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Experimental Research

Effects of pinealectomy on morphological features of blood vessel in chicken: an electron microscopic and stereological study

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ABSTRACT

Although the effects of melatonin on vascular smooth muscle and vascular reactivity are described in last decade, the effects of melatonin on vascular tissues are still vague. The current study investigated the effects of pinealectomy on the blood vessel wall in the chicken. Fifteen chicks were divided into three groups, namely unoperated control, sham-operated and pinealectomized chicks. At the end of the experiment, a quantitative structural analysis of 15 sciatic artery samples from these groups was investigated. Effects of pinealectomy were quantified using volume fraction approach of stereological method. In this study, total cross-sectional area of the artery in pinealectomy group was significantly higher than those in unoperated control and sham-operated animals ($p < 0.05$). However, surgical pinealectomy procedure resulted in a significantly decreased thickness of the tunica adventitia of the vessel wall, in contrast to those of tunica media ($p < 0.01$). The present results indicate that pinealectomy had a vasodilator effect on the sciatic artery. In the light of current study, the structural changes in the vessel demonstrated by quantitative morphometric methods have been interpreted as a reflection of the role of melatonin on vessel wall and vascular reactivity, but this suggestion need to be validated in the human setting.

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1. Introduction

Major secretory product of the pineal gland, a neuroendocrine transducer with neuronal input and endocrine output, is melatonin (N-acetyl-5-methoxytryptamine) known as the hormone of darkness. Although the effects of the pineal hormone melatonin on vascular smooth muscle and vascular reactivity are described in last decade, morphological changes in the vessel are not yet clearly established (Shibata et al., 1989; Weekley, 1995; Muck et al., 1996; Mahle et al., 1997; Doolen et al., 1998; Monroe and Watts, 1998; Anwar et al., 2001; Vandeputte et al., 2001; Pache et al., 2002). Several studies have shown that pinealectomy reduces vascular wall thickness and melatonin treatment restores normal wall thickness and luminal diameter via melatonin receptors (Regrigny et al., 1999; Regrigny et al., 2001). Thus, melatonin deficiency was found to lead atrophy of the vascular wall in rat cerebral and caudal arteries (Hadju et al., 1990; Regrigny et al., 1999; Anwar et al., 2001; Regrigny et al., 2001; Masana et al., 2002). It has also been shown that the effects of melatonin depend on the presence of the vascular endothelium (Geary et al., 1998; Monroe and Watts, 1998; Anwar et al., 2001; Okatani et al., 2001a; Okatani et al., 2001b; Pogan et al., 2002; O'Rourke et al., 2003). Indeed, the growing knowledge about this substance is reflected in the steadily increasing number of publications. To our knowledge, however, there is no experimental study in the literature, which specifically addresses the effects of melatonin deprival following pinealectomy on the structural features of the blood

vessels, which reveals the role of melatonin on the layers of the vessel wall and its lumen. This study was conducted to explore the effects of melatonin deprivation produced by pinealectomy on the histopathological features of the blood vessels at light and electron microscopic levels, using the methods of quantitative stereological analysis.

2. Material and Methods

2.1. Animals and Experimental Protocol

Experiments were performed on Hybro Broiler chickens (weighing 40-70 g). Animals were obtained from a local hatchery (Institute of Agricultural Research of Erbeyli, Aydın, Turkey). They were kept in individual cages under constant laboratory conditions at 20 to 22ºC room temperature, exposed to 12 h of light (lights on at 6 AM and off at 6 PM), and allowed free access to food and fluid. Experiments were performed in accordance with the guidelines of the Ethical Committee of Ege University School of Medicine. Newly hatched chicks were divided into unoperated control group (n=5), sham-operated group (n=5) and surgical pinealectomy group (n=5) on the day of experiment, maintained for 8 weeks under the above regimes.

