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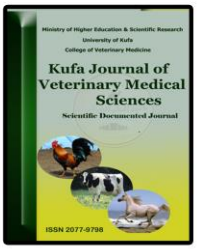

Histological and Immunohistochemistry Studies of Syrinx Calcification in the Laying Hens (*Gallus gallus domesticus*)

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Abstract

The aim of this study is to detect calcification of syrinx and determination its appearance in four ages of groups of laying hens is a kind red. Using special dyes to detect deposits of calcium salts as well as using immunohistochemistry method, showing that the calcification of the syrinx at the age of 6 months appeared as small calcified foci in the middle part of it only, while part of the tracheal and bronchial of the syrinx did not get in it any calcification. At the age of 18 months, syrinx appeared many calcified foci in the middle part, as well as the emergence of calcification is simple for the first time in the upper part (tracheosyringeal) cartilage of it. With age progress, the syrinx appears fully calcified in the middle part (pessulus) of it, as well as the emergence of calcified spots for the first time in the first three cartilaginous rings of the brochosyringeal cartilage from him.

Key words: Calcification, Immunohistochemistry method, Von kossa, Syrinx

دراسة نسيجية ونسيجية كيميائية مناعية لتكلس عضو التغريد في الدجاج البياض

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الخلاصة:

أن الهدف من هذه الدراسة هو كشف التكلس الحاصل في عضو التغريد وتحديد ظهوره في اربعة مجاميع عمرية
من الدجاج البياض ذي النوع الاحمر .بأستخدام صبغات خاصة لكشف ترسبات أملاح الكالسيوم وكذلك بأستخدام طريقة الفحص النسيجي الكيميائي المناعي ,تبين ان التكلس في عضو التغريد بعمر 6 اشهر ظهر بشكل بؤر صغيرة متكلسة في الجزء الوسطي منه فقط ,في حين الجزء القصبي والقصيبي التابع لعضو التغريد لم يحصل فيه اي تكلس .في عمر 18 شهرا "ظهرت في عضو التغريد بؤر متكلسة كثيرة في الجزء الوسطي ,كذلك ظهور تكلس بسيط لأول مرة في الجزء العلوي(القصبي)منه .مع تقدم العمر يظهر الجزء الوسطي من عضو التغريد متكلس بالكامل ,كذلك ظهوربقع متكلسة لأول مرة في الحلقات الغضروفية الثلاثة الاولى فقط من الجزء السفلي(القصيبي)منه..

Introduction

Birds change from mammals because of specific structures in their respiratory system (1). The avian respiratory system consists of nasal cavity, larynx, trachea, syrinx, bronchi, lungs and multiple air sacs (2). Chief roles of respiratory system in birds are interchange gases inflows and

outflows, stability temperature in birds body also support to generation of sound, air moved across breathing passage since the nasal cavity to the larynx then carry on by the trachea and go into syrinx in addition to bronchi (3). In birds, the syrinx is the vocal structure of songbirds (4). It's located by the level of the 2nd or 3rd thoracic vertebrae and between trachea and primary bronchus, also connected with the clavicular air sac close to the heart at the dorsal lateral of stomach then ventrally of the esophagus (5). Syrinx is lying in the connection of the trachea and two bronchi and so it belongs to the collection of trachea bronchial syrinx (4). Syrinx is the greatest essential and greatest widely studied structure in the birds sound production, additional that the structure is essential in sound generating furthermore indicated important data around the classification of birds because of the differences in composition of the structure in diverse kinds, German anatomist categorized birds species via the anatomy of syrinx previously on 1878 (6), cited by (7). Syrinx comprises of specialized cartilaginous structure tracheosyringeal cartilage, bronchosyringeal cartilage as well as middle syringeal cartilage (tympanum) (8). The number of tracheosyringeal cartilage different as of one to six in chickens (9). In chickens, the cartilaginous bronchosyringeal comprised of 7 cartilage rings in the form of a"C" shape (10). Mucosa of the syrinx involves either: stratified squamous (11). Columnar (12, 13), or pseudo stratified epithelium (14). Calcium (Ca) is one of the most significant elements that have an important role in the major biological functions of the bird's body, where it works to maintain the organization of egg production in his body (15). Furthermore, the calcium set up more than third of all minerals within the adult bird's body (16). Numerous causes' influence of the calcification development comprising age, hormones, nutrition calcium, vitamin D and parathyroid hormone plays a chief part in calcification (17). According to (18), in experiments conducted on chickens found that when giving large amounts of vitamin D3 gives indications comprises a syndrome classified by irregular calcium deposition in these soft tissue then to end with calcification, calcium deposits are frequently found in the urinary as well as respiratory tract.

