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DIAGNOSIS OF THE ELASTIC CARTILAGE IN *XIPHOPHORUS HELLERII* FISH

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ABSTRACT : Some studies tackled the elastic cartilage ofteleost, but they do not give an importance to specify its type especially in *X. hellerii* fish. There are many types of elastic cartilages in the teleost skeleton, therefore; the current study tackled diagnosis of the elastic cartilage, the study of its distribution and its type in the body of *X. hellerii* fish, depending on characteristics of chondrocytes andextracellular matrix (ECM) in the stained tissue sections with the normal stain and special histochemistry stain. It turns out that the fish skeleton has very small specific areas of elastic cartilage in the visceral skull, which centered in the gill arches and the hyoid arch only. The current study distinguished one type of elastic cartilage, which is elastic /cell-rich Cartilage (ECRC). The ECRC characterized that its extracellular matrix is a few pigmentation with black color with verhoeff stain which is special toelastic fibers. Its cells are shrunken and its quantity exceeds extracellular matrix quantity. ECRC type is founded between hyaline cartilage. The extracellular matrix of elastic cartilage (ECRC) overlaps with the extracellular matrix of hyaline cartilage without boundaries between them. It has been noticed it is in small areas between those hyaline cartilages in articulated areas with each other.

Key words : Cartilage, elastic cartilage, *Xiphophorus hellerii*.

INTRODUCTION

Generally, structural tissue are classified in vertebrates to bones and cartilages. While these tissues in fish have secondary types of bones and many types of cartilages.

Each one of them has distinguished with special characteristics have been derived from bone and cartilage with their general known characteristics (Witten *et al*, 2010).

The division of cartilage histologically in vertebrates depends on the fibers type, which are found in the ground substance to hyaline cartilage, elastic cartilage and fibrocartilage (Gartner, 2015).

As other vertebrates, in fish the cartilage is divided according to its fiber and its extracellular matrix (Benjamin, 1990). In addition, chondrocytes gave extra characteristics to the tissue sections which enabled the researchers to distinguish many kinds of cartilages. Each one of them is diagnosed in certain areas of the fish skeleton, one of these cartilages was elastic cartilage.

The elastic cartilage consists of chondrocytes, which surrounded by extracellular matrix (ECM) this in turn mainly consists of two components determine the physical and chemical characteristics of the cartilage. The first component is represented by collagenous network, which is responsible about the tensile strength of extracellular matrix. The second component is proteoglycans especially aggrecan and hyaluronan which are responsible about the cartilage ability of bearing and distribution pressure (Grassel and Aszodi, 2017). Thus, it could be distinguished from other types of cartilage, depending on the ECM components and histological structure. This kind also characterized by existence of elastic fibers, which contains elastic protein and chondroitin sulfate (Pawlina and Ross, 2018).

The elastic cartilage is of many types in teleost as elastic/ cell-rich cartilage (ECRC), which its extracellular matrix was marked with abundance of elastic fibersand the untransparent and un shrunk cartilaginous cells and in its histological sections (Benjamin, 1990). The distribution of elastic cartilage in the teleost was different according to different species (Hall, 2005). Benjamin (1990) clarified that the (ECRC) is found in (sensor horns) barbells and maxillary oral valves of catfish.

Al Ali (2008) pointed out that the first appearance of elastic cartilage in *Poecilias phenops* fish was in embryo of 6mm length. Its differentiation started in hyoid arch inarticular area of cartilage of hyomandibula -syplectic cartilage and interhyal cartilage in one side and interhyal cartilage and the hyperptrophic cartilage in other side.

Also in this age, the elastic cartilage appearedamong the articular area cartilagein each one ofgill archin the first four gill arches of the pharyngeal bottom area. The lastelastic cartilage differentiation was in embryo of 7-8 mm. length. This elastic cartilage was located in the confined area among the basibranchial, basal hyoid and ceratonyal. The type of those elastic cartilages wasn't diagnosed.

Than the above, it has turned out the necessity what Witten *et al* (2010) pointed out about the locating and the time of appearing of many cartilage types in the teleost, to understand the developing process, which occurs inside fish skeleton and the functional importance for each type. In addition to that fish have a variety group of specialist cartilages which clearly different from those cartilage in upper vertebrates (Makhtar, 2017).

Therefore, the current study tackled diagnosis the elastic cartilage (ECRC) in *X. hellerii* fish in order to provide information about elastic cartilage which may benefit the histological, functional and pathogenesis studies.

MATERIALS AND METHODS

Bringing and upbringing fish

The mature *X. hellerii* fish (male and female) has been obtained from shops of ornamental fish in Basra. Those fish were transported safely to fish farming laboratory in the animal house of Biology Department in Faculty of education for Pure Sciences; after they were put in airtight plastic bags, which contains oxygenated water. The male was 8 cm.length and the female was 10 cm. length.

Their weights were 3.7-2.5 gm. Then they were put in a special glass basins which its capacity is 150. Those basins were in laboratory full of pure water, this water was founded by Reverse Osmosis Process (RO). The PH was (8.1 -7) those basins were cleaned well by using $NaCl_2$ before putting fish and supplied with electric filters to pure water. Electric pneumatic pumps were used to provide dissolved oxygen. A temperature scale were affixed on basins to observe water temperature. The temperature were between (28-25^{0}C).

