Özgün Deneysel Çalışma

Melatonin Administration Prevents the Disruptive Effects of Traumatic Brain Injury in Ovariectomized Rat Brain

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✔ **Objective:** Effect of melatonin treatment on ovariectomized rat brain after traumatic brain injury (TBI) was investigated with diffusion-weighted imaging (DWI).
Methods: Twenty-four young Wistar-albino rats were studied. 18 of them were bilaterally ovariectomized, and the remaining 6 were surgically incised but not ovariectomized. After 7 days postoperatively, they were assigned to four groups with equal number of animals. Groups were named as Group 1, sham operated; Group 2, ovariectomized; Group 3, ovariectomized + TBI; Group 4, ovariectomized + TBI + treated with melatonin. Group 3 received vehicle (0.1 % etanol) whereas group 4 had received 4 mg/kg melatonin intraperitoneally. Drug administration started immediately before injury and continued for 7 days. DWIs were obtained one week post injury, and apparent diffusion coefficient (ADC) maps were constructed.
Results: There is no significance between the ADC values of sham operated and ovariectomized rats (p=0,861). The placebo treatment group (group 3) had lower ADC values than ADC values of sham and ovariectomized groups but the difference was not statistically significant (p=0.146 and 0.197). ADC values in rats with melatonin treatment were higher than the placebo group (p=0,002) and are similar to sham group (p=0,062) that implied a physiological state. TBI resulted in the decreased ADC values that are compatible with cytotoxic edema. The results after one week show a significant increase in ADC values which is concordant with effective treatment of melatonin.
Conclusion: Traumatic brain injury generates an initial period of cerebral cytotoxic edema. Melatonin administration prevents the disruptive effects of TBI in ovariectomized rat brains.

Key words: Ovariectomy, traumatic brain injury, melatonin, diffusion weighted imaging

Melatonin Ovarektomi Yapılmış Sıçanlarda Travmatik Beyin Hasarını Önlemektedir

✔ **Amaç:** Overektomi yapılmış sıçanlarda melatoninin travmatik beyin hasarına etkisinin difüzyon ağırlıklı görüntülerle incelenmesi hedeflenmiştir.
Yöntem: Çalışmada 24 adet genç Wistar-Albino sıçan kullanılmıştır. Onsekiz sıçana iki yanlı overektomi uygulanmış, 6'sına aynı kesi yapılmış ancak overektomi gerçekleştirilmemiştir. Cerrahiden yedi gün sonra eş sayılı dört grup oluşturulmuştur. Grup I kontrol grubu (sham), Grup II yalnız overektomi yapılmış grup, Grup III overektomi sonrası travmatik beyin hasarı oluşturulmuş grup, Grup IV overektomi ve travmatik beyin hasarı sonrası melatonin uygulanmış grup olarak belirlenmiştir. Grup III'e sadece peritoniçi %0.1'lik etanol verilirken (taşıyıcı), Grup IV'e taşıyıcı ile birlikte 4 mg/kg melatonin uygulanmıştır. İlaç tedavisi hemen travma sonrası başlatılıp yedi gün sürdürülmüştür. Difüzyon ağırlıklı görüntüler hasardan bir hafta sonra alınmış, belirgin difüzyon katsayılı (BDK) (apparent diffusion coefficient) haritalar çıkartılmıştır.
Bulgular: BDK ile ilgili olarak ilk iki grup arasında anlamlı bir farklılık yoktur (p=0.861). Plasebo tedavili grupta (Grup III) ilk iki gruba göre daha düşük BDK değerleri elde edilirken farklılık istatistiksel anlam göstermemiştir (p=0.146 ve p=0.197). Melatonin tedavili gruptaki BDK değerleri plasebo tedavili gruptan daha yüksek olup (p=0.002) fizyolojik durumu simgeleyen ilk gruba (sham) benzerlik göstermektedir (p=0.062). Travmatik beyin hasarı, sitotoksik ödem ile uyumlu azalmış BDK değerleri vermektedir. Bir hafta sonraki sonuçlar melatoninin etkin tedavi gücü ile uyumlu olarak BDK'da anlamlı artış göstermektedir.
Sonuç: Çalışmamızdaki radyolojik değerlendirmeler ışığında melatonin uygulaması, overektomi yapılmış sıçanlarda travmatik beyin hasarının tahrip edici etkilerini engellemektedir. Bu sonuç daha geniş deney grupları ile sınanmalıdır.

