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Surgical pinealectomy accelerates intervertebral disc degeneration process in chicken

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Abstract Despite the importance of intervertebral disc (IVD) degeneration both in research and clinical practice, the underlying biological mechanism of this phenomenon remains obscure. The current study investigated the effects of neonatal pinealectomy on the development of IVD degeneration process in chicken. Thirty chicks (3 days of age) were divided into two equal groups: unoperated controls (Group X) and pinealectomized chicks (Group Y). Pinealectomies were performed at the age of 3 days. At the age of 8 weeks, magnetic resonance imaging examination of one animal in each experimental group was taken. At the end of the study, serum melatonin level was determined by using ELISA method and histopathological or biochemical examination of specimens from all subjects was done. The results of biochemical analyses were compared using Mann–Whitney *U* test, whereas The Chi-square test was adopted for the histological findings. In this study, the serum melatonin levels in Group Y were significantly lower than those

in Group X ($P < 0.001$). Similarly, scoliosis was developed in 14 out of 15 (93%) in Group Y. Hydroxyproline content of IVD tissue was high in Group Y compared with the values in Group X, although there was no significant difference. Histologically, an appearance of normal IVD was observed in Group X, while the presence of a degenerated IVD was observed in Group Y. From the results of the current study, it is evident that surgical pinealectomy in new-hatched Hybro Broiler chicks has a significant effect on serum melatonin level as well as on the development of IVD degeneration and spinal malformation. In the light of these results from present animal study, melatonin may play a role in the development of IVD degeneration in human beings, but this suggestion need to be validated in the human setting.

Keywords Chicken · Disc degeneration · Pinealectomy · Magnetic resonance imaging · Intervertebral disc · Melatonin

Introduction

Intervertebral disc (IVD) degeneration is a heterogeneous and multifactorial process that is a major problem for the working population [1–3, 6, 9, 21, 28, 30, 35, 37, 39, 42]. It manifests itself as early as in the second decade of

life and the percentage of individuals with the degenerating IVD increases with age [21, 28, 30, 35]. Therefore, the early phases of the degeneration process are frequently observed among children and adolescents [21, 28, 35, 37, 39]. In past decades, the understanding of IVD degeneration and spinal malformations have

received much attention because of its clinical importance. In 1997, Crean et al. [11] reported that the diseases of the IVD, degenerative IVD disease and scoliosis, are both characterized by changes in the extracellular matrix (ECM) components that will affect the mechanical function of the tissue. In particular, the association of degenerative IVD disease and changes in the collagenous matrix has been stressed [4, 18, 27, 28]. Although there is no doubt that genetic predisposition and ageing are the most important determinants, the precise cause for the dramatic degenerative changes in the IVD tissue is poorly understood [6, 9, 30, 42]. Recent research has focused on understanding the biology of the IVD degeneration [14–16, 21, 31]. These have suggested that the biologic treatment of degenerated IVD, including gene therapy, cell-based tissue engineering, and the application of mesenchymal stem cells may be an opportunity to halt or slow the evolution of IVD degeneration [14–16, 31]. Till date, however, there is no effective therapy to reduce the degeneration process, a condition which is very detrimental from the point of socio-economic costs [14, 15].

Melatonin (*N*-acetyl-5-methoxytryptamine) is a multifunctional agent which is secreted from pineal gland to the blood in a circadian manner with a peak at the dark period of the light–dark cycle [5, 7, 23, 32, 34]. Moreover, there is an age-dependent alteration of its circadian secretion during a human lifetime. It has been demonstrated that melatonin levels progressively decline with age, such that in elderly humans melatonin levels are much lower than that of young individuals [32, 34]. These findings suggest the involvement of melatonin in age-related disorders such as the IVD degeneration. Since the first experiment by Thillard [38] in new-hatched chicks, the observation that neonatal pinealectomy leads to an experimental scoliosis in young chickens similar to those in human caused extensive research as an attractive model [5, 7, 19, 23–25, 41, 45]. As melatonin is the main secretory product of the pineal gland, studies to determine the cause of the observed spinal deformities after surgical pinealectomy have focused on melatonin. Furthermore, it has been shown that melatonin has a role on fibroblast collagen production in experimental studies [12]. However, the possible role of melatonin on the IVD biology has not been studied so far. The aim of this study was to determine the effects of neonatal pinealectomy on the development of the IVD degeneration process in chicken.