2.2. Operative Procedure

At 3 days of age, the chicks were pinealectomized under the general anesthesia of intraperitoneal sodium pentobarbital (Nembutal sodium®, Abbott Laboratories Comp., İstanbul-Türkiye, 40 mg/kg). Briefly, the part under surgical intervention after shaving was disinfected by using polyvidon iyod. In aseptic conditions, a 2-cm midline incision was made through the skin above the superior sagittal sinus and was extended posteriorly to just below the confluence of sinuses and a skull flap was raised with a scalpel. The pineal gland, which lies just beneath the dura mater and between two cerebral hemispheres and cerebellum, was taken out by using a microsurgical forceps after cutting from its pedicle, as previously described (Turgut et al., 2003). The skin was then sutured with vicryl 6/0.

2.3. Histological Assessment

The experiments were ended at 8 weeks after pinealectomy procedure or sham operation and 5 animals from each experimental group were randomly selected and sacrificed for histopathological evaluation at the end of the experiment. In each animal, right sciatic artery was exposed via posterior thigh approach and a segment of artery in 10 mm length proximal to the proximocaudal branch of the femoral artery was carefully removed. Then, the excised segments were cut into peaces of equal length followed by fixation with 2% gluteraldehyde buffered in cacodylate 0.1M and 2% paraformaldehyde solution (pH 7.4) for 24 hours after fixation. Once they were fixed, they were rinsed in cacodylate buffer (pH 7.4) twice and then tissue blocks were cut. Following this step, specimens were postfixed in 1% osmium tetroxide for 2 hours, dehydrated in an ascending alcohol series and took into propylene oxide twice. After this step, the tissues were embedded in epoxy resin. Following the hardening, serial semi-thin sections of 1-µm thickness were cut by using a LKB 11800 ultramicrotome (Bromma, Sweden). The resins were removed from epoxy embedded tissue sections. Then, the sections were stained with 1% toluidine blue and examined by light microscopy. Completeness of pinealectomy was confirmed by absence of any pineal tissue remnant in the surgical pinealectomy area in all chickens.

2.4. Stereological Analysis

Ten sampled sections, 1-µm-thickness, from each artery were analyzed in a systematic random manner. A test point grid was used to estimate area fraction of the tunica media (TM) including tunica intima, tunica adventitia (TA) and vessel cross sectional area (CSA) of vessel using a modified light microscope (Kaplan et al., 2001; Kaplan et al., 2005). A test point grid, printed on a transparent sheet (Fig. 1), was inserted into the eyepiece and two dial indicators attached to the stage of microscope were used (Kaplan et al., 2001; Kaplan et al., 2005). Systematic random area sampling ($200x200\mu m^2$) on the section was done by means of dial indicators. This ensures that all locations within a vessel cross section are equally represented and that the profile of vessel is sampled with an equal probability regardless of shape, size, orientation and location of it. During each step of sampling, the points that were superimposed on interested region of vessel were counted (SOIF, 40×; NA=0.65; 400x). The area fraction of TM within the wall of the artery was estimated using the following formula:

Area fraction of TM (tm, vessel wall) = The unit of the area fraction is %.

The mean CSA of the artery was estimated by superimposing of a test point grid on the vessel (40× SOIF, NA=0.65; 400x) (Gundersen, 1986). Total point numbers superimposed on the lumen were multiplied by

