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Histological and immunohistochemical studies on adrenal gland of immature and mature dogs.

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ABSTRACT

In this study, fourteen male dogs in two groups (immature and mature ages) are used to study the histological structure of the adrenal gland and immunohistochemical reactions of vascular endothelial growth factor, Beta catenine, and Synaptophysin. Histologically the adrenal gland consists of stroma and parenchyma, the capsule of immature dogs is delicate but thick at mature ages, and the cortex is arranged into Zona Arcuta, Faciculata and Reticularis. ZA arranged in arch-like structures that appear more in the mature ages. ZF arranged into cords of highly vacuolated cells than ZA and ZR and between these cords blood sinusoid appears more distinct in the immature one. The medullary cells are arranged into follicles around blood sinusoids found between cortical cells of immature glands and centrally localized in the glands of mature ages. Immunohistochemically, VEGF gives a strong reaction in cortical and medullary cells of immature ages and a moderate reaction in mature ones. Beta catenine shows strong reactions in adrenal gland of immature ages and a weak reaction of immature ones. Synaptophysin gives a strong reaction in medulla of immature age and a weaker reaction in mature ones. From these results, we conclude that the Beta catenine localization associated with zonation of the adrenal gland of dogs and VEGF localization and higher expression in immature animals might be related to angiogenesis of adrenal gland blood vessels. Similarly, the localization and the strong expression of Synaptophysin in the medulla of immature animals might related to the neuroendocrine differentiation of developing medullary cells.

1.INTRODUCTION

Dog as a pet animal provides companionship in our homes and makes us feel less alone [**1**], dogs are widely used as lab animals **[2]** and in forensic medicine**[3].**The adrenal gland is one of the endocrine systems that secrete various hormones in different conditions **[4]**. It regulates multiple physiological functions such as metabolism and stress response. Insufficient normal adrenal function will result in disruption of carbohydrate metabolism, and electrolyte levels resulting in death **[5]**. In dogs, the adrenal gland has clinical importance where the increase or decrease of its hormonal activities results in common disorders; Cushing and Addison diseases [**6, 7]** respectively. Histologically, the adrenal gland comprises two separate portions; the cortex and medulla, which originate from the mesoderm and ectoderm, respectively **[8].** In the dog, cortical cells are arranged into three zones; arcuata, fasciculata, and reticularis, extending from the adrenal capsule to the medulla **[9]**. Most of these cells are rich in mitochondria, smooth endoplasmic reticulum **[8]** additionally, cells of zona fasciculate are rich in numerous lipid droplets so-called "clear cells or spongiocytes" Functionally, cells of zona arcuata, fasciculate,andreticularis secrete mineralocorticoids, glucocorticoids, androgen **[10]**.The adrenal medulla is composed of two types of cells: chromaffin and ganglionic **[8]**. These medullary cells are arranged in cords that contact fenestrated capillaries with a wide lumen. They are innervated by preganglionic sympathetic axons and are divided into two types: adrenaline-secretingcells and noradrenaline-secreting cells **[11]**.

Normal aging of the adrenal gland is associated with changes in its structure and function. The most common structural changes that occur in adrenal glands are lipofuscin and hemosiderin accumulation and fatty changes **[12]**. These various morphological changes of the adrenal gland that occur during aging are associated with alterations in hormonal output, such as, an increase in glucocorticoid secretion and a decrease in adrenal androgen levels **[13]**.

Many studies discussed histological changes in the adrenal gland of different species with age; in cattle ***[14, 5]***, *in buffalo* ***[15, 16]***, *in camel* ***[17,18]***, *in* sheep and goat **[19,20]**, in pig **[21,22]**. However, in dogs, little investigations on histological changes of the adrenal gland depending on maturity were reported **[18]** .Therefore, the present study aimed to investigate histological and ultrastructural changes in the adrenal glands of immature and mature male dogs. Moreover, immunohistochemical changes of synaptophysin (SYNP), B-catenin, and vascular endothelial growth factor (VEGF) expressions in the adrenal gland were evaluated.