Anahtar kelimeler: Difüzyon ağırlıklı görüntüleme, melatonin, overektomi, sıçan, travmatik beyin hasarı

Traumatic brain injury (TBI) produces a cascade of events that lead to the destruction of brain tissue and subsequent cognitive deficits. Currently, an effective and well established treatment of TBI does not exist. Therefore, most of the research on TBI has concentrated on neuroprotection in an attempt to preserve brain function. We have shown that melatonin administration prevents the disruptive effects of pinealectomy on brain tissue [1]. In addition, using MRS we have shown that several markers of neuroplasticity and neurogenesis are increased in melatonin treated rats [1]. Progesterone and 17b-estradiol influence neurogenesis and neuronal differentiation [2-7]. Estrogen has been reported to maintain cerebral blood flow in posttraumatic injury as an antioxidant and ameliorate excitotoxic injury. Progesterone acts by reducing immune-inflammatory reactions, membrane lipid peroxidation, and cerebral edema. Progesterone also stimulates the remyelination of damaged neurons [2-5]. Wagner et al. have shown that progesterone and allopregnanolone are potent neuroprotectants in TBI [3]. In contrast to above mentioned facts, clinical studies indicate that outcome for females may be worse due to the increased risk for women from the point of functional outcome after TBI [2-5]. Reiter et el [8]. reported that melatonin is an effective free radical scavenger and highly neuroprotective substance with antioxidant properties. The protective role of melatonin in neuropathology is widely accepted by researchers that study brain aging, Alzhemier's diseae, Parkinson's disease and stroke, where an increased rate of cell death occurs [9]. According to the free radical theory of aging, reactive oxygen species (ROS) initiate degradative processes that contribute to the development of aging. In this way, the antioxidative properties of melatonin suggest that it might exhibit an antiaging effect [10]. In addition to antioxidant potential, several possible mechanisms are considered to be involved in the melatonin neuroprotection, including maintenance of cellular glutathione homeostasis [11], inhibition of activation of NF-kB [12] and changes in gene expression of antioxidant enzymes [13]. Diffusion-weighted magnetic resonance imaging (DWI) is an effective in-vivo tool to assess microstructural changes of the brain parenchyma. It was mainly used to detect ischemia-related changes; and now, it is used to detect very subtle abnormalities such as developmental disorders and infections [14-19]. The technique measures random molecular movement of water in tissues and detect any alteration of that movement during cytotoxic and/or vasogenic edema [15,18,19]. Apparent diffusion coefficient (ADC) maps are an extension of DWI and are used to quantify these diffusional changes [16,17]. The aim of the present study was to answer the question of whether melatonin is also neuroprotective in the case of traumatic neuronal damage, which is not primarily related to oxidative stress. For this purpose, we investigated the efficacy of melatonin treatment in ovariectomized rat brain after traumatic brain injury by using DWI.

MATERIAL and METHODS

A total of 24 young Wistar-albino rats, of which 18 animals were submitted to bilateral ovariectomy and 6 rats were submitted to the same surgical incision but without ovariectomy were studied. After 7 days, rats were assigned to four groups of 6 animals each. Group 1, sham operated; group 2, ovariectomy; group 3, ovariectomy + TBI; group 4, ovariectomy + TBI + melatonin. Animals in group 3 and 4 received traumatic brain injury. Rats in group 3 received vehicle (0.1 % ethanol) whereas melatonin group had received 4 mg/kg melatonin intraperitoneally. Melatonin (Sigma Chemical Co., St. Louis, Missouri, USA) was dissolved in ethanol and diluted in saline to give a final concentration of 1% ethanol. Because of the very variable melatonin dosage schemes reported in literature, we administrated melatonin at the dose of 4 mg/kg which concentration was previously used for blocking production of ROS successfully [20]. Melatonin or vehicle administration started immediately before injury and continued for 7 days. DWIs were obtained one week post injury,

and apparent diffusion coefficient (ADC) maps were constructed.

