

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

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**Abstract:** *The aim of this study is to detect calcification of trachea and determination its appearance in different age of groups of laying hens. Using special dyes to detect deposits of calcium salts as well as using immunohistochemistry method, showing that the calcification in upper part of trachea does not appear at the age of 6 months, while in the lower part of which appeared simple calcification in the form of small foci. With age progress, the calcification of the hyaline cartilage of the trachea appears as foci with varying size carrying within them dark color calcium deposits. The calcified foci become occupy the large size of tracheal rings and degeneration and death of cartilaginous cells with appear in other somewhere of tracheal rings.*

**Keywords:** Calcification, Von Kossa, Immunohistochemistry, Hyaline Cartilage.

## 1. Introduction

Birds change from mammals because of particular 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]. Trachea was a tube detected in the ventro - medial ingluviens and go into the thoracic cavity when creating the syrinx, responsible for defense appliance to the respiratory system and doings as a resonator to the sound created via the syrinx [4]. The trachea consists of cartilage rings [5]. The numbers of cartilage rings in the trachea differs by reason of the size of neck, frequently trachea is consisting of 108-126 rings [6]. Histologically, trachea was lined by ciliated, pseudo stratified columnar epithelium, lamina-propria was composed of loose connective tissue also include alveolar mucous glands; the sub mucosa was continuous with the perichondrium of the cartilaginous rings and obtainable elastic fibers [7]. Basal cell of the epithelium contain round or irregular nuclei, normally the cells of the mucous gland are mucous secreting cells [8]. Epithelium lining of the trachea is mucous form excretion through either intraepithelial simple alveolar mucous glands or goblet cell, the upper part of trachea includes many simple alveolar mucous glands while in lower part of it the glands are replaced by goblet cells which form intraepithelial glands [9]. Lamina propria comprised collagen fibers, elastic fibers, blood vessels and nerve fibers [10]. 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 [11]. Furthermore, the calcium set up more than third of all minerals within the adult bird's body [12]. The concentration of calcium ions (Ca<sup>2+</sup>) increases during the daily cycle of females laying hens to producing egg and it lowers during the calcification of egg [13]. Numerous causes' influence of the calcification development

comprising age, hormones, nutrition calcium, vitamin D and parathyroid hormone plays a chief part in calcification [14]. According to [15], in experiments conducted on chickens found that when giving large amounts of vitamin D<sub>3</sub> 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. The histochemical and immunohistochemistry study of the cartilage provides with essential data around numerous physiological besides pathological procedures in this tissue [16].

## 2. 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 trachea samples isolated from upper and lower part of cervical region 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 hyaline cartilage. Briefly, sections from embedded laying hens' trachea 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, then 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 BSbuffer & 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.

### 3. Results and Discussion

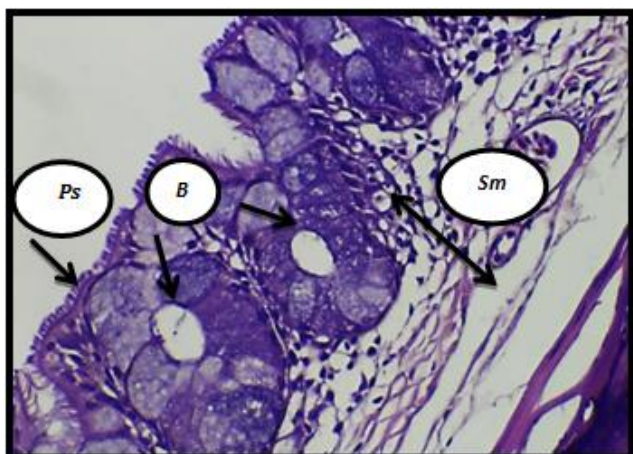
#### 3. 1. Histological & immunohistochemistry results of trachea a- At 6 month's age

The study revealed that the mean of body weight of laying hens in this age was about (1861±27. 29g) (Table 1).

