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Histological and Immunohistochemistry Studies of Aorta artery Calcification in the Laying Hens

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Abstract: *A histological and immunohistochemical has been conducted to detection the calcification in the aorta artery of laying hens of red type (Gallus gallus domestic).The study included taking (24) healthy hens birds, They were divided into four groups (6 months, 12 months, 18 months, and 24 months), according to their ages. A histological study has been made by using two dyes, hematoxylin and eosin to show the general histological construction and Van Kossa tincture to show the deposition of calcium salts in thetissue. Also, the immunohistochemical technique was conducted by using primary and secondary antibodies show mild calcification appear as block spot in aorta artery at 6 months age.At the age of 12 months, the appearance of calcification in the aorta artery is more than the previous age. While at the age of 18 months, calcification appears as a small focus scattered in the arterial wall. With advancing age, the appearance of calcification is more clearly. Where at the age of 24 months it appears in the form of large black patches or tarnishes in the aorta artery*

Keywords: *laying hens, calcification, aorta, van kossa, immunohistochemical*

I. Introduction

The greatest number of fowls is to be found in South-East and East Asia, frequently utilized for egg [1]. Fowls have an exceptionally proficient system for diffusing oxygen into the blood, fowls have a ten times more prominent surface region to gas exchange volume than mammals, subsequently, fowls have more blood in their capillaries per unite of lung than a mammal[2]. The cardiovascular system conveys nutrient and oxygen to tissues while simultaneously expelling metabolic rubbish including carbon dioxide [3];[4].Mineral constitute around 4% of vertebrate creatures of which calcium and phosphorus make up more than half of this sum [5]. These two minerals contain more than 70% of the mineral content of the creature body [6]; [7].Calcium is one of the key component required for upkeep and egg production. It assumes a noteworthy part in a wide assortment of biological function [8]The arterial calcification has been perceived for over 200 years [9];[10]. Vascular calcification was initially recorded by Mönckeberg in the nineteenth century, and for a long time [11].Calcification was thought to be an inactive procedure that happened as a nonspecific reaction to tissue injury or necrosis [9] ;[10].Vascular calcification can be classified into four primary sorts depending upon its area: atherosclerotic intimal calcification, medial calcification (Mönckeberg's sclerosis), cardiac valve calcification and calcific uremic arteriolopathy[12].Arterial calcification happens with advanced age, atherosclerosis, metabolic disorders, including end stage renal illness and diabetes mellitus, and some genetic disorders [13].Arterial calcification adds to hypertension and expanded dangers of cardiovascular events, leading to morbidity mortality[14]. Calcification is an unmistakable element of atherosclerotic lesions in individuals [15]. and has additionally, been watched morphologically in atherosclerotic plaques from pigeons [16]. Spontaneous (non-induced) atherosclerosis in the chicken was initially portrayed in 1914 [17].Calcification is a vital part of the atherosclerosis procedure and for the most part, it is being created inside the intimal layer of the vessels wall [18]. Different avian species, for example, pigeons, turkeys and chicken which have been appeared to be the helpful empirical creature for induction of atherosclerosis [19].

II. Materials and Methods

2.1.The Experimental Animals and Samples collection

Twenty four healthy fowls were gathered form poultry fields in Waist city .They were splitting into four groups regarding their different ages (6 months, 12 month, 18 month, 24 month), every gathering has six fowls.Every fowl anesthetized by chloroform inward breath. The fowls were left for 2-5 min to finish anesthesia. The thoracic bowlwas opened and aorta artery samples were gathered from the laying hens specifically after sacrificing these fowls.The aorta artery samples were keeped in 10% formalin in for (72) hr, they were cleaned up by water for 2-3, and transported the samples to numerous histologicaltechniques 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.