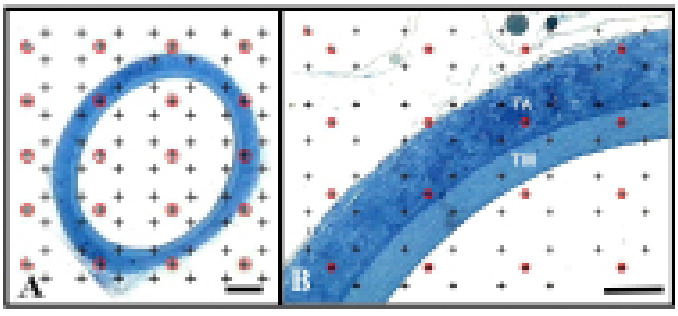

Fig. 1- A test point grid superimposed on the section of artery for estimation of the cross-sectional area (CSA) of vessel (a) and area fraction of tunica media (TM) and tunica adventitia (TA) (b). Each sampled sections were examined by a step (200 µm x 200 µm) that was determined in a systematic random sampling manner. In each step of area sampling, points that are superimposed on interested regions, TM and TA, were counted. The area fraction of each layer in the wall of the vessel was estimated by means of dividing the total point numbers that are superimposed on interested layer by the total points that are superimposed on both layers. The mean CSA of the arterial lumen was estimated by superimposing of a test point grid on the vessel lumen (40× Sofia, NA=0.65; 400x). Total point numbers superimposed on the lumen of artery were multiplied by representing area of each point [a(p)] to estimate CSA of the vessel (a). Toluidine blue, scale bars for (a) = 250 µm and for (b) = 50 µm.

representing area of each point [a(p)=36 μm2] to estimate CSA of the vessel (Fig. 1). Coefficient of variation (CV) for stereological analysis was also estimated (Table 1) (West et al., 1991; Schmitz, 1998; Schmitz and Hof, 2000).

Table 1. The Mean Coefficient of Variation for All Groups of Chickens at 8 Weeks After Pinealectomy (CSA, cross-sectional area; CV, coefficient of variation; TM, tunica media; TA, tunica adventitia)

Parameters related with vessel wall and lumen	CV of unoperated control group	CV of sham-operated control group	CV of pinealectomy group
TA / Vessel wall	0.05	0.17	0.08
TM / Vessel wall	0.11	0.14	0.10
TA / TM	0.15	0.30	0.18
TM / TA	0.17	0.33	0.19
CSA	0.60	0.29	0.31

2.5. Statistical Analysis

Results are expressed as means + standard deviation (SD). CSA and area fraction values of the vessel wall and lumen were compared by the Mann-Whitney U test. A p value ≤ 0.05 was accepted as significant. Additional details are provided in the text and legends to figures.

3. Results

All chickens showed no evidence of gross neurophysiologic deficit and no wound infections were noted in the postoperative period. Neither feeding habits and behavior nor the various growth parameters such as body weight of the animals were affected by the operation. At the end of the experiment, histological examination of the brains of the animals in surgical pinealectomy group revealed that the pineal gland had been removed at surgery and no extraneous tissue had been left behind or had regenerated.

3.1. Light Microscopy Findings

Vessel of the control group consists of concentric layers or "tunics" of different tissue types (Fig. 2), that are tunica intima

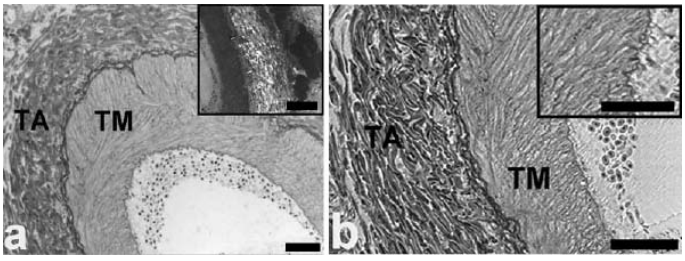

Fig. 2- Micrographs of the control artery (a, b). TM, tunica media; TA, tunica adventitia. Inset shows a magnified area of junction between tunica intima and media. Scale bar = 70 μm.

which composes of the inner cell layer of endothelium, and a relatively thin supporting connective tissue. The second of these was the TM, the middle muscular and/or elastic layer, containing smooth muscle and elastic tissue in varying proportions. The TA was the outer, fibrous connective tissue layer. In the vessel lumen, many nucleated erythrocytes were seen. Internal and external elastic membranes of the vessel were obvious.

In sham-operated control group, three layers that are the tunica intima, TM and TA were also found. There was no obvious qualitative difference from the vessel of control group (Fig. 3).

