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Research article

Immunohistochemical study of TH, DBH and PNMT in the adrenal medulla of the adult jungle crows (*Corvus macrorhynchos*)

Kober, A. K. M. H.[1*], Aoyama, M.[2], Sugita, S.[2]

[1]*Department of Dairy and Poultry Science, Chittagong Veterinary and Animal Sciences University, Khulshi, Chittagong-4225, Bangladesh*

[2]*Department of Animal Science, Faculty of Agriculture, Utsunomiya University, 350 Minemachi, Utsunomiya-shi, Tochigi 321-8505, Japan*

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*Corresponding author:
Email: humayuna2002@yahoo.com
Tel.: +8801712164794

A B S T R A C T

The localization of 3 catecholamine-synthesizing enzymes, tyrosine hydroxylase (TH), dopamine ß-hydroxylase (DBH) and phenylethanolamine N-methyltransferase (PNMT), were investigated in the adrenal medulla of the jungle crow (*Corvus macrorhynchos*) using the immunohistochemical peroxidase-antiperoxidase method. TH-immunoreactivity (IR) was observed in almost all adrenal medullary cells, whereas DBH- and PNMT-IR were not observed in the adrenal medullary cells. These results indicated the localization of TH enzyme, but not DBH and PNMT, in an active form within the adrenal medullary cells of the jungle crow.

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INTRODUCTION

The biosynthesis of catecholamines in the adrenal medulla results from a series of consecutive steps catalyzed by major 3 enzymes: tyrosine hydroxylase (TH), dopamine ß-hydroxylase (DBH) and phenylethanolamine N-methyltransferase (PNMT). The first step is the conversion of tyrosine into dopa by TH. DBH is needed to convert dopa to norepinephrine (NE), and PNMT is required for the conversion of norepinephrine to epinephrine (E). Physiological actions of the catecholamines are diverse. The NE functions primarily as a neurotransmitter. Both NE and E influence the vascular system, whereas epinephrine affects metabolic processes, such as carbohydrate metabolism. In avian species, normal adrenal medullary tissues produce varying amounts of catecholamines (Ghosh, 1977, 1980). While the concentrations of catecholamines in extracts of serum and tissues have been measured, the immunohistochemical detection of catecholamines in tissue sections has not been examined

extensively. Recently, Kober *et al*. *(*2010) reported the cellular localization of three catecholamine biosynthesizing enzymes (TH, DBH and PNMT) in chicken adrenal medulla and suggested that the majority of medullary cell are E-containing cells (Kober *et al*., 2010). Ohmori *et al.* (1997) and Ohmori (1998) stated that TH-immunoreactivity (IR) was observed in almost all adrenal medullary cells. In addition, many medullary cells exhibited PNMT-IR, although some cells were PNMT-immunonegative, and PNMT is considered a marker of E synthesis. However, most of these studies focused on the adrenal glands of domestic birds, and little attention has been paid to the adrenal glands of wild birds, including that of the jungle crow. The jungle crow is a common bird in Japan. They live in an environment separated from that of domestic birds, and may have different immunohistochemical characteristics of their adrenal glands that are different from that of domestic birds. It is assumed that there might exist different mechanisms involved in synthesizing catecholamine in the wild birds. So it is important to determine what kinds of catecholamine-synthesizing enzymes are localized in the adrenal medullary cells of the jungle crow. Therefore, the present study was carried out in order to demonstrate the cellular localization of TH, DBH and PNMT and to identify NE and E cells in the adrenal medulla of adult jungle crows by immunohistochemical methods.

MATERIALS AND METHODS

Collection of birds and tissue processing

Six adult healthy Japanese jungle crows (3 males, 3 females) weighing average 600-800 gm were used for this study. The jungle crows were captured from the forests in Ueno, Heirin Temple and the Experimental Farm of Utsunomiya University located in Moka City, Japan. All the birds were fed a commercial diet and water was provided ad libitum during the pre-experimental period. All of the jungle crows were cared for according to guidelines suggested in the care and use of laboratory animals at Utsunomiya University. The capture of crows was permitted by Saitama Prefecture (Permit for the Catching of Wild Animals No. 01-02) and Tochigi Prefecture (Permit No. 0010). The jungle crows were considered as adult by observing the rudimentary size of the bursa of Fabricius and the black color of the upper palate of the beak. All procedures involving animals were carried out in accordance with the Animal Protection Regulations of Japan.

For histomorphological analysis, all of the jungle crows were killed by an overdose of pentobarbital sodium (30 mg/kg body weight; Dainippon Pharmaceutical, Osaka, Japan). The birds were then perfused transcardially with a Ringer solution and fixed by perfusion with Zamboni's solution. Then, the adrenal glands were post-fixed with Zamboni's solution overnight at 4^{o}C. Tissue blocks were prepared and embedded conventionally in paraffin. Serial sections were cut at a thickness of 5 µm in a coronal plane using a microtome (Sakura sledge microtome IVS-400; Sakura Seiki, Tokyo, Japan) and mounted on glass slides.

