Osseous Microanatomy and Histochemical Characterization in Species, Gender Determination and Age Estimation

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Abstract

Background: Bone is a tissue of particular interest, because the hard tissue of the skeleton is normally well preserved and details of microscopic structure can be seen in specimens many centuries old. The microscopic structure of bone can be utilized for identification of unknown bone samples in forensic odontology. Physical anthropologists may encounter complete or fragmentary specimens of bone in which standard anthropometric methods provide inconclusive or questionable information. If the histological structure of such material could reveal, with reasonable accuracy, details of species, age, and gender, it would be a useful and valuable laboratory tool. **Materials and Methods:** A cross-sectional analysis of formalin fixed paraffin embedded sections from biopsied lesions (5 cases each of goat bone, 5 cases of chicken bone specimen, 5 cases of human male bone specimen, 5 cases of human female bone specimen) was done. The variation of the trabeculae by measuring the average width of trabeculae, the average marrow space, and the ratio of the above two through morphometric analysis was assessed. The observational percentage variation amongst the species by number of osteons, osteocytes shape i.e., -spindle, elliptical and round, presence of inflammation was noted. The stroma and hard tissue for Collagen characterization (Special stains)Type, orientation and maturity using Picrosirus stain, Sub intimal fibrosis surrounding the bone using Van Gieson, Percentage of mature and immature bone pattern using Azan stain was analyzed. **Result:** Chicken bone shows absence of osteons in comparison to human bone with increased number of osteocytes. Goat bone shows plexiform pattern with scattered haversian canals more comparable to human bone architecture. Average human Haversian canal diameter was greater than non-human mammalian species. Azan staining helped in differentiation of mature from immature bone. Picrosirus staining helped in establishing collagen type, orientation and thickness. Sub intimal fibrosis surrounding the bone was checked using Van Gieson staining. **Discussion:** The complexities of bone tissue and its growth processes are such that misinterpretations of a critical nature can be made if the observer is not well informed on the multitude of basic details dealing with Osteogenesis, remodelling, and comparative histology. **Conclusion:** Bone histomorphological assessment is an effective method for species identification, gender determination and age estimation by fragmentary osseous remains.

Keywords: Age Estimation, Gender Determination, Histomorphological Characterization, Osseous Microanatomy, Species Identification

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Introduction

Histological analysis of bone plays a significant role in the history of the field of forensic odontology[1,2]. Forensic Odontology in combination with forensic anthropology proves to give excellent results in victim identification. Bone has a remarkable property to repair the micro damage, by responding to load-bearing, and by accomplishing the process of remodelling[3]. Bone remodelling occurs throughout life in humans and involves a coupled sequence of cellular activation, Resorption, and formation of bone by altering the balance of essential nutrients in the body by regulating serum concentration by protecting bone from natural biomechanical forces that causes microscopic damage, and by repairing this to prevent it[4,5]. The continuous production of osteons by the bone remodelling process results in a strong correlation between the age of an individual and the number of osteons per unit area in a cross-section of their bone[6]. In situations where the skeletal remains are usually highly fragmented, degraded or burnt and unidentifiable, it plays a crucial role in traditional 2D histological approaches to differentiate amongst species type. Staining of bone specimens allows the researcher to evaluate the location of vascular channels, the orientation of cortical bone, along with osteons orientation[7]. Various methods can be employed for determining the various histological characteristics of tissue, some of which includes ground section preparation for measuring haversian canal diameter, calculating the inter-osteon width, using panel of histological stains including variety if trichrome stains, and other special stains, Immunohistochemical markers for calculating vascularity for comparison purposes and other parameters. Hence, for visualization of the internal microanatomy of the tissue, these techniques provide sensitive data to visualize bone samples in more detailed and simplified form[8]. In a country like India where more than 60 percent of the people work for the agriculture and poultry industry, it is of utmost importance for the odontologist to differentiate between animal and human bone[9]. Amongst the critical evaluations for microbiologist is to differentiate between mammalian and poultry bones which is usually done on the basis of two criteria by looking at the macroscopic details, for instance the colour and fragments shape, size and shape of osteocytic lacunae. The shape of osteocyte lacuna in both poultry and mammals is often seen in different ways elliptic, round and spindle shaped[10].

Thus the present study was undertaken to determine species, gender and age estimation in a mass disaster site using simple histochemical techniques.

