Morphological Changes and Vascular Reactivity of Rat Thoracic Aorta Twelve Months After Pinealectomy

Pinealektomiden On İki Ay Sonra Sıçanların Torasik Aortalarındaki Morfolojik Değişiklikler ve Vasküler Reaktivite

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ABSTRACT Objective: Melatonin, a hormone produced by the pineal gland, has been suggested to protect against development of hypertension and atherosclerosis. In this study, the effects of long-term melatonin deficiency for twelve months after pinealectomy on the α-adrenergic-contractions induced by phenylephrine, endothelium-dependent relaxation responses to acetylcholine and the morphological changes in the rat thoracic aorta were studied. **Material and Metods:** Rats were pinealectomized twelve months before the beginning of the vasomotor studies. Rings of arteries were mounted in isolated tissue baths for the measurements of isometric contractile force. The contractile responses to phenylephrine and endothelium-dependent relaxation responses to acetylcholine in the vessels were evaluated. Endothelial function was evaluated by vascular relaxation to acetylcholine. Histological examinations demonstrated the alterations of tunica media in the vessels of pinealectomized rats. **Results:** Thick and thin areas were observed in the transverse sections of vessels and the ratio of the widest media thickness to the narrowest was found significantly increased in pinealectomized group (2.85 ± 0.56) when compared to the control group (1.65 ± 0.10). In addition, α-smooth muscle actin and elastic lamellae staining of the media were attenuated in pinealectomized rats. Although contractile responses of vessels to phenylephrine in pinealectomized rats were lower than control group, significant difference was found for only one concentration (3x 10-8 mol l-1) of phenylephrine. There was no difference between the relaxation responses to acetylcholine in pinealectomized and control groups. **Conclusion:** These results show that long-term melatonin deficiency may cause some morphological changes in the tunica media of vessels. However, the function of endothelium and vascular responsiveness to ∝-adrenergic stimulus seem to be mostly protected.

Key Words: Melatonin; aorta, thoracic; rats

ÖZET Amaç: Pineal bezden üretilen bir hormone olan melatoninin hipertansiyon ve ateroskleroza karşı koruyucu olduğu ileri sürülmüştür. Bu çalışmada sıçan torasik aortalarında, pinealektomi sonrası on iki ay süreyle uzun süreli melatonin eksikliğinin, fenilefrinle oluşturulan α-adrenerjik kasılmalar, asetilkolinle oluşan endotelyuma bağlı gevşemeler ve morfolojik değişiklikler üzerine etkileri çalışıldı. **Gereç ve Yöntemler:** Sıçanların pineal bezleri vazomotor çalışmaların başlamasından on iki ay önce çıkarıldı. Arter halkaları izometrik kasılma gücün ölçümü için izole doku banyosuna asıldı. Damarlarda fenilefrinle oluşturulan kasılma cevapları ve asetilkolinle oluşan endotelyuma bağlı gevşeme cevapları değerlendirildi. Pinealektomize sıçanların damarlarındaki tunika media tabakasının değişiklikleri histolojik olarak muayene edildi. **Bulgular:** Kontrol grubu (1.65 ± 0.10) ile karşılaştırıldığında damarların transvers kesitlerinden elde edilen kalın ve ince alanlar ve media tabakasının en geniş kalınlığının en dara oranı pinealektomize grupta (2.85 ± 0.56) anlamlı olarak artmış bulundu. Ayrıca, medianın α-düz kas aktin ve elastik lamel boyaması pinealektomize sıçanlarda azaldı. Her ne kadar pinealektomize sıçanlardaki arterlerin fenilefrine kasılma cevapları kontrol grubundan daha düşük olsa da fenilefrinin yanlızca bir konsantrasyonunda (3x 10-8 mol l-1) anlamlı farklılık bulundu. Pinealektomize ve kontrol grupların asetilkoline gevşeme cevapları arasında anlamlı farklılık yoktu. **Sonuç:** Bu sonuçlar uzun süreli melatonin eksikliğinin damarların tunika mediasında bazı morfolojik değişikliklere neden olabileceğini göstermektedir. Bununla birlikte endotelyumun fonksiyonu ve ∝-adrenerjik uyarılara damarsal cevap verirlilik çoğunlukla korunmuş gibi görünmektedir.