Materials and methods

Animals and tissue preparation

Twenty four birds of laying hens were collected from poultry fields of Wasit city, the birds were divided according to age into four groups (6 months, 12 months, 18 months and 24 months) each group were six birds, all birds should be clinically healthy and devoid of any type of injuries. All laying hens was anesthetized with chloroform dropped in cotton pad kept about the face, after that the syrinx samples were cut off from the thoracic region of the laying hens and

were fixed in 10% formalin for (72) hours, then and there washed up in tap water for 2-3 hours and then moved the samples to numerous histological techniques as followed: dehydration, clearing, infiltration, embedding, cutting and staining with hematoxylin and eosin (H&E) stain for appearing the general structure of the tissue, in addition with von kossa stain to detect deposits of calcium salts in paraffin sections.

Immunohistochemistry technique

The standard biotin free one- step HRP polymer anti- mouse, rat and rabbit IgG (H+L) with DAB immunostaining

procedure were used to detect calcium deposits in the cartilage of syrinx. Briefly, sections from embedded laying hens' syrinx were dewaxed, hydrated in a graded decreasing series of alcohol solutions and then washed 3 times in distilled water. Antigen retrieval by immersed slides in jar containing citrate buffer solution (PH= 6). Slides were washed with phosphate buffer saline (PBS) with PH 7.2 for 5 minutes; sections were incubated with peroxidase block for 5-10 minutes at RT, and then were washed with distilled water 3times, also slides were washed with PBS. Subsequently, sections were incubated with protein blocking solution 5-10 minutes at RT, and then incubated with primary antibody (diluted of 1:500) for 30 minutes at RT. Slides were washed with PBS 5-7 times, and then incubated with one -step HRP polymer for 30 minutes at RT, hen slides were washed with PBS, also slides were washed with distilled water 2-3 times afterward, add few drops of ready to use DAB reagent on tissue slides (was used by mixed well 1 ml of reagent BS buffer & substrate and 50 microliter of reagent C chromogen) for 6-10 minutes at RT, then washed with PBS and were washed in distilled water. Later, section was incubated with hematoxylin stain 30- 60 seconds subsequently; slides were washed with distilled water and mounted with D.P.X. mounting medium.

Results and discussion

Histological and immunohistochemistry results of syrinx

a-At 6 month's age

The study revealed that the mean of body weight of laying hens in this age was about (1861±27.29 g) (Table 1), and the histological results with H&E stain of the longitudinal section of the syrinx at this age showed that the skeleton of the syrinx comprised three diverse cartilage groups: tympanum, tracheosyringeal and bronchosyringeal cartilage. The tunica mucosa was lined by ciliated pseudo stratified epithelium with goblet cells. The lamina propria and submucosa comprised loose connective tissue. These results are perfectly similar to the finding of several authors like (19), in ostrich and (20), in common bulbul and pigeon. With calcium detecting stain von kossa and over and above H&E stain, showed a simple calcification occurred in the (pessulus) central part of the syrinx only in the form of small foci deposited calcium salts within, while other parts as tracheosyringeal and the bronchosyringeal were not showed any singes of calcification within (Fig. 1, 2). On the other hand, the consequences of immunohisto-chemistry method of the syrinx were appeared the calcification in the form of foci brown in color in the middle part of it only. The ratio of calcification of the syrinx at this age is relatively assessed (+) as summarized in (Table 2).

