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S Jaiswal

Department of Veterinary
Anatomy, C.V.A.Sc.,
GBPUA&T, Pantnagar,
Uttarakhand, India

I Singh

Department of Veterinary
Anatomy, C.V. A.Sc.,
GBPUA&T, Pantnagar,
Uttarakhand, India

D Mahanta

Department of Veterinary
Anatomy, C.V.A.Sc.,
GBPUA&T, Pantnagar,
Uttarakhand, India

S Sathapathy

Department of Veterinary
Anatomy, C.V.A.Sc.,
GBPUA&T, Pantnagar,
Uttarakhand, India

M Mrigesh

Department of Veterinary
Anatomy, C.V.A.Sc.,
GBPUA&T, Pantnagar,
Uttarakhand, India

S Tamil Selvan

Department of Veterinary
Anatomy, C.V.A.Sc.,
GBPUA&T, Pantnagar,
Uttarakhand, India

Correspondence

I Singh

Department of Veterinary
Anatomy, C.V. A.Sc.,
GBPUA&T, Pantnagar,
Uttarakhand, India

Histological, histomorphometrical, histochemical and ultrastructural studies on the heart of Uttara fowl

S Jaiswal, I Singh, D Mahanta, S Sathapathy, M Mrigesh and S Tamil Selvan

Abstract

Present study was carried out on 24 birds of different age groups of Uttara fowl and elucidates age-related changes in the heart through histological, histomorphometrical, histochemical and ultrastructural studies. Atrial and ventricular wall was comprised of inner endocardium, middle myocardium and outer epicardium. Myocardium was the thick layer which composed of striated and branched cardiac muscles cells with centrally placed nucleus. Numerous purkinje fibres and blood vessels present in the connective tissue network of myocardium. Gomori's method revealed a dense network of reticular fibres between the cardiac muscle fibres. Weigert's stain gave positive reaction for elastic fibres in atrial wall and wall of blood vessels. Masson's trichrome revealed positive reactions for collagen fibres in sub endocardial layer. Ultrastructural studies showed the presence of intercalated disc, branching patterns of cardiac muscles cells, supply of coronary vessels on ventricular wall, pectinate muscles folds and endocardial cells projecting towards the luminal surface of atria.

Keywords: Histological, histomorphometry, histochemical, scanning electron microscopy, heart, Uttara fowl

Introduction

Uttarakhand has a great diversity of flora and fauna. The poultry population of Uttarakhand state is 26.01 lakh which has increased by 7.01% per annum while the population of desi fowl in the state decreased 28.67% from 2003 to 2007. The per capita annual egg and meat consumption in the state is 26 eggs and 1.15 kg/head which are less than national level as well as the recommended level of the Indian council of medical research [6]. There are many species of birds reared in Uttarakhand. One of the local breed of bird found in Uttarakhand is Uttara fowl. It is generally reared under a backyard system in the Kumaon division of Uttarakhand state. It is of local importance in the region as it gives nutritional as well as economic security to the rearing families [13]. Local hill fowl meat is more chewy and tasty and is very popular among hilly rural areas [8]. The circulatory system comprises both the blood and lymphatic vascular systems. The blood vascular system is composed of the following structures, the heart which pumps the blood, the arteries which carry the blood with nutrients and oxygen to the tissues, the capillaries by which the interchange between blood and tissues takes place and the veins which convey the blood to be pumped again to heart [5].

Materials and methods

The present study was carried out on 24 birds of different age groups of Uttara fowl and elucidates the age-related changes through histological, histomorphometrical, histochemical and ultrastructural studies. For carrying out the histological, histomorphometrical and histochemical studies birds were divided into four age groups viz. day old, 7, 28 and 112 days old. Collected tissue specimens were fixed in 10% neutral buffered formalin to prevent tissue from putrefactive and autolytic conversion and to safeguard the cell constituents. For histological studies the fixed tissue was processed by following steps:

• Washing

The fixed tissues were washed overnight under running tap water.

- **Dehydration**

The tissue was taken to ascending gradient of alcohol (viz. 50%, 70%, 80%, 90%, 95% and 100%) to eliminate water from them.

