Saja Kareem Al-Etbee et al /J. Pharm. Sci. & Res. Vol. 11(7), 2019, 2783-2790

ISSN:0975-1459
Journal of Pharmaceutical Sciences and Research
www.jpsr.pharmainfo.in

Histological and Immunohistochemical Study of Valves Calcification in the Bulls

Saja Kareem Al-Etbee[1] and Ahmed Mahdi Al- Badri[2*]

[1] *Department of Biology, Collage of Science, Wasit University, Wasit, Iraq.*

[2] *Assist. Professor, Department of Biology, Collage of Science, Wasit University, Wasit, Iraq.*

Abstract:

A histological and immunohistochemical study was appeared the general structure and detect the calcification in the aorta, pulmonary, bicuspid and tricuspid valves of calves. The study included taking (18) healthy hearts from massacres of Wasit. They were divided into three groups, according to animals' ages (1 year, 2 years and 3 years). Six heart for each group. The heart samples were anatomized and taken parts from the four types of valves (aorta, pulmonary, bicuspid (mitral) and tricuspid). All these samples were fixed in formalin 10%. A histological study has been made by using three types of dyes, hematoxylin and eosin to show the general histological structure, Van Kossa and Alizarin Red stain to detect the deposition of calcium salts in the tissue. Also, the immunohistochemical technique was conducted by using primary and secondary antibodies show mild calcifications appear in the valves at 1 year old. The result showed at the age of 2 years, the appearance of calcification in valves were more than the previous age, it appeared in the form of brown blots spread through all layers of the valves. With advancing age, the appearance of calcification was more clearly, as in 3 years old which appears in the form of large brown patches in all types of valves.

Keywords: Calcification, Valves, Von Kossa, Alizarin Red, bulls.

INTRODUCTION

Cattle are the most common type of large domesticated ungulates (although there are wild varieties as well) [1]. The cardiovascular system is the vehicle for carrying of blood in the body, carrying nutrients to the organs and body tissues and eliminating several waste products [2]. The circulatory system consists of the four chamber heart (with the four heart valves), the general vessels that convey the blood to and collect the blood from the peripheral organs, and the pulmonary vessels that carrying the blood through the lung for exchange of oxygen and carbon dioxide [3]. Mammalian hearts have four valves with mainly similar structures and locations [4]. The atrioventricular valves are located between the right atrium and right ventricle (tricuspid valve) and between the left atrium and left ventricle (mitral valve), whereas the semi-lunar valves found between the right ventricle and pulmonary artery (pulmonary valve) and between the left ventricle and the aorta (aortic valve) [5]. Minerals found about 4% of vertebrate creatures of which phosphorus and calcium make up more than half of this sum [6]. These two minerals contain more than 70% of the mineral content of the body [7], [8]. Calcium is necessary to main body processes such as blood coagulation, cell membrane permeability, cardiac rhythm control, ossification, activation and secretion of hormones and enzymes, nerve and muscle excitation. Phosphorus is the second most abundant mineral in animals and 80% of this element is found in teeth and bones [9]. The calcification is accumulation of calcium salts in a body tissue. It normally occurs in the development of bone, but calcium can be deposited abnormally in soft tissue causing it to harden [10]. Several causes effect on the calcification development parathyroid hormones, comprising age, vitamin D and high levels of calcium nutrition which plays a primary role in calcification [11]. Cardiac valves Calcification can lead to regurgitation, stenosis or both [12]. Calcific deposits generally produce valvular dysfunction only after the bioprostheses have been in place for several years [13]. The aims of this present study its detect spontaneous calcification of heart valves and determine its appearance, in different ages of males cattle (bulls) by using histological and immune histochemical technique.

MATERIAL AND METHOD

The Samples collection

18 samples of bull's heart were collected from healthy animals of wasit massacres. The samples were divided according to age of animals into three groups (1 year, 2 years and 3 years) depending on teeth equation, each group contained 6 hearts, all hearts should be clinically healthy and devoid any type of injuries. These heart samples were anatomized and taken parts from the four types of valves (aortic valve, pulmonary valve, bicuspid (mitral) valve and tricuspid valve). The samples were kept in 10% formalin in for (72) hr., they were cleaned up by water for 2-3, and transported 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 Alizarin Red and Von kossa stain to detect deposits of calcium salts in paraffin sections.

Immunohistochemistry technique

Charge slides which comprising wax embedded valve tissues briefly, were dewaxed in absolute toluene for 30 minute. The tissue was placed in reducing concentration of absolute ethanol for 10 minutes, 90% one minute and 70% one minute. Washed slides in 2-3 times in clean water. The procedure should be based on manufacture, antigen retrieval by immersed slides in citrate buffer solution then heated in oven to (100 ºc). The slides incubated for 30 minute to cool at room temperature (RT). Washed slides with phosphate buffer saline PBS 3 times. The sections incubated with protein blocking solution for 10 minutes at

RT. The Slides incubated with primary antibody for 30 minute at RT. The negative control was treated without adding the primary antibody. Subsequently, the slides washed with PBS 7 times. The slides incubated with one - step HRP polymer for 30 minute at RT. Slides were washed seven times with PBS also slides were washed with distilled water 3 times. Add little drops of DAB reagent on tissue slides and delay for 10 minutes at RT. Slides were washed 7 times with PBS and washed in distilled water. Later, sections incubated with hematoxylin for 60 second. Subsequently; slides washed with distilled water until it ran clear and mounted with D.P.X., mounting medium and enclosed by coverslip.