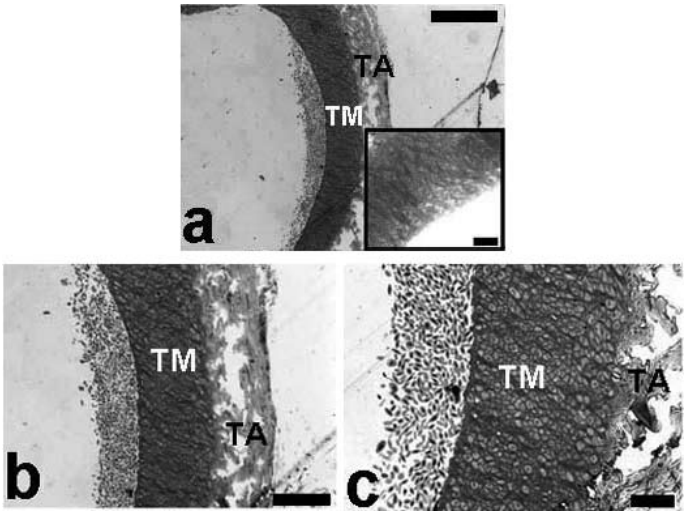

Fig. 3- Micrographs of the arteries of sham operated chicks (a-c). TM, tunica media; TA, tunica adventitia. Inset shows a magnified view of the tunica media in (a). Scale bar = 60 μm.

In the sections of surgical pinealectomy groups' vessel, three layers of vessel wall were also seen. A conspicuous difference that is inner elastic lamina of the arterial wall was not observed. In addition the external elastic lamina was not observed unambiguously in comparison of the controls. Smooth muscle cells filled the media. The TA was more thickened from the controls (Fig. 4).

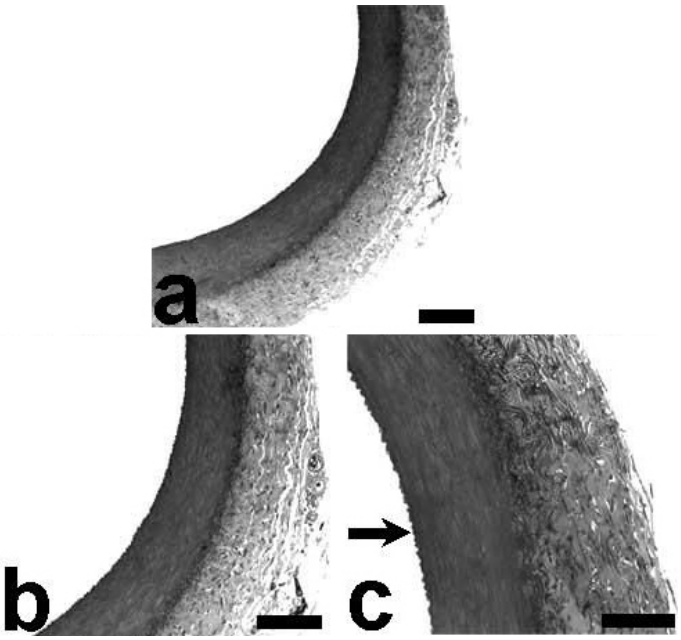

Fig. 4- Arteries of pinealectomy chicks (a-c). Arrow signs endothelia. Scale bar = 80 μm.

3.2. Electron Microscopy Findings

In the arteries of control chicks, the TM was composed almost smooth muscle (Fig. 5). A few fine elastic fibers were scattered among these cells. Internal elastic membrane was clear. In the lumen of artery, many nucleated erythrocytes were found. A result of muscle contraction endothelium of the wall was seen regular in shape.

In the sham controls, the tunica intima consists of a single layer of regular endothelial cells (Fig. 6a, b) and distinct lamina propria. A thin internal elastic membrane is present, beneath which lies the TM that is seen to consist