Trauma Model

TBI was performed as described by previously [21]. Rats were preoperatively anesthetized by i.p. application of a mixture consisting of ketamin hydrochloride (75 mg/kg) and xylazine hydrochloride (8 mg/kg). Briefly, after a sagittal scalp incision, rats were immobilized and brain damage was induced via a cortical contusion using a pneumatic piston (5-mm diameter, 4 m/sec, 250 msec) to a depth of 3 mm. The entire procedure was completed within 10 min.

Imaging Sequence

MRI examination was conducted on 1.5 T scanner with 32 mT/m gradient force (Gyroscan Intera Master, Philips, Best, The Netherlands) that was used for recent studies performed on rats [1]. A quadrature birdcage coil was used for signal acquisition. Axial T1 weighted spin echo and T2 weighted half Fourier single shot turbo spin echo (HASTE) and DWI sequences were obtained. DWI were performed with a fat suppressed, multishot spin echo planar imaging (EPI) sequence with parameters outlined below: TR/TE=2191/81 ms, EPI factor: 77, slice thickness: 3.0 mm, slice gap: 1. 0 mm, number of signal acquisition: 1, FOV: 230x230 mm, matrix size: 77x256. B values of 0, 500 and 1000 s/mm^2 were used for automated apparent diffusion coefficient (ADC) maps. EPI images were cine-reviewed to reveal any evidence of subject motion and the acquisition was repeated when necessary.

Histological Analysis: At the end of experiment the brains were removed from the skull, and fixed in 10 % neutral buffered formalin solution and then embedded in paraffin as usual. Serial sections were cut using the microtome at a thickness of 4 µm and stained with hematoxylin&eosin. The histologic sections were examined for the presence of intersititial edema and vascular dilatation with a microscope and photographed.

Image Analysis

Trace ADC values were measured from cerebral parenchyma. The borders of cerebral parenchyma were manually traced on magnified T1 weighted images to obtain region of interest (ROIs) (Figure 1). These images were primarily used to define small rat brain that may be difficult to delineate on poor quality EPI images. ROIs were then automatically transferred on to ADC maps. To further avoid the CSF effects on diffusion measurements, linear ROIs were used when necessary, and placements were verified on ADC maps. Each brain was measured three times and their average was calculated.

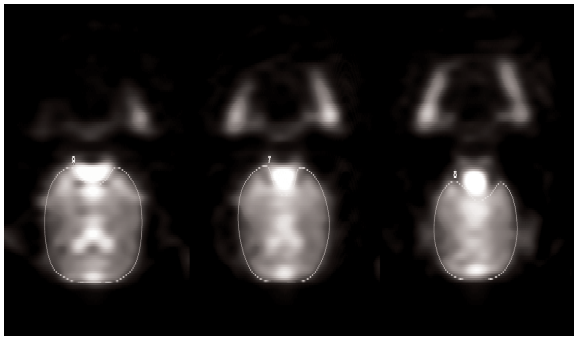


Fig. 1. Manually traced region of interest showing the borders of rat brains on T1 weighted images.

Statistical Analysis

Statistical analyses were performed using The Statistical Package for Social Sciences (SPSS) (version 13.0). Results are given in the text as mean±standard error (SE). ADC value differences between the groups were tested using one-way ANOVA and post-hoc multiple comparisons (Least significant difference). Highest acceptable significance level was defined as 0.05.