Methodology of Statistics

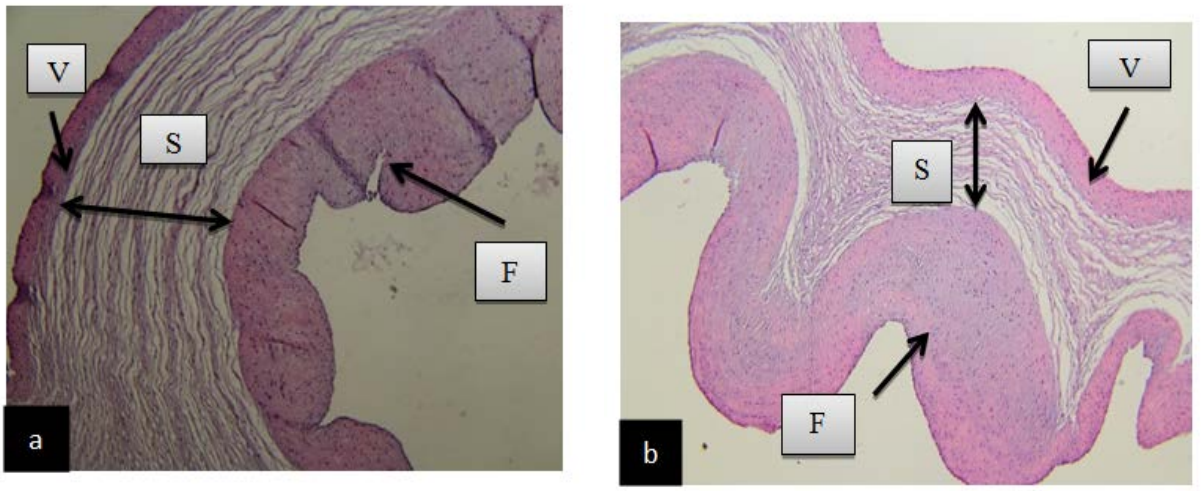
Computer Assisted digital image analysis (Digital morphometric study)

The slides were photographed by using canon digital camera installed on Mejia microscope with 1/2 X photo adaptor, with using (40 X) objective. The result images were analyzed on Intel Core I3 based computer using Video Test Morphology software (Russia), with a specific built-in routine for area, % area measurement and object counting.

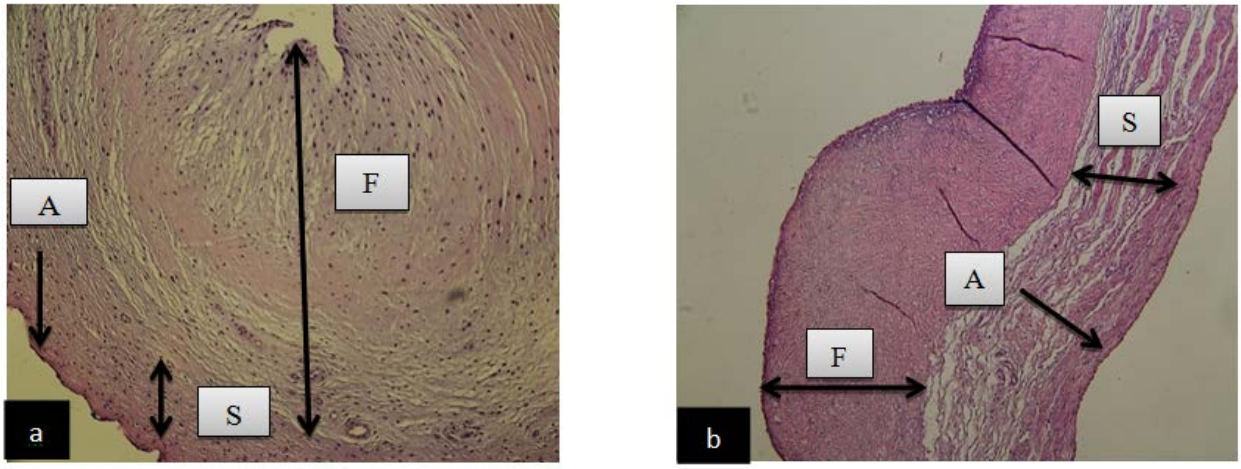
RESULT AND DISCUSSION

Histological and Immunohistochemical results at 1 year old.

The histological results of routine stain (hematoxylin and eosin) was appeared the general structure of valves, which consist from three layers, fibrosa layer; spongiosa layer and aventricularis layer (in pulmonary and aortic valve) and atrialis layer (in the bicuspid and tricuspid valves) (Fig. 1, 2). The fibrosa was composed of bundles of collagen fibers that arranged both longitudinally and radially, and the spongiosa was a thin lucent layer containing delicate collagen fibers and scattered fibroblasts. The ventricularis and atrialis were less dense, longitudinally arranged, thin and mainly composed of elastic fibers. These facts were compatible with human [14] and pigs [15].

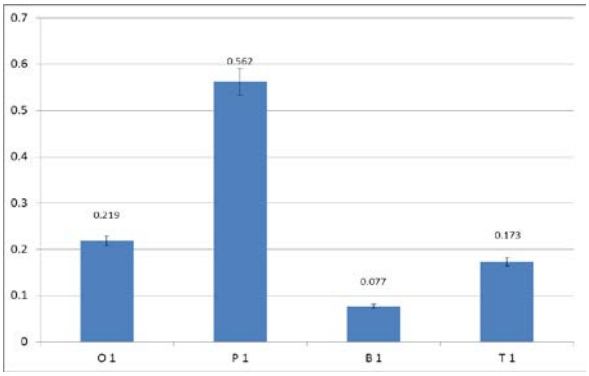


(Figure 1 a,b): Cross section of the valves of bull at aged 1 year showing (a): layers of the aortic valve, fibrosa (F), spongiosa (S), and ventricularis (V), stained with (H&E 100 X),(b) layers of the pulmonary valve, fibrosa (F), spongiosa (S), and ventricularis (V), stained with (H&E 400 X).

