both mitochondria and live cells.[35] KA treated tissues weremeasured as 100 % of whole area in 18 hr after KA-injury and then the intensity changes were measured at 24 hr after melatonin

treatment to standardize every intensity of DCFH-DA. The intensity was decreased in 50 % in 0.01 mM melatonin treated group, 45 % in 0.1 mM melatonin treated group and 20 % in 1 mM melatonin treated group compare to the KA only treated group. This indicates that neuronal damage by KA injury is significantly related with ROS, and it also supports the previous report on melatonin that suggests the melatonin acts as a scavenger that reduces ROS generated by KA. However, The PI uptake and cresyl violet staining results dependent on the melatonin concentration unlike the ROS generation was increased as the concentration of melatonin increased.

Western blot was carried on to detect 5-LO, caspase-3, calmodulin, and SOD-2 to measure protein expression. 5-LO is a protein related with lipid peroxidation. In other studies, KA-induced damage showed upregulation of 5-LO expression in limbic system. In addition, increased endogenous ROS was upregulation of 5-LO expression.[36] In this study, 0.1 mM and 1 mM of melatonin significantly reduced the 5-LO expression.

Caspase-3 plays a critical role in apoptosis. From signaling pathway, caspase-3 participates in various apoptosis pathways including KA-induced injury. From our study, melatonin gradually reduced the caspase-3 activity, especially, that 1 mM melatonin generated significant changes in caspase-3 expression.

Calmodulin is an important signaling enzyme. It is activated by increase in cytosolic Ca^{2+}. Calmodulin expression is suitable for represent a pro-oxidant effect of melatonin. Normally, calmodulin binds with calmodulin inhibitor but melatonin blocks the calmodulin-calmodulin inhibitor complex and then calmodulin that in turn, inhibits pro-oxidant action.[37] In this result, calmodulin was reduced at 48 hrs after melatonin treatment compare to the KA-injury groups. 24 hr had a tendency to reduced the expression, but there was no significance.

SOD-2 protects neuronal cells against KA-induced damage and the activation of SOD-2 is known to removes ROS.[38,39,40] In global ischemia, SOD-2 expression level was higher than peroxisome treated groups, and compare to the isoproterenol induces injury model, melatonin treated groups had lower expression level than isoproterenol only treated group. Naturally the more ROS generated, the more requirement of SOD was produce and the KA-injury group demonstrates more SOD-2 expression than melatonin treated groups.[41] Also, SOD-2 is late phase protein similar to calmodulin because the result of 48 hr after melatonin treatment showed significance but not in the result after the 24 hr. From this western blot analysis demonstrated that quantification of 5-LO, caspase-3, calmodulin and SOD-2, was lower than that of KA only treated group. It means melatonin has neuroprotective effects that regulate viability of cells against ROS and apoptosis.

In second experiment, melatonin secreted from pineal gland, was used and the amount of PI uptake demonstrated a tendency to decrease in both fetal and adult cell co-culture groups compared to KA only-treated group. However the changes had little significance in CA1 region, but the changes in CA3 region was statistically significant. The amount of PI uptake was 20 % lower in adult cell co-cultured group than KA only treated group at 24 hr and 15 % lower than KA only treated group at 48 hr.

In cresyl violet stain result, pineal cell co-culture indicates neuronal cell protective effect by demonstrating 50 % to 60 % of enhancement of cell viability compared with KA only treated group, and indicated a similar significance with PI uptake.

Pineal cells reduce ROS generation to 50 % compared to KA only treated group in DCFH-DA fluorescence.

Melatonin secreted from pineal cell demonstrated considerable antioxidant effect which is similar to the treatment of 1 mM of melatonin, but there was no outstanding difference between fetal and adult cell co-culture group. 5-LO expression level was significantly lower than the KA only-treated group in fetal and adult cells co-cultured groups. Caspase-3 level also demonstrated statistical significance in two groups, but calmodulin and SOD-2 has no significance at 24 hr after in melatonin treatment groups. In contrast, SOD-2 expression at 48 hr after in melatonin treatment has statistical significance in all cell types, and from all of the results, of only calmodulin has significance in adult group. Protein expression was decreased in fetal and adult cell groups in western blot, and the significant difference between fetal cell and adult cell group was not demonstrated. On the other hand, fetal and adult cells effects were better than normal group.

Based on above, this study demonstrated that the effect of the melatonin on KA induced oxidative damage by different concentrations of melatonin, and the cell type. 0.01, 0.1, and 1 mM treatment of melatonin significantly attenuated neuronal damage and generation of ROS, and higher concentration of the melatonin was more effective than that of lower concentration. Moreover, melatonin secreted from pineal cell has also had protective effects. These results suggest melatonin could be one of the most effective antioxidants.