RESULTS

Table 1 provides the descriptive statistics and results of univariate analysis of variance (*post hoc* LSD) between the experimental groups. Parenchymal ADC values in sham group (n=6) were between 783 and 861 mm^2/sx10^{-3} (Mean: 822±15 mm^2/sx10^{-3}, SD: 36.95 mm^2/sx10^{-3}). In the ovariectomy group these values were

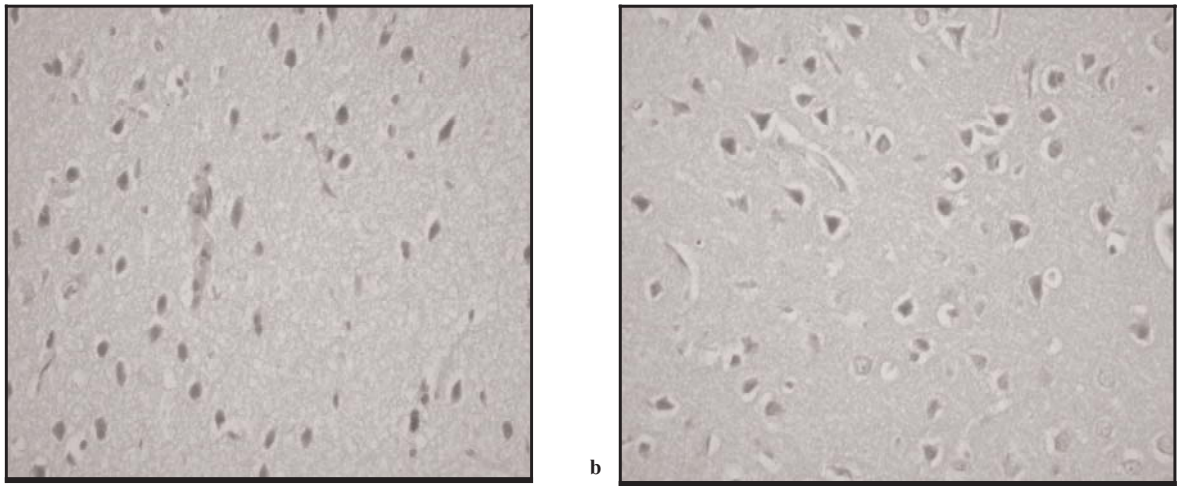
Table 1. Summary table for means±SE and significance levels determined using univariate analysis of variance for ADC values for each of the experimental groups (n=6 rats per group).

Groups (n=6)	ADC
I- Sham	822±15 $mm^2/s \times 10^{-3}$
II- Ovariectomy	816±29 $mm^2/s \times 10^{-3}$
III- Ovariectomy+TBI+ 0.1% etanol	767±26 $mm^2/s \times 10^{-3}$
IV- Ovariectomy+TBI+melatonin	895±30 $mm^2/s \times 10^{-3}$
P values*	
I vs II	,861
I vs III	,146
I vs IV	,062
II vs III	,197
II vs IV	,044
III vs IV	,002

between 741 and 890 $mm^2/sx10^{-3}$ (Mean: 816±29 $mm^2/sx10^{-3}$, SD: 71.29 $mm^2/sx10^{-3}$). ADC values in ovariectomy+TBI+ 0.1 % etanol group were between 698 and 835 $mm^2/sx10^{-3}$ (Mean: 767±26 $mm^2/sx10^{-3}$, SD: 65.36 $mm^2/sx10^{-3}$). Ovariectomy+TBI+melatonin group, these values were between 817 and 972 $mm^2/sx10^{-3}$ (Mean: 895±30.18 $mm^2/sx10^{-3}$, SD: 73.94 $mm^2/sx10^{-3}$). Four experimental groups were evaluated for differential mean ADC values. There was no significance between the ADC values of sham operated and ovariectomized rats (p=0.861). The placebo treatment group had lower ADC values than sham and ovariectomy groups but the difference was insignificant (p=0.146 and 0.197). Despite this fact, these results had pointed to cytotoxic brain edema formation due to traumatic insult (Fig.2a). ADC values due to melatonin treatment are significantly higher than the placebo treatment group (p=0.002) and were similar to sham (control) group (p=0.062) (Fig. 2b). TBI resulted a decrease in ADC values which indicates cytotoxic edema and melatonin had reversed that situation. Histopathology seen in TBI rats corresponded well with the pathology observed with DWI. TBI resulted in a decrease in tissue cellularity indicating edema in the cortex. Overall, treatment with melatonin resulted in a small decrease in the tissue cellularity of cortex over that seen with trauma alone.