Materials and methods

Experimental animals and groups

A total of 30 new-hatched Hybro Broiler chicks (3 days of age; 40–70 g) of both sexes were obtained from a local

hatchery (Institute of Agricultural Research of Erbeyli). All the chickens were kept in individual cages under constant laboratory conditions at 20°–22°C room temperature, a 12-h light–dark cycle (6 a.m.–6 p.m.), with cool white fluorescent bulbs. They were given free access to commercial chicken diet that did not contain melatonin and serotonin, and water ad libitum. The chicks were divided randomly into two groups: Group X, intact control group of 15 chicks in which no surgical intervention was performed; and Group Y, surgical group of 15 chicks in which pinealectomy procedure was performed. The experimental protocol was reviewed and approved by the Ege University School of Medicine Animal Care and Use Committee.

Pinealectomy procedure

Surgical pinealectomy procedure was performed under the general anesthesia of intraperitoneal sodium pentobarbital (Nembutal sodium®, Abbott Laboratories Comp., Istanbul, Türkiye, 40 mg/kg body weight). The part under surgical intervention after shaving was disinfected by using polyvidon iodine. In aseptic conditions, a 2-cm midline incision was made at the junction of frontal and parietal bones over lambdoid fontanel and a skull flap was raised with a scalpel. Then 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. The skin was sutured with vicryl 6/0.

Melatonin assay

Eight weeks following the pinealectomy procedure, 5 ml of blood sample was taken immediately from a peripheral wing vein of each chicken using a 20-gauge needle at 1 p.m.–3 p.m. and put into a glass tube without heparin, and stored at 4°C for 24 h. Then, blood samples were centrifugated at 3,000 rpm for 20 min and the collected serum was stored at –85°C until assayed. Melatonin concentrations in serum samples were measured via enzyme-linked immunosorbent assay (ELISA) using commercial kit (IBL, Cat. No. RE 540 21, Hamburg, Germany). All samples from each subject were assayed in duplicate to ensure reliability.

Magnetic resonance imaging examination

Magnetic resonance imaging (MRI) examination of one animal in each experimental group was performed using a 3-in surface coil. The surface coil was placed behind the lumbar region of vertebral column in 1.5-T MRI imager (Signa, Horizon, GE Medical Systems,

Milwaukee, WI, USA) to obtain T2-weighted images of the IVD in the sagittal plane. In each animal, the signal intensities of three consecutive IVDs in the lumbar region were measured on the midsagittal sections as an expression of IVD degeneration. After imaging, the animals were sacrificed for subsequent biochemical or histopathological evaluation.

Gross examination

At the end of study period, the chickens in each group were killed using an overdose of anesthetic after collection of blood samples and/or MRI examination and their weights were determined as an index of growth. Vertebral columns of the chickens were dissected out along with the thoracic cage and photographs were taken for comparison before biochemical and histopathological evaluations.

Tissue collagen measurement

In each experimental group, ten animals were randomly selected for collagen assay in the IVD material. Hydroxyproline was chosen to determine the collagen content of the IVD tissue. Aminoacid hydroxyproline was determined as previously described by Reddy and Enwemeka [33]. The presence of the aminoacid hydroxyproline in collagen (about 11–13%) is a unique feature because this aminoacid occurs in only a few other proteins like elastin. Therefore, hydroxyproline has been used for many years as a means of determining the amount of collagen present in a tissue. For the assay, O-ring screw-capped high temperature glass tubes were used. Aliquots of standard hydroxyproline and test samples were then mixed with a buffered chloramine-T reagent and oxidation was allowed to proceed for 25 min at room temperature. The chromophore was then developed with the addition of Erlich's reagent, and the absorbance of reddish purple complex was measured at 550 nm using a spectrophotometer. Absorbance values were plotted against the concentration of standard hydroxyproline, and the presence of hydroxyproline in unknown tissue extracts was determined from standard curve. The collagen content of the IVDs was calculated assuming that 12.5% of collagen is hydroxyproline [13]. Results of biochemical study were expressed as $\mu\text{g}/\text{mg}$ of tissue weight.