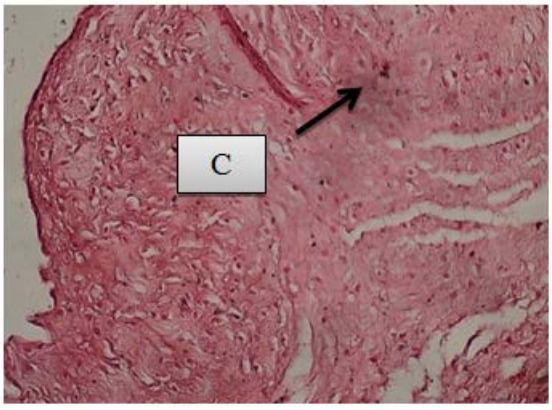


(Figure 2 a,b): Cross section of the valves of bull at aged 1 year showing (a): layers of the bicuspid valve, fibrosa(F), spongiosa (S), and atrialis (A), stained with (H&E 200 X),(b): layers of the tricuspid valve, fibrosa (F), spongiosa (S), and atrialis (A), stained with (H&E 100 X).

The histological technique by using calcium detecting stains (Von kossa and Alizarin Red stain) were showed small brown points of calcification which spread in aortic valve, pulmonary valve, bicuspid valve and tricuspid valve (Fig. 3, 4, 5, 6). By immunohistochemical method with antibody (S100 Calcium Binding Protein B), as well the results of this technicality was appeared the calcium deposit as small points (Fig. 7, 8). Statistically, the ratio of calcification of valves at this age of bulls was estimated as follows [0.219 in aortic valve (O), 0.562 in pulmonary valve (P), 0.077 in bicuspid valve (B) and 0.173 in tricuspid valve (T)] as summarized in (chart 1), (Fig. 5.b, 6.b).

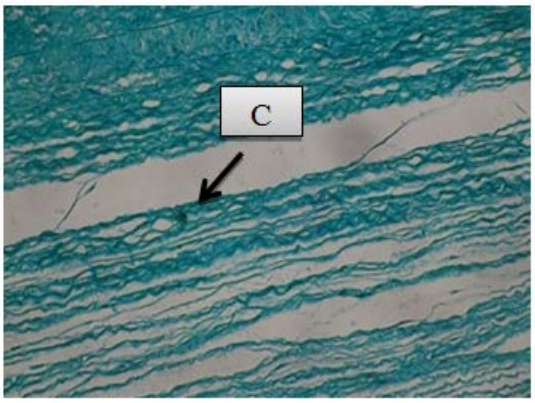


(Chart 1): Chart showing the relative proportions of calcification the four types of valves ,aortic valve (O), pulmonary valve (P), bicuspid valve (B) and tricuspid valve (T) in bulls at age one year old.

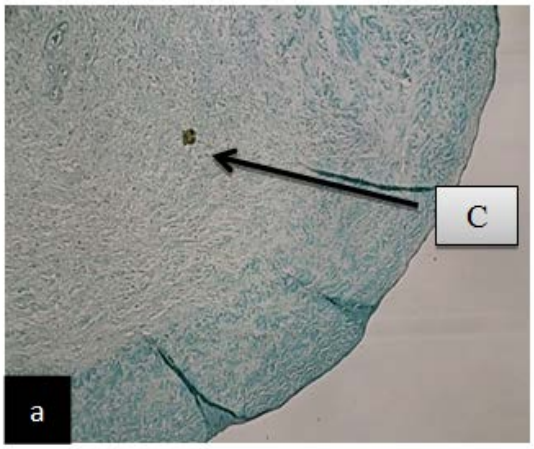


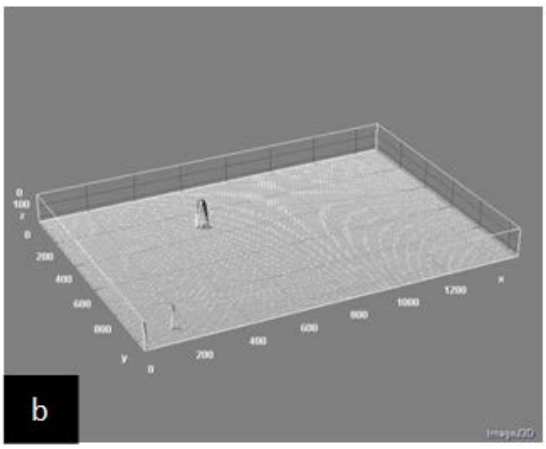
(Figure 3): Cross section of aortic valve of bull at aged 1 year showing the calcification (C) in small brown point stained with (Van Kossa 400 x).

All these results were agree with some authors like Gott *et al.* [16] who showed mitral valves of sheep were 14 to 24 weeks of age had thickened, immobile, calcified leaflets, which stained with von Kossa's reagent; however Bernaccae *et al.* [17] who found focal calcium deposition in a bovine pericardial valve by Alizarin Red stain. On the other hand Schoen *et al.* [18] was demonstrated by light microscopy that calcification initially appeared as small, clearly intrinsic nodules that were scattered throughout the cusps of aorta of sheep with 3 to 4 months of age, otherwise, the earliest cuspal calcific deposits were noted in the fibrosa layer but later deposits expanded spongiosa layer of aortic valve. Wirrig *et al.* [19] who described the calcification of aortic valve of mice at 3 weeks aged as brown staining by von Kossa stain.