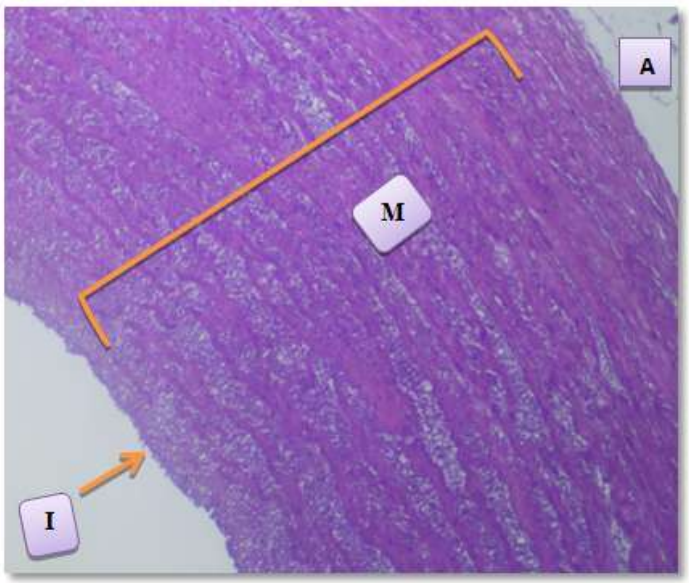
2.2. Immunohistochemistrytechnique

Immunohistochemically staining was performed utilizing the Calcium Channel, Voltage –Dependent, gamma Subunit 5(CACNG5)(AA43-296) antibody.The tissues were de-waxed by doused slides in toluene and afterward rehydrated by absorbing combined concentrations of ethanol, followed by a wash in 2-3 times in clean water.Antigen retrieval by immersed slides in jar containing citrate buffer solution (PH= 6). Position the slides in a. washed slide with Phosphate Buffer Saline (PBS) for 5min. exogenous peroxidase activity by incubating the piece in peroxidase block (H_20_2) for 5-10 min at RT, and then were washed with distilled water 3times, also slides were washed with PBS. Incubate segment in protein blocking solution for 5-10 min at RT.Then, the area was incubated in primary antibody, not supplied, just buffer is supplied for dilution for (20-30min) at room temperature, slides were washed with PBS five to seven times incubated with one- step HRP polymer secondary for 20-30 min at room heat, followed by five to seven times washed in PBS and washed slide with filtered water two to three times.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.

III. Result and Discussion

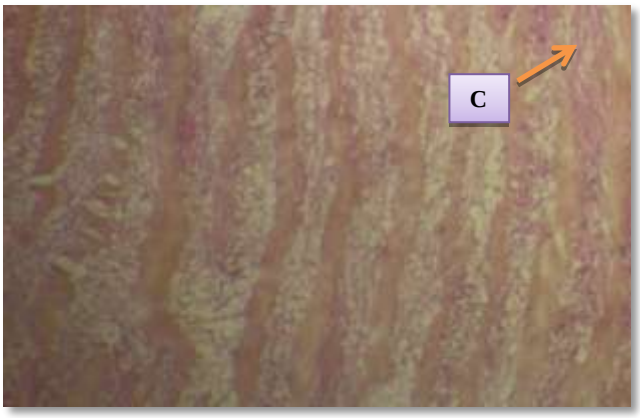
3.1. Histological and Immunohistochemical results at 6 month old.

The histological examination by using hematoxylin and eosin stain was described the general composition of the elastic arteries and showed that the wall of aorta artery consists from the tunica intima, which is single layer of endothelium cells adjacent to arterial lumen. Tunica media which is composed of concentric stratums of rotaring layers of involuntary smooth muscle fibers. These muscular layers possessing elastic fibers lamina. The third layer of the vessels is adventitia, which consists of connective tissue with different thickness in the aorta artery (Figure 1) .These results are corresponded with findings obtained in the arterial wall of the elastic arteries of birds by [20; 21].

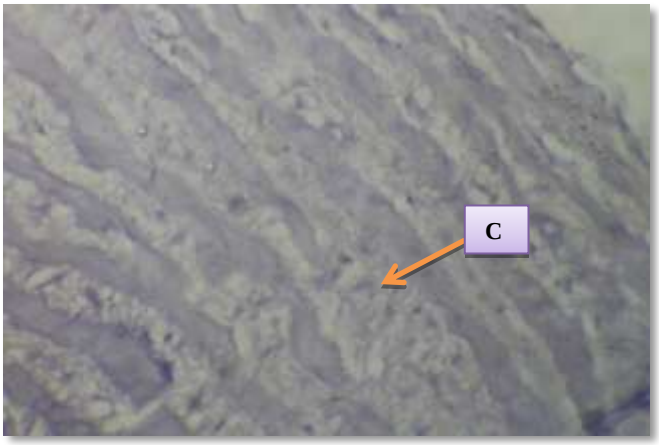


(Figure 1): Gross section of the aorta artery of chicken at aged 6 month showing wall layers of aorta, Intima (I), Media (M), Adventitia (A), Stained with(H&E 20 X)