Anahtar Kelimeler: Melatonin; aorta, torasik; sıçanlar

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Melatonin, a hormone produced by the pineal gland, is released with a circadian rhythm, with high levels at night.[1] Melatonin has been suggested as a potent antioxidant that may protect the development of hypertension and atherosclerosis. Decreased melatonin synthesis has been determined in patients with hypertension or coronary artery disease.[2,3] Melatonin administration has been observed to reduce blood pressure and decrease catecholamine levels in human subjects.[4,5] It has also been reported that melatonin reversed N-methyl-l-arginine induced blood pressure (BP) elevation, and increased carotid artery blood flow in rats.[6,7] Impaired endothelial function which may play a role in the pathophysiology of hypertension[8] and atherosclerosis,[9] was shown to be restored by melatonin probably due to its antioxidative properties.[10,11] Moreover, age-related changes in the amplitude of the melatonin rhythm in humans of advanced age have been reported.[12] Thus, it could be suggested that decreased melatonin serum concentrations with aging may result in endothelial dysfunction and promote hypertension or atherosclerosis.

On the other hand, an interesting involvement of melatonin in the cardiovascular regulation of rats was suggested by pinealectomy experiments that resulted in a temporary hypertension.[13] Increased BP levels have been reported to return to the normal range two months after pinealectomy in rats.[13-16] Cunnane et al. reported that vasoconstrictor responses to norepinephrine, serotonin and angiotensin II in pinealectomized (Px) rats were greater than that of control rats a week after pinealectomy, i.e. in the short-term period of pinealectomy.[17] Zanoboni et al. also showed the wall thickening and lumen narrowing of the rat arterioles 90 days after pinealectomy.[13] However, there is no data about the effects of decreased melatonin levels on the endothelial function, vascular reactivity and morphology of vessels in a longer period after pinealectomy.

The present study was designed to investigate the effects of reduced melatonin levels twelve months after pinealectomy, which is a quite long period for rat studies, on the α-adrenergic-induced contractions, endothelium-dependent relaxations and morphological changes in the rat thoracic aorta.

MATERIAL AND METHODS

ANIMALS

Twenty male Wistar rats weighing 150-200g were placed in a quiet and temperature (21 ± 2°C) and humidity (60 ± 5%) controlled room in which a 12-12 h light-dark cycle was maintained.

All experiments in this study were performed in accordance with the guidelines for animal research from the National Institutes of Health and were approved by the Commitee on Animal Research at Inonu University, Malatya.

PINEALECTOMY

Rats were pinealectomized twelve months before the beginning of the vasomotor studies and morphologic evaluation of their aortas, in order to constitute a long term melatonin deficiency since the amplitude of the melatonin rhythm has been shown to be reduced by 60–100% after pinealectomy in previous studies.[1]

Pinealectomy was performed for ten rats as described by Kuszak and Rodin.[18] Rats were anesthetized with ketamine hydrocloride (75 mg kg^{-1}) and xylazine (8 mg kg^{-1}) before the operation. The entire procedure was completed within 15 min. Pinealectomy was confirmed by the histological evaluation of the gland for each animal. Ten rats, as a control group, underwent sham operations.

VASOMOTOR STUDIES

Freshly harvested thoracic aortas of rats were cleaned of fat and connective tissues. Two rings (each approx. 0.5 cm long) were prepared from a segment of thoracic aorta. Preparations were mounted in 40-ml organ baths containing Krebs-Henseleit buffer (mmol l^{-1}: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11, pH 7.4) maintained at 37°C and oxygenated with 95% O_2, CO_2 mixture. The preparations were suspended under 0.5 g resting tension which was determined in the baseline studies and equilibrated for 60 min,

with replacement of bathing fluid every 15 min. Isometric tension studies were performed using a Harvard Universal model oscillograph (Harvard Apparatus, Inc., Massachusetts, U.S.A).

Cumulative concentration-response curves to phenylephrine (Phe, 10^{-8} to 10^{-4} mol l^{-1}) were established. The vessels were then submaximally precontracted with Phe (typically 3´10^{-7} mol l^{-1}), and endothelial function was evaluated by vascular relaxation to acetylcholine (Ach, 10^{-8} to 10^{-4} mol l^{-1}). Nitric oxide (NO) mediation of ACh responses was confirmed by blocking ACh-induced relaxations by N-methyl-l-arginine (L-NAME, 10^{-3} mol l^{-1}), a specific competitive inhibitor of NO synthase.

Contractile responses were measured as force (g) and expressed as a percentage of the maximal contraction or, for relaxations, as a percentage of the precontracted tension.