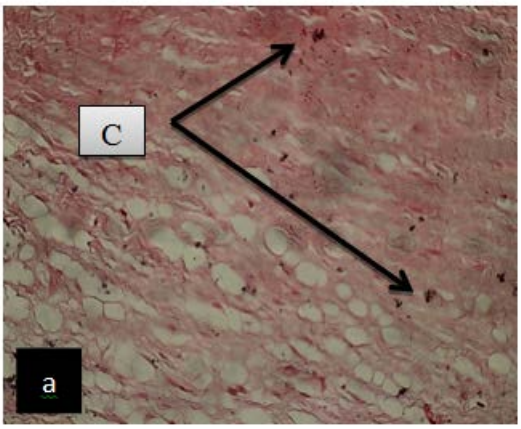


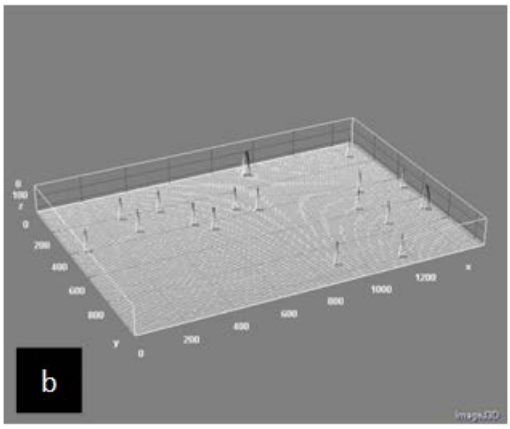
(Figure 4): Cross section of pulmonary valve of bull at aged 1 year showing the calcification (C) in small brown point stained with (Alizarin Red 400 x).



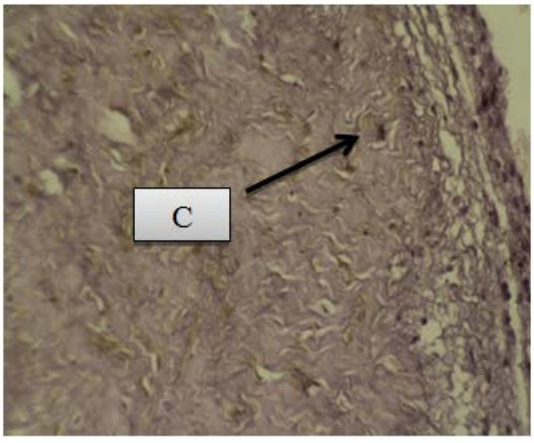


(Figure 5a,b) (a): Cross section of bicuspid valve of bull at aged 1 year showing the calcification (C) in small brown point stained with (Alizarin Red 400 x). (b): Surface plot of bicuspid valve of bull at aged 1 year showing the percentage of calcification for the tissue.

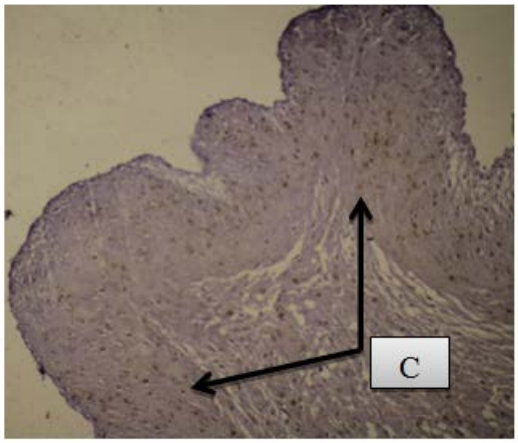




(Figure 6a,b) (a): Cross section of tricuspid valve of bull at aged 1 year showing the calcification (C) in small brown points stained with (Van Kossa 400 x). (b): Surface plot of tricuspid valve of bull at aged 1 year showing the percentage of calcification for the tissue.



(Figure 7): Cross section of pulmonary valve of bull at aged 1 year showing, calcification (C) in the forms of spread small points Immunohisto-chemistry 400x).

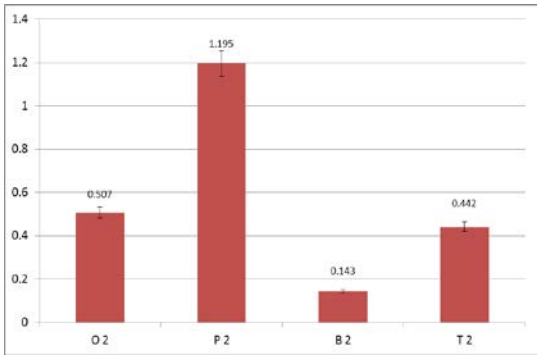


(Figure 8): Cross section of bicuspid valve of bull at aged 1 year showing, calcification (C) in forms of spread small point (Immunohisto-chemistry 200x)

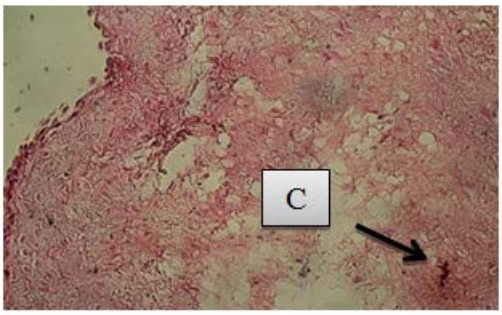
Histological and Immuniohistochemical results at 2 years old.

The histological findings with von kossa stain and Alizarin stain noticed the aortic valve, pulmonary valve, bicuspid valve and tricuspid valve were calcified, and these calcifications lesion were more than that observed in the same valves of former age (1 year old) (Fig. 9, 10, 11, 12). Also, the immunohistochemical technicality was showed the calcification apparent as dark brown spots which disarray in all layers of these valves (Fig. 13, 14). The proportion of calcium deposit in the aortic valve, pulmonary valve, bicuspid valve and tricuspid valve was estimated respectively [0.507 in (O), 1.195 in (P), 0.143 in (B), 0.442 in (T)] as summarized at (chart 2), (Fig. 10.b).