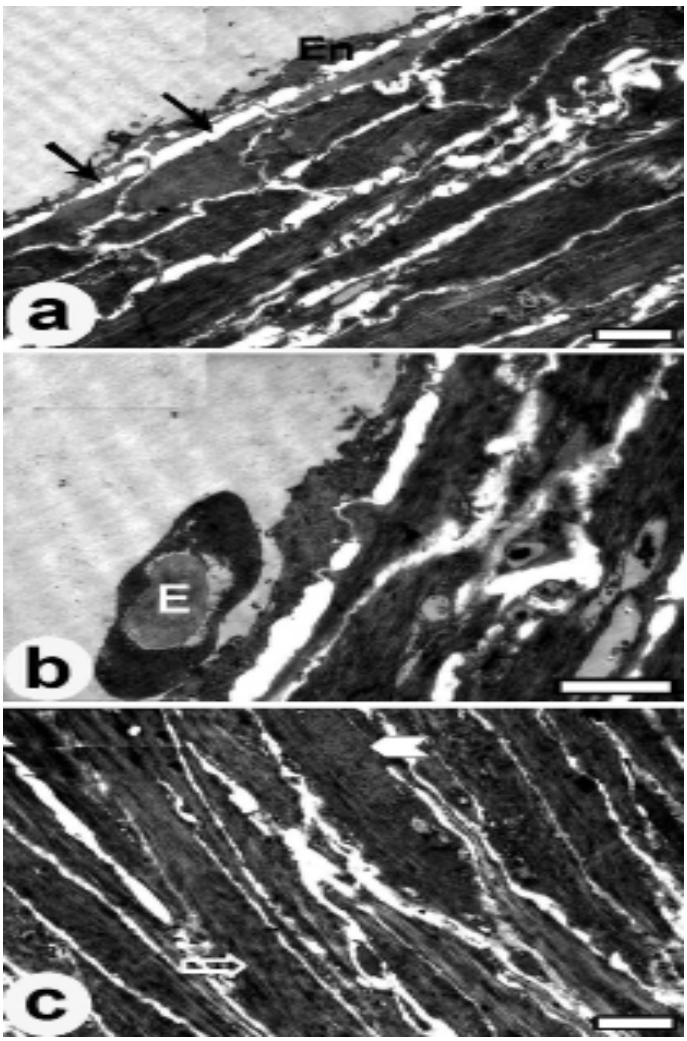

Fig. 5- Electron micrographs of the control chicks'arteries (a-c). Back arrows and transparent arrows show internal elastic membrane and smooth muscle cell in tunica media (TM), respectively. White arrow head reveals nucleus of the smooth muscle cell in (c). Scale bar = 5 μm.