HISTOLOGY

For light microscopic evaluation, aorta sections were fixed in 10% phosphate buffered formalin. Paraffin-embedded specimens were cut into 5mm thick sections and stained with Hematoxylin–eosin (H-E), Masson's trichrome stain, Verhoeff's elastic stain and anti a-smooth muscle actin immunostaining kit (Sigma Chem. Co. St. Louis). The aorta sections were examined with Leica DFC 280 light microscope by an experienced observer unaware of the animal treatment groups. Leica Q Win Plus Image Analysis System (Leica Micros Imaging Solutions Ltd.; Cambridge, U.K.) was used for morphometric analysis. Ten different sections were measured (μm) for each rat. The measurements were applied to tunica media in 10 different fields for each section. The ratios of the minimum and maximum values for each section were selected for statistical analysis.

DRUGS

Phe, ACh and L-NAME were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All drugs were dissolved in distilled water.

Statistical Analysis

Results were expressed as the arithmetic mean ± standart error of the mean (SEM) of the number of experiments. Distribution of the samples in the groups was analyzed with one sample of Kolmogorov-Smirnov test. Statistical analysis of the data was performed by using Student's t test for vasomotor findings and by Mann-Whitney U test for histological findings. The differences between groups of data were considered to be significant when $p< 0.05$.

RESULTS

There was no difference between the body weights of Px (167 ± 6.9 g) and control (173 ± 7.5 g) groups before the pinealectomy or sham operation. Although body weights of Px rats seemed to be increased, no significant difference was demonstrated between Px (414 ± 15 g) and control (398 ± 12 g) animals twelve months after the operation.

Phe produced concentration-dependent contractions in thoracic aortas isolated from control and Px rats. Although contractile responses of vessels to Phe in Px rats were lower than control group, statistically significant difference was found for only one concentration (3x 10^{-8} mol l^{-1}) of Phe (Figure 1). On the other hand, maximum contractile responses to Phe in the thoracic aortas of Px rats (0.91 ± 0.11 g) were not significantly different from the control rats (0.93 ± 0.07 g).

Endothelium-dependent relaxation responses to Ach also tended to decrease in Px rats, while no significant difference was determined between two

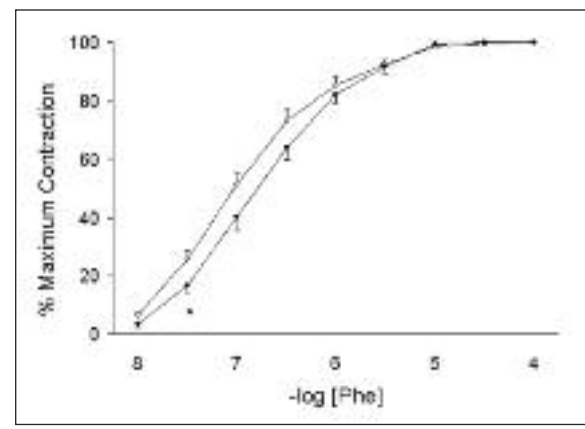

FIGURE 1: Concentration-response curves for phenylephrine (Phe)-induced contractions in isolated aorta rings of control (open circles) and pinealectomized (solid circles) rats. Each point represents the mean ± SEM bars of 10 experiments. *Significantly different from the values of control group.

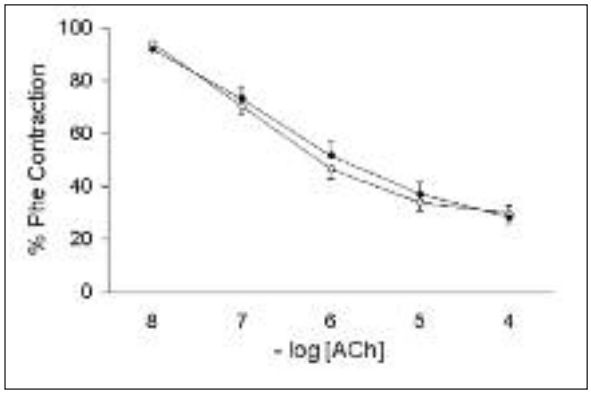

FIGURE 2: Concentration-response curves for endothelium-dependent relaxations induced by acetylcholine (ACh) in isolated aorta rings of control (open circles) and pinealectomized (solid circles) rats. All vessels were submaximally precontracted by 3x10-7 mol l-1 phenylephrine (Phe). Each point represents the mean ± SEM bars of 10 experiments.

groups (Figure 2). Relaxations to Ach were completely inhibited by L-NAME, thus confirming their dependence on NO production (data not shown).