DISCUSSION

Antioxidants may have a beneficial effect on many age-related diseases. In the free radical theory of aging, it is suggested that accumulated free radical damage may be responsible for degenerative process during aging [22]. Aging in normal animals with an intact pineal gland is associated with increased oxidative damage accompanied by elevated lipid peroxidation products and morphological changes [23]. Melatonin is involved in a variety of important physiological responses such as immunologic, regulation and neuroprotection [8]. Moreover, melatonin enhances the production of antioxidant defense system and stabilizes cell mem-



a b

Fig. 2. TBI resulted in a decrease in tissue cellularity indicating edema in the cortex of the ovariectomized rats (a). Treatment with melatonin resulted in a small decrease in the tissue cellularity of cortex over that seen with trauma alone (b) (H&E, X200).

brane fluidity against oxidative stress by reducing lipid peroxidation thus helping neurons resist oxidative damage [24].

TBI in ovariectomized rats resulted in a pattern of changes in ADC values. In the cortex of ovariectomized and injured rats ADC values were found to be decreased at the end of first week. This was compatible with restricted diffusion that reflects energy failure and cytotoxic edema. The cellular mechanisms underlying cytotoxic edema are complex, and are currently thought to be the result of ischemia that disrupts energy metabolism, causing a failure of various ion pumps [25]. However, the restriction was not prominent, and it was not observed in injured but melatonin administered rats. This difference underlines the protective effects of melatonin in TBI. This protection may be in two different ways. Melatonin may either prevent the formation of edema or it accelerates its resolution. This hypothesis must be validated through a new set of experiment in which rats would test before and after the injury. In the cortex a significant increase in ADC was observed in the group given the combination of TBI and melatonin, suggesting that melatonin may be neuroprotective. The cellular mechanisms underlying this apparent increase in water diffusion remains to be resolved. A significant increase with regard to the vehicle group values was also observed, reflecting an increased molecular motion. These increased values implied disrupted cerebral vasoregulation and an increase in interstitial volume during melatonin treatment. In vasogenic edema the water accumulates prevalently in the extracellular space with an increase in isotropic diffusion in white matter and gray matter, and an increase in ADC values and mean diffusivity [18,19,26]. Treatment with melatonin appeared to be effective in attenuating brain edema in ovariectomized rat brain after TBI. Thus, the increase in ADC values in the melatonin-treated rats was probably due to its antioxidant and free radical-scavenging effects. There is evidence that melatonin helps the cell to deal with oxidative stress. Barlow et al. [27] demonstrated an increase in cerebral glutathione peroxidase activity in melatonin treated rats. Okatani et al. [28] found increase in glutathione peroxidase and superoxide dismutase activity in the brain of fetal rats whose mothers had received melatonin. Recent study demonstrated that oxygen-glucose deprivation induced cortical neuronal cell death was prevented by melatonin at least in part [29].

Experimental models suggest that gonadal hormones are neuroprotective. Ovariectomy alone did not result in measurable changes in ADC values. This suggests that a relatively brief period of menopause was not sufficient to produce microstructural alterations, at least as visualized by DWI. In the present study the addition of traumatic brain injury resulted in only a small and non-significant decrease in ADC values. Considered together these results indicate that the predominant response after traumatic brain injury in ovariectomized rats is a reduction in the ADC reflecting decreased water diffusion associated with cytotoxic edema. Recently combined estrogen-progestin therapy also fails to prevent mild cognitive impairment in postmenopausal women aged 65 years or older [30]. Yun et al. [31] reported that melatonin may modulate cognitive plasticity, independent of the effects of sex steroids.

This is the first study demonstrates that the development of brain edema following TBI can be prevented in vivo in ovariectomized rats using melatonin. TBI resulted in an early decrease in ADC values indicating cytotoxic edema in the cortex that was followed at 1 week by an increase in the ADC that was associated with melatonin treatment. Overall, the addition of melatonin to traumatic brain injury resulted in a significant increase in the ADC values that was associated with decrease in the magnitude of brain edema. These results reflect the presence of transient microstructural alterations that develop during TBI [32]. This attests to the fact that physiological levels of melatonin may be relevant in normally reducing free radical dam-

age to key neural structures. Melatonin has neuroprotective properties and it therefore may prove to be a useful therapeutic agent in the treatment of postmenopausal TBI. These findings provide a starting point for human studies on the neuroprotective effects of melatonin.