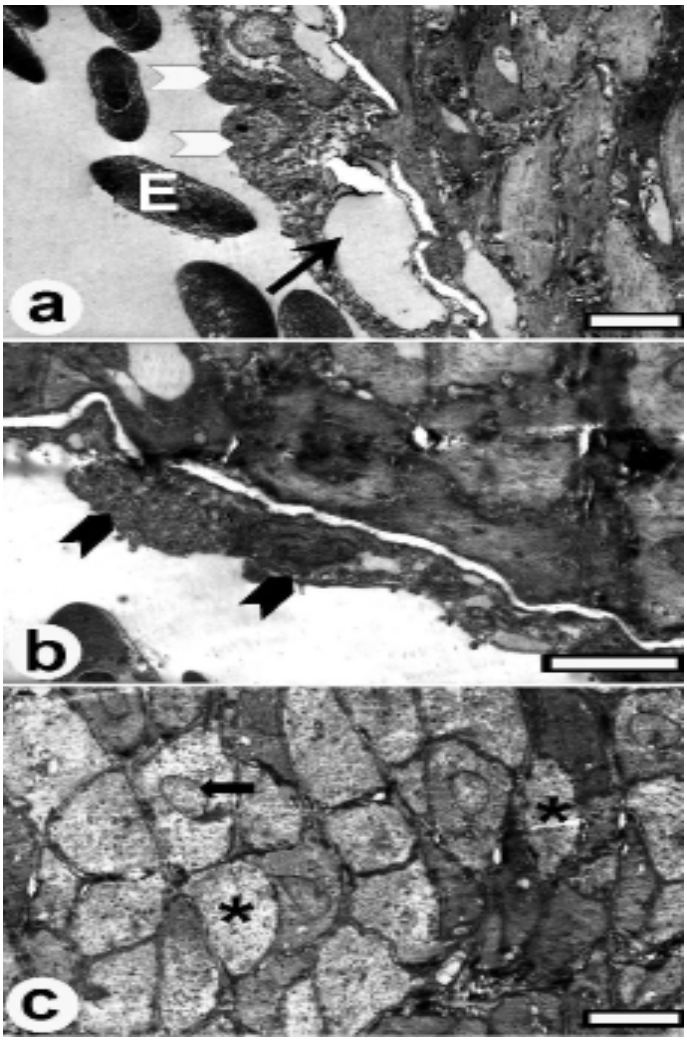

Fig. 6- Sham control chicks' arteries (a-c). Large arrow, capillary at lamina propria in (a); E, erythrocyte; asterisk, smooth muscle cell in tunica media (TM). Small arrow, nucleus of the smooth muscle cell in (c) and black or white arrow head shows endothelia in (a) and (b). Scale bar = 8 μm.

of tightly packed concentric layers of smooth muscle fibers (Fig. 6a, c). In the vessel sections of chicks in pinealectomy group, endothelium with vacuolar degeneration was generally irregular in shape (Fig. 7). Many endothelium cells have dark cytoplasm that is indicating the necrosis. Internal elastic membrane of these vessels was wavy appearance considering existence of vasoconstriction. In TM inter-cellular adhesions were loosen and it may be a sign of the vascular hypertrophy. Besides perivascular fibril accumulation was determined.

3.3. Vessel Cross Sectional Area

Quantitative stereological evaluations for CSA and area fraction values of the vessel wall and lumen were performed in the segment of sciatic artery in the three groups of chickens. The quantitative results of CSA for all groups were summarized in Table 2. The mean CSA of the vessel was greatly increased in surgical pinealectomy group as compared with unoperated control and sham-operated animals ($p<0.05$). The results of the mean volume fraction values of each layer of the vessel wall for all groups of chickens were also summarized in Table 3. A significant difference was observed between unoperated control and pinealectomized, and also sham-operated and pinealectomized chicks for all anatomical parameters ($p<0.01$). Interestingly, surgical pinealectomy procedure resulted in a significantly decreased thickness of TA, in contrast to those of TM.

Table 2. Comparison of the Mean Cross-Sectional Area for All Groups of Chickens at 8 Weeks After Pinealectomy (CSA, cross-sectional area; SD, standard deviation)

Groups	CSA (Mean ± SD) (mm2)
Unoperated control group (n = 5)	0.606 ± 0.306
Sham-operated control (n =5)	0.643± 0.086
Pinealectomy group (n = 5)	0.924 ± 0.294†

Values are expressed as the percentage (Mean ± SD).
† Significantly different from unoperated control and sham-operated control groups ($p < 0.05$).

Table 3. Comparison of the Mean Area Fraction Values of Each Layer of the Vessel Wall for All Groups of Chickens at 8 Weeks After Pinealectomy (TA, tunica adventitia; TM, tunica media; SD, standard deviation)

	Unoperated control group (n = 5)	Sham-operated control group (n = 5)	Pinealectomy group (n = 5)
TA / Vessel wall	0.683 ± 0.03	0.455± 0.034†	0.569 ± 0.04††
TM / Vessel wall	0.317 ± 0.36	0.544± 0.034†	0.431 ± 0.04††
TA /TM	2.199 ± 0.35	0.873± 0.115†	1.347 ± 0.24††
TM / TA	0.467 ± 0.08	1.264± 0.186†	0.770 ± 0.15††

Values are expressed as the percentage (Mean ± SD).
† Significantly different from unoperated control group ($p < 0.01$).
†† Significantly different from unoperated control and sham-operated control groups ($p < 0.05$).