The histological preparation results with calcium detecting stain (van kossa stain) in the present work revealed that first evidence of the present the calcification emergence as very small black point which spread with wide apart in the layers of tunica media in aorta artery. The immunohistochemical examination by using calcium primary antibody appeared tenuous calcium deposit with dark color in the tunica media of both aorta and pulmonary arteries (Fig 2). Relatively the aorta artery calcification was estimate as (+) at this age of laying hens chicken (Table 1) This result was similar to finding of the [22], who classification the calcium atherosclerosis as seven lesions in psittiacine, which ages ranged from (10 weeks to 48 years), there are no calcium deposit was observed in the elastic arteries at type I,II lesions., but mild positive staining for calcification by van kossa stain was at type III lesion.



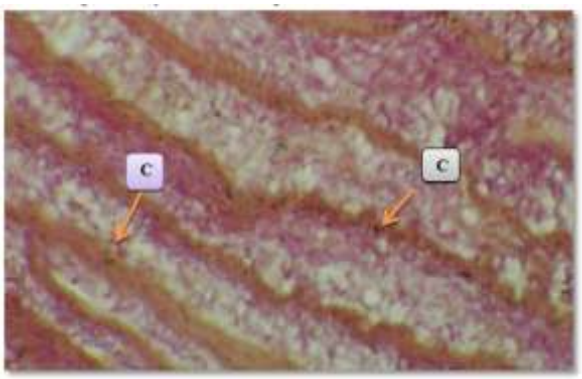
(Figure2): Gross section of the aorta artery of chicken at aged 6 months showing the calcification (C) in small black point stained with (Van Kossa 40 x)



(Figure 3): Immunhistochemistry in cross section of the aorta arteries of chicken at age 6months, showing, calcification(C) in the forms of small black point (40X).

3.2. Histological and Immuniohistchemical results at 12 months old.

Through this age of present work, the hematoxylin and eosin preparation of aorta artery section was illustrated the normal common histological structures of these artery. The section of aorta artery of chicken at this age were coloring by van kossa stain, showed that calcification is relatively more than the preview age (6 month old) and the calcification lesion occupies a large portion of the smooth muscle layer of aorta artery (Fig 4). To promote of the results at above, the immunohistochemical technicality appeared that the more dark brown blotches which disarray in the tunica media of aorta artery comparison with previous scattering of age 6 months (Fig 5). The calcification ratio in the aorta at this age of present was estimated as (++) as showing in (Table 1). This finding are similar to the documented by [22] in Psittacine, he mentioned in the type IV,V atherosclerotic lesions the modrate plaque calcification in aorta, pulmonary and brachiocephalic arteries.

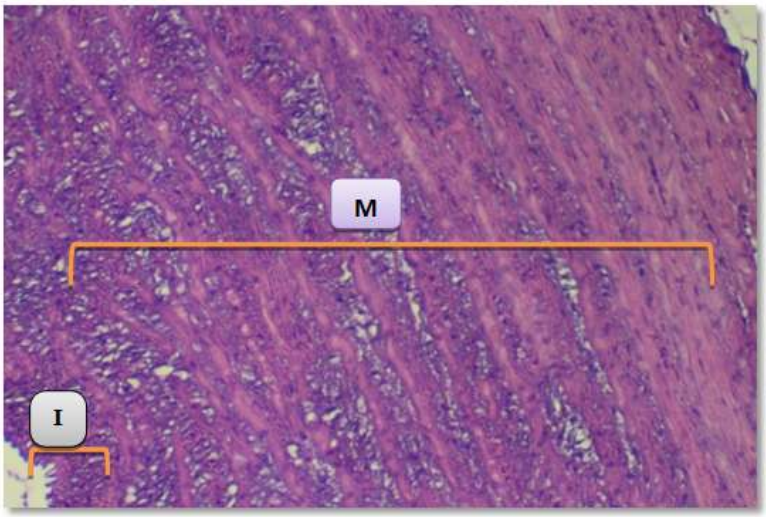


(Figure4): Gross section of the aorta artery of chicken at aged 12 months, showing the calcification (C) in the from black point spread in media layer stained with (Van Kossa 40 X)