Fish were fed once a day by using provender (animal and vegetarian) before one hour of replacing water, that is by replacing two-thirds of water each 48 hour after feeding to have a healthy environment for fish.

Preparation of tissue sections

Three replicates were taken (male, female) of *X. hellerii* fish to study the distribution of the elastic cartilage in it. The samples were fixed by using formalin 10% concentration for 24hour then the samples were washed by using tab water to remove formalin. After washing many times for 12hour dehydration was done by passing those samples in series of ascending concentrations of ethyl alcohol 70%, 90%, 100%, respectively.

Then they were cleaned by Xylene to remove alcohol residue. Those samples were transported to molten paraffin wax, in (60-58^{0}C) temperature in electric oven for four hours. The wax was replaced each two hours to remove the xylene residue totally. Those samples are buried in metal molds taking into account directing the sample to cut it in different levels. Those samples are refrigerated to get wax templates which are ready to cut (Kiernan, 2012). Those wax templates were cut to get strips with sequential sections by using microtome with 6 µm. thickness. The histological sections were carried on clean slides to be ready for the staining stage. Those stained tissue sections were carried with Canada balsm material then covered with cover.

Hematoxylin and Eosin (H&E) stain were used as a normal stains, verhoeff elastic stain was used as a special stain to diagnose elastic cartilage (Humason, 1972). Those tissue sections were examined by microscope of Leica and photographed by using digital camera.

RESULTS

The current study results showed that the elastic cartilage (ECRC) in *X. helleri* fish is found within the fish skeleton supporter. It represents a few proportion of it comparing with the hyaline cartilage. It occupied a small areas among the hyaline cartilage centered inarticular areas of those cartilage. It was in the head area of fish within the fish skeleton supporter of the visceral skull only. The current study diagnosed existence of one type of elastic cartilage in *X. hellerii* fish, which is elastic cell-rich cartilage (ECRC), it was founded in the gill arches IIII and hyoid arch only. It was noticed that it exists in small areas among the hyaline cartilage in articulate area with each other.

The elastic/ cell-rich cartilage (ECRC) distinguished by containing much quantity of chondrocytes and a little extracellular matrix colored with verhoeff stain in bold black color and pink color with H & E stain. Those cells seemed buried at the middle of this material. It was spherical to oval shape. It was less shrunken than it is in the hyaline cartilage, which appeared it inside gaps. The nucleus of the oval cell with fine granular chromatin and homogeneous cytoplasm while the elastic cartilage circumference ECRC cells seem as if it shaped in regular rows. It looks compressed and elongated into an oval

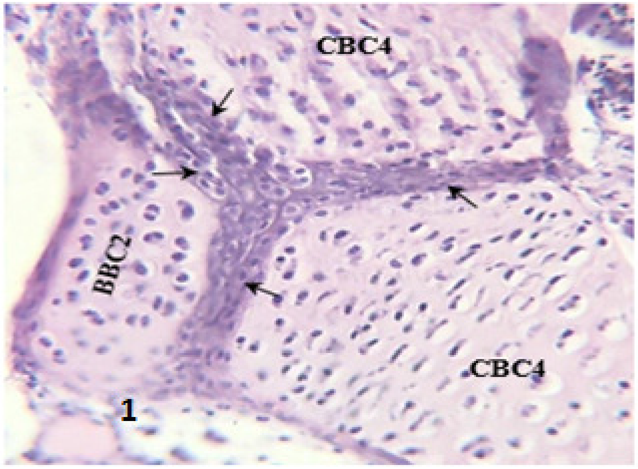


Fig. 1 : Explains a frontal section of fish head is passing in the level of the ceratobranchials IIII (CBC4) explains the basibranchial cartilage II (BBC2) with the ceratobranchials IIII (CBC4).

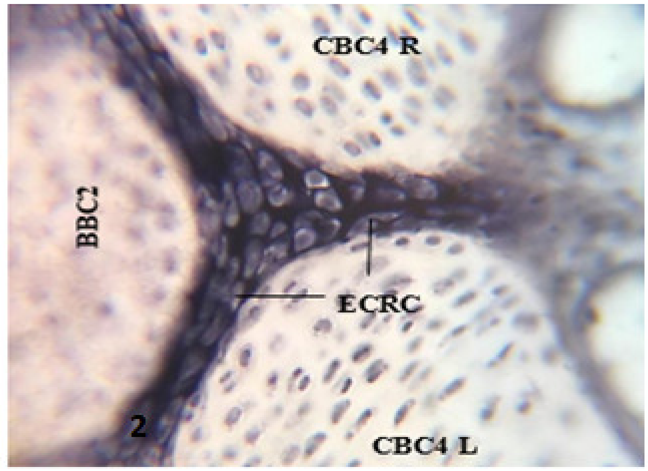


Fig. 2 : Explains the elastic cartilage ECRC which is confined between the ceratobranchial IIII from the right side (CBC4R) and the ceratobranchial IIII from the left side (CBC4L) and the basibranchial II (BBC2), which located in front of it, Verhoeff stain, 1000X.