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

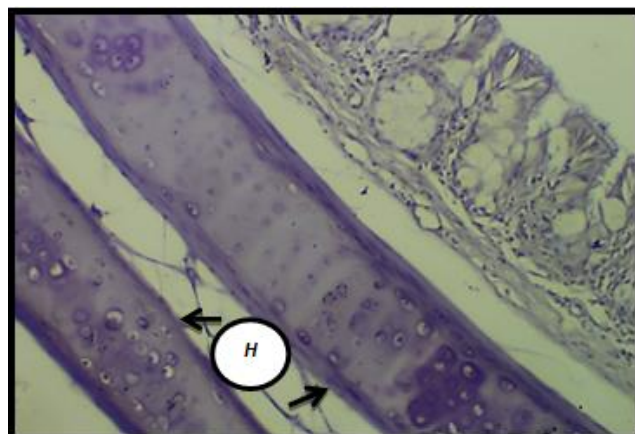
The Age of Laying Hens	Body weight ( Mean ± SE )
6 months	1861 ± 27. 29
12 months	1325 ± 63. 29
18 months	2209 ± 115. 6
24 months	1867 ± 125. 9

The histological examination by hematoxylin and eosin (H&E) staining through cross and longitudinal section of upper part of trachea it was appeared that the trachea of laying hens in this age was seemed as unfilled hyaline cartilaginous tube and it was lined by ciliated, pseudo stratified columnar epithelium. These results were agreed with [17], in Japanese quail; [18] and [19], in goose. In the upper part of trachea many simple branched tubular mucous glands full the epithelium (Fig. 1).



**Figure 1:** Cross section of the upper part of trachea in the Laying hens (at 6months age) showing the trachea lined by ciliated, pseudostratified epithelium (Ps), Branched tubular mucous glands (B) full the submucosal layer (Sm) (H&E stain, X40).

These results are similar to [10, 20 and 8], who studied the trachea in turkey and pigeon. On the other hand, the microscopic examination with von kossa stain addition to H&E stain showed absence of any indication of calcification in the upper part of the trachea in this time of age but only it shows the essential histological structure of the trachea. Immunohistochemistry method by using primary antibody calcium channel, voltage dependent, gamma subunit 5 (CACNG5) and secondary antibody one-step HRP polymer anti- mouse, rat and rabbit IgG (H+L) with DAB were observed results approximately similar to what appeared in histological study, when appeared the upper part of the trachea without any indication of calcification in this lifetime (Fig. 2).



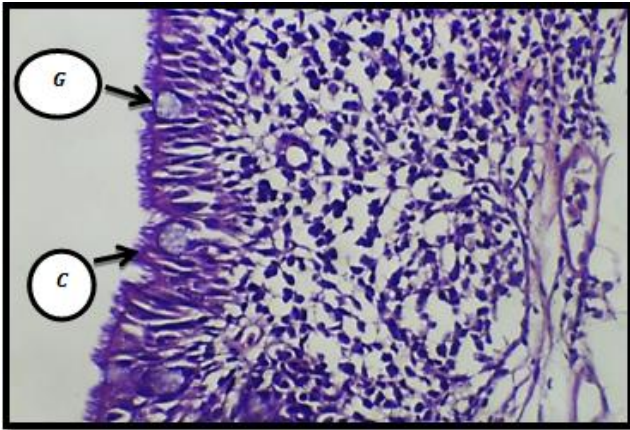
**Figure 2:** Immunohistochemistry in cross section of upper part of trachea (at 6months age) showing the hyaline cartilage (H) without any calcification (X40)

Relatively, can consider that the percentage of calcification at this age estimated (-) (Table 2).