- **Clearing**

The clearing was done using Xylene-I and Xylene-II solutions each for 30 minutes to eliminate alcohol from dehydrated tissue and to make the tissue translucent.

- **Impregnation**

Then tissue was immersed in paraffin immersion I, II and III for 1 hour each at 62 degree Celsius to ensure the penetration of paraffin into tissues and for the removal of reagent.

- **Embedding**

The tissue was arranged in steel molds in desired orientation and tissue cassette was fixed over the mold. Then melted paraffin wax was poured over cassette in order to make paraffin blocks. Soon after embedding, molds were transferred to a refrigerator for hardening.

- **Sectioning and staining**

- After block preparation, the tissue sections were cut in 4-5 microns thickness by Leica microtome, Japan and stained by
- Hematoxylin and eosin stain to study general histoarchitecture^[1].
 - Gomori's staining for reticular fibers^[1].
 - Weigert resorcinol staining for elastic and collagen fibers^[1].
 - Masson's trichrome staining for collagen fibers^[9].

Thereafter, the stained tissue sections were examined under the Microscope (ECLIPSE 80i) and photomicrography was performed.

Histomorphometrical Parameters

The histomorphometry of stained histological sections are carried out after calibration with ocular to stage micrometer scale^[3]. The following parameters were recorded:

- The thickness of wall on left and right atria.
- The thickness of wall of left and right ventricles.
- The thickness of interventricular septum.
- The width of epicardium in left and right atrium.
- The width of myocardium in left and right atrium.
- The width of endocardium in left and right atrium.
- The width of epicardium in left and right ventricles.
- The width of myocardium in left and right ventricles.
- The width of endocardium in left and right ventricles.

The data were analyzed by applying 'Student's T test' for comparison in 2 parameters and 'one way ANOVA' for comparison in three parameters to compare area related variables within different age groups according to the standard procedure outlined by^[14].

Histochemical Studies

Histochemical studies were rendered on collected tissue samples. After fixation, dehydration and paraffin embedding the procedures for following techniques were performed:

- Combined alcian blue – PAS staining for acid and neutral mucins^[1].

Ultrastructural Studies (SEM)

Scanning Electron Microscope (SEM) studies was carried out

as per standard method at SEM facility GBPUAT Pantnagar. Electron microscopic studies were carried out as per standard method described by. Electron microscopic manual of AIIMS, New Delhi,^[10, 2] with some modifications.

1. Fixation

The tissue samples collected for SEM studies were fixed using Karnovsky's fluid (2.5% glutaraldehyde+2% paraformaldehyde) for 24 hours at 4 °C immediately after washing in 0.1M PBS (pH 7.4).

2. Washing

The fixed tissues were subjected to washing with 0.1M phosphate buffer, three changes of 15 minutes each at 4°C.

3. Dehydration

To remove water from the tissues, ascending grades of acetone (30%, 50%, 70%, 80% and 90%) were used for 10 minutes at 4°C which was followed by dehydration in dry acetone once again for 10 minutes at room temperature.

4. Drying

The CPD (Critical Point Drying) was performed on tissues by using the critical point drier. The sample was transferred from acetone to a chamber with liquid carbon dioxide under pressure. When the dehydrating agent i.e. liquid CO₂ had completely impregnated the tissue, the chamber was warmed upto critical point where liquid CO₂ changed to Gaseous CO₂ which was then released gradually to avoid condensation and the tissue sample was completely dry.

5. Mounting

The dried specimens were firmly earthed to brass stubs with the help of carbon tape. This adhesive material provides an electronic conductive medium between tissues and stub. The stubs were ready for the next stage of sputter coating.

6. Sputter Coating

A sputter coating device was used for metal coating of specimens. A uniform coating was done by evaporating Gold under vacuum. A coating of around 35 nm thickness was obtained within 3-5 minutes. This coating of conductive material protected the specimen from the high energy electron beam as well as helped in the conduction of electrons and prevented charging which in turn facilitates better quality images at higher magnification.

7. Viewing

Specimens were viewed under variable pressure scanning electron microscope.