Immunohistochemistry

Simple immunohistochemical staining was performed using the peroxidase-antiperoxidase (PAP) method (Sternberger, 1979). In short, after dewaxing, rehydrated sections were treated with 3% hydrogen peroxide (H_2O_2) in methanol for 30 minutes at room temperature to block the endogenous peroxidase activity, followed by rinsing for 15 minutes in 0.01M phosphate buffered saline (PBS) at pH 7.4. Background staining was prevented by incubating in 2% normal goat serum and 2% bovine albumin. The sections were incubated at 4^{0}C for 24 hours in rabbit anti-bovine TH, DBH, and PNMT polyclonal antibodies (LS-C 232, DZ1020, and PZ 1040; Biomol International Inc., USA) diluted to 1:500-1000 with PBS pH 7.4 containing 2% normal goat serum and 2% bovine albumin. Subsequently, the sections were rinsed 3 times in PBS for 15 minutes. The sections were incubated for 20 minutes in goat-anti-rabbit IgG (ICN Pharmaceuticals Inc., Aurora, USA) diluted 1:500 in PBS. Thereafter, the sections were incubated for 20 minutes in rabbit ABC complex (ICN Pharmaceuticals Inc., Aurora, USA) diluted 1:500 in PBS. The immunoreactions were visualized using a freshly prepared solution of 3, 3'-diaminobenzidine tetrahydrochloride (DAB, Dojin Laboratories, Kumamoto, Japan; 10 mg in 50 mL of 0.05 M Tris–HCl buffer pH 7.6 containing 1.5 mL H_2O_2 at 0.03 %, and 0.15g ammonium nickel at 0.3%). Lastly, all sections were dehydrated by ascending concentrations of alcohol and mounted. The sections were observed using a light microscope (Olympus BX51). Some sections were incubated without primary antibody as negative controls. We did not find any expression of the enzymes in the control sections.

RESULTS

Immunohistochemical observation

Tyrosine hydroxylase (TH)

When sections of the jungle crow adrenal glands were incubated with antibody to TH, specific reaction was observed in the adrenal medullary cells (Figure A, D). TH- IR was observed in most adrenal medullary cells (Figure A, D). Staining was limited to the cytoplasm of medullary cells and was evenly distributed. TH-IR medullary cells were either solitary or formed large clusters (Figure A). A considerable number of medullary cells showed strong TH-IR, and these cells were distributed throughout the adrenal gland. TH-IR cell clusters were seen as islets, isolated islets, large islets and elongated islets (Figure D).

Dopamine-beta-hydroxylase (DBH)

DBH-IR was not observed in the adrenal medullary cells, and which did not coincide with TH-IR-containing cells clusters (Figure B).

Phenylethanolamine-N-methyltransferase (PNMT)

PNMT-IR was not observed in the adrenal medullary cells, which did not coincide with TH-IR containing cells clusters (Figure C).

DISCUSSION

In this study, it was observed that TH-IR medullary cells were intermingled with cortical cells in the jungle crow adrenal gland. TH, which is a key enzyme in the synthesis of catecholamine, was localized in the adrenal medullary cells. TH-IR was observed in adrenal medullary cells of the jungle crow adrenal gland. These observations were consistent with the descriptions of medullary cells in jungle crow adrenal medulla (Kober *et al.,* 2012), in which TH-IR is detected in almost all medullary cells. In the present study, it was identified that staining was limited to cytoplasm of medullary cells and evenly distributed. This result is similar to that of Kober *et al*. (2010) and Ohmori *et al*. (1997) who showed that TH staining was evenly distributed in their cytoplasm in medullary cells of the chicken adrenals. But in this study, DBH-IR was not detected in the adrenal medullary cells, which did not coincide with that of TH-IR cell clusters. This result did not correspond with our previous study in chicken (Kober *et al.,* 2010), and it suggests that DBH-IR is detected in all medullary cells of chickens by using same anti-Bovine DBH. These unexpected phenomena may be explained by the possibility of existence of DBH in inactive form in all adrenomedullary cells of Jungle crow.