REFERENCES

1. **Hasçalık S, Çelik O, Karakaş HM, Parlakpınar H, Fırat AK. Özşahin M.** Protective role of melatonin in pinealectomized rat brains: in vivo magnetic resonance spectroscropic analysis. J Pineal Res 2005; 39:342-5.
2. **Wagner AK, Hammond FM, Sasser HC, Wiercisiewski D, Norton HJ.** Use of injury severity variables in determining disability and community integration after traumatic brain injury. J Trauma 2000; 49:411-9.
3. **Wagner AK, Kline AE, Sokoloski J, Zafonte RD, williams M, Capulong E, Dixon CE.** Intervention with Environmental Enrichment after Experimental Brain Trauma Enhances Cognitive Recovery in Male but not Female Rats. Neurosci. Lett 2002; 334:165-168.
4. **Wagner AK, Bayır H, Ren D, Puccio A, Zafonte RD, Kochanek P.** Relationships between Cerebrospinal Fluid Markers of Excitotoxicity, Ischemia, and Oxidative Stress After Severe TBI: The Impact of Gender, Age, and Hypothermia. J. Neurotrauma 2004; 21:125-136.
5. **Wagner AK, Wiillard LA, Wenger MK, Bolinger BD, Kline AE, Zafonte RD, Dixon CE.** Evaluation of Estrous Cycle Stage and Gender on Behavioral Outcome after Experimental Traumatic Brain Injury. Brain Research 2004; 998,113- 21.
6. **Garcia-segura LM, Azcoitia I, Doncarlos LL.** Neuroprotection by estradiol. Prog Neurobiol 2001; 63:29-60.
7. **Wang L, Andersson S, Warner M, Gustafsson JA.** Estrogen actions in the brain. Sci STKE 2002; 25, 2002:PE29.
8. **Reiter RJ, Acuna-Castroviejo D, Tan DX, Burkhardt S.** Free radical-mediated molecular damage. Mechanisms for the protective actions of melatonin in the central nervous system. Eur J Pharmacol 2001; 428:169-75.
9. **Sainz RM, Mayo JC, Rodriguez C, Tan DX, Lopez-Burillo S, Reiter RJ.** Melatonin and cell death: differential actions on apoptosis in normal and cancer cells. Cell Mol Life Sci 2003; 60:1407-26.
10. **Harman D.** The free radical theory of aging. Antioxidant Redox Signal 2003; 5:557-61.
11. **Floreani M, Skaper SD, Facci L, Lipartiti M, Giusti P.** Melatonin maintains glutathione homeostasis in kainic acid-exposed rat brain tissues. FASEB J 1997; 11:1309-15.
12. **Mohan N, Sadeghi K, Reiter RJ, Meltz ML.** The neurohormone melatonin inhibits cytokine, mitogen and ionizing radiation induced NF-kappa B. Biochem Mol Biol Int 1995; 37:1063-70.
13. **Antolin I, Rodriguez C, Sainz RM, Mayo JC, Uria H, Kotler ML, Rodriguez-Colunga MJ, Tolivia D, Menendez-Pelaez A.** Neurohormone melatonin prevents cell damage: effect on gene expression for antioxidant enzymes. FASEB J 1996; 10,882-90.
14. **Hossmann KA, Hoehn-Berlage M.** Diffusion and perfusion MR imaging of cerebral ischemia. Cerebrovasc Brain Metab Rev 1995; 7:187-217.
15. **Le Bihan D, Breton E, Lallemand D, Aubin ML, Vignaud J, Laval-Jeantet M.** Separation of diffusion and perfusion in intravoxel incoherent motion MR imaging. Radiology 1988; 168, 497-505.
16. **Moseley ME, Cohen Y, Mintorovitch J, Chileuitt L, Shimizu H, Cohen Y, Weinstein PR.** Early detection of regional cerebral ischemia in cats: comparison of diffusion-and T2-weighted MRI and spectroscopy. Magn Reson Med 1990; 14:330-346.