Tunica intima, media and adventitia of all rat thoracic aorta specimens showed normal histology in the control group (Figure 3a). In the Px group, the intima and adventitia were also normal. No evidence of disruption and atherosclerosis was detected in the intima (Figure 3b). However, the thickness of the media in Px rats showed alterations. In the transverse sections, thickness of the vessel wall was not the same along its diameter; thick and thin areas were observed (Figure 4a). The ratio of the widest media thickness to the narrowest was assessed and found significantly increased in Px group (2.85 ± 0.56) compared to that in control group (1.65 ± 0.10). The aortas of two rats in Px group showed prominent wall thinning (Figure 4b).

Although the elastic lamellae of control group were stained prominently by Verhoeff's elastic stain, this staining was weak in the vessels of Px rats (Figure 5). The anti α-smooth muscle actin staining was strongly positive (+++) in the control group, whereas the Px group showed weak positive (+) staining (Figure 6).

DISCUSSION

In the present study, histological examination revealed the tunica media changes in the rat thoracic aorta due to long-term melatonin deficiency. In the transverse sections of the vessels, twelve months after pinealectomy, thin and thick media sections were observed and the ratio of the media thickness, i.e. the ratio of the widest to the narrowest measurement, was significantly increased compared to that of control rats. Regrigny et al. reported reduced wall thickness in the cerebral arterioles of rats one month after pinealectomy.[19] They also observed that treatment with melatonin restored normal wall thickness. On the other hand, Zanoboni et al. observed arteriolar wall thickening and lumen narrowing in the kidneys of Px rats 90 days after pinealectomy.[13] Since the thoracic aorta is an elastic arteria and its media histology is different from organ arteries, this study demonstrated for the first time the effects of long-term pinealectomy on the morphology of elastic arteries.

We determined histological alterations in the elastic lamellae and smooth muscle cells of rat thoracic aorta after pinealectomy. Elastic lamellae and smooth muscle cells are the basic components of the tunica media in elastic arteries. Vascular smooth muscle cells are capable of many functions including synthesis of collagen, elastin and proteoglycans. They also constitute an important element in normal vascular repair and in pathologic processes such as atherosclerosis.[20] In the Px group, we observed that smooth muscle cells weakly stained with anti α-smooth muscle actin. Elastic lamellae of the media were also thin and weakly stained with elastic stain. These alterations might be occurred as a result of the smooth muscle cell damage as well as impairment in the function of these cells.

Another finding of the current study was that the responsiveness to Phe, an μ_1-selective adrenergic agonist, was attenuated in the aortic rings of Px rats as compared to controls, although statistically significant difference was found for only one concentration of Phe. This result is different from the finding that demonstrated an enhancement of μ_1-adrenergic-induced contractions in rat vessels one week after pinealectomy.[18] In our previous study, we observed that reduced melatonin levels in the second month following pinealectomy did

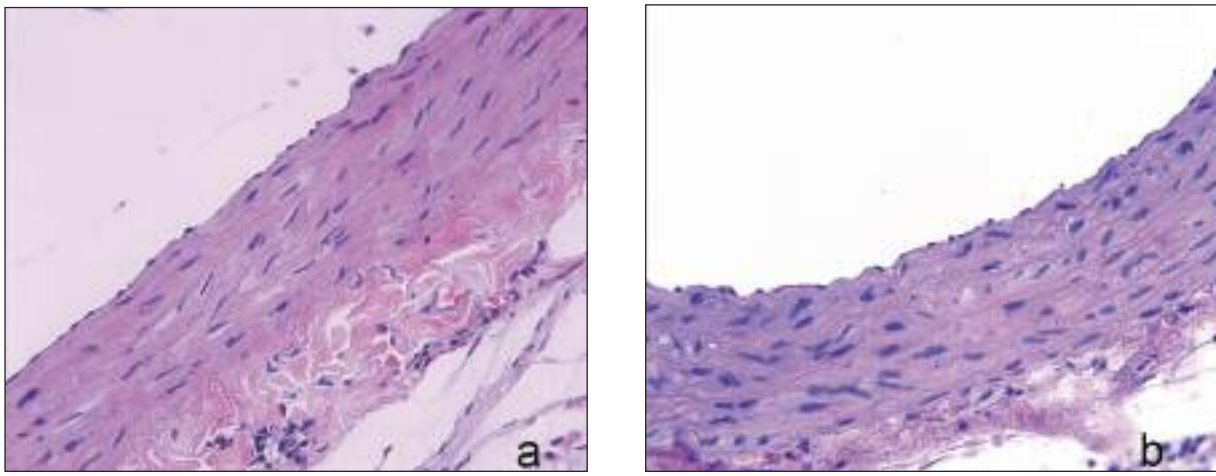

FIGURE 3: Photomicrographs of thoracic aorta in control **(a)** and pinealectomy **(b)** groups. **a:** Tunica intima, media and adventitia appear normal. Hematoxylin-eosin X132. **b:** Tunica intima is normal. No evidence of atherosclerosis and disruption are seen. The media alteration is not detectable in this figure. Hematoxylin-eosin X132.