(Figure 5): Immunohistochemistry in cross section of the aorta artery of chicken at aged 12 months showing, calcification (C) in the forms of small black point (40x)

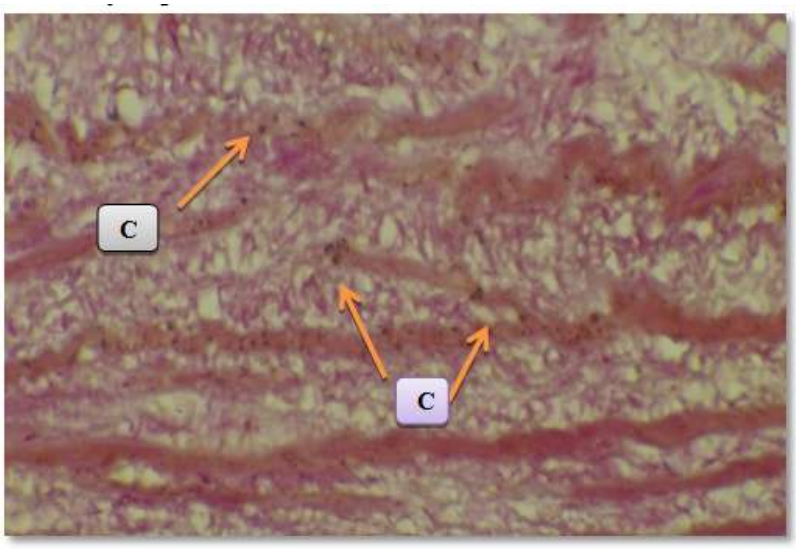
3.3.Histological and Immuniohistochemical results at 18 months old.

The results of the routine histotechnique with hematoxylin and eosin showed that the tunica media of aorta arterycomposed involuntary smooth muscle fibers with elastic lamina(Fig 6). Generally, these findings are identical to [22], who studied the arteries in psittacinewho described the intima was made out of a solitary layer of straightened extended endothelial cells. The media was made out of concentric lamellae of flexible filaments and shaft formed smooth muscle cells (SMCs) installed in a ground substance made out of collagen, versatile strands, and extracellular grid. A very much portrayed inside versatile lamina, which indicates the outskirt between the intima and the luminal side of the media and is considered part of the media. [23], was recognized no lipid or calcium stores were noted inside the intima and media on the histological areas with hematoxylin and eosin and histochemical stains. In accordance with specialty activate of hematoxylin and eosin stain, its don't show any calcium solidity in this tissue.



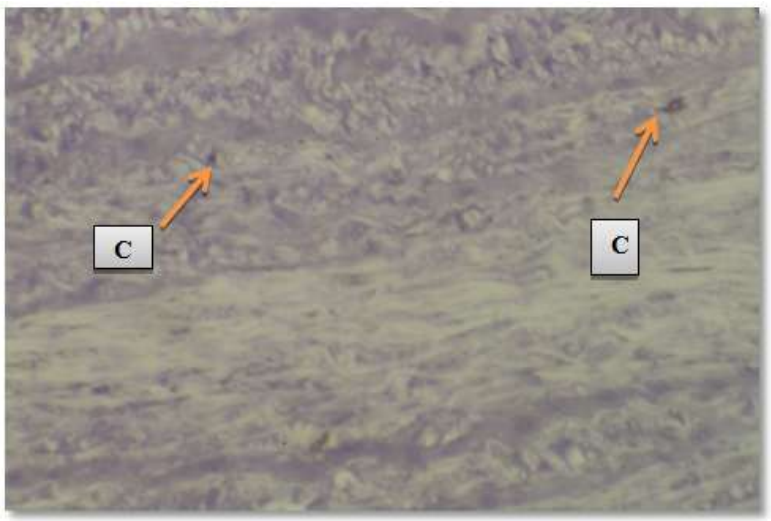
(Figure 6): Gross histological section of the aorta artery of chicken age (18) months, showing wall layers of aorta, Intima (I), Media (M), stained with(H&E, 20X)

Nevertheless, the tissue section which treated with van kossa procedure technique and the results were occurred that the calcifications at this step age are comparatively is much more than the former ages of this work where the light microscope finding showed the calcium condensation appearance as small foci which widely scattered in the tunica media of the aorta artery (Figure 7).