2-MATERIALS AND METHODS

1-Animals

Fourteen healthy male local dogs were used in the present study. The animals were collected from Sheibin El-kom, Menoufia governorate, Egypt, and were brought into Histology department, Faculty of Veterinary Medicine, Menoufia University, Egypt. The collected dogs were divided into 2 groups (n= 7/group) according to their ages; immature (less than 1 year old), and adult (2-3 years old). The ages of different animal groups were determined as outlined by ***[*23*]***.

2-Sampling

After veterinary inspection, dogs were euthanized with Ketamine (100 mg/Kg) and xylazine (10mg/Kg) IM injection ***[*24*]***. Animals were dissected from the abdominal area. Both adrenal glands of each animal were collected for histological, and immunohistochemical examinations.

3- Histological examination.

The collected adrenal gland specimens were fixed in 10% neutral buffered formalin for 48-72 hours. Following fixation, the specimens underwent a series of dehydration steps using increasing concentrations of alcohol. Subsequently, the specimens were cleared in xylene and embedded in paraffin wax. Thin sections of 5µm thickness were prepared using a microtome ***[*25*]*** Sections were stained with H&E for a general histological view and Masson's trichrome for collagen fiber identification.

4-Immunohistochemistry

Paraffin 5µm thick sections on the positive slide were dewaxed in xylene and rehydrated through decreased concentrations of ethanol. Antigen unmasking was performed using 10 mM citrate buffer (pH 6.0) for 20 min. Endogenous peroxidase activity was blocked by using 3% H_2O_2 in methanol for 10 min and then soaked with phosphate-buffered saline (PBS) (pH 7.2–7.4) three times for 5 min. The sections were then pre-incubated with 0.25% trypsin-EDTA for 10 min to block non-specific antigens. The tissue sections were allowed to react overnight at 4°C with anti-SYP (dilution 1/100; Abcam) β-catenin (dilution 1/200, Abcam), and VEGF (10 pg/ml) antibodies. The slides were then incubated at room temperature for 1h, rinsed with PBS three times, and incubated with appropriate

biotinylated secondary antibodies for 30 min. For visualization, a commercial ABC kit was used and incubated with DAB for 5 min. Sections were counterstained with hematoxylin.

3.RESULTS

Histologically, the adrenal gland of dogs consists of stroma and parenchyma. The stroma represents the fibrous CT capsule and septa (Figs 1A, B). The adrenal capsule of immature dogs consisted of delicate dense collagenous CT (Figs 1C, E) but mature dogs had thicker capsules (Fig 1D). Also, dense collagenous CT septa extended into the parenchyma where it extensively extended in mature dogs rather than immature ones (Figs 1E, F). The cortex is subdivided into 3 distinct zones: ZA, ZF, and ZR (Fig 2). ZA consisted of columnar cells in arching structures in immature and mature dogs but at mature age, these arching appears longer with small masses might be present (Figs 2A, B). ZF consisted of polyhedral vacuolated cells characterized by wide blood sinusoids between the cells in immature dogs (Fig 2C) and narrower blood sinusoids in mature dogs (Fig 2E). ZR of immature and mature dogs was characterized by vacuolated cells arranged in the form of a network (Figs 2C, E).

The medulla of the adrenal gland consists of follicles of basophilic cells, arranged around blood sinusoids. In immature dogs, they were scattered among vacuolated cells of ZF while in mature dogs, they are localized at the center of the gland (Figs 2C, D).For VEGF immunohistochemical expression, the adrenal medulla of both immature and mature dogs shows a positive reaction (Fig 3A, B). However, immature dogs expressed stronger VEGF than mature dogs (Fig 3C, D).Beta catenine was localized immunohistochemically to ZA, ZF, and the medulla of both immature and mature dogs (Fig 4A, B). Meanwhile, strong immunoreactions were identified in cells of ZA, ZF, and medulla of immature dogs (Figs 4C, E, G) respectively compared to weak reactions in cells of ZA, ZF, and medulla of mature dogs (Figs 4D, F, H).