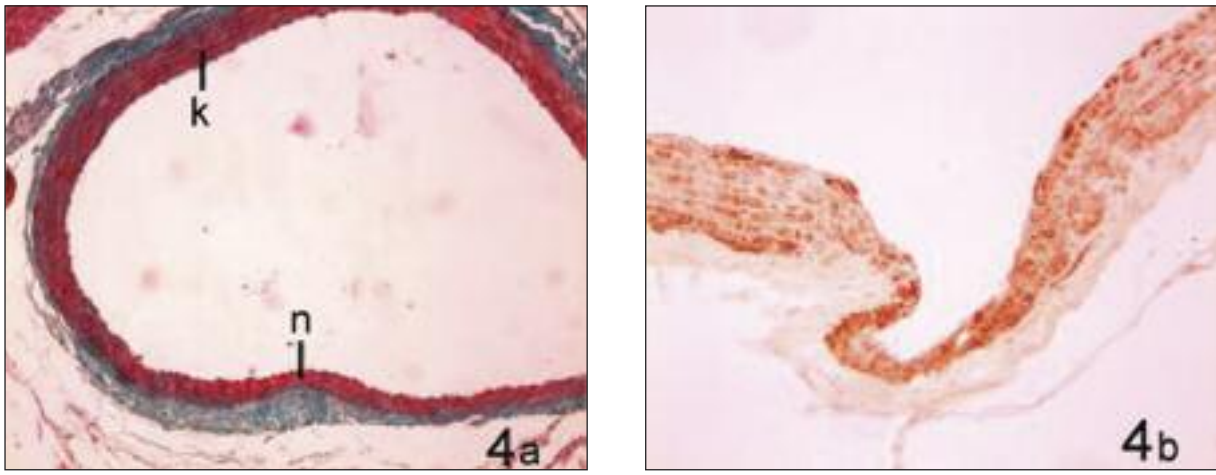

FIGURE 4: Photomicrographs of thoracic aorta in pinealectomy group. **a:** Thick (k) and thin (n) tunica media are seen. Masson's trichrome stain X33. **b:** Prominent reduction in media thickness is visible. Anti α-smooth muscle actin immunostaining X66.

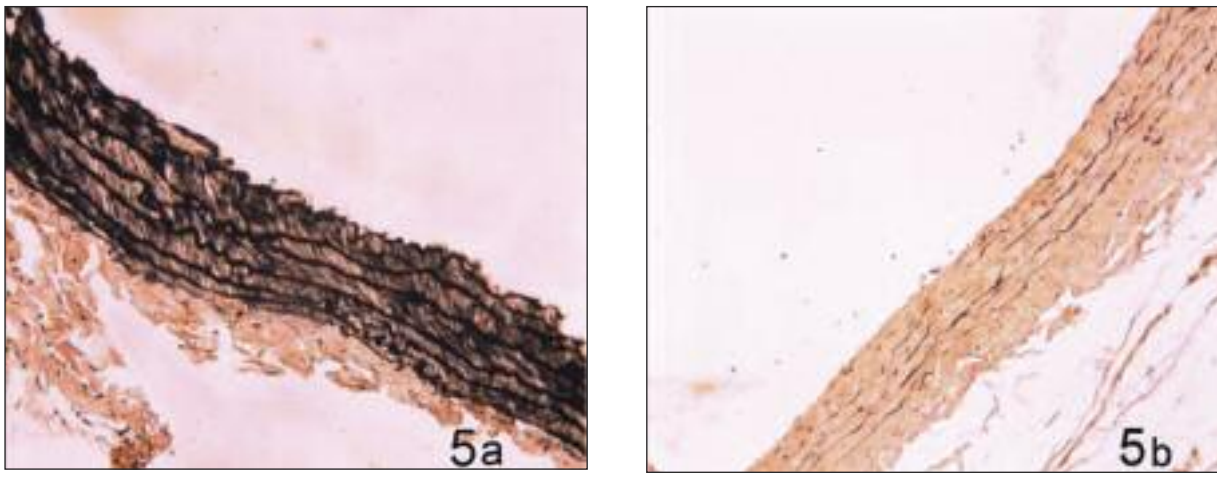

FIGURE 5: Photomicrographs of thoracic aorta in control (a) and pinealectomy (b) groups. a: Thick and continuous elastic lamellae are stained prominently. Verhoeff's elastic stain X66. b: Thin elastic lamellae are stained weakly. Verhoeff's elastic stain X66.