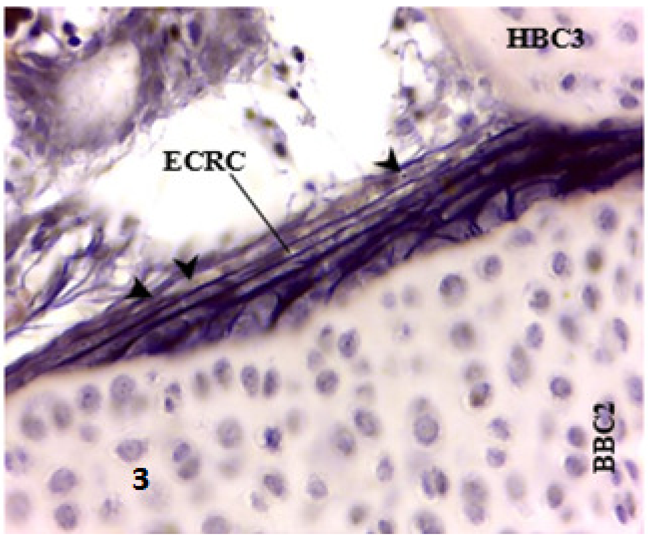


Fig. 3 : A section explains the continuation of the elastic cartilage ECRC in the hyoid arch with the hyaline cartilage interhyal cartilage (INH), notice that the Interhyal cartilage didn't ossify in the close area from the elastic cartilage ECRC (Arrows).

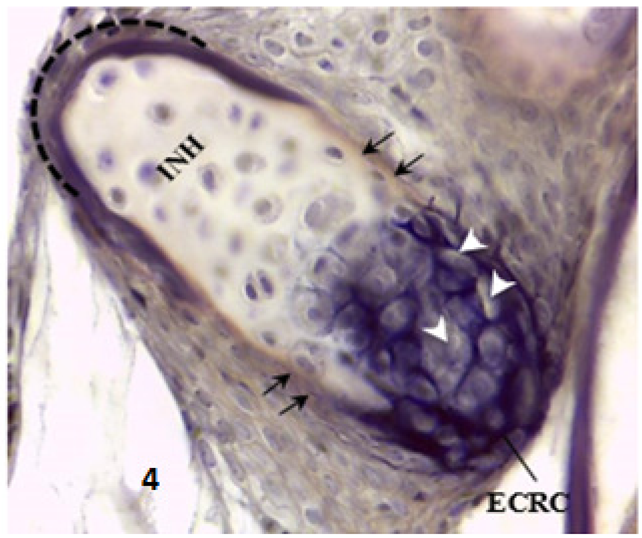
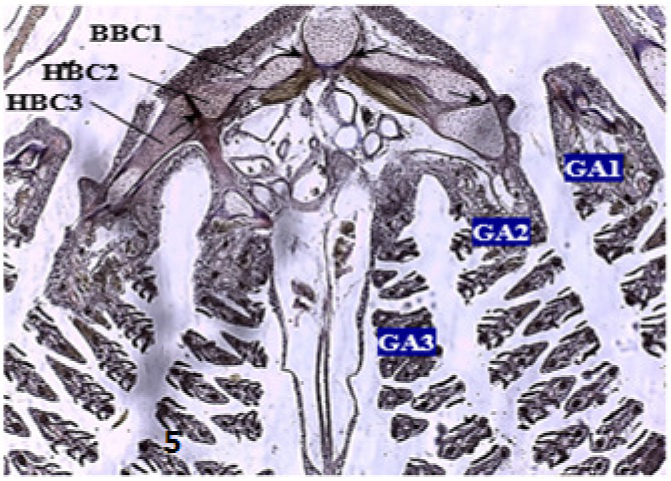


Fig. 4 : A section explains the elastic cartilage's cover ECRC (Arrows), which surround the basibranchial cartilage II (BBC2) in the gill Arch III (GA3), notice the elastic fibers which are skinny strings. Verhoeff stain, 1000X.

surrounded by an extracellular matrix abundant with elastic fiber. It was noticed there wasn't clear interval boundaries between ECRC and the hyaline cartilage since the extracellular matrix of (ECRC) overlaps with extracellular matrix of the hyaline cartilage gradually without interval boundaries from other tissue (Figs. 1, 2 and 3) as well the ECRC surrounded by perichondrium, which is rich with elastic fibers, which seems in thin filaments (Fig. 4).