SYN is detected in the medullary cells of the adrenal gland and within a few positive cells mass among ZA is identified in both immature and mature dogs (Fig 5A, B). Strong SYN immunoreactions of cells pass extend from capsule to ZA were seen immature dog, but a moderate immunostaining was observed in the gland of mature dogs. (Fig 5C, D). Similarly, medullary cells of immature dogs show stronger immunostaining than mature dogs (Fig 5E, F).

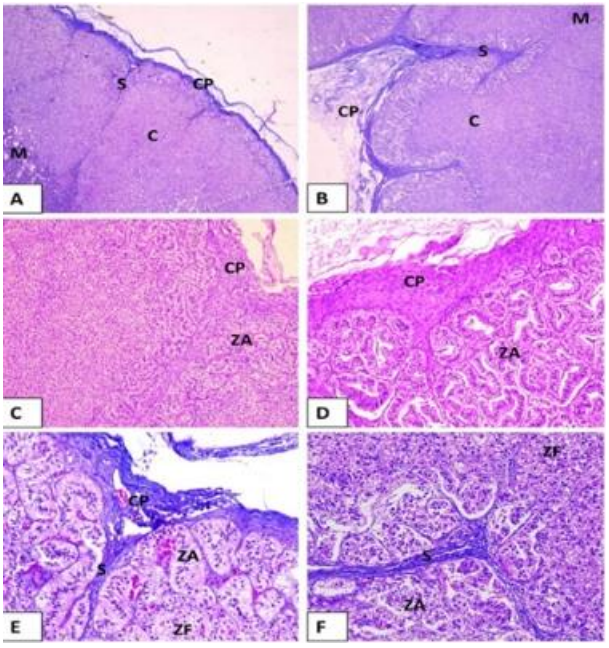

Figure 1: Photomicrograph showing the general features, parenchyma and fibrous stroma of the adrenal gland of immature (A, C, E) and mature (B, D, F) dogs. Delicate capsule in immature dog (A, C) but thicker capsule in mature (B, D). (A and B, Masson trichrome, X4) but (C and D, H&E, X10). (E) showing less septa extend from capsule in immature dog than mature (F). Masson trichrome, X20. CP, capsule; S, septa; C, cortex; M, medulla; ZA, zona arcuata; ZF, zona fasciculate.

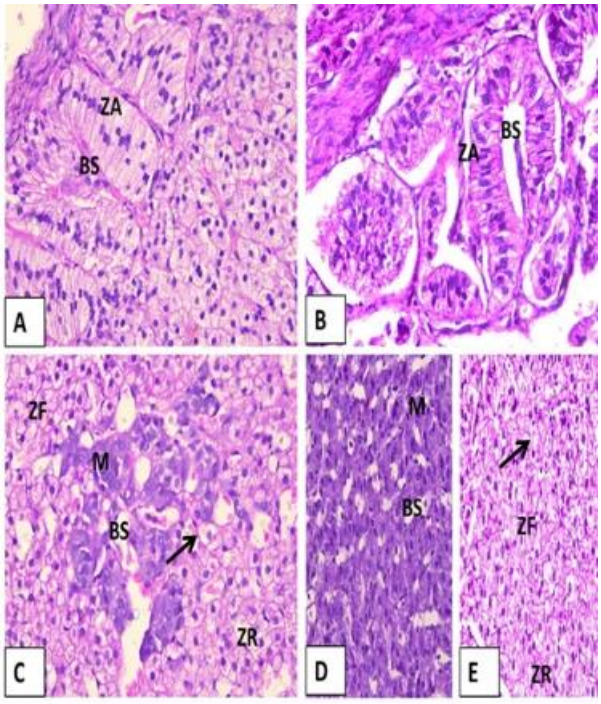

Figure 2: Photomicrograph showing different zones of cortex and medulla. ZA, zona arcuata; ZF, zona faciculata; M, Medulla; arrow, vacuolated cells; BS, blood sinusoids. H&E, X40. ZA of immature (A) is arching structure, but mature (B) long arching and small masses may be present. (C) showing wide sinusoids between vacuolated cells of ZF in immature dog. But narrower sinusoids in mature dogs (E). Cells of ZR of immature (C) and mature dog (E) are arranged in form of a network. The medulla of the immature gland (C) and mature (D) among ZF and the center of gland respectively. Blood sinasoid (Bs), cells of zona faciculata in immature dog surround basophilic cells of the medulla (C) (arrow), zona reticularis (ZR)