(Figure 7): Gross section of the aorta artery of chicken at age (18) months, showing the calcification (C) in the from black small foci stained with (Van Kossa 40x)

Whereas the immunohistochemical technique with calcium primary antibody (CACNG5) and secondary antibody (HRP-anti Mouse, Rat and Rabbit polymers) showed that the calcification at this age appeared as brown to black small blots or blotches spread through the smooth muscle fibers of tunica media stratum of the aorta artery (Figure 8).Accordingly, the calcification ratio of this age was more than which appeared during the previous age 12 months and relatively was estimated (+++) as (Table1). These results were corresponding with the same author who are mentioned the advanced calcification lesions (IV,V) and described ultrastructural by transmission electron microscope that accumulation of dense calcium granules in the tunica media, descending aorta and brachiocephalic arteries. As well, this result of our work was similar to finding of [24] who determined the calcium content in the smooth muscle of the aorta layers of pigeon with normally occurring atherosclerosis.

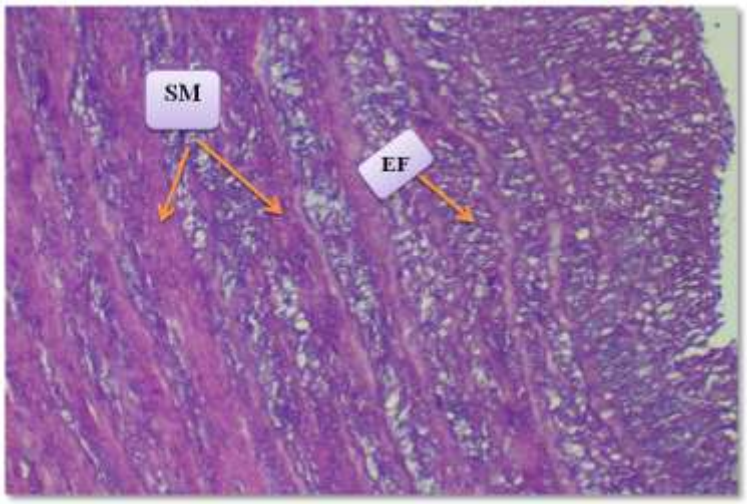


(Figure8): Immunohistochemistry in cross section of the aorta artery of chicken at aged (18) months showing, calcification (C) in the forms of small black foci, (40x).

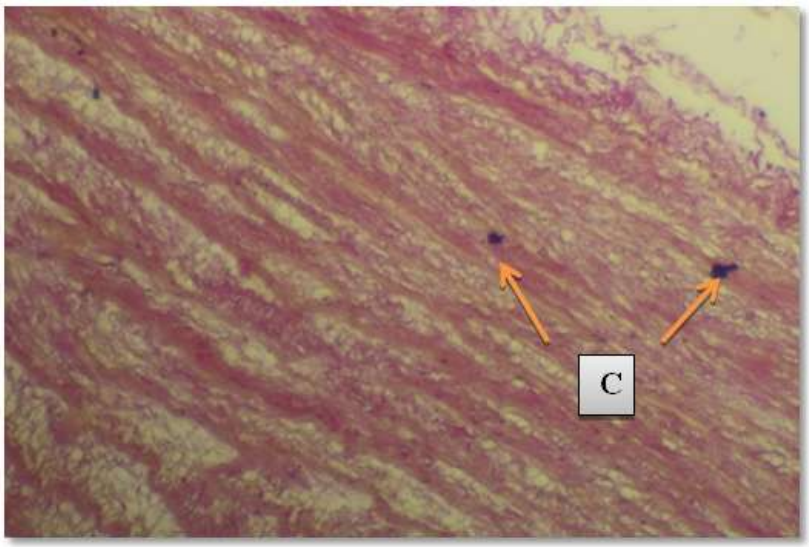
3.4. Histological and Immunohistochemical results at 24 month old