b- At 12 month's age

The average of body weight was about (1325 ± 63.29 g), and the histological structure of syrinx in this age showed the tympanum consists of 5 tracheal rings. These results are parallel to (21), who reported that the tympanum comprises of 5 tracheal rings in chickens, on the other hand, its disagree with (19), which documented the number of it 3 in the ostriches and (22), who recorded 2 in Japanese quails. Pessulus was placed caudo-medial side of tympanum consist of hyaline cartilage. Like to [23], but differ from (1, 5), who said that the pessulus in the mallard was comprised of bone tissue. Tracheosyringeal cartilages were formed from 4

cartilaginous rings. The number of this cartilage approves with (9), who determined the number of these cartilages diverse from one to six in chickens. However, vary with (24), who stated that the number of tracheosyringeales cartilage might be 8 and the bronchosyringeal cartilage consists of 7 cartilage rings in the form of a "C" shape. This observation was equivalent with (10), which studied it in sea gulls, but disagrees with (25), who recorded that the number of bronchosyringeal cartilage in turkeys was 4 cartilage rings. In addition, von kossa and H&E stain results showed that there were calcification occurred in the (pessulus) central part of the syrinx in the form of foci more than that seen in previous old and not have any calcification on other cartilaginous parts (tracheosyringeal and bronchosyringeal) cartilage of the syrinx (Fig.3), the calcification like enough rises with advance age, at least up one year.Immunohistochemistry technique of the syrinx found occurrence the calcification in the middle part of its only and both the tracheosyringeal and bronchosyringeal any calcification did not happen with them. The proportion of calcification of syrinx at this age is relatively projected (+ +) as summarized at (Table 2). This statement came matching what he noted by (26), in common murres at one year old. The syringeal calcification in birds with increase age possibly occurs but is weakly recorded (27, 28 and 29).

c- At 18 month's age

In this age, showed the mean of body weight was about (2209 ± 115.6 g), and the histological sections with von kossa stain and H&E stain showed the occurrence of calcification in the most central part of the syrinx (pessulus) and debuting occurrence of simple calcification in the upper part of the syrinx (tracheosyringeal cartilage), while the bronchosyringeal part appeared as hyaline cartilage without any calcification (Fig. 4, 5). To promote the von kossa stain, the histological sections of the syrinx treated with labeled calcium antibody and appeared the calcium deposits in the tracheosyringeal cartilage which show as brown foci colors (Fig. 6). The amount of calcification in these histological section estimated (+ + +). This findings was established through the preceding studies completed by (30), in male mallards and (31), in pigeons. On the other hand, (26), mentioned that the posterior tracheal rings was at all entirely cartilaginous anterior to the tympanum of the syrinx, although little calcification patch occurred in adult murres bird.

However, all these events of advance hyaline cartilage calcification which take place in most parts of respiratory system was probably as a result of forward bird s in age, lack of egg production and take fodder with high levels of calcium components at this age.

d- At 24 month's age

At this age, appeared the average of the body weight was about (1867± 125.9 g) (Table 1), and the results of syrinx sections which treated with calcium detecting method, showed advance stage of pessulus ossification (Fig.7) as well as, firstly appearance of calcification centers in the left & right bronchosyringeal part of the syrinx (Fig. 8). While the antibody- antigen detecting method explained calcification morbidity in the first bronchosyringeal ring more than that in the second and third rings and doesn't occurrence calcification in other bronchosyringeal rings (Fig.9). At this order age of the present work the all

skeletal parts of the syrinx become calcified when compared with the other previous ages and can be relatively estimated (+ + + +) (Table 2). These results was parallel to the findings of (28), who described in some birds the mineralization with arrangement occurred in the middle part of the syrinx (pessulus) and in the first bronchosyringeal ring, and it was happened caudally in other cartilaginous bronchi. However, (26) mentioned in murres bird that the first bronchosyringeal cartilage is very large modified and calcified ring and the site of trachea laterals muscle inserts and can move through vocalization. The manner and timing of calcification development was shown in syrinx at different age stages, but the calcification not speared caudally in bronchi rings toward the lungs because it might affect the mechanism of respiration and gas exchange.

Table 1: shows the mean of body weight (gm.) in the four ages of laying hens.