The ECRC is diagnosed in the four gill arches in articular areas of cartilage where ossification occurs, in the first three gill arches, the ECRC is diagnosed among hypobranchial and the first three ceratobranchial respectively in one side and the first three hypobranchial and the first and second basibranchial respectively on other side (Figs. 5, 6, 7 and 13). In gill arches I & II, the ECRC appeared among basibranchial I, hypobranchial I and hypobranchia lI, ceratobranchial I (Figs. 7, 8, 9 and 10) and between hypobranchial II, ceratobranchial II (Figs. 5, 6 and 11). In the gill arches III, the ECRC is found among basibranchial II, hypobranchial III (Figs.12, 13 and 14) in one side and between hypobranchial III, ceratobranchial III on other side (Figs. 13, 15 and 16). Also ECRC seems surrounding the basibranchial II as a cover over all its sides, generally it was the biggest elastic cartilage in fish which extends along the confined area



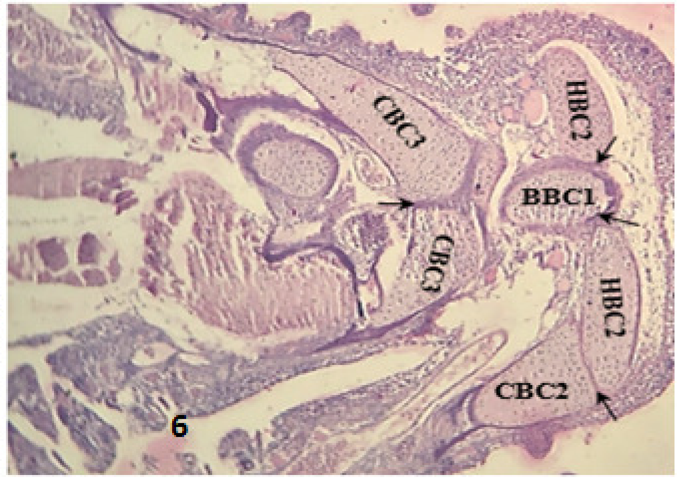
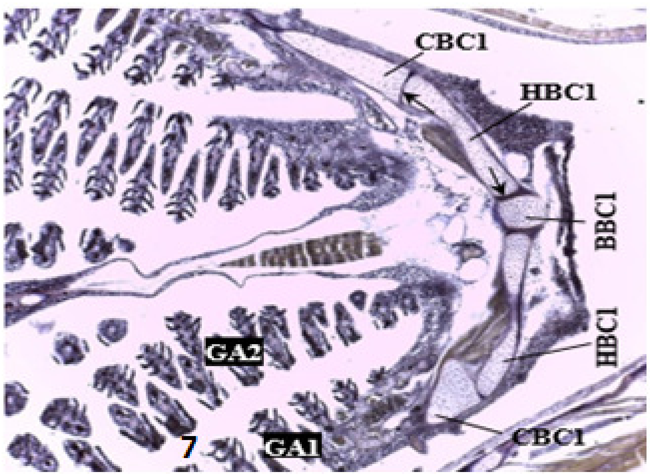


Fig. 5 : A frontal section in fish head is passing in the gill arches II area (GA2) and the gill arches III (GA3), a part of the gill arch I (GA1) appears, arrows point out to the articular ossification cartilages area which have the elastic cartilage ECRC. Verhoeff stain, 100X.

Fig. 6 : A frontal section in phargeal bottom area of the fish explains the existence of the elastic cartilage in articular gill arches areas,the arrows point out to it's existence areas between the basibranchial I (BBC1) and hypobranchial cartilage II (HBC2). H&E Stain, 100X.



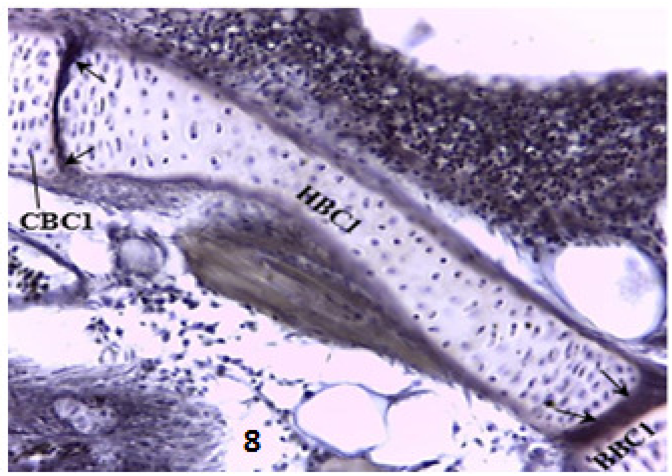


Fig. 7 : A frontal sectionin the fish head is passing in the gill arches area which appear in it gill arch III (GA3) and a part of gill arch II (GA2), arrows point out to the elastic cartilage existence areasECRC. Verhoeff stain, 100X.

Fig. 8 : A frontal section explains the elastic cartilage ECRC between the hypobranchial I (HBC1) and the cevatobranchial I (CBC1) of the gill arch1 (GA1).Verhoeff stain, 400x.

between basibranchial II and hypobranchial III from front and ceratobranchial IIII from back. Its cells seem oval to elongated line up as rows surrounding with extracellular matrix stained with black color and verhoeff stain (Figs. 4, 13 and 14).