**Table 2:** Show the relative proportions of calcification of the trachea of four ages of laying hens.

Age of laying hens	Upper part of trachea	Lower part of trachea
6 months	-	+
12 months	+	++
18 months	++	+++
24 months	+++	++++

This result at this stage was identical to [21], who mentions the pattern and timing of tracheal calcification in golden comet bird and observed that the first rings of trachea were not involved any mineralization in early age studied at 112 day after hatching. The lower part of tracheal sections with stain by H&E was showed the existence of the epithelium surrounding the cartilage as well as a fully cilia surrounding epithelium and the great numbers of goblet cells occupied by mucins to form intraepithelial glands present in the submucosal layer (Fig. 3).

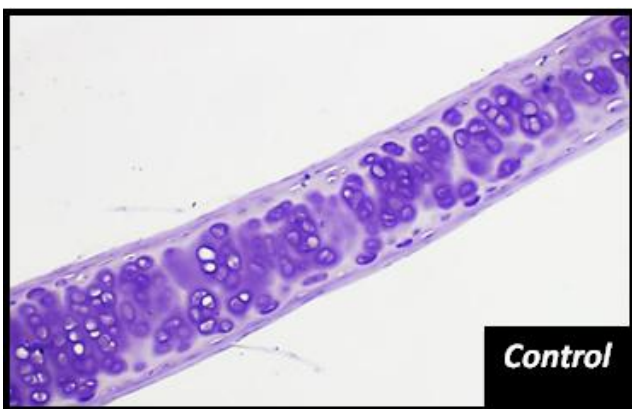


**Figure 3:** Cross section of the lower part of trachea in the Laying hens (at 6months age) showing the cilia surrounding the epithelium(C), Groups of goblet cell (intraepithelial glands) (G) (H&E stain, X40)

These consequences reinforced as a result of the same remark uttered by [9], in general birds. With von kossa stain addition to H&E stain appeared the emergence of calcification in the cartilage in a few small foci deposited inside calcium salts of brown to dark in color. Likewise, immunological results of the lower part showed a slight calcification in the form of foci varies in color from light brown to dark deposits calcium salts inside it. These occurrences of calcification in lower part of trachea relatively are valued (+). This result was parallel to his record [21], who reported that the mineralization of caudal tracheal rings sequence at 112 days after hatching in domestic fowl was frequently gotten by way of small separate foci.

**b- At 12 month's age**

The average of body weight was about (1325 ± 63. 29 g). The microscopic results with von kossa stain as well as H&E stain and with immunohistochemistry process to detect the deposition of calcium salts in the hyaline cartilage found there was no any calcification when the primary antibody was absent from the control section (Fig. 4).



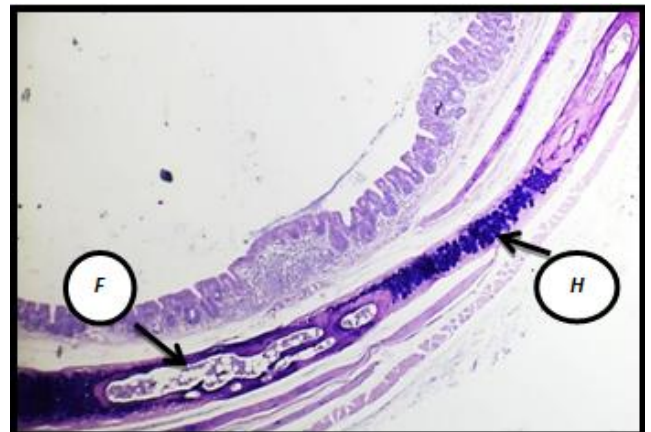
**Figure 4:** Negative control image show that there is no calcification in the upper part of trachea (at 12 months age) when the primary antibody was absent from the control section (Secondary antibody HRP polymer, X20).

While in the upper part of the trachea appeared beginning stage of calcification in the form of dark brown stains color inside cartilage (Fig. 5).