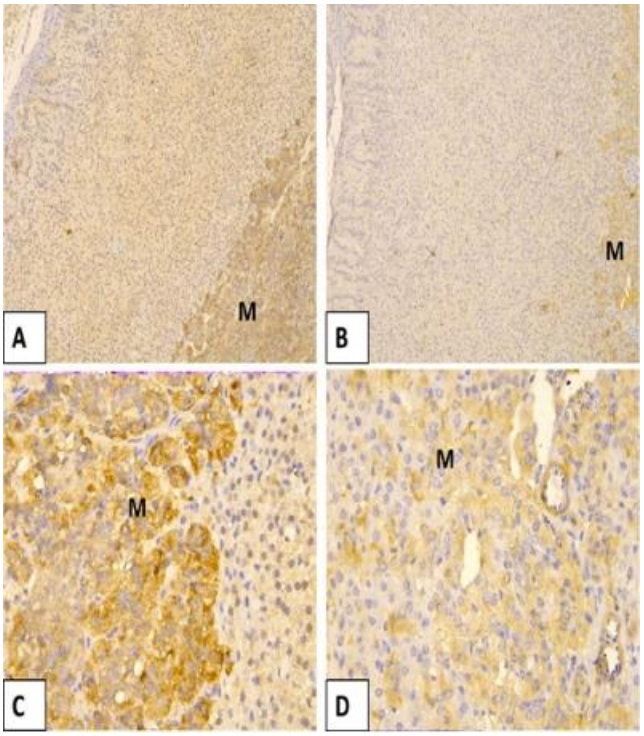

Figure3: Photomicrograph showing immunohistochemical reaction of VEGF in dog adrenal gland. (A and B) showing general view of VEGF expression in adrenal of immature and mature dogs respectively. X10. (C and D) are higher magnification showing strong positive reaction of medulla of Immature dogs (C) but moderate reaction at medulla of mature dog (D). X40.