In gill arches fourth, the ECRC is diagnosed among ceratobranchial IIII from right side and ceratobranchial IIII from the left side with basibranchial II, which is located infront of it (Figs. 1, 2, 13, 14 and 17). While in hyoid arch, the ECRC is diagnosed in one area of the gill arches which represented the articular area among the hyaline cartilage which is hyomandibula –syplectic cartilage and Interhyal cartilage, it was in very small areas (Figs. 3, 18 and 19). It was noticed that the ECRC didn't ossify, in addition the hyaline cartilage area didn't ossify too which was in contact with elastic cartilage (Fig. 3).

DISCUSSION

The previous studies dealt with diagnosis the cartilage in teleost, those studies showed the existence of many types of cartilage in different fish (Witten *et al*, 2010). The main reason behind that variety was the difference of the components of extracellular matrix whence its chemical structure and its fiber content in addition to its distributionand its quantity nature (Young *et al*, 2013).

In teleost eight types of cell-rich cartilage and three types of matrix- rich cartilage were diagnosed (Benjamin, 1990). One of those cartilage was ECRC which is

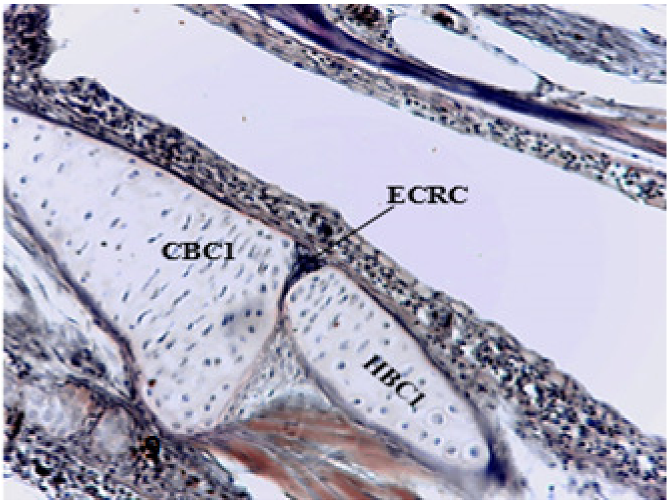


Fig. 9 : A section explains the hypobranchial cartilage I (HBC1), it is noticed the existing of elastic cartilage ECRC between hypobranchial cartilage I (HBC1) and the basibranchial 1 (BBC1) in one side, Verhoeff, 400X

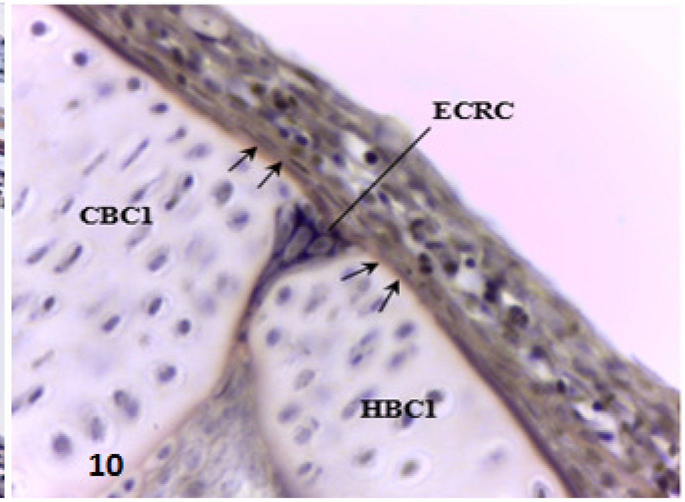


Fig. 10 : A section explains the elastic cartilage ECRC between the hypobranchial I (HBC1) and the ceratobranchial 1 (CBC1) of the Gill arch I (GA1), Verhoeff stain, 1000x.

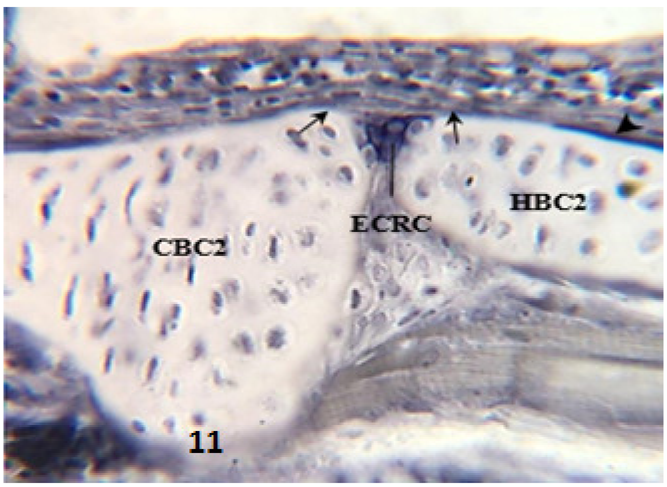


Fig. 11 : The elastic cartilage of type ECRC appear between hypobranchial II (HBC2) and ceratobranchial II (CBC2) of the gill arch II (GA2), Verhoeff, 1000X