8. Photography

The inbuilt provision of digital camera was used for photography. The digital pictures were stored on compact disc.

Results and discussion

The structural characteristics of Uttara fowl heart have been examined in the present study which was conducted to evaluate the age related histological, histomorphometrical, histochemical and ultrastructural changes in the heart of Uttara fowl. Apparently healthy Uttara fowl birds of day old, 7, 28 and 112 days were procured from the Instructional Poultry Farm, Nagla and their heart was collected from apparently healthy birds to perform various studies.

a. Histological studies

a. 1. Heart

The heart wall consisted of three layers i.e. inner endocardium, middle myocardium and epicardium. The myocardium was the thickest layer of heart which mainly composed of striated and branched cardiac muscle cells with centrally placed nucleus (Fig. 1). The epicardium had a thin layer of mesothelial cells along with loose connective tissue surrounded by adipocytes. [12] Observed that the avian heart has an outer fibrous layer called as pericardium. [4] Also reported presence of the fibrous layer of pericardium as outer layer of heart.

a. 2. Pericardium

In the present study the pericardium had a parietal layer as simple squamous epithelium rested over a thin layer of loose connective tissue. The visceral layer formed epicardium that too had a thin loose connective layer covered by simple squamous epithelial cells i.e. mesothelial cells. In between these two layers liquor pericardi was present. Similar findings were reported in domestic fowl by [12, 4].

a. 3. Endocardium

The endocardium was the inner layer of the heart attached to the myocardium. It was made up of simple squamous epithelium. The endocardium was thin layer in all the age group of Uttara fowl and was divided into three layers i.e. endothelium, sub endothelium and sub endocardium. Purkinje fibers and collagen fibers were present in the sub endocardial layer of endocardium. Similar finding was observed by [4].

a. 4. Myocardium

In the present study the myocardium was the thickest layer of the heart present between the endocardium and epicardium in all the age group of Uttara fowl. There was a gradual increase in the thickness of the myocardium in both atrium and ventricle from day old to 112 days old birds.

a. 5. Atrial myocardium

Muscle fiber bundles were arranged longitudinal as well as in oblique fashion separated by loose connective tissue. Elastic fibers were abundant in between the atrial myocardial cells. The loose connective tissue had capillary network. The atrial muscle cells were small with centrally placed nucleus. The myocardium was striated and arranged in linear array that branches and anatomizes in specific pattern giving the appearance of a sheet. The similar findings were also observed by [11] in rats. The pectinate muscle folds were observed in the atrial wall. Small blood vessel was observed in atrial myocardium of day old, 7, 28 and 112 days of were having tunica intima, tunica media, tunica externa, elastic fibers and collagen fibers. The interatrial septum was thin separating the two atrial chambers. The thickness of the atrial myocardium increased with the advancement of age.

a. 6. Ventricular myocardium

In the present study it was found that there was a gradual increase in the thickness of the ventricular myocardium from day old, 7, 28 and 112 days in the heart of Uttara fowl. The amount of interstitial connective tissue was more in the right ventricle than that of the left ventricle. The ventricular muscle cells were larger than that of atrial muscle cells. The myocardial muscles of ventricle entered in the cardiac valves. Also found in the study of [4]. Purkinje fibres were present in between the sub endocardium layer of endocardium and

myocardium (Fig. 1). The purkinje fibres were also found in the study of the heart of birds by [7]. The blood vessel was observed in ventricular myocardium of day old, 7, 28 and 112 days of was having tunica intima, tunica media, tunica externa, elastic fibres and collagen fibres. The left ventricular myocardium was thicker than the right ventricular myocardium from day old, 7, 28 and 112 days in the heart of Uttara fowl. The myocardial cell was striated and arranged in a linear array that branches and anatomizes in specific pattern giving the appearance of a sheet (Fig. 21). The similar findings were also observed by [11] in rats. The purkinje cells were also observed in the myocardium of day 28 Uttara fowl.

a. 7. Epicardium

In the present study it was found that epicardium was outer layer of heart beneath the visceral layer of pericardium. It was also found in the study of [12] that the epicardium was outer layer of the heart. It was found that the epicardium was the visceral serous pericardium. It is made up of simple squamous epithelium. The increase in thickness of epicardium in all the age group of Uttara fowl was very minute which was almost negligible. Similar finding was observed by [4].