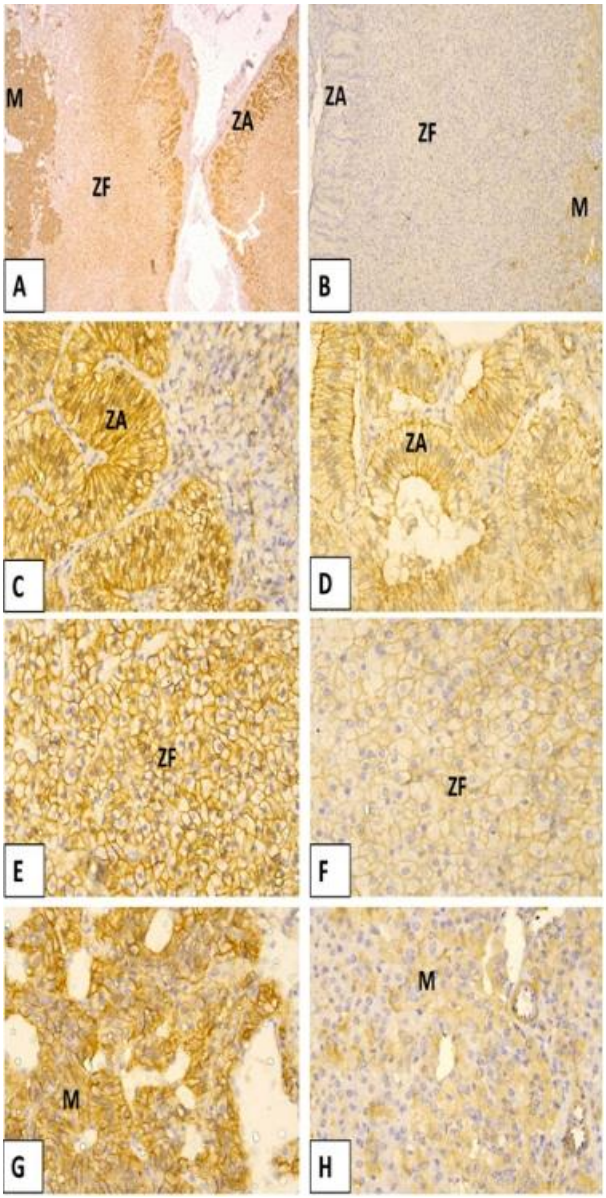

Figure 4: Photomicrograph showing immunohistochemical reaction of Beta catenine in adrenal gland (A, C, E, G are immature and B, D, F, H are mature). (A and B) showing a general view of the adrenal expression of Beta catenine that is localized to ZA, ZF, and M. X4. (C, E, G) showing strong reactions in ZA, ZF, and M of Immature dogs respectively compared with weak reaction in ZA, ZF, and M of Mature dogs (D, F, H). X40

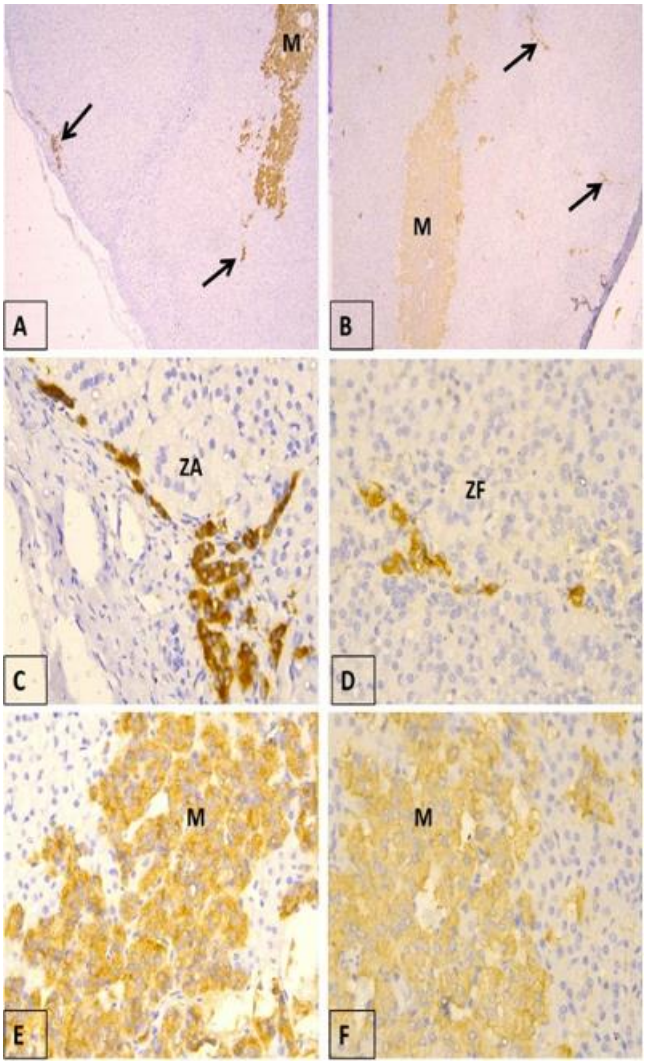

Figure 5: Photomicrograph showing immunohistochemical reaction of SYN in adrenal gland of dog (A, C, E are immature and B, D, F are mature). (A and B) showing general view of adrenal expression of SYN in both immature and mature dogs. X4. (C and D) showing strong reaction of nerve branch that pass from capsule and ZA of Immature dog (C) and moderate at mature (D). (E and F) showing strong positive reaction at medullary cell at Immature dog (E) and moderate at M of Mature dog (F)

4.DISCUSSION

According to the results of this study: The adrenal gland stroma consisted of a capsule and septa; the adrenal capsule increased in thickness with age so at young age dogs the adrenal gland had a delicate capsule and at a mature age, the capsule became thicker. Similarly, CT septa extended into parenchyma at a mature age than a young age and this agrees with the results of **[26, 9]** in their studies in the adrenal gland of dogs, these results disagree with**[18]** in camel where the authors illustrate that adrenal gland capsule is thick at immature age compared to other ages.