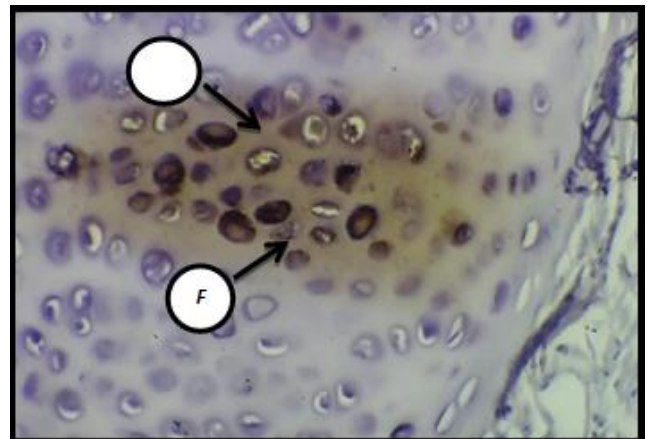


**Figure 5:** Cross section of the upper part of trachea in the Laying hens (at 12 months age) showing calcium salts deposition (C) in hyaline cartilage (H) (Von kossa stain, X20).

So that, this first calcification amount at this part of trachea comparatively valued (+). While in the lower part of trachea appeared calcification as foci in much area up 3-5 zones along the tracheal rings are deposited inside these foci calcium salts with brown dark pigmentation (Fig. 6, 7).



**Figure 6:** Cross section of the lower part of trachea in the Laying hens (at 12 months age) showing the calcification in form of foci (F) in thehyaline cartilage (H) (H&E stain, X4).



**Figure 7:** Immunohistochemistry in cross section of the lower part of trachea (at 12 months age) showing calcification in form of foci (F) scattered inside cartilage (X100).

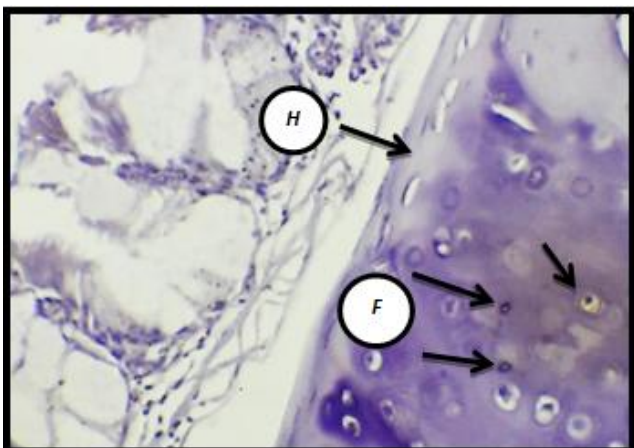
The tracheal calcification morbidity in this age are increasing and can relatively represent (+ +) compared with that remarked in the previous age (6 months). These findings are similar to [21], who mentioned the mineralization pattern which noted in golden comet bird at (140-182) days after hatching.

#### c- At 18 month's age

In this age, showed the mean of body weight was about ( $2209 \pm 115.6$  g). Histological and immunological results were identical in terms found different size of calcium deposits with dark to brown color spread in the upper part of trachea (Fig. 8, 9).



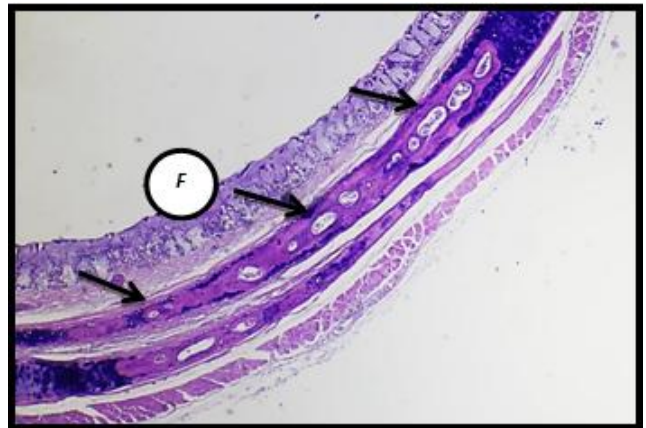
**Figure 8:** Longitudinal section of upper part of trachea (at 18 months age) showing calcification as form of foci (F) present in overlapping hyaline cartilage (Von kossa stain X4).