The Age of Laying Hens	*Body weight Mean ± SE*
6 months	1861 ± 27.29
12 months	1325 ± 63.29
18 months	2209 ± 115.6
24 months	1867 ± 125.9

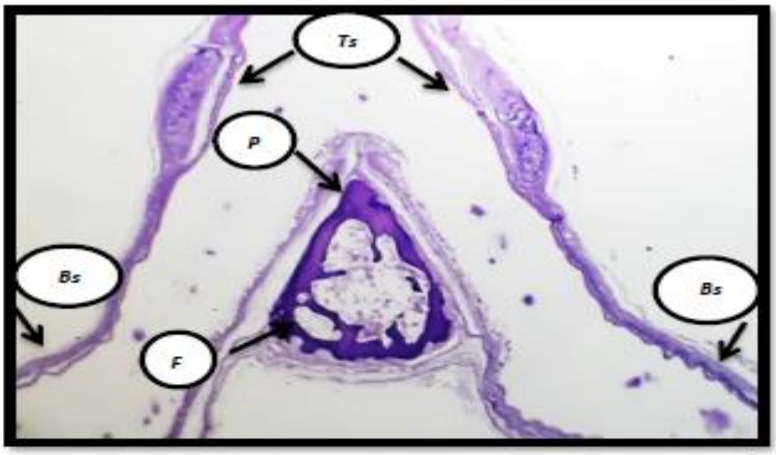

Figure 1: Longitudinal section of the syrinx in the Laying hens (at 6months age) showing the tracheosyringeal cartilage (Ts), Psseulus calcification (P) as foci in form (F), Right & left bronchosyringeal cartilage (Bs) (H&E stain, X4).

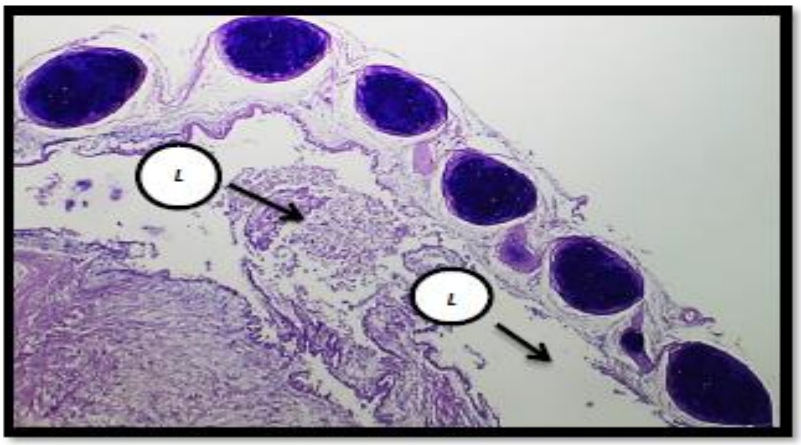

Figure 2: Longitudinal section of the syrinx in the Laying hens (at 6months age) showing the rings of brochosyringeal cartilage of the syrinx without any calcification, Lumen of bronchosyringeal cartilage (L) (H&E stain, X4).

Figure 3: Longitudinal section of the syrinx (at 12 months age) showing pessulus calcification in form of foci (F), Tracheosyringeal cartilage (Ts) without any calcification (Von kossa stain, X4).

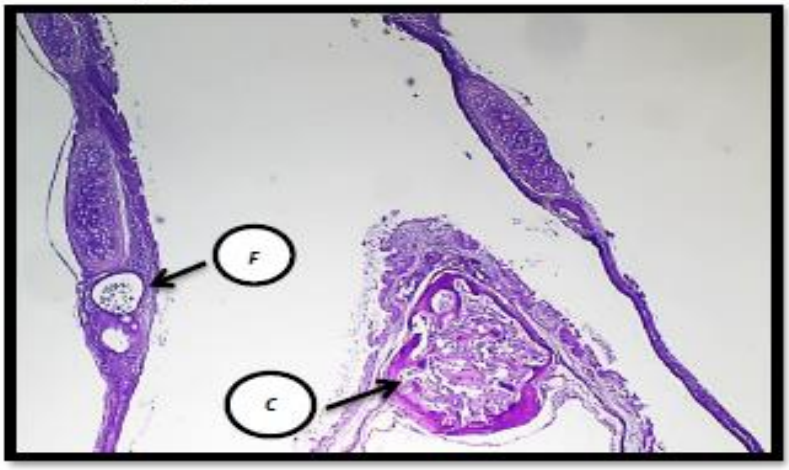

Figure 4: Longitudinal section of the syrinx in the Laying hens (at 18 months age) showing pessulus calcification (C), calcification foci in tracheosyringeal cartilage (F) (H&E stain, X4).