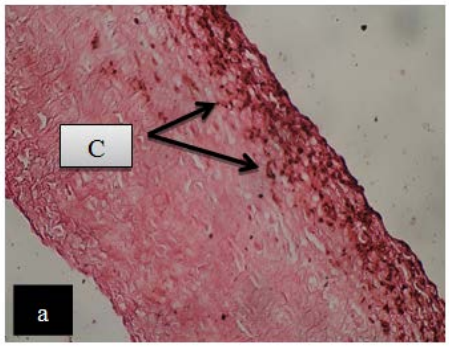
This result is agreed with Simmons [20] who described the calcification in aortic valve of male pigs as nodules which detected by alizarin red stain. On the other hand Schoen and Levy [21] were demonstrated in sheep the calcification of aortic valve is diffuse, involving the entire cross section, most predominantly in the innermost and outermost 20% of the media which stained with von Kossa's reagent. Our results which indicate to progress values of calcification lesions in all types of values at this age (2 years old) were supported by yamate *et al.* [22], the incidence and severity of the lesions increased with age. Moreover, the statistical findings were showed increase of calcific deposit areas in aortic and pulmonary valves than the bicuspid and tricuspid valves which present between atrium and ventricle champers of heart (chart 2). This pathological variation because of aortic and pulmonary valves can be affected by physiological and pathological arterial (aortic and pulmonary) calcification. This strongly suggests was predicable by Beaufrere *et al.* [23] he was mentioned in type of vertebrata the calcification type (V) is commonly found in aortic and pulmonary large arteries at the base of heart.

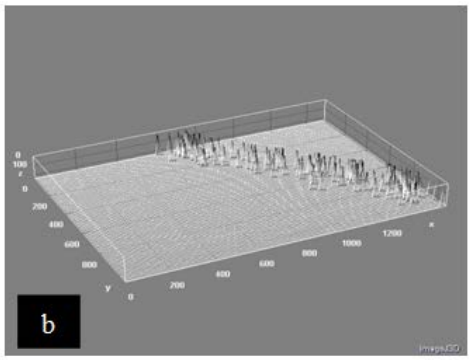


(Chart 2): Chart showing the relative proportions of calcification the four types of valves, aortic valve (O), pulmonary valve (P), bicuspid valve (B) and tricuspid valve (T) in bulls at age two years old.

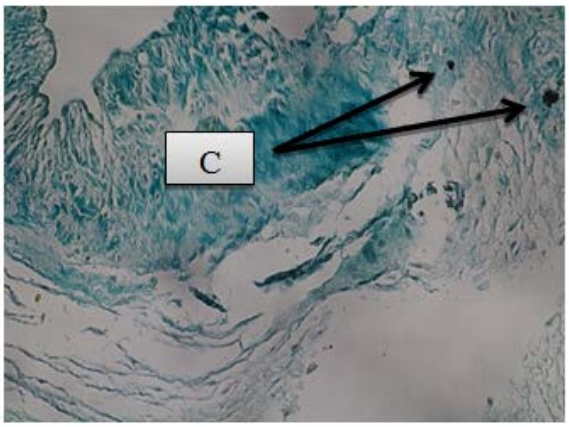


(Figure 9): Cross section of aortic valve of bull at aged 2 years showing the calcification (C) in brown black spot stained with (Van Kossa 400 x).

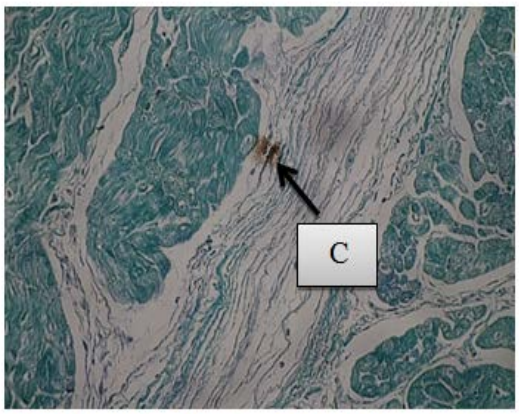




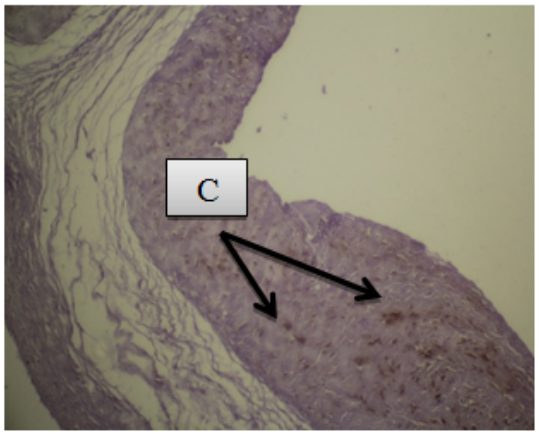
(Figure 10) (a): Cross section of pulmonary valve of bull at aged 2 years showing the calcification (C) in many of brown spots stained with (Van Kossa 400 x). (b): Surface plot of pulmonary valve of bull at aged 2 years showing the percentage of calcification for the tissue.



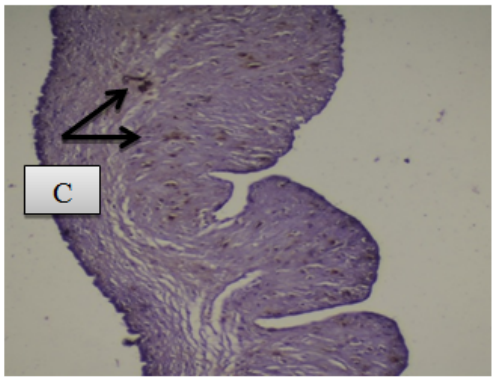
(Figure 11): Cross section of bicuspid valve of bull at aged 2 years showing the calcification brown spots stained with (Alizarin Red 400 x).



(Figure 12): Cross section of tricuspid valve of bull at aged 2 years showing the calcification (C) in brown spots (C) in stained with (Alizarin Red 400 x).



(Figure 13): Cross section of pulmonary valve of bull aged 2 years showing, calcification (C) in the forms of spread small points (Immunohistochemistry, 200x).