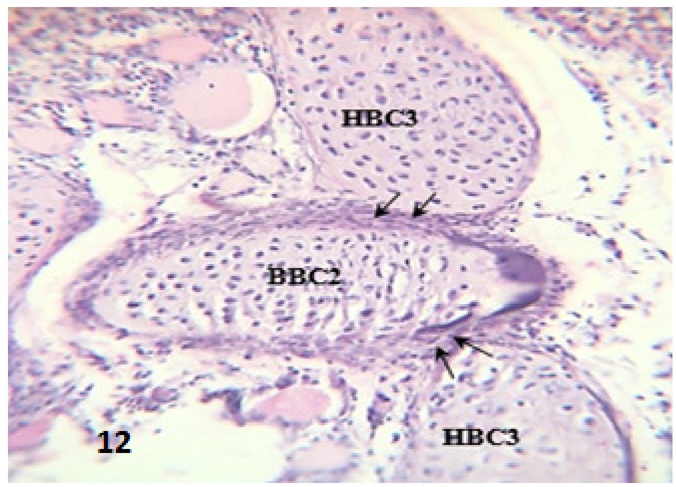


Fig. 12 : Explains the basibranchial cartilage II (BBC2) and the hypobranchial cartilage III (HBC3) of the gill arch III (GA3), Verhoeff stain, 400x.

diagnosed by the current studyin *X. hellerii* fish. Thus, the current study is very interested to diagnose the elastic cartilage in *X. hellerii* fish because the studies of diagnosing this cartilage of teleost and determine its type and its distribution are rare.

The current study depends in diagnosing elastic cartilage on a group of diagnostic qualities which represented by the abundance of its chondrocytes. The shrinkage of chondrocytes and the existence of elastic fiber was the main reason of distinguishing that type among the other rest of cartilage types like hyaline- cell cartilage (HCC) and elastic hyaline –cell cartilage (ECC).

In spite the cell of ECRC shrinking but that shrinking seems less than what is it in real hyaline cartilage cells. It is worth mentioning that cells shrink is because of extracellular matrix nature where the chondrocytes is buried. Whenever the extracellular matrix was more hardness, the chondrocytes appeared clearly shrunken in the tissue sections (Frantz *et al*, 2010). Thisrefers in one hand to the difference in the extracellular matrix nature of ECRC from extracellular matrix of the real hyaline cartilage.

As the ECRC similar to Zellknorpel (ZK) and fibro/ cell-rich cartilage (FCRC) whene rapprochement cells and the quantity of extracellular matrix. ECRC is diagnosed and distinguished by the existence of elastic fiber in it.

Al Ali (2016) diagnosed ZK in *Poecilias phenops* fish in the first four gill arches. He showed also it didn't exist in gill arches V and in other different parts of the

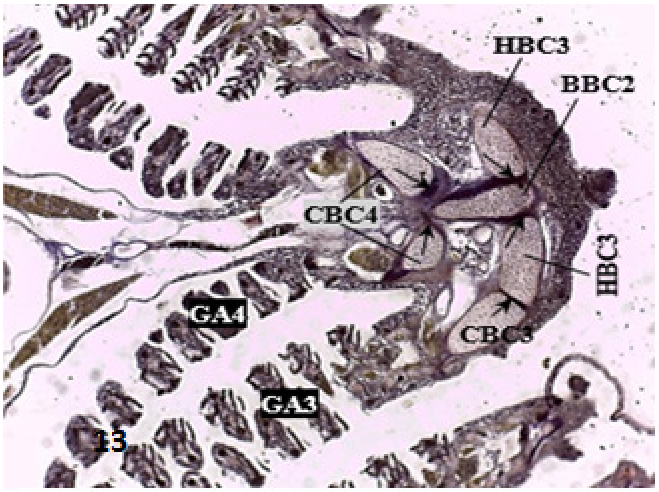


Fig. 13 : A frontal part in the phargeal bottom of fish explains the elastic cartilage ECRC (the arrows) in the articular areas of gill arch 3 between (BBC2) and the hypobranchial III (HBC3), Verhoeff, 100X.

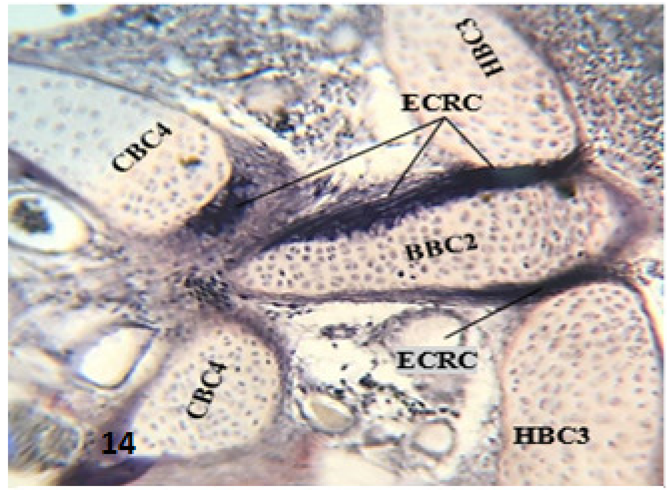


Fig. 14 : Explains the existing areas of the elastic cartilage ECRC in the articular areas of basibranchiale cartilage II(BBC2) with the hypobranchial cartilage III (HBC3), Verhoeff stain, 400x.