Ⅴ. CONCLUSION

In conclusion, from this experiment using PI, cresyl violet staining and DCFH-DA support that KA-induced damage is fully related in ROS generation. ROS act as a neurotoxin molecules in the hippocampus. Melatonin is one of the various antioxidants, and from this study, proper concentration of melatonin reduced the ROS generation and neuronal cell death. So, melatonin is a proper antioxidant on KA-induced oxidative stress in hippocampus. Also, melatonin secreted from pineal cells shows similar results compare with each concentrations of melatonin treatment. In addition, the results from co-culture indicates that more effectiveness than each concentrations of melatonin treatment. It suggests that stimulation of pineal gland to accelerate melatonin secretion is possibily one of the useful means of alleviating the neuronal disorders which was generated by oxidative stress.

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ABSTRACT (IN KOREAN)

해마절편 모델에서 kainic acid에 의해 유발된 산화적 스트레스에 미치는 melatonin의 신경보호 효과

<지도교수 이 배 환>

연세대학교 대학원 의과학과

김 형 아

뇌는 고도로 산소를 소비하는 기관으로 자유전자가 다량 존재한다. 이런 자유전자들은 세포 내 구성성분인 단백질, 지질 그리고 DNA를 공격하여 그 기능을 변성하거나 소실하게 만들며 최종적으로는 여러 가지 질병을 일으킨다. 이러한 산화적 손상으로 인한 질병으로부터 세포 및 조직을 보호하기 위해 자유전자 scavenger로 다양한 산화적 손상모델에서 ROS를 효과적으로 제거하는 항산화 물질로 알려져 있다. 멜라토닌은 산화환원과정을 거치지 않는 방법을 통해 free radical을 제거할 뿐만 아니라 반응을 하고 난 후에도 안정한 최종산물을 만들어 terminal 항산화제라고 불리기도 한다.

이 연구의 목적은 다양한 뇌신경 질환에 영향을 미치는 KA에 의해 유도된 산화적 스트레스에 멜라토닌의 효과를 설명하고자 한다. 이를 위해 다른 농도의 멜라토닌과 송과체 세포로부터 분비되는 멜라토닌을 KA로 산화적 손상을 일으킨 해마절편모델에 사용하였다. 해마절편

배양에는 생후 6~8일 된 쥐가 사용되었고 3 주간 배양하였다. 18 시간 동안 5 uM의 KA를 처리하여 생성된 뉴런 손상과 활성산소 종의 생성을 각각 PI uptake와 DCHF-DA 광도를 통해 알아보았다.멜라토닌의 보호작용을 관찰하기 위해 두 가지 실험을 수행하였다.먼저, KA에 의해 손상을 입은 해마절편모델에 0.01, 0.1, 1 mM의 멜라토닌을 처리하여 농도에 따른 멜라토닌의 효과를 알아보았다. 두 번째로, 송과체 세포로부터 분비되는 멜라토닌의 효과를 관찰하기 위해 KA로 손상을 준 해마절편모델과 동시배양방법을 이용하였다.

실험 Ⅰ은 0.1과 1 mM의 멜라토닌이 PI uptake 결과에서 멜라토닌을 처리한 후 24, 48 시간에서 효과적으로 뉴런세포를 효과적으로 보호하는 것을 알 수 있었다 또한, cresyl violet염색을 이용하여 살아있는 뉴런세포의 수를 측정한 결과 멜라토닌을 처리한 후 48시간이 지난 결과가 PI uptake 결과와 연관성이 있음을 알 수 있었다. DCHF-DA 광도 측정에서는 KA만 처리한 군과 비교해 멜라토닌 처리 후 24시간이 지났을 때 0.01, 0.1, 1mM의 멜라토닌을 처리한 군의 활성산소 종의 발생 정도가 감소한 것을 알 수 있었다. 또한, 5-lipoxigenase(5-LO), caspase-3, calmodulin, SOD-2 단백질의 발현 정도를 측정하기 위해 western blot을 수행하였다. 5-LO, caspase-3, calmodulin, SOD-2의 발현은 비슷한 경향을 보였다. 모든 발현이 KA에 의한 손상을 준 군에서 증가하였고, 멜라토닌을 처리한 군은 KA로 손상을 준 군에 비해 감소되었다.

실험 Ⅱ, fetal cell과 adult cell을 이용하여 멜라토닌을 동시배양으로 처리한 후 48시간에서 뉴런세포의 사멸이 감소하였다. PI uptake와 cresyl violet염색은 뉴런세포의 사멸과 살아있는 세포를

확인하기 위해 수행하였다. Fetal과 adult세포 동시배양 군은 KA손상에 의한 세포사멸로부터 보호하였다. DCHF-DA 측정을 통해, 그 감도가 fetal 과 adult 세포와 24시간 동시배양 후 감소하였다. 5-LO, caspase-3, calmodulin, SOD-2는 KA만 처리한 군과 비교해 유의미하게 감소하였다. 따라서, 이런 결과들은 송과체 세포로부터 분비되는 멜라토닌이 산화적 손상을 효과적으로 감소시킨다는 것을 나타낸다.

이 결과로, 해마절편모델에서 멜라토닌이 KA로 유도된 산화적 손상에 효과적이라는 것을 시사한다.

핵심이 되는 말: 멜라토닌, kainic acid, organotypic hippocampal slice culture, 산화적 손상, 활성산소 종