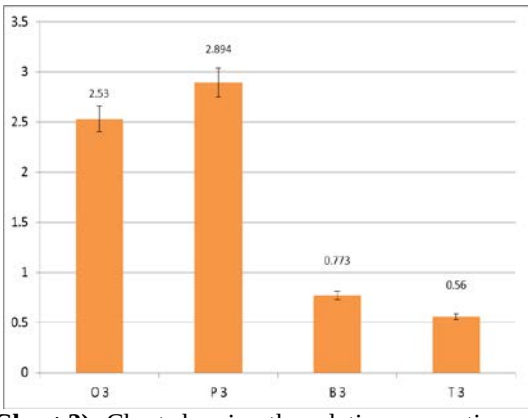
(Figure 14): Cross section of tricuspid valve of bull at age 2 years showing, calcification (C) in the forms of spread small points (Immunohistochemistry 200x).

Histological and Immunohistochemical results at 3 years old.

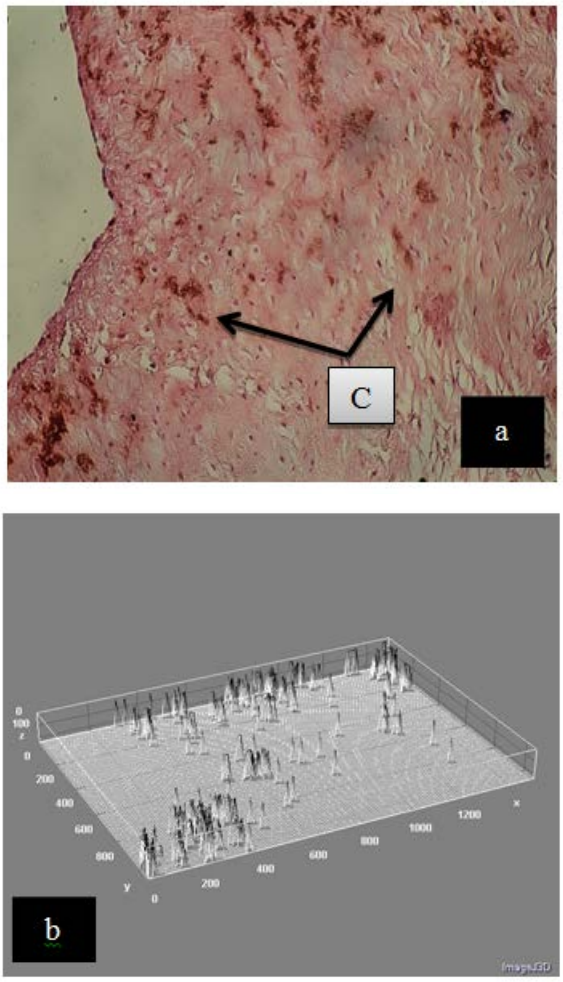
With Von Kossa stain, the histological sections was described the evidently calcification in aortic and tricuspid valves in multi place which appeared as brown spots (Fig. 15, 16). While at employment with alizarin red stain the calcification were appeared in pulmonary and bicuspid valves as very broad brown to black and red spots which occupied the wideness of valves structures (Fig. 17, 18). On the other hand, the results of immunohistochemical technique were used at this age likewise were evidenced densely spreads of calcification through each valves with different average (Fig. 19, 20), which parallel the histological findings. All the methods of this work, were indicated to that most parts of the four valves structure at this age become calcified when compared with the previous ages of the current experiment, and can be estimated [2.53 in (O), 2.894 in (P), 0.773 in (D), 0.56 in (T)] (chart 3), (Fig. 15.b).

This result was agreed with Hofmann *et al.* [24] who showed that high levels of calcium deposition in the valves of adult mice at aged (5-13) months old, detected by Von Kossa and Alizarin Red stain. Weiss *et al.* [25] was reported occurrence the abundant of calcification in valve leaflet tissue and at the attachment points to the aorta of old mice (range 17 to 25 months), Von Kossa staining of valve tissue was showed mineralization of tissue appears as discreet dark foci.

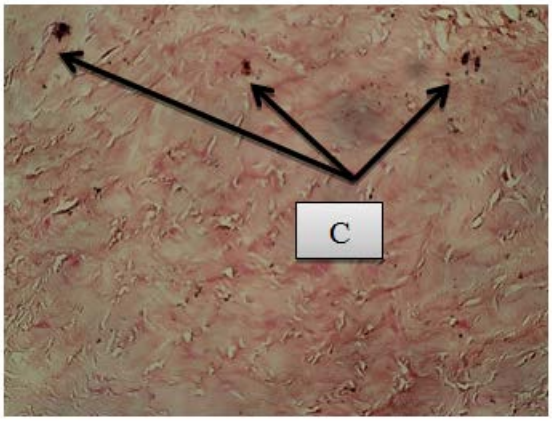
We reported on the appearance and evaluation of calcium deposit in the four types of heart valves including (aorta, pulmonary, bicuspid and tricuspid) in the males of cattle. Our result were revealed that high rate of calcification of valves, especially with advance age (chart 4).Thinking that this increase in the valves calcification probably is due to influence of powerful stress on the valves through mechanical heart function. In addition, natural synthesis of connective tissue of valves may all play role and were allowed in facilitate the elevate calcification in the valves. Moreover, and reinforcement of this view, [26] were mentioned a strong relationship between calcification and mechanical stress in biological artificial heart valves in bovine. The valves calcification has been occur in parts of highest stress-press where connective tissue fibers breakdown [27, 28].