Histopathological evaluation

The remaining five animals from each experimental group were used for histopathological evaluation. In each animal, the spine including three consecutive IVDs

in the lumbar region was cut into blocks of equal length followed by fixation with 10% buffered formaldehyde. The blocks were then decalcified in 5% hydrochloric acid. A representative midsagittal section was taken from each and were embedded in paraffin wax. The tissue sections were stained with hematoxylin and eosin (HE) for light microscopic observation. The sections were examined by histologists unaware of the experimental group with light microscopy for the following indicators of disc degeneration process: (1) cellular degeneration; (2) granular matrix changes; (3) mucous degeneration; (4) fibrocartilage fibrillation [46, 47]. In each animal from both groups, all parameters for each IVD were semiquantitatively evaluated ranging between "absent," "slight," "moderate," and "marked," as a modification of those described elsewhere [21].

Data analysis

Data were expressed as arithmetical mean $\pm$ standard error of measurement (SEM) for each group. Comparisons of weights, length of spines, serum melatonin levels, and collagen content of the IVD tissue were made by Mann–Whitney *U* test, whereas Chi-square test was used for the histological findings. A *P*-value of less than 0.05 was considered significant.

Results

In this study, all animals showed no evidence of gross neurophysiological deficit and no postoperative wound infections. In both groups, the chickens outwardly showed normal growth as measured by body weight. No significant difference could be detected in the mean weight of the chickens between the two groups (data not shown).

Magnetic resonance imaging findings

MRI images show the normal IVD in Group X as having high signal intensity of the lumbar IVDs on T2-weighted sagittal image, while the degenerated disc in Group Y showed low signal intensity (Fig. 1). The change in signal intensity correlates with the decrease in water content of IVD tissue, suggesting the development of degeneration. In Group X, the average signal intensity (193.3 ± 11.4) was significantly larger than those found in group Y (112.4 ± 10.5) (Table 1).

Serum melatonin level

The average serum melatonin level from the chickens subjected to pinealectomy in Group Y was low

Fig. 1 Magnetic resonance image (T2-weighted sagittal slice) through the lumbar spine of **a** a control chicken in Group X showing normal IVD, **b** a pinealectomized chicken with degenerated IVDs and scoliotic curvature in Group Y

Table 1 Signal intensity of IVDs on lumbar MRI in both groups of chickens at 8 weeks of age after neonatal pinealectomy

Groups	<i>n</i>	T2 signal intensity ^a
Group X	3	193.3 ± 11.4
Group Y	3	112.4 ± 10.5

Values are expressed as means ± SEM

^a Mean signal intensity of the three consecutive IVDs assessed by MRI of the lumbar spine in both groups of chickens

(18.7 ± 2.4). In contrast, the average value for Group X was significantly higher than those in group Y (31.3 ± 0.6, $P < 0.001$) (Table 2).

Macroscopic observations

All chickens in both groups were examined by visual observation and palpation for the presence of spinal malformation. Fourteen out of the 15 cases (93%) in Group Y developed scoliotic curvature (Fig. 2).

Table 2 Serum melatonin levels in both groups of chickens at 8 weeks after neonatal pinealectomy

Groups	<i>n</i>	Serum melatonin (pg/ml)
Group X	15	31.3 ± 0.6
Group Y	15	18.7 ± 2.4 ^a

Values are expressed as means ± SEM

^a Significant different from controls ($P < 0.001$)

Tissue hydroxyproline content

Hydroxyproline content in the IVD was high in the chickens subjected to pinealectomy in Group Y compared with control values in Group X, although there was no significant difference between these groups ($P > 0.05$) (Table 3).

Fig. 2 A typical specimen example of a chicken spine at the end of experiment showing **a** straight in Group X, **b** severe scoliotic curve in Group Y

Table 3 Hydroxyproline contents of the IVD tissue in both groups of chickens at 8 weeks after neonatal pinealectomy

Groups	<i>n</i>	Hydroxyproline content (mg/100 mg tissue)
Group X	10	1011.4 ± 176.9
Group Y	10	1226.9 ± 211.5

Values are expressed as means ± SEM

Light microscopy

Histologic examination of the specimens revealed an appearance of normal IVD and parts of adjacent vertebrae of chickens in Group X, while the presence of a degeneration of IVD in Group Y. Furthermore, the area of the cartilage endplate regions adjacent to the degenerated IVD in a pinealectomized chicken in Group Y was increased compared with those in Group X (Fig. 3). Results of semiquantitative evaluation of IVDs of animals from each experimental group for histological degeneration process were given in Table 4. In Group Y, degenerative changes were marked in two IVDs (13%) and mild or moderate in the remaining 12 IVDs (80%). In contrast, out of three consecutive IVDs of a chicken without spinal deformity from Group Y, two showed mild degenerative changes and the remaining one exhibited no sign of degeneration, indicating that the histological changes of degeneration may be a secondary effect of spinal deformity. Statistically, there was a significant difference between Group X and Group Y ($P < 0.05$).