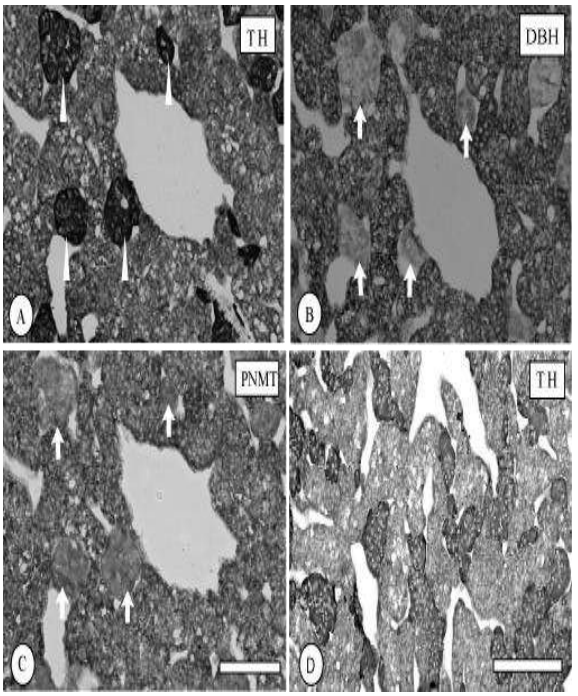

Figure: Immunoreactivity using antisera against three catecholamine biosynthesizing enzymes in the adrenal gland of the Jungle crow. Parts of three consecutive paraffin sections (A, B and C) were immunostained with antiserum to tyrosine hydroxylase (TH, A), dopamine β-hydroxylase (DBH, B), and phenylethanolamine N-methyltransferase (PNMT, C), respectively. TH immunoreactivity (IR) was observed in most adrenal medullary cells and a considerable number of medullary cells showed strong TH-IR (arrowheads in A). DBH- and PNMT-IR were not observed in medullary cells and that cells did not coincide with TH-IR cells (arrows in B, C respectively). Scale bar in A, B & C indicates 50 µm. (D) Sections immunostained for TH. TH-IR cell clusters were seen as islets, isolated islets, larger islets and elongated islets. Scale bar indicates 100 µm.

In addition, PNMT-IR was not detected in the adrenal medullary cells. In contrast to chickens and mammals, where distinct PNMT positive and negative cells have been identified, such cytoenzymologic classification was not obtained. The present result did not correspond with that of Kober *et al.* (2010) and Ohmori *et al*. (1997), where they show that adrenal medulla in the chicken contains many PNMT-IR and some PNMT-immunonegative cells. On the other hand, our result corresponds with that of Guha *et al*. (1992), who suggested that distinct PNMT-IR cells indicating E cells and PNMT-immunonegative cells indicating NE cells could not be obtained in the adrenal medullary

cells in the four avian species (pigeon (*Columba livia***)**, house crow (*Corvus splendens*), owl (*Bubo virginianus*) and woodpecker (*Campephilus principalis*)). It may be significant to note that they were unable to identify E and NE synthesizing cells in the four avian species. According to our previous study, when the adrenal glands of chickens were incubated with same anti-Bovine PNMT antibody, many medullary cells exhibited PNMT-IR, but some cells were PNMT-immunonegative. Using PNMT antibody, chicken adrenal medullary cells were divided into two types: one type showing PNMT-positive (presumably the E cells), and the other type showing PNMT-negative (presumably the NE cells) (Kober *et al.,* 2010). These studies show that the bovine PNMT acts as a marker enzyme in differentiating E and NE cells in the chicken adrenal medullary cells. However, there exist several explanations for this anomaly. One possibility is that PNMT might be inactive, or alternatively, might not use to synthesize epinephrine, as suggested by Foster *et al*. (1985). Furthermore, the PNMT might exist in all adrenomedullary cells of jungle crow, and only in some cells it exists in an active form and plays role in conversion of NE to E (Ghosh *et al.,* 2001). This discrepancy may also be explained that the DBH and PNMT level may be too low to be detected by our technique, although in the chicken adrenal medulla showed DBH-or PNMT-IR by this technique. In addition, DBH- or PNMT-immunoreactive cells of adrenal medulla might not immunoreactive to the antibodies used in this study. In the frog adrenal medulla, the properties of the PNMT found in both medullary cells and neurons indicate that this enzyme is a different protein from mammalian PNMT (Wurtman *et al*., 1968). In relation to this study, the properties of the PNMT in the jungle crow adrenal medullary cells may be different from that of bovine PNMT. This anomaly may also be explained by the fact that the antibodies against bovine antigens may not be suitable for identifying these avian proteins, either they do not recognize the avian homologue or gene sequence are not homology with the Jungle crow.

CONCLUSION

In conclusion, immunohistochemical features of the adrenal gland of the Jungle crow were similar to the general avian adrenal structures but showed species-specific differences. PNMT-IR was not observed in the adrenal medullary cells; therefore, PNMT may not act as a marker enzyme in differentiating the E and NE cells, similar to results in pigeon, house crow, owl and woodpecker but different from chicken. DBH and PNMT may exist as different form compared with domestic birds like the chicken. All of the above explanations for this anomaly, however, are speculative; the real phenomena of this discrepancy of the present findings in the Jungle crow adrenal medullary cells remain unclear. However, further investigations in this area may bring forth useful information.

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