17. **Verheul HB, Bala'zs R, Berkelbach Van Der Sprenkel JW, Tamminga KS, Van Lookeren Campagne M.** Comparison of diffusion-weighted MRI with changes in cell volume in a rat model of brain injury. NMR Biomed 1994; 7:1-5.
18. **Fırat AK, Karakaş HM, Fırat Y, Yakıncı C.** "Quantitative evaluation of brain involvement in ataxia telangiectasia by diffusion weighted MR imaging", European Journal of Radiology 2005; 56:192-196.
19. **Karakaş HM, Bayındır Y, Fırat AK, Yağmur C, Alkan A, Kayabaş U.** Cerebral diffusional changes in the early phase of anthrax: is cutaneous anthrax only limited to skin? J Infect 2006; 52:354-8.
20. **Parlakpınar H, Sahna E, Özer MK, Özuğurlu F, Vardı N, Acet A.** Physiological and pharmacological concentrations of melatonin protect against cisplatin-induced acute renal injury. J Pineal Res 2002; 33:161-166.
21. **Strauss KI, Barbe MF, Marshall RM, Raghupathi R, Mehta S, Narayan RK.** Prolonged cyclooxygenase-2 induction in neurons and glia following traumatic brain injury in the rat. Neurotrauma 2000; 17:695-711.
22. **Harman D.** The free radical theory of aging. Antioxid Redox Signal 2003; 5:557-61.
23. **Sahna E, Parlakpınar H, Vardı N, Ciğremiş Y, Acet A.** Efficacy of melatonin as protectant against oxidative stress and structural changes in liver tissue in pinealectomized rats. Acta Histochem 2004; 106:331-6.
24. **Savaskan E.** Melatonin in aging and neurodegeneration. Drug development research 2002; 56:482-490.
25. **Schaefer PW, Grant PE, Gonzalez RG.** Diffusion-weighted MR imaging of the brain. Radiology 2000; 217:331-345.
26. **Fonda C, Gasparotti R, Villari N.** Diffusion-weighted MR of the brain: methodology and clinical application. Radiol Med 2005; 109:155-197.
27. **Barlow-Walden LR, Reiter RJ, Abe M, Pablos M, Menendez-Pelaez A, Chen LD, Poeggeler B.** Melatonin stimulates brain glutathione peroxidase activity. Neurochem Int 1995; 26:497-502.
28. **Okatani Y, wakatsuki A, Kaneda C.** Melatonin increases activities of glutathione peroxidase and superoxide dismutase in fetal rat brain. J Pineal Res 2000; 28:89-96.
29. **Harms C, Lautenschlager M, Bergk A, Freyer D, Weih M, Dirnagl U, Weber JR, Hortnagl H.** Melatonin is protective in necrotic but not in caspase-dependent, free radical-independent apoptotic neuronal cell death in primary neuronal cultures. FASEB J 2000; 14:1814-24.
30. **Shumaker SA, Legault C, Rapp SR, Thal L, Wallace RB, Ockene JK, Hendrix SL, Jones BN, 3rd, Assaf AR, Jakson RD, Kotchen JM, Wassertheil-Smoller S, Wactawski-Wende J, Whims Investigators.** Estrogen plus progestin and the incidence of dementia and mild cognitive impairment in postmenopausal women: the Women's Health Initiative Memory Study: a randomized controlled trial. JAMA 2003; 289:2651-62.
31. **Yun AJ, Bazar KA, Lee PY.** Pineal attrition, loss of cognitive plasticity, and onset of puberty during the teen years: is it a modern maladaptation exposed by evolutionary displacement? Med Hypotheses 2004; 63:939-50.
32. **Önal Ç, Ünal F, Turantan MI, Üzüm G, Hasanoğlu A, Kaynar MY.** The effect of intraventricular albumin in experimental brain oedema. Acta Neurochir (Wien) 1997; 139(7):661-669.