**Figure 9:** Immunohistochemistry in cross section of the upper part of trachea (at 18 months age) showing the foci of calcification (F) scattered in the hyaline cartilage (H) (X40).

Therefore, the calcification morbidity in the upper part of trachea at this age can be relatively estimated (+ +). This case was associated to what has been observed by [22], where remarked that the upper part of the trachea occurred in which calcification in the form of patches scattered inside the cartilage in common murres at the age more of

one year. In the lower part of trachea and with immune histochemical technique, von kossa stain and H&E stain noticed the tracheal rings were calcified in 4-8 foci occupies the wide area of the cartilaginous rings, the number of these foci more than that observed in the same part of the former age (Fig. 10).



**Figure 10:** Cross section of the lower part of trachea in the laying hens (at 18 months age) showing calcification in form of group's foci (F) in the cartilage (H&E stain, X10).

Accordingly, relatively the percentage valued (++ +) (Table 2). This result was equivalent to the result reached by [22], who revealed that the posterior most tracheal rings were more calcified in age more of one year of murres bird, therefore concluded that the calcification probable rises with age.

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 birds 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 ( $1867 \pm 125.9$  g) (Table 1). In this advanced old of laying hens, immune histochemical results of the upper part of trachea were confirmed the histological results where showed the presence of evidently calcification in multi place of hyaline cartilage rings which appeared as medium and large foci filled by calcium deposits with dark brown colors (Fig. 11, 12).



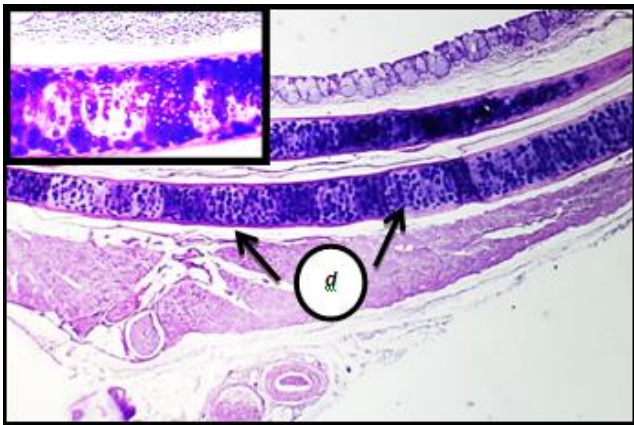
**Figure 11:** Cross section of the upper part of trachea (at 24 months age) showing the calcification in form of group's foci (F) in the cartilage (H&E stain, X10).

foci (F) medium and large in size in the hyaline cartilage (H&E stain, X10).



**Figure 12:** Cross section of the upper part of trachea in the Laying hens (at 24 months age) showing the calcification in form of group's foci (F) medium and large in size in the hyaline cartilage (Von kossa stain, X10).

As well as to occupancy calcification foci wide space of cartilaginous rings, the results described the stage of cell atrophy and death in most remainder part of hyaline cartilage tracheal rings (Fig. 13, 14).

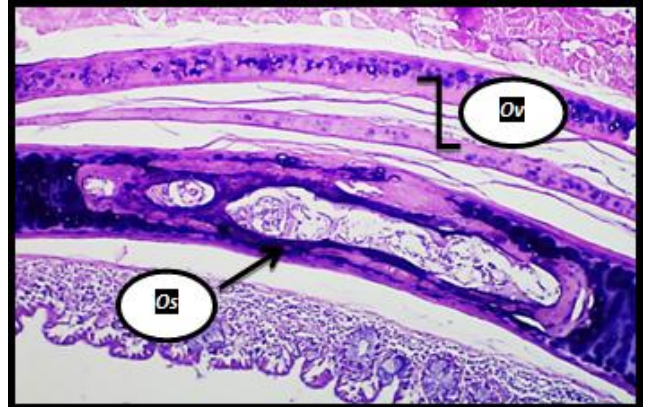


**Figure 13:** Cross section of the upper part of trachea (at 24 months age) showing stage of atrophy and death (d) in hyaline cartilage cells (H&E stain, X4 & X20)