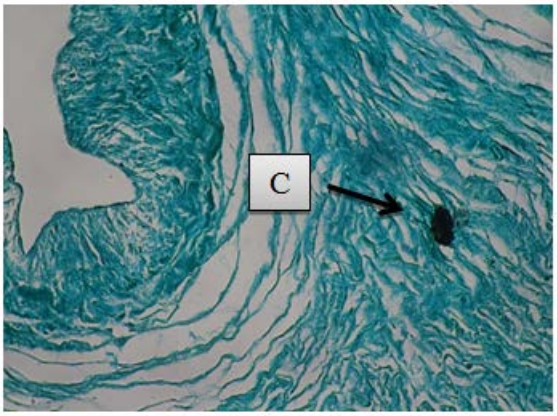
(Chart 3): Chart showing the relative proportions of calcification the four types of valves,aortic valve (O), pulmonary valve (P), bicuspid valve (B) and tricuspid valve (T) in bulls at age three years old.



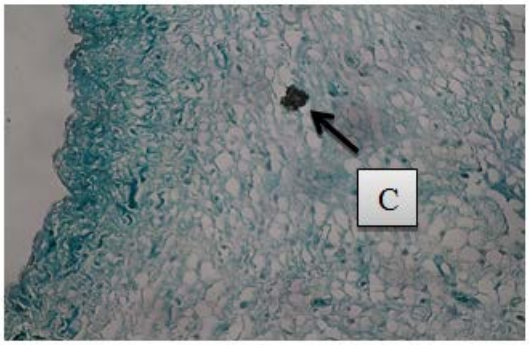
(Figure15) (a): Cross section of aortic valve of bull at aged 3 years showing the calcification (C) in spread of brown spots stained with (Van Kossa 400 x). (b): Surface plot of aortic valve of bull at aged 3 years showing the percentage of calcification for the tissue.



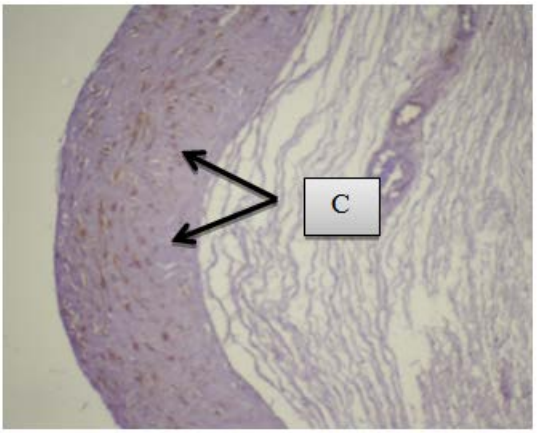
(Figure 16): Cross section of bicuspid valve of bull at aged 3 years showing the calcification (C) in large brown spots stained with (Alizarine Red 400 x).



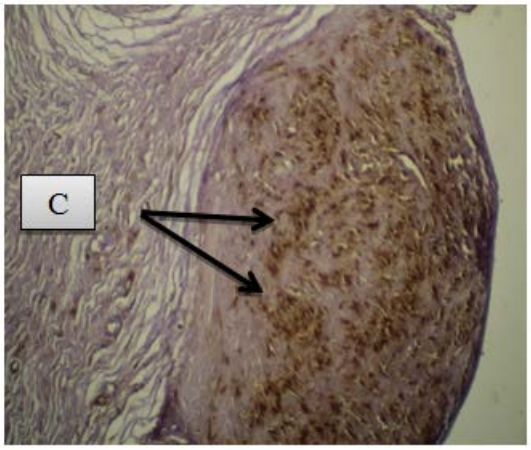
(Figure 17): Cross section of pulmonary valve of bull at aged 3 years showing the calcification (C) in spread of black spot stained with (Van Kossa 400 x).



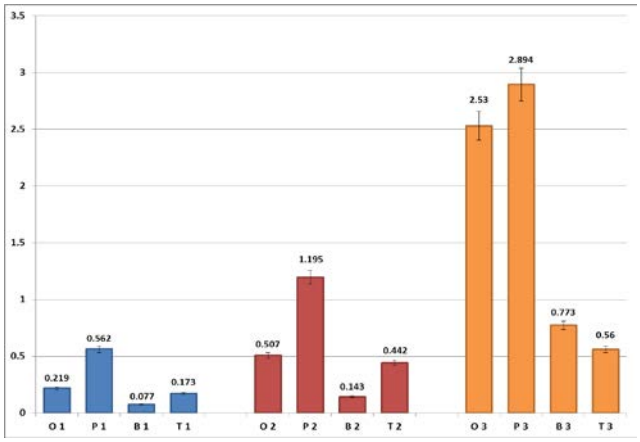
(Figure 18): Cross section of tricuspid valve of bull at aged 3 years showing the calcification (C) in large brown spot stained with (Alizarin Red 200 x).



(Figure 19): Cross section of aortic valve of bull at aged 3 years showing, calcification (C) in the forms of spread points (Immunohistochemistry 200x).



(Figure 20): Cross section of pulmonary valve of bull at aged 3 years showing, calcification(C) in the forms of spread points (Immunohistochemistry 200x).



(Chart 4): Chart showing the progress of calcification ratio in four types of bulls valves, aortic valve (O),pulmonary valve (P), bicuspid valve (B) and tricuspid valve (T) with forward age.

CONCLUSION

Histologically, Hematoxylin and Eosin stain showing the valves consist from three layers, fibrosa layer; spongiosa layer and aventricularis layer (in pulmonary and aortic valve) and atrialis layer (in the bicuspid and tricuspid valves). Van Kossa and Alizarin Red stain and immunhistochemical technique showing the different valves calcification proportion with variants grade ages. The spontaneous calcification is mild in young age (1year) which appeared as small points in valves. This calcification ratio was increased at 2 years old of calves and which appeared as large brownish spots at (3 years) of age. The calcification of aorta and pulmonary valves is more than the calcification of bicuspid and tricuspid valves.