Table 2: show the relative proportions of calcification ofthe syrinx of four ages of laying hens.

Age of laying hens	*Syrinx*
6 months	+
12 months	++
18 months	+++
24 months	++++

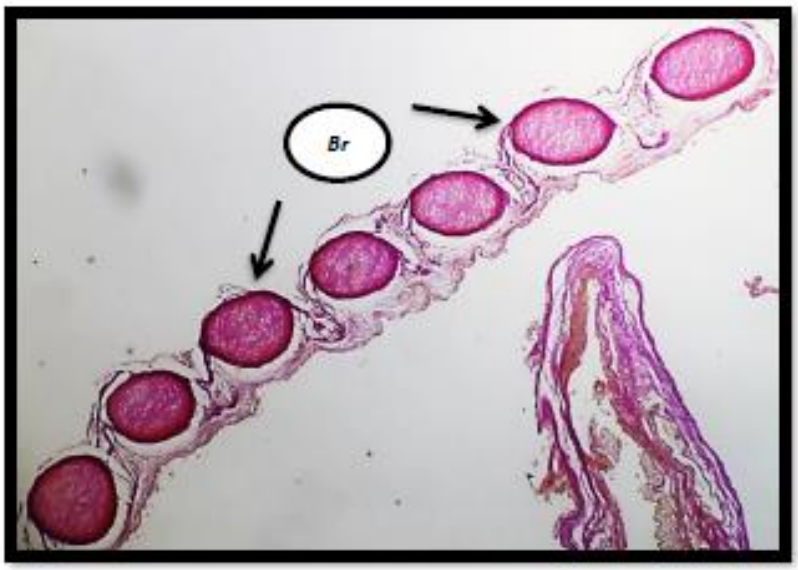

Figure 5: Longitudinal section of the syrinx in the Laying hens (at 18 months age) showing the 7 bronchosyringeal cartilage rings (Br) without any calcification (Von kossa stain, X4).

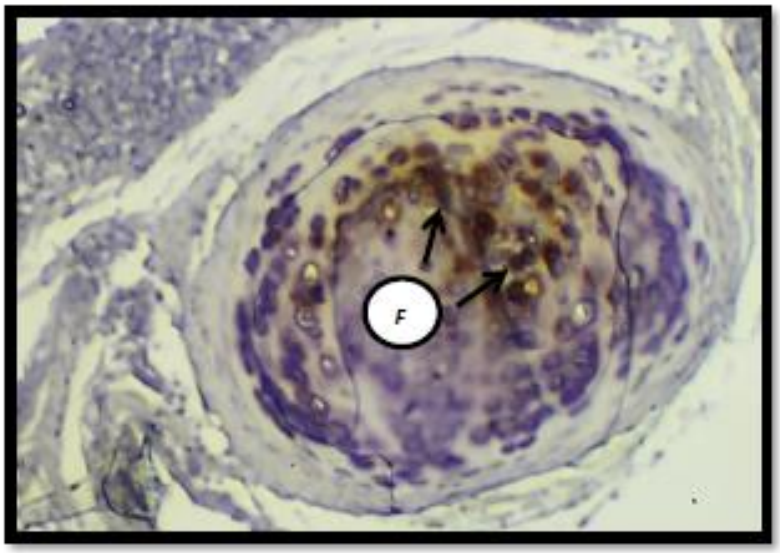

Figure 6: Immunohistochemistry in longitudinal section of the syrinx (at 18 months age) showing the foci of calcification (F) in tracheosyringeal cartilage (40X).

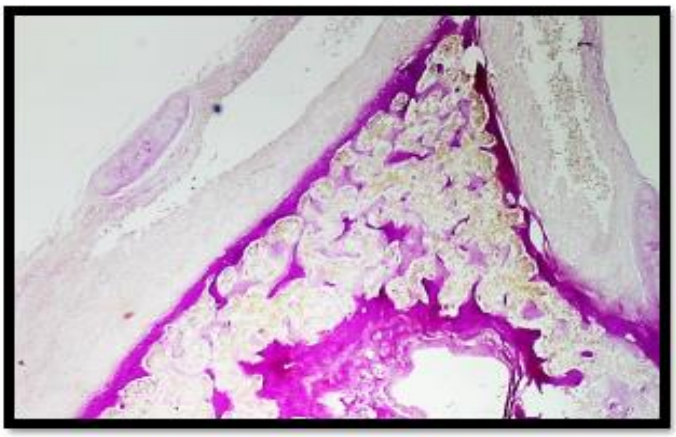

Figure 7: Longitudinal section of the syrinx in the Laying hens (at 24 months age) showing full ossified the middle part of the syrinx (pessulus) (Von kossa stain, X4).