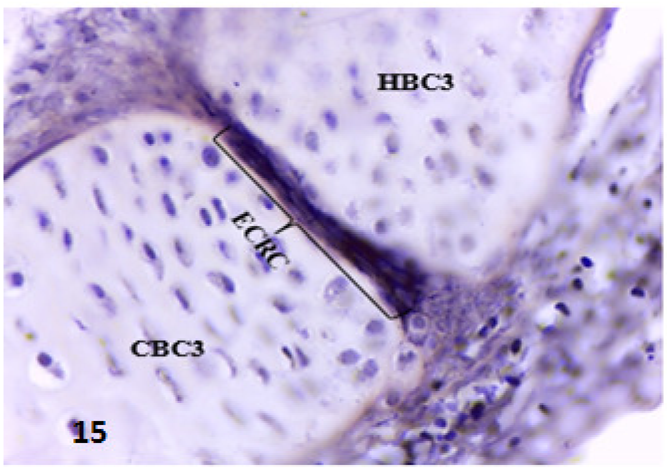


Fig. 15 : Explains the hypobranchail cartilage III (HBC3) and the ceratobranchial cartilage III (CBC3), the arrows confine between them the existing, area of the elastic cartilage ECRC. H&E stain, 400x.

Fig. 16 : A frontal section that explains the elastic cartilage ECRC between hypobranchail III (HBC3) and the ceratobranchial III (CBC3) of the gill arch III (GA3), notic the existing of cartilage ECRC in the articular area. Verhoeff stain, 1000x.

body. It was in two positions in each gill arches they are pasal plate of gill filament and its thorn (Benjamin, 1990) pointed out that FCRC is found between maxilla and premaxilla in many teleost as *Badis badis*, *Telmatherina ladigesi, Sphaerichthysos phromenoides* and *Macropodus opercularis.*

The current study results, showed elastic /cell-rich cartilage ECRC continuity with hyaline cartilage. The nearby parts from ECRC which didn't ossify, did not also ossify. This may reflex some sort of, the alternate relationship nature between the two types of cartilage, which refers to the alternate affection between them.

In addition to the internal environment role where ECRC is found to determine the histological structure nature. It also reflexes some sort of functional relationship between the two types of cartilage. It is usually installation is compatible with job performance (Gillis *et al*, 2006).

When the histochemicalmethodis used the boundaries appear between the extracellular matrix of the hyaline cartilage and the extracellular matrix of elastic cartilage when, it was being stained with Verhoeff stain which is special for the elastic fibers, in spite of the continuity of the extracellular matrix in both the hyaline and elastic cartilage. This means special genes have differentiated in the chondrocytes of elastic cartilage, which led to this difference in extracellular matrix. Those genes specialize to excrete elastic fibers. The existence of ECRC in the qualities which the current study has diagnosed in the articular areas of ossification hyaline cartilage was to provide the required flexibility for the movement of those

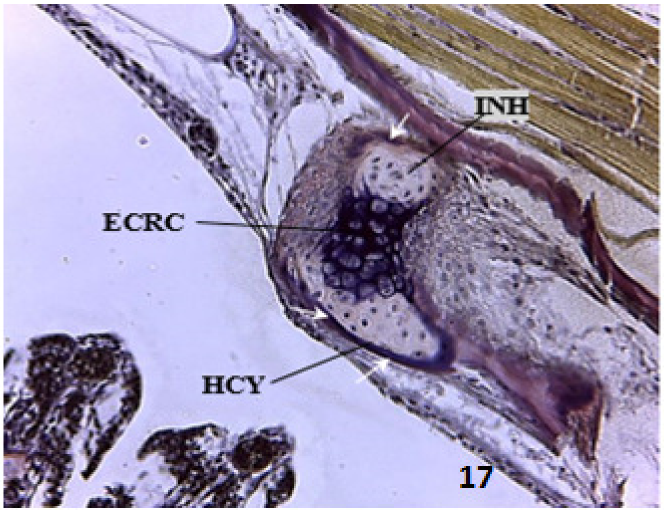


Fig. 17 : A frontal section in the fish's head explains the elastic cartilage ECRC (the arrow) between basibranchial II (BBC2) and hypobranchial III (HBC3) and ceratobranchial IIII (CBC4) and basibranchial II (BBC2) of the gill arch IIII (GA4). Verhoeff stain, 100x.

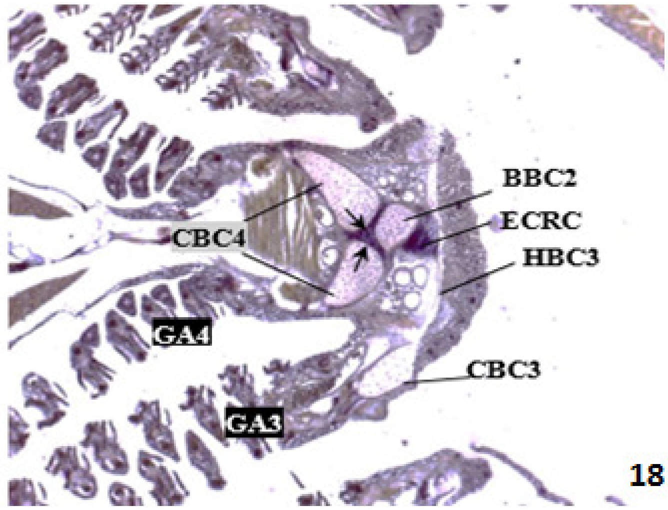


Fig. 18 : A frontal section in the fish's head explains the elastic cartilage ECRC between compositions of hyoid arch between the hyomandibula- syplectic cartilage (HCY). Verhoeff stain, 400x.