**Figure 14:** Longitudinal section of the upper part of trachea in the Laying hens (at 24 months age) showing stage of atrophy (a) and death of hyaline cartilage cells (Von kossastain, X4)

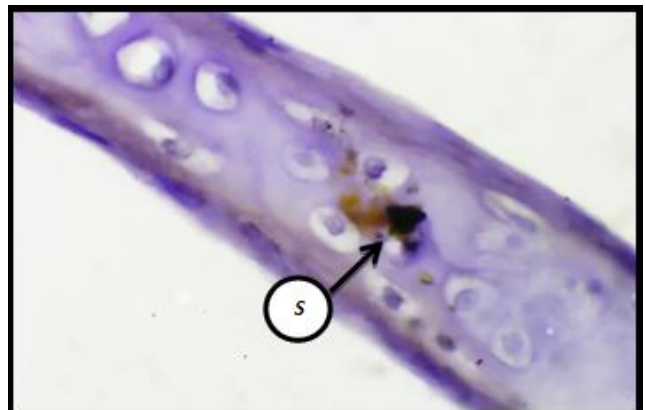
histological and immune histochemical findings of lower part of trachea at this age showed that the calcification foci are more than which appeared in upper part, and these foci become combined or inserted and occupies more than the quarter diameter of the tracheal rings (Fig. 15, 16, 17, 18).



**Figure 15:** Cross section of the lower part of trachea in the Laying hens (at 24 months age) showing combined ossified foci (Os) in overlapping cartilage rings (Ov) (H&E stain, X10).

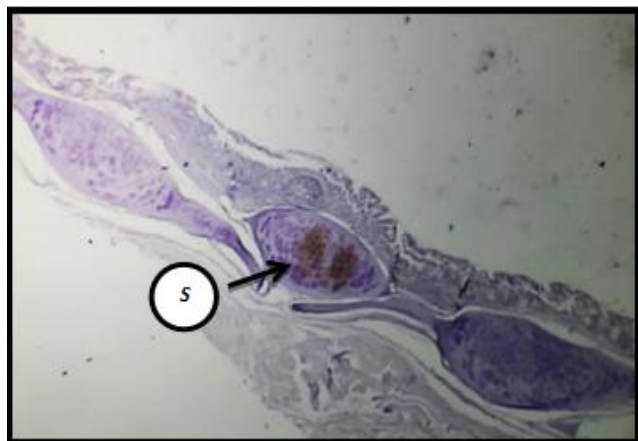


**Figure 16:** Cross section of the lower part of trachea in the Laying hens (at 24 months age) showing cartilage ossification (Os) in overlapping tracheal rings (Ov) (Von kossa stain, X10).



**Figure 17:** Immunohistochemistry in cross section of the lower part of trachea (at 24 months age) showing spots calcification (S) in the hyaline cartilage (X 40)

Comparatively can estimation the level of calcification in this age period at (+ + +) as summarized in (Table 2). The



**Figure 18:** Immunohistochemistry in longitudinal section of the lower part of trachea (at 24 months age) showing calcification in the form of brown spots (S) inside cartilage (X4)

Thus, all these sites of calcium precipitate in the hyaline cartilage of the lower part of trachea relatively estimated (+ + + +). Each result of tracheal calcification at this age of laying hens equivalent with [21], he mentioned distribution of calcification in sequent tracheal rings of golden comet bird at 2 years old.

The great development of the calcification ratio of hyaline cartilage of the respiratory tract at this advanced in months (24 months) might have been resultant of compilation motives in addition to toward age, the secondary ossification of the skeleton has been completed, the end of the egg production period for these birds and an imbalance in the metabolism of calcium in order to give laying hens large amounts of calcium in food to re-stimulate egg production.

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