REFERENCES

1- Herzog H. Some we love, some we hate, some we eat: Why it's so hard to think straight about animals. New York, NY: Harper. 2010: Pp. 237-263.

2- Levick JR. An Introduction to Cardiovascular Physiology, Arnold. 1991: Pp.1-12.

3- Guyton AC and Hall E. Textbook of Medical Physiology, Elsevier lnc. 2006: Pp: 161-170.

4- Evans HE. The heart and arteries, in Miller's Anatomy of the Dog. (Miller, M.E. and Evans, H.E., eds.), Saunders, Philadelphia, PA, (1993); 3rd Ed: Pp. 586-602.

5- Iaizzo PA. Heart valves From Design to Clinical Implantation. Springer Science+Business Media New York. (2013); 1st Ed: Pp. 3-25.

6- Cromwell GL and Coffey RD. Phosphorus-a key essential nutrient, yet a possible major pollutant-its central role in animal nutrition. Biotechnology in the feed industry. 1991:Pp. 133-145.

7- Maynard LA, Loosli JK, Hintz HF and Warner RG. Animal Nutrition.. McGraw-Hill Book Company. New York, USA. 1979; 7th Ed: Pp. 220 –223.

8- Singh KS and Panda B. Poultry Nutrition.. Kalyani Publishers. 1996; 3rd Ed: Pp.104-113.

9- Sobiech P, Rypuła K, Wojewoda-Kotwica B, Michalski S. Usefulness of calcium-magnesium products in parturientparesis in HF cows. J. Elem. 2010; 15(4): 693-704.

10- Miller JD. Cardiovascular calcification: Orbicular origins. Nature Materials. 2013; 12: 476-478.

11- Perry TW. Animal Life-Cycle Feeding and Nutrition. Academic Press, INC.New York, USA. 1984:Pp. 22- 27.

12- Thandroyen FT, Whitton IN, Pirie D, Rogers MA and Mitha AS. Severe calcification of glutaraldehyde-preserved porcine xenografts in children. Am J Cardial. 1980; 45:690.

13- Ferrans VJ, Boyce SW, Billingham, ME, Jones M, Ishihara T and Roberts, WC. Calcific deposits in porcine bioprostheses: structure and pathogenesis. Am J Cardiol. 1980; 46: 721.

14- Gross L amd Kugel M A. Topographic anatomy and histology of the valves in the human heart. American Journal of Pathology. 1931; 7(5):445–474.

15- Christie GW, Barratt-Boyes BG. Mechanical properties of porcine pulmonary valve leaflets: How do they differ from aortic leaflets? Ann Thoracic Surg. 1995; 60: 195–9.

16- Gott JP, Chih P, Dorsey LMA, Jay JL, Jett GK, Schoen FJ, Girardot JM and Guyton RA. Calcification of porcine valves: a successful new method of antimineralization. Ann Thorac Surg.1992; 53:207-16.

17- Bernacca GM, Fisher AC, Wilkinson R, Mackay TG and Wheatley DJ. Calcification and stress distribution in bovine pericardial heart valves. Journal of Biomedical Materials Research. 1992; 26: 959-966.

18- Schoen FJ, Hirsch D, Bianco RW and Levy RJ. Onset and progression of calcification in porcine aortic bioprosthetic valves implanted as orthotopic mitral valve replacements in juvenile sheep. The Journal of Thoracic and Cardiovascular Surgery. 1994; 108:880-887.

19- Wirrig EE, Gomez MV, Hinton RB and Yutzey KE. COX2 inhibition reduces aortic valve calcification in vivo. Arterioscler Thromb Vasc Biol. 2015; 35(5): 938-947.

20- Simmons CA, Grant GR, Manduchi E and Davies PF. Spatial Heterogeneity of Endothelial Phenotypes Correlates With Side-Specific Vulnerability to Calcification in Normal Porcine Aortic Valves. Circulation Research. 2005; 96:792-799.

21- Schoen FJ and Levy RJ. Tissue Heart Valves: Current Challenges and Future Research Perspectives. J. Biomed Mater Res. 1999; 47: 439–465.

22- Yamate, J, Tajfma M, Maruyama Y and Kudow S. Observations on soft tissue calcification in DBA/2NCrj mice in comparison with CRJ: CD-l mice. Laboratory Animals. 1987; 21: 289-298.

23- Beaufrere H, Ammersbach M, Reavill D, Garner M, Heatley J, Nevarez J. *et al.*. Prevalence and risk factors in psittacine atherosclerosis: a multicenter case-control study. J. Am. Vet. Med Assoc. 2013; 242(12):1696-1704.

24- Hofmann JJ, Briot A, Enciso J, Zovein AC, Ren S, Zhang ZW, Radtke F, Simons M, Wang Y and Iruela-Arispe M L. Endothelial deletion of murine Jag1 leads to valve calcification and congenital heart defects associated with Alagille syndrome. Development.2012; 139: 4449-4460.

25- Weiss RM, Ohashi M, Miller JD, Young SG and Heistad DD. Calcific Aortic Valve Stenosis in Old Hypercholesterolemic Mice. Journal of Circulation. 2006; 112: 2065-2069.

26- Bernacca GM, Fisher AC, Wilkinson R, Mackay TG and Wheatley DJ. Calcification and stress distribution in bovine pericardial heart valves. Journal of Biomedical Materials Research. 1992; 26: 959-966.

27- Whalen RL, Snow JL, Harasaki H and Nose Y. Mechanical strain and calcification in blood pumps. Trans. Am. Sac. Artif Int. Organs, 1980; 26: 487-492.

28- Deck JD, Thubrikar MJ, Nolan SP, and Aouad J. Role of mechanical stress in calcification of bioprostheses, in Cardiac Bioprostheses: Proceedings of the Second International Symposium, L. H. Cohn and V. Galluci(eds.), Yorke Medical Books, New York, 1982: Pp. 293-305.