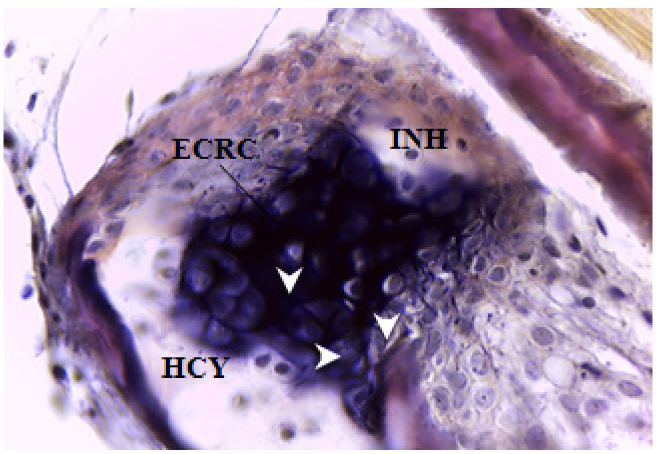


Fig. 19 : A magnified section from figure(18) explains the elastic cartilage ECRC in the hyoid arch between the interhyal cartilage (INH) and hyomandibula- syplectic cartilage (HCY), the arrows point out to cells of the shrunken elastic cartilage ECRC. Verhoeff stain, 1000x.

structures. The gill arches cartilages are regarded as part of the fish skeleton, which belongs to visceral skull (Hilton, 2011). Those structures are being exposed directly to water currents during swimming and breathing therefore; the presence of ECRC in the gill arches probably gives flexibility to its skeleton to avoid tensile and tension, which may be caused by current water of the gills and thus rapture them. As well, the hyaline cartilage distributes the high pressure power which generates as reaction during the sudden and fast movement (Guilak *et al*, 1994). This means the presence of ECRC in the Articular areas is because of fish functional requirements during the growth and development stages (Benjamin, 1990). Also, the presence of elastic cartilage between hyomandibula-syplectic cartilage and interhyal cartilage be as flexible cushion to provide adequate flexibility for hyomandibula-syplectic cartilage, interhyal cartilage and ceratohyal cartilage, during feeding process because interhyal cartilage is articulated with hyomandibula- syplectic cartilage from one side and ceratohyal cartilage from other side which provides flexibility to compositions, which connect with it thus it avoids damage for both of them.

The small areas, which filled with ECRC of fish skeleton are adequate to perform its job among the hyaline cartilage, this agrees with what Benjamin (1986) had mentioned who diagnosed it as small pieces in several fish like *Tanichthys albounbes*, *Pangasius sutchi*, *Clarias batrachus* and *Corydoras metae*.

Also he diagnosed the elastic cartilage ECRC in sensor horns and in the upper jaw of catfish. That refers to its distribution in the fish skeleton is differ according to fish type (Benjamin, 1990). Al Ali (2008) referred that presence of elastic cartilage in small pieces among the hyaline cartilages in *Poecilia sphenops* fish but he didn't diagnose the type of elastic cartilage

The current study didn't diagnose elastic hyaline – cell cartilage (ECC), which belongs to hyaline–cell cartilage (HCC) and the elastic cartilage which elastic/ interstitial- rich cartilage, which is similar to the real elastic cartilage which is exist in upper vertebrates like human. Perhaps, the reason is specificity of fish type or the evolutionary ladder of fish.

Benjamin (1986) showed that thee lastic hyaline – cell (ECC) exist in parts of oral sucker in *Gyrinocheilus aymonieri* fish while it is not exist in *Labeo bicdor* fish and *Botia horae* fish. As well, the normal elastic cartilage existsin some parts of body which need to support and high flexibility like nasal flap as in *Hybopsis gelida* and *Hybopsis aestivalis* fish (Branson, 1963).

CONCLUSION

The fish skeleton has very small specific areas of elastic cartilage in the visceral skull which centered in the gill arches and the hyoid arch only. It was distinguished one type of elastic cartilage which is elastic /cell-rich Cartilage (ECRC). The ECRC characterized that its extracellular matrix is a few pigmentation with black color. Its cells are shrunken and its quantity exceeds extracellular matrix quantity. ECRC type is founded between hyaline cartilage. The extracellular matrix of elastic cartilage (ECRC) overlaps with the extracellular matrix of hyaline cartilage without boundaries between them. It has been noticed it is in small areas between those hyaline cartilages in articulated areas with each other.

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