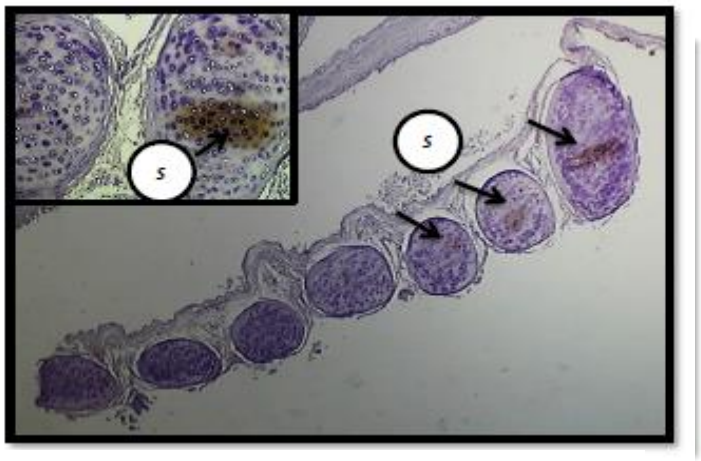

Figure 9: Immunohistochemistry in longitudinal section of the syrinx (at 24 months age) showing calcification as spots in form (S) s (at inside 3 rings of bronchosyringeal cartilage (X4 & X20). ilage

Conclusions

In this study, slight calcification was denoted in syrinx in the 6 months age of chickens but with age progress the calcification of the hyaline cartilage of the syrinx appear as foci with varying size carrying within them dark color calcium deposits. The calcified foci become occupy the large size of the syrinx, and in the older age of this work very high calcification morbidity show in the most parts of syrinx skeleton. The pattern of the syrinx calcification was increased with advance age of birds concurrent with decrease of egg production and take nutrition with elevated calcium.

References

1- Tasbas M, Haziroğlu RM, Ozcan Z (1986): Penguenin dilive ön solunum yollarnnnn (larynx cranialis, trachea, syrinx) anatomik ve histolojik yapnsn üzerinde bir çalnşma. Ankara Univ Vet Fak Derg, 33, 240-261.

2- Dyce, K.M., Sac W. O. Wensing C. J. G. (2010): Textbook of

VeterinarAnatomy.4thEditio Saunders Elsevier. Pp: 799-804.

3- Fedde MR. (1998): Relationship of structure and function of the avian respiratory system to disease susceptibility. Poult Sci. 77, 1130-1138.

4- Fletcher, N. H. (1989): Acoustics of bird song – some unresolved problems. Comments Theor Biol. 4, 237–251.

5- Frank T, Probst A, König HE, Walter I (2007): The syrinx of the Male Mallard (*Anas Platyrhynchos*) special anatomical features. Anat Histol Embryol, 36, 121-126.

6- Muller JP, (1878): On certain variations in the vocal organs of the Passeres that have itherto escaped notice, London: Macmillan.

7- AL-Bishtue, Abdulla Aaid Hade. (2014): Anatomical investigations of the syrinx (voice box) of the adult male West African guinea fowl (*Numida meleagris galeata*) in the AL-Najaf AL-Ashraf province. AL -Qadisiya Journal of Vet. Med. Sci. Vol. 13 No. 2. Pp: 100-105.

8- Larsen ON, Goller F, (2002): Direct observation of syringeal muscle function in songbirds and a parrot. J Exp Biol, 205. Pp: 25–35.

9- Getty R, Sisson and Grossman's (

(1975: The Anatomy of the Domestic Animals. 5thed, W.B aunders Company, Philadelphia. Vol. 1-2. Pp: 1884-1917.