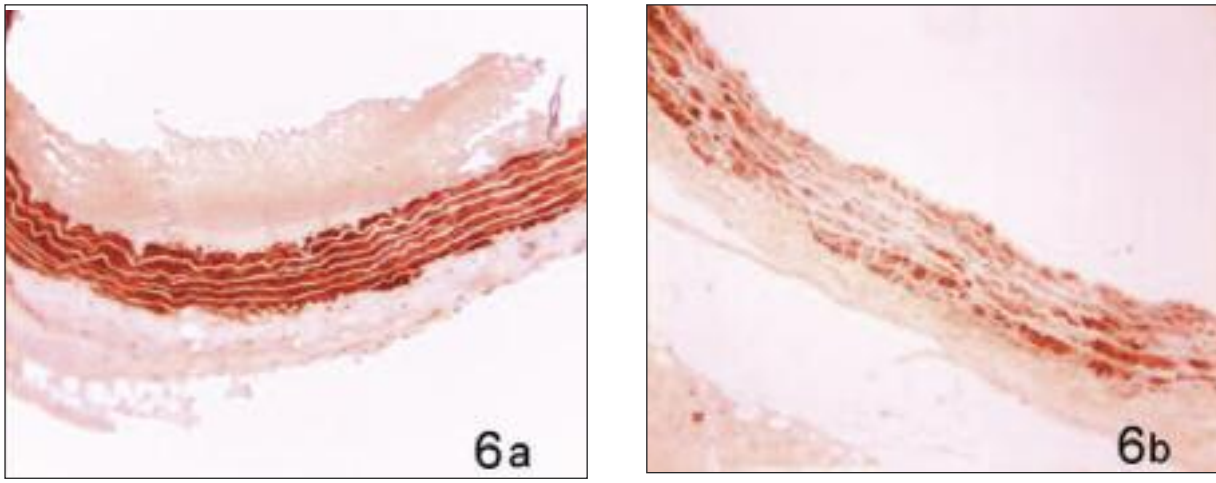

FIGURE 6: Photomicrographs of thoracic aorta in control (a) and pinealectomy (b) groups. a: The smooth muscle cells are stained strongly. Anti α-smooth muscle actin immunostaining X66. b: The smooth muscle cells are stained weakly. Anti α-smooth muscle actin immunostaining X66.

not modify the vascular reactivity to various vasoconstrictor agents such as phenylephrine, 5-HT, clonidine, AT-II and vasopressin.[16] As the α-smooth muscle actin is the major actin isoform of vascular tissue and contributes to cell-generated mechanical tension,[21] decreased α-smooth muscle actin staining of rat aorta observed in the present study may explain the reduced responses to Phe following long-term melatonin deficiency.

We also demonstrated decreased elastin component of thoracic aorta in Px rats. Elevated arterial stiffness was shown to be related to extracelluler matrix elastin/collagen ratio and accepted as an important predictor of cardiovascular events. Drugs which reduce the arterial stiffness have been shown to increase the elastogenic matrix profile in aortic smooth muscle cell culture.[22]

On the other hand, we hypothesized that long-term melatonin deficiency would cause an endothelial dysfunction, since the oxidative stress over the antioxidant capacity of the body is accepted to lead to endothelial dysfunction,[9-11] and total antioxidant capacity of rat serum was shown to be related to the serum concentrations of melatonin.[23] However, this possibility is not likely because we demonstrated completely intact vascular endothelium histologically twelve months after pinealectomy, and we could not show any significant reduction in the endothelium-dependent relaxation responses to Ach.

In conclusion, the present study demonstrated that long-term melatonin deficiency for twelve months following pinealectomy caused some significant changes in the tunica media of the rat thoracic aorta such as altered media thickness and decreased α-smooth muscle actin and elastic lamellae staining. On the other hand, the integrity and function of endothelium and vascular responsiveness to μ-adrenergic stimulus seem to be mostly protected. The importance of the morphological changes in the tunica media of vessels is not clear.

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