The adrenal gland cortex consists of Zona Arcuta, Zona Fasciculata, and Zona Reticularis. In the dog, the Zona Arcuta consisted of columnar cells in arching structure *in both*

immature and mature ages but the arching structure appeared longer in the mature age this agrees with the results of ***[9]*** *in dog but this disagrees with* ***[19, 20]*** *in goat and sheep respectively, where they illustrate that Zona glomerulosa cells are arranged as clusters not as arch structures like dogs so called zona glomerulosa, not zona arcuta.*

Zona fasciculata consisted of polyhedral highly vacuolated cells arranged in cords and the blood sinusoids between them were observed wider in immature dogs than mature one and this agree with ***[26]*** *in dog and also agree with* ***[27,5]*** *at pig and cattle respectively.*

Zona reticularis consists of vacuolated cells arranged in the form of reticulum in the immature and mature age of dogs this agrees with the results of most studies ***[21 ,8,9]*** *in the adrenal gland of pig, Persian squirrel anomalous, and dog respectively.*

The present result showed that the adrenal medulla consisted of basophilic cells arranged in follicles around large blood sinusoids in immature age, where there were a large amount of medullary parenchymal cells still isolated within the cortex, especially among vacuolated cells of zona fasciculata, in the mature animals these basophilic cells found at the center of gland arranged into follicles around large blood sinusoids this results agree with ***[26] [*** *in their study on adrenal gland of dogs at different ages, but this disagree with study of* ***[21]*** *on adrenal gland of new natal and adult guinea pig where he mentioned that the adrenal medulla cells arranged in follicles around large blood sinusoids in the center of the gland at new natal and adult age.*

VEGF is an angiogenic factor that is expressed in steroidogenic organs such as the adrenal gland, in the adrenal gland of a dog there was a strong positive reaction in the immature age rather than a moderate positive reaction in the mature age. These observations might be associated with developmental activity of the immature age where the newly formed blood vessels are more required than the adult gland. This observation coincides with ***[28]*** *who stated that the VEGF plays a crucial role in promoting angiogenesis and also our study agrees with studies of* ***[29]*** *in humans with normal and adrenocortical tumors of the adrenal gland. On other hand* ***[30]*** *in the rat, showed that VEGF gives a positive reaction only at the medulla and with no reactivity at the cortex.*

Beta catenine is an important factor that has an impact role on adrenal zonation. So, the present study showed a strong positive reaction of beta catenine in the immature age gland at cortical and medullary cells. At a mature age, there are moderate to weak positive reactions. These results agree with the results of ***[31,32]*** *in rat and human adrenal glands respectively.*

Synaptophysin is a neurosecretory protein that is used as a high-sensitivity immunohistochemical marker. In his study, there were strong positive reactions at the adrenal medulla of immature dogs and moderate reactions in mature dogs besides positive immunostaining in a few cortical cells in immature and mature dogs. Since synaptophysin is responsible for neuroendocrine differentiation it appears more at immature age gives strong positive reactions at immature than mature ones, these results agree with ***[33,34]*** *at human adrenal gland.*

5-CONCLUSIONS

2. *Finally, we can conclude that ZA cells are arranged in arch-like structures mainly established in the gland of mature animals and might be associated with a previous higher expression of Beta catenine, the most factor related to adrenal gland zonation. VEGF gives a strong positive reaction at cortical and medullary cells in the gland of immature animals rather than a moderate reaction in mature ones and that might be associated with the crucial role in promoting angiogenesis of the developing adrenal gland. Synaptophysin gives a strong positive reactions in medullary cells of immature age and a weaker reaction in mature animals and that is associated with the previously proven role of synaptophysin as neuroendocrine differentiation that stimulates the differentiation of neural origin medulla into neuroendocrine cells.*

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Authors' declarations

Publication consent

Each author has demonstrated their consent for the publication of the current manuscript

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All data of this study is provided.

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