In the present study, the myocardium revealed weak reaction for reticular fibers in day old and day 7 birds but in case of day 28 and day 112 birds, myocardial cells of both ventricular and atrial wall revealed a dense network of reticular fibers. Moreover, mild reaction was present around the blood vessels in all the age groups and black coloured fibers were seen (Fig. 2).

Masson's trichrome stain shows an intense reaction to collagen fibers present within the sub endocardial and sub endocardial layer in case of interventricular septum and ventricular wall (Fig. 3). Collagen fibers are also present in the tunica media and tunica externa layers of blood vessels present within the myocardium. The amount of collagen fibers is abundant in case of aorta, pulmonary artery and pulmonary veins compared to the other blood vessels present in the myocardium. [4] Observed that sub endothelial layer composed of collagen and elastic fibers along with few numbers of smooth muscles cells. Again, the fibrous portion of interventricular septum composed of bundles of collagen fibers.

Elastic fibers were less frequent in the ventricular wall than the atrial wall. [4] Observed that atrial wall was abundant in elastic fibers which arranged parallelly to the surface of endocardium. However, the Weigert's stain gave positive reaction for elastic and collagen fibers around blood vessels present within the myocardial cells and for the elastic and collagen fibers present around the aorta, pulmonary artery and pulmonary veins in all age groups. Elastic fibers are more abundant in the tunica intima and tunica media layers of the aorta and pulmonary artery compared to the other blood vessels present in the myocardium (Fig. 4).

b. Histomorphometric studies

In the present study after staining the tissue samples with hematoxylin and eosin staining method, histomicrometry was done at day old, 7, 28 and 112 days Uttara fowl. The thickness of the right auricle was $1476.33 \pm 21.49 \mu$, $1665.53 \pm 46.02 \mu$, $2296.2 \pm 96.04 \mu$ and $3712.33 \pm 152.9 \mu$. The thickness of the left auricle was $1028.35 \pm 13.11 \mu$, $1229.2 \pm 23.13 \mu$, $1831.73 \pm 46.09 \mu$ and $1972.167 \pm 107.58 \mu$ (Table. 1).

In this study the thickness of the left ventricle layers was recorded The epicardial thickness was $40.13 \pm 5.73 \mu$,

40.133.62 μ , 43 \pm 3.84 μ and 60.2 \pm 3.84 μ respectively at day old, 7, 28 and 112 days showing the gradual increase in the thickness of the epicardium (Table. 2). The myocardial thickness was observed which was 1423 \pm 29.97 μ , 1519.74 \pm 22.24 μ , 2593.14 \pm 68.93 μ and 4884.4 \pm 56.61 μ respectively at day old, 7, 28 and 112 days indicating the gradual increase in the thickness of the myocardium with the advancement of age (Table. 2). Whereas the endocardial thickness was recorded as 37.26 \pm 5.28 μ , 40.13 \pm 3.62 μ , 45.86 \pm 3.62 μ and 60.2 \pm 3.84 μ at day old, 7, 28 and 112 days showing the gradual increase in the thickness of the endocardium as the age of the bird advanced. The reason for this increase in thickness might be due to increase in number and thickness of individual fiber as the age advanced. Statistically there was no significant ($p>0.05$) difference between epicardium, myocardium and endocardium in all the age groups of Uttara fowl bird (Table. 2).