The routine histological examination by using hematoxylinand eosin stain habitually was appeared only the general structure of aorta artery (Figure 9). The structure of these arteries wall consists from normal tunica intima which particularized with simple squamous epithelium cells. The tunica media is made out of substituting layer of smooth muscle strand and flexible lamina, the adventitia is comprise of connective tissue layer changing in thickness. All these finding werementioned in the various species of old age birds [25] in pigeon, [26] in chicken, [27] in parrot and [28] in japanese quails. However, the histological examination for

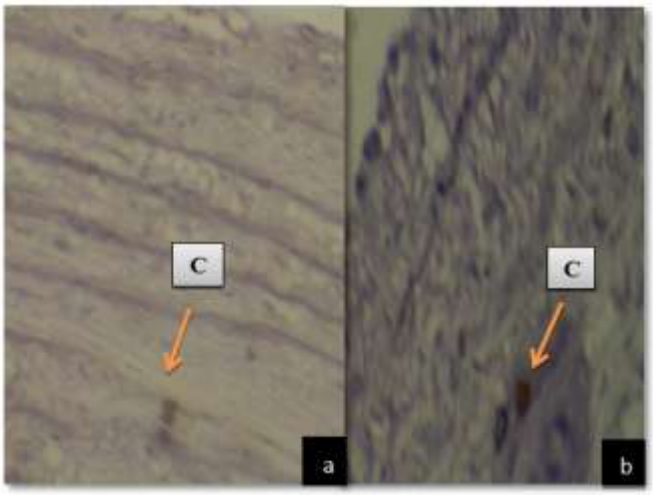
the artery section which treated by calcium detecting stain (van kossa) elucidated that the calcification in the aorta artey appear as clear big foci of calcium combines or form large blots with blackish or brownish color (Figure 10).Identically as results above, the immunohistochemical procedure for paraffin section of these arteries revealed that the calcium accumulations in tunica media of the blood vessels appear rather large blackish spots and widish in section compare with that calcium gather which arise in the previous age of this project (Figure 11). Thus and depending on the histological and immunohistochemical inspections, the calcification morbidity of the aorta artery relatively evaluated as (++++) at 24 months age of laying hens chicken. These results are properly compatible with several resent reviews [29] who described the calcification lesion type (VII) through which peristaltic large calcium plaques in the wall of major arteries of psittacine birds, whilst he documented in (2013) seven lesion type of atherosclerosis in tunica media, Type (V) calcification fibro atheroma is commonly found in the large arteries at the base of heart, pulmonary arteries and ascending aorta of amazon parrot. However several studies [30, 31] were demonstarted these lesion appear with less frequently in the abdominal aorta and peripheral arteries. In the light of the results at this age (24 months old) of chicken, the profusely advanced large blood vessels calcification was occurs, firstly due to forward of birds age and give diet with high level of calcium (up 100 kg/ one ton nutrition) which genitive lipid or fatty quantities, simultaneity with great reduction of eggs production at these aged birds. Furthermore, the elevated serum calcium and phosphates are strongly correlated with mineralization and have direct effect on vascular smooth muscle calcification [32].



(Figure 9): Gross section of the aorta artery of chicken at aged (24) months, showing Elastic Fiber (EF), Smooth muscle (SM), stained with (H&E 40x).



(Figure 10): Gross section of the aorta artery of chicken at age (24) months showing, the calcification (C) in the fromof large blots in media layer stained with (Van Kossa, 20x).



(Figure11): Immunohistochemistry in cross section of aorta artery of chicken at aged (24)months showing calcification (C) in forms of large spots, a (40x), b (100x)

(Table 1): The relative proportions of arterial calcification in laying hens

Chicken laying hens age	Aorta artery
6 month	+
12month	++
18 month	+++
24 month	++++

IV. Conclusion

Histologically, the (H&E) stain showing the general structure of aorta artery wall which consist from tunica intima, media and adventitia. Van Kossa stain and immunhistochemical technique appear the different blood vessels calcification proportion with variants grade ages.The calcification is mild in young age (6 month) which appear as small scattered spot in tunica media of blood vessels.This calcification ratio was increased at 12 and 18 months of age birds and which appeared as large black blotches at (24 months) of age.Commonly, the blood vessels calcification occurs in birds with advance age which accompaniment reduction in eggs production.

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