Discussion

The IVD has a complex structure which contains very few cells embedded in an ECM [11, 14–17]. These cells are very important for the function of maintaining and repairing the ECM by synthesizing macromolecules and proteinases for its breakdown [11, 17]. When there is an imbalance between ECM synthesis and breakdown, its composition and organization alters and the cellular

Table 4 Histological findings in the IVD tissue from both groups of chickens at 8 weeks after neonatal pinealectomy

Groups	<i>n</i>	Degenerative changes (%) ^a			
		Absent	Slight	Moderate	Marked
Group X	15	6 (40)	6 (40)	3 (7)	0 (0)
Group Y	15	1 (7)	4 (27)	8 (53)	2 (13)

^a There was a significant difference between Group X and Group Y ($P < 0.05$)

repair responses are inadequate [11]. The degeneration process, which consists of biological changes in nucleus pulposus and gross structural changes in anulus fibrosus and cartilage endplate, rapidly begins [9, 29, 42]. Degenerated IVDs have only a small hydrostatic region, whereas a healthy IVD contains a soft and highly hydrated central region known as the nucleus pulposus [1–3]. This alters the mechanics of the vertebral column and leads to painful and disabling conditions. Today, the biology of the degenerative IVD disease remains one of the most controversial topics in the spine literature [29].

Radiologically, MRI is a sensitive measure of the IVD degeneration process [22]. On MRI, degenerative changes are seen as loss of T2 signal intensity from the nucleus pulposus which is the only sign of aging [6]. In clinical practice, signal intensity has been used as an indicator for IVD degeneration [6, 22, 37]. In the current study, T2-weighted sagittal scans of the chicken spine revealed a degenerated IVD in pinealectomized chicken in Group Y, whereas a normal IVD finding in control group. As expected, this finding showed a positive correlation with the increased collagen content measurement in the IVDs of pinealectomized chickens compared with those of the control group. Our results were most probably related to the development of degeneration process in the avian IVD tissue. In a previous study, Antoniou et al. [4] found that the synthesis of Type II collagen was higher in the scoliotic IVDs compared with the normal ones. In general, our findings suggest that degenerative changes such as increased collagen content

Fig. 3 Histological examination of sagittal section of IVD (*id*) in **a** intact control (Group X) and **b** pinealectomy (Group Y) groups. HE stain; scale bars are 500 micron. Note the existence of an increase in the cranial and caudal cartilage endplate areas of degenerated IVDs in a pinealectomized chicken in Group Y (*arrows*)

and scoliotic curve may be due to melatonin deprivation. Thus, it is likely that the chicken provides a reliable and useful animal model to characterize the biological effects of melatonin on the development of the IVD degeneration. In the chicken, the pineal gland is a small organ attached by a long pineal stalk to the third ventricle, lying in the space between the cerebral hemispheres and the cerebellum deep to the skull [20]. Surgical removal of the gland or the section of its stalk, known as pinealectomy, is always accompanied by a significant decrease in serum melatonin levels [7].