In the present study the thickness of the right ventricle layers was recorded after staining the tissue with hematoxylin and eosin at the desired part of the heart was taken for staining and histomorphometry of the different age groups bird i.e. day old, 7, 28 and 112 days Uttara fowl. The epicardial thickness was 31.53 \pm 5.28 μ , 40.13 \pm 3.62 μ , 45.86 \pm 3.62 μ and 60.2 \pm 3.84 μ respectively at day old, 7, 28 and 112 days showing the gradual increase in the thickness of the epicardium. The myocardial thickness was observed as 1372.96 \pm 29.97 μ , 2703.6 \pm 22.24 μ , 1975.16 \pm 68.93 μ and 2909.63 \pm 56.61 μ respectively at day old, 7, 28 and 112 days indicating the gradual increase in the thickness of the myocardium with the advancement of age. Whereas the endocardial thickness was recorded as 34.4 \pm 4.44 μ , 40.13 \pm 3.62 μ , 43 \pm 3.84 μ and 54.4 \pm 6.94 at day old, 7, 28 and 112 days showing the gradual increase in the thickness of the endocardium as the age of the bird advanced. The reason for this increase in thickness might be due to increase in number and thickness of individual fiber as the age advanced.

c. Histochemical studies

PAS and Alcian blue activity was observed very weak in all age groups in both ventricular and atrial wall of Uttara fowl (Fig. 5).

d. Ultrastructural studies

Scanning electron microscopic (SEM) examination of ventricular wall of day 112 Uttara fowl birds revealed the branching pattern of myocardial cells and presence of intercalated disc (Fig. 6). Again, SEM studies of atrial wall showing the presence of pectinate muscles folds as well as the endocardial cells projecting towards the atrial chamber (Fig. 7). SEM studies of the right ventricular wall showing the coronary vessels which supply circulation to the endocardium, myocardium and epicardium of the right ventricular wall (Fig. 8).

Conclusion

The present study showed that the epicardium of the heart of Uttara fowl was externally covered by thin layer of pericardium which composed of a layer of mesothelial cells, connective tissues and adipocytes. The myocardial connective tissues were having numerous purkinje fibers and blood vessels. The blood vessels that observed within the myocardium having tunica intima, tunica media, tunica externa and connective tissue fibers. In other age groups the distribution of reticular fibers was sparse. Elastic fibers were less frequent in the ventricular wall than the atrial wall.

Elastic fibers were more abundant in the tunica intima and tunica media layers of aorta and pulmonary artery compared to the other blood vessels present in the myocardium. Masson's trichrome stain showed an intense reaction for collagen fibers present within the sub endothelial and sub endocardial layer in case of interventricular septum and ventricular wall. PAS and Alcian blue activity was observed very weak in all age groups in both ventricular and atrial wall of Uttara fowl. The scanning electron microscopic structure of the hearts of Uttara fowl was successfully studied in this case.

Table 1: Thickness of left and right auricle (μ) at different age groups of Uttara fowl (mean \pm SE)

Age group	Thickness of right auricle (μ)	Thickness of left auricle (μ)
Day old	1476.33 \pm 21.49	1028.35 \pm 13.11
Day 7	1665.53 \pm 46.02	1229.2 \pm 23.13
Day 28	2296.2 \pm 96.04	1831.73 \pm 46.09
Day 112	3712.33 \pm 152.9	1972.167 \pm 107.58

$P>0.05$, ** highly significant ($P\leq 0.01$): Different superscript within rows indicates highly significant difference between thickness of auricle

Table 2: Thickness of epicardium, myocardium and endocardium of left ventricle (μ) at different age groups of Uttara fowl (mean \pm SE)

Bird no/parameter (day) (4x)	Thickness of left epicardium (μ)	Thickness of left myocardium (μ)	Thickness of left endocardium (μ)
Day old	40.13 \pm 5.73	1423 \pm 29.97	37.26 \pm 5.28
Day 7	40.133.62	1519.74 \pm 22.24	40.13 \pm 3.62
Day 28	43 \pm 3.84	2593.14 \pm 68.93	45.86 \pm 3.62
Day 112	60.2 \pm 3.84	4884.4 \pm 56.61	60.2 \pm 3.84