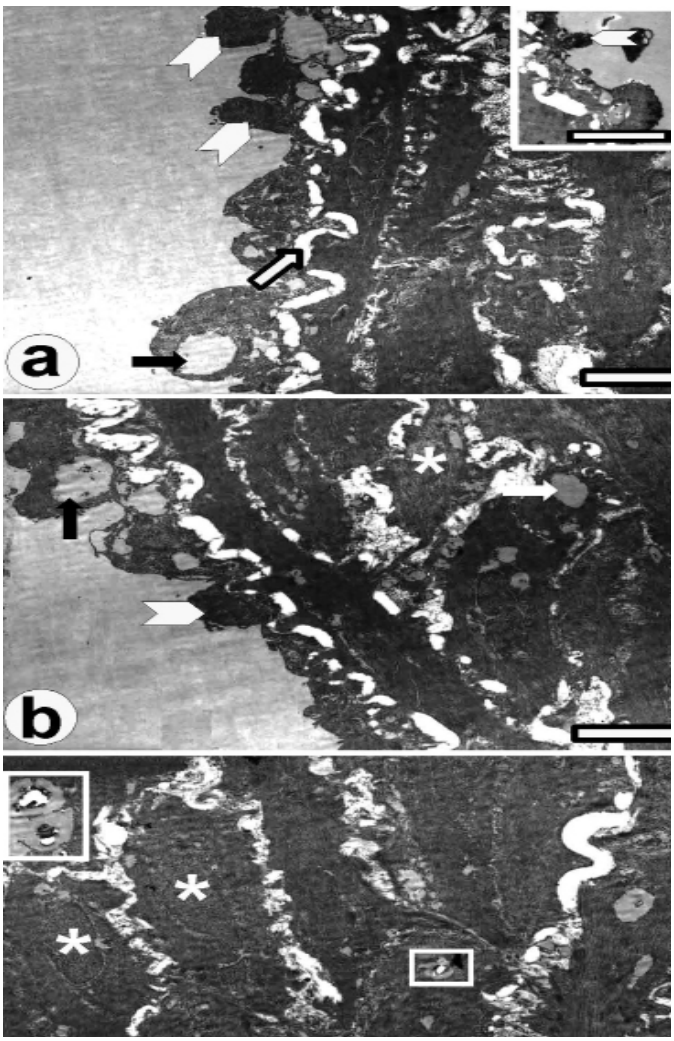

Fig. 7- Micrographs of the pinealectomy artery were shown in (a), (b) and (c) as following. Black arrows; vacuoles in (a) and (b). Transparent arrow; wavy appeared internal elastic membrane, arrow heads; electron dense stained cells indicating necrosis, asterisks; smooth muscle cells, inset points a shrunken necrotic cell in (a) and perivascular fibrillar accumulation in (c). Scale bar = 12 μm.

4. Discussion

The present study shows that pinealectomy procedure resulted in some morphological alterations of the vessel. Recently, Regrigny et al., (2001) demonstrated that there was a decrease in cerebral arteriolar wall thickness in melatonin deficient rats, suggesting a trophic effect of melatonin on the arterial wall mass. Moreover, it is well known that the vessels undergo atrophy of the wall during aging (Hadju et al., 1990) and it has also been reported that melatonin levels decline with age (Sack et al., 1986; Mishima et al., 2001). Therefore, the involvement of melatonin in age-related vessel atrophy is possible. In agreement with this, (Hadju et al., 1990) observed an atrophy of the cerebral arteriolar vessel wall associated with a decrease in passive distensibility in old rats. It is possible that structural changes in the layers of the vessel wall due to melatonin deprival are important in passive distensibility. We demonstrated that melatonin deprival caused a widening of the lumen of blood vessel. In our study, the mean CSA of the vessels in pinealectomy group was significantly larger than the values of the unoperated control and sham-operated animals. This finding is in accordance with those reported for the vasodilator effect of melatonin by some researchers (Shibata et al., 1989; Weekley, 1995; Monroe and Watts, 1998; Anwar et al., 2001). An interesting new finding is that pinealectomy results in a decreased thickness of the TA of the vessel wall, in contrast to that of the TM. This result may come from the dilatation of vessel, i.e. increasing CSA of it, since the major contributor of the vessel wall is known the TA.