In the current study, the chickens in the control group exhibited no spinal malformation, but a spinal malformation was observed in 93% of the pinealectomized chickens. In the literature available, the production rate of spinal lateral curvature after surgical pinealectomy varied from 52% to 100%, owing to the differences in the observation periods [5, 7, 10, 19, 23, 25, 30]. To our knowledge, limited experimental studies of spinal malformation development following neonatal pinealectomy in the chickens, including ours, have been reported in the literature available [5, 10, 19, 24, 25, 45]. All of them have demonstrated that spinal malformation did not develop in all cases after surgical ablation of the pineal gland in new-hatched chicks, even though serum melatonin levels were significantly low [5, 7, 19, 45]. To date, however, there is no study investigating the effects of neonatal pinealectomy on the development of IVD degeneration in animals in the literature. Interestingly, experimental pinealectomy induced various spinal malformations such as lateral curvature and axial rotation in both chicken and bipedal rats, as seen in idiopathic scoliosis in humans [26]. It was speculated that dysfunction of specific melatonin receptors in the spinal cord due to decreased melatonin production might be responsible for the development of various vertebral deformities such as scoliosis [23, 44]. In our previous study, we demonstrated that pineal gland transplantation following pinealectomy in young chickens has no significant effect on the development of spinal malformation and serum melatonin level [41]. Our study was sharp contrast to the results of Machida et al. [24], who had demonstrated that transplantation of the pineal gland after pinealectomy in new-hatched chicks leads to reestablishment of normal serum melatonin levels and a significant reduction of scoliotic curvature. The reason for the differences between these two studies is not obvious; thereby, the role of melatonin in the development of malformations of the vertebral column in chickens after pinealectomy remains controversial.

In this study, we observed that surgical pinealectomy procedure leads marked histological degenerative changes in IVD tissue of pinealectomized chickens. Furthermore, there was a prominent increase in the area of the cartilage endplate regions of degenerated IVDs in pinealectomized chickens compared with those in the

intact control group. This histological finding was consistent with our previous evidence related to increased vascular channel count in the cartilage endplate in the presence of IVD degeneration process [40]. However, the current study has certain limitations. One of them was the small sample size for the evaluation of signal intensity, although three consecutive IVDs in each group were investigated. Only one animal from each group was examined using MRI because of the cost of the examination. Obviously, it was also more informative to perform a stereological analysis for further evaluation of some histological parameters, instead of only semi-quantitative evaluation of IVD tissue. Another criticism was the need for a longer observation period for assessment of the natural course of IVD degeneration in chickens. Although the common bipedal feature of chickens and humans is critical for the development of lateral curvature and axial rotation in the spinal column, there is a phylogenetic difference between avians and mammals. Furthermore, it is also possible that age-related degenerative changes may develop in the IVD of chickens. It is noteworthy that in the current study, this phenomenon has been observed by neurosurgeons in neither children nor adults after different surgical approaches for lesions of the pineal region [43]. Such a confusing disparity between avian and mammalian species warrants additional study in future. Therefore, it seems difficult to directly extrapolated the results of the experimental studies involving chickens to the clinical studies in human beings. Importantly, the collagen content of IVD tissue is also considered to play a regulatory role in osteoblastic growth and differentiation [36]. Our radiological, biochemical, and histological findings confirmed that surgical pinealectomy procedure accelerates IVD degeneration process in chicken. In a previous study, we demonstrated that the use of exogenous melatonin reduced the cartilage endplate vascularity of degenerated IVDs, suggesting that this neurohormone may have an osteoinductive effect on bone formation [40]. More studies are needed on the role of melatonin, acting via specific melatonin receptors, in the development of IVD as well as bone mineralization after neonatal pinealectomy in chickens.

Conclusion

Considering the current data available, it is now clear that: (1) surgical pinealectomy is always accompanied by a significant decrease in serum melatonin levels in young chickens; (2) pinealectomy procedure may produce various spinal malformations such as scoliosis in chickens; (3) neonatal pinealectomy is associated with the development of degenerated IVD in the pinealectomized chickens during 8-week follow-up; (4) the histological features of IVD degeneration in melatonin-deficient young chickens

are similar to age-related alterations in the IVDs of humans; and (5) the underlying mechanism of IVD degeneration and spinal malformation observed at the end of our experiment is probably a significant reduction of serum melatonin level after neonatal pinealectomy. In conclusion, the current study provided evidence that melatonin has a significant implication in both degenerative IVD disease and various spinal malformations such as scoliotic curvature. This information will aid in targeting appropriate treatment for IVD degeneration and spinal malformations. In the current study, the mechanisms by which neonatal pinealectomy procedure in chicken results in the development of IVD degeneration

are not known, and further studies are needed to clarify the possible role of melatonin in this phenomenon. In future, it will be interesting to elucidate the changes in the distribution pattern of the major collagen types in the IVD tissue using specific immunohistological markers during the evolution of the degeneration process observed after neonatal pinealectomy.

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