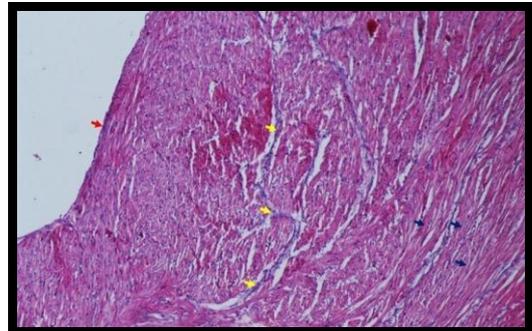


Fig 1: Photomicrography showing endocardium (red arrow), purkinjefibres (yellow arrow) and myocardial fibres (black arrow) in the right ventricular wall of day 112 Uttara fowl (H&E, X 10)

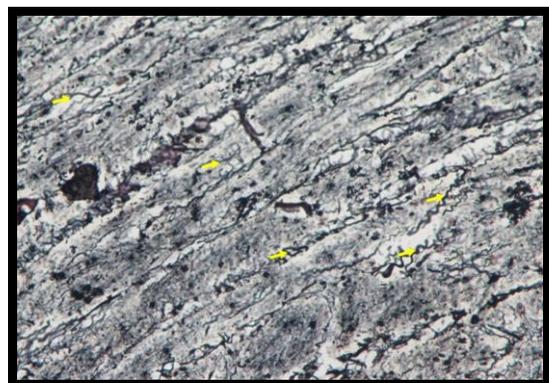


Fig 2: Photomicrography showing reticular fibres (yellow arrow) in the ventricular wall of day 112 Uttara fowl (Gomori's method \times 40).

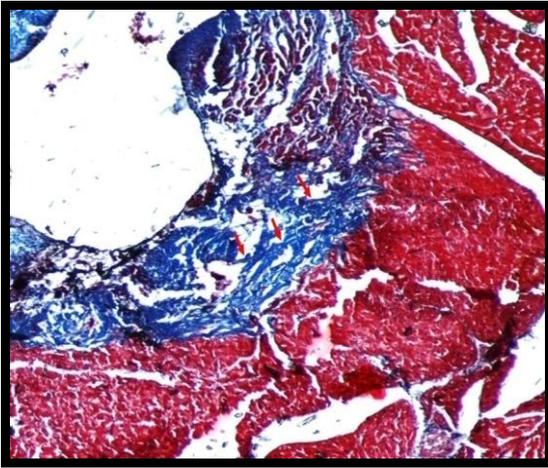


Fig 3: Photomicrography showing collagen fibres (red arrow) in the interventricular septum of day 28 Uttara fowl (Masson's Trichrome, X 10)

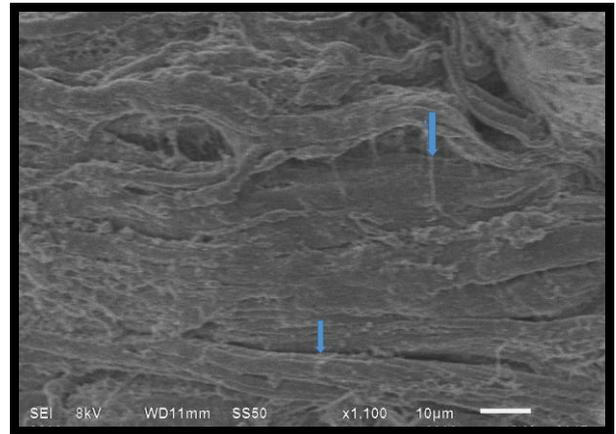


Fig 6: Scanning electron micrography showing cardiac muscles fibres with intercalated disc (blue arrow) in ventricular wall of day 112 Uttara fowl (*1,100, 8 kV)

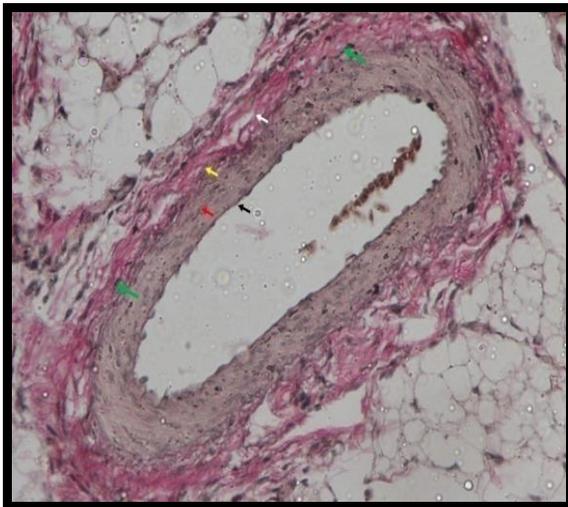


Fig 4: Photomicrography showing tunica intima (black arrow), tunica media (red arrow), tunica externa (yellow arrow), elastic fibres (green arrow) and collagen fibres (white arrow) in the aorta of day 28 Uttara fowl (Weigert's method, X 40).