10- Nazan Gezer Ince1, Gulsun Pazvant, Hasan Aipak, (2012): Anatomical features of the syrinx in sea gulls. 59, 1-3.

11- Bacha W.J (1990): Color Atlas of Veterinary Histology Philadelphia, Lippincot Williams &Wilkins. P.187.

12- Benks W.J (1993): Applied Veterinary Histology. London, Mosby. P. 404.

13- Campbell T.W (1995): Avian Hematology and Cytology. Iowa, State University Press. P. 55.

14- Scala, G., M. Corona and G.V. Pclagalli, (1990): The structure of the syrinx in the duck 20. Dyce,K.M., W.O. Sack and C.J.G. Weaning, 1987. (*Anas platyrhinchos*). Anat. Histol. Embryol.,Textbook of Veterinary Anatomy. edition, London. 19, 135-142.

15- Elaroussi, M.A., Forte, L.R., Eber, S.L. & Biellier, H.V. (1994): Calcium homeostasis in the Laying hens. 1. Age and dietary calcium effects.Poult.Sci.73, 1590-1596.

16- Klasing, K.C., (1998): Comparative Avian Nutrition. CABI Publishing, Walling ford, UK. Pp: 238-248.

17- Perry,T.W. (1984): Animal Life-Cycle Feeding and Nutrition. Academic Press, INC.New York, USA. Pp: 22-27.

18- Leeson, S. & Summers, J.D. (1988): Some nutritional implications of leg problems with poultry.British Veterinary Journal 144, 81-92.

19- Yildiz, H., A. Bahadir and A. Akkoc, (2003): Applied Veterinary Histology. On themorphological structure of syrinx in 3 edition, Mosby Yearbook, St. Louis, pp: 404. Ostriches (Struthio Camelus). Anat. Histol. Embryol., 22. Deeming, D.C., 1999. The Ostrich Biology. 32, 187-191.

20- Al- badri, A.M.S., Jassim, B.A. and Abbas, J.G. (2014): Macroscopic study of syrinx in the common bulbul (*Pycnontus barbatus*) and indigenous pigeon (*Columba domestica*) AL-Qadisiya Journal of Vet. Med. Sci. Vol. 13. No.1. Pp: 88-93.

21- King AS (1989): Functional Anatomy of the Syrinx. In: Form and Function in Birds. Academic Press, London. Pp: 105-182.

22- Cevik Demirkan A, Hazıroğlu RM, Kürtül I (2007): Gross morphological and histological features of larynx, trachea and syrinx in *japanese quail*. Anat Histol Embriyol, 36, 215-219.

23- Gaunt, A.S., Gaunt, S.L.L. and Hector, DH (1976): Mechanics of the Syrinx in *Gallus gallus*. I. A Comparison of Pressure Events in Chickens to Those in Oscines. The Condor. 78. 208-223.

24- Lockner FR, Youngren OM, (1976): Functional Syringeal Anatomy of the Mallard. I. In situ Electromyograms during ESB Elicited Calling, The Auk, 93, 2, Pp: 324-342.

25- Khaksar .Z , Tavakol Kookhdan . E and Parto. P, (2012): A Study on Anatomy and histological structure of larynx in adult male and female turkeys. Journal of Zoology 7 (3), 245-250.

26- Edward H. Miller. Sampath S. Seneviratne. Ian L. Jones. Gregory J. Robertson. Sabina I. Wilhelm (2008): Syringeal anatomy and allometry in murres (Alcidae : Uria) 149, 545- 554.

27- Fitzgerald TC (1969): The Coturnix quail: anatomy and histology. Iowa State University Press, Ames. Pp: 253-255.

28- Hogg D.A. (1982): Ossification of the laryngeal, tracheal and syringeal cartilages in the domestic fowl: J. Anat. 134, 57-71.

29- McLelland, J. (1989): Larynx and trachea, in '(King & McLelland 1989), chapter 2. Pp: 69–103.

30-Warner R.W. (1972): The anatomy of the syrinx in passerine birds. J Zool 168, 381-393.

31- Frank, T., Walter, I., Probst, A. and Konig, H.E (2006): Histological aspects of the syrinx of the male mallard (*Anas platyrhynchos*).Anat. Histol. Embryol. 35, 396-401.