The pineal hormone melatonin is synthesized in the pinealocytes of the pineal gland with an endogenous rhythm and has a role in many physiological and pathological processes in human. At present, there are a number of conditions, which are said to be improved by administration of melatonin (Joo et al., 1998; Cuzzocrea et al., 2000; Sun et al., 2002; Pei et al., 2003). Recently, Pei et al., (2003) suggested that treatment with melatonin significantly reduced both the infarct volume and the brain nitric oxide production due to cerebral ischemia in rat stroke models (Anwar et al., 2001) reported that melatonin has an endothelium dependent vasorelaxant effect due to lowered calcium content in vascular tissue. Then, Monroe and Watts, (1998) reported that melatonin could inhibit vascular reactivity or vascular tone in the rat aorta, probably due to its effect on endothelial nitric oxide activity (Weekley, 1995) examined the influences of several drugs on the melatonin-induced vasorelaxation of isolated rat aorta and speculated that melatonin may exert part of its vasoactive actions by an interaction with perivascular nerve terminals. In rabbit basilar arteries, it was found that melatonin has a vasorelaxing action due to inhibition of Ca2+ channels (Shibata et al., 1989). Our findings are consistent with the results of these studies. On the other hand, it was reported that melatonin improved Ca2+ signaling in dysfunctional endothelial cells characterized by an overproduction of free radicals and thereby evoking endogenous vasoconstrictor responses to sympathetic outflow (Muck et al., 1996; Vandeputte et al., 2001; Pogan et al., 2002). In an in vitro study on vascular reactivity in human umbilical artery, Okatani et al., (2001b) indicated that melatonin might potentiate vascular tension in the artery by scavenging endogenous nitric oxide. Geary et al., (1998) suggested that melatonin inhibits endothelial K+ channels to decrease flow-induced release of nitric oxide as well as block smooth muscle K+ channels to enhance vascular tone. A few recent publications indicated a vasoconstrictive effect of melatonin in cerebral, caudal and coronary arteries via activation of either melatonin 1 or melatonin 2 membrane receptors in experimental animals and humans (Laitinen et al., 1992; Viswanathan et al., 1993; Doolen et al., 1998; Regrigny et al., 1999; Ekmekcioglu et al., 2001; Vandeputte et al., 2001; Masana et al., 2002; Pogan et al., 2002; Chucharoen et al., 2003; O'Rourke et al., 2003). Regrigny et al. (2001) demonstrated that melatonin deprival makes the arteriolar wall thinner and decreases distensibility of the cerebral arteriolar wall. Thus, it is evident that the effects of melatonin on vascular tissues are still vague.

However, a detailed description of the effects of pinealectomy on the structural features of the vessel wall has not been reported in the literature. In the current investigation, we describe the effects of melatonin on the vessel wall and CSA of its, using a quantitative stereological method. The experimental data provide information supporting the role of melatonin in the vessel morphology and/or vascular reactivity. Based on our results, it is apparent that the chicken is a useful experimental model for the investigation of the effects of melatonin on the layers of the vessel wall and its lumen. However, the present study has certain limitations. First, the sample size was not large, although the vessel structure of each animal in all groups was investigated. Second, only one type of vessel, the sciatic artery, was studied and it can not be concluded if the blood vessel changes are specific for the studied artery or if there is a general change in all arterial vessels in the body. Logically, not all vessels of the animals in the study could be examined and a different effect on other vessels is possible for other types of vessels, particularly arteries of the muscular versus those of the elastic type, but also veins, arterioles, venules, capillaries as well as vessels of different organs and tissues. Third, measurements on fixed vessels are discussed in terms of vessel wall dynamics, which is not clearly justified. Fourth, the vessel morphology is different between chickens and human. There is no doubt that the sciatic artery of birds may not be comparable to that of quadrupeds. In the present study, an indirect action of could be responsible for its vasodilator effect. It is likely that melatonin's receptors in the vasculature play a role in the effects as we observed. In the study, however, the mechanisms by which pinealectomy procedure in chickens result in the development of vasodilatation and morphological changes are not known, and further studies are needed to clarify the possible role of melatonin behind this phenomenon. In future, it will be interesting to quantify the melatonin receptors in the layers of the blood vessel wall by autoradiography and/or radioreceptor assay. Furthermore, further analysis such as distribution of cellular and matrix components in the vessels that would provide some depth to the study.

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