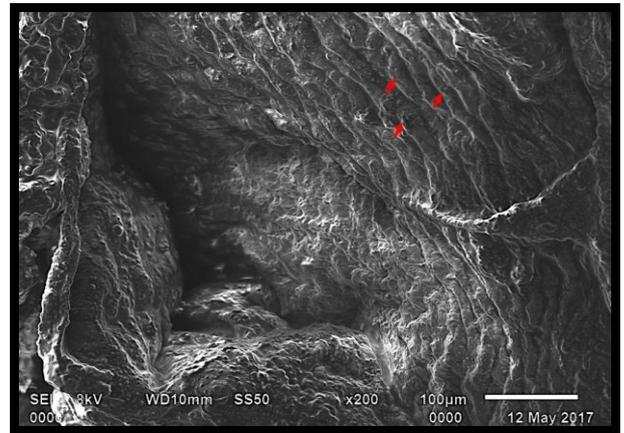


Fig 7: Scanning electron micrography showing luminal surface of atrium with endocardial cells (red arrow) projecting towards the chamber and folds of pectinate muscles (white arrow) in day 112 Uttara fowl (* 200, 8kV)

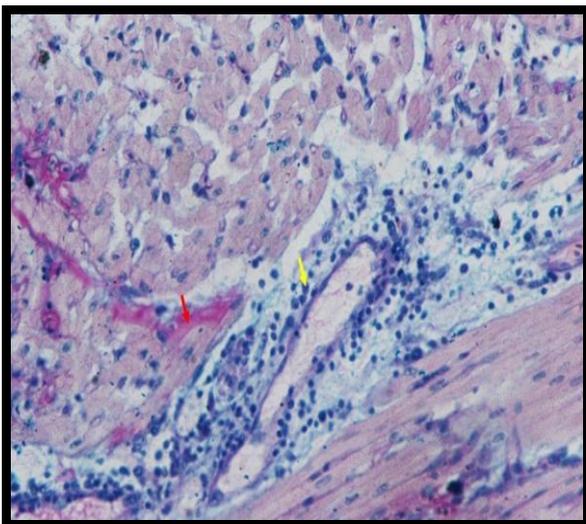


Fig 5: Photomicrography showing medium PAS and Alcian blue reaction in the left ventricular wall of day 112 Uttara fowl (PAS & Alcian Blue method, X 40)

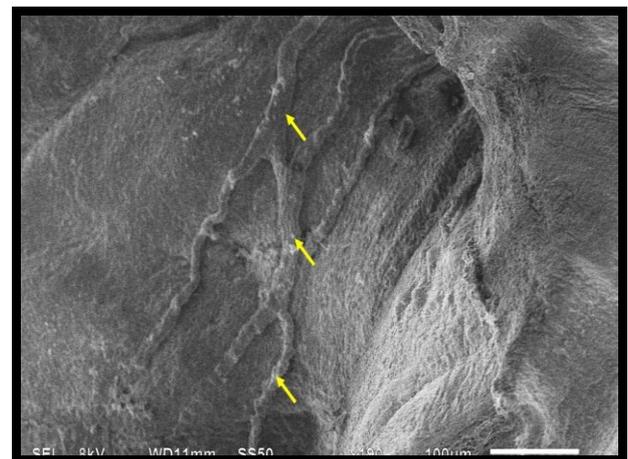


Fig 8: Scanning electron micrography showing coronary supply (yellow arrow) in the right ventricular wall of day 28 Uttara fowl (* 190, 8kV)

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