Materials and Methodology

Sample

A retrospective study was conducted in the Department of Oral and Maxillofacial Pathology and Microbiology after obtaining consent from the Institutional Review Board according to their ethics for working. The research sample includes histological collection of bone specimens of various animals from poultry (Chicken, Goat) and human Jaw bones.

Specimens were fixed in neutral buffered formalin and decalcified in 10% nitric acid solution (29195, Qualigens, India) before preparation of paraffin blocks. Four serial sections 4 µm thick were obtained from each paraffin block using a rotary microtome (Leica RM2125 RT, Leica Microsystems, Germany) and were stained manually with H&E, Azan, picrosirius red stains. Sections were deparaffinized in xylene, rehydrated through a descending alcohol concentrations to distilled water (DIH20) and stained using a routine protocol with Harris hematoxylin (H3136, Sigma-Aldrich) and eosin (E4009, Sigma-Aldrich) according to Wilson and Gamble. Stained sections were then dehydrated through ascending alcohol concentrations, cleared in xylene and a cover glass mounted with DPX (340, 517, Central Drug House Pvt. Ltd., India).

The following histomorphometric variables were collectively analyzed:

Trabecular variation (Using morphometric analysis)

- Average of trabecular width, marrow width and their ratio

Vascularity (Using immunohistochemistry for Factor VIIIAg)

- Counting the mean vessel density

Percentage variation (Observational)

- Number of Osteons
- Number of osteocytes

- Shape of osteocytes lacunae -spindle, elliptical and round

Collagen characterization (Special stains)

- Type, orientation and maturity using **Picrosirus stain**
- Sub intimal fibrosis surrounding the bone using **Van Gieson**
- Percentage of mature and immature bone pattern using **Azan stain**

Evaluation of Stained Sections

All stained sections were evaluated for staining intensity and contrast. Each sample was assessed using a binocular microscope at 40×magnification (Olympus BX53) and its image was taken using a digital camera (Olympus EPL3) connected to the microscope.

For Factor VIII Ag

For Factor VIII antibody, the deparaffinized tissue sections were placed in 10 mmol/L citrate buffer, pH 6.0, and heated to cycles of 95°C and 98°C for 13 minutes. Immunohistochemical staining for these proteins was performed by the avidin-biotin complex procedure with a streptavidin-biotin complex peroxidase kit. Primary monoclonal antifactor VIII (AM016-5M, Biogenex Ind. Pvt. Ltd.) along with secondary antibody-poly HRP secondary detection system (QD400-60KE, Biogenex Ind. Pvt. Ltd.) was used. Normal mucosa for the expression of FVIII was used as a positive control. The mean numbers of the stained blood vessels were then noted in the same ten fields of highest vascularization and determined as the micro vessel density for each case.

Preparation of Picrosirus Red Stain and Staining Procedure

The paraffin embedded tissue sections were deparaffinized and rehydrated as described previously. Sections were then immersed in 0.1% Direct Red 80/Sirius Red F3B (35780, Sigma-Aldrich, USA) dissolved in saturated aq. picric acid solution for 1 hr at room temperature (RT) followed by differentiation in 0.5% glacial acetic acid (537020, Sigma-Aldrich), then dehydrated through ascending concentrations of alcohol, cleared in xylene and a cover glass mounted with DPX[11]. Slides were visualized under polarizing microscope.

Staining Protocol

The paraffin embedded tissue sections were deparaffinized and rehydrated as described previously. Sections were immersed in celestine blue hematoxylin and then differentiated with 1% acid alcohol and washed in DIH2 0. Slides were immersed in Solution A for 5 min, rinsed in water and transferred to Solution B for 5 min. Slides were drained then placed into Solution C for 2–3 min followed by dehydration, clearing and cover slipping as described for Picrosirus red.

Azan

Each tissue was passed through xylene and descending grades of ethanol, then rinsed in distilled water for 1 min and placed in direct red solution for 45 min, rinsed twice under running water. Section differentiation via 0.1% aniline blue alcohol solution was done for 3 min and stopped by placement in acetic alcohol solution. They were placed in 5% aqueous phosphotungstic acid for 10 min, rinsed in distilled water and placed for 5 min in Aniline Blue-Orange mixture and briefly rinsed again in distilled water. Xylene was finally used 3 times for 2 min each to clear the slides and cover slips placed.

Results

Species Identification

Mean trabecular width, mean marrow width and their ratio were calculated using morphometric software using a binocular microscope at 40×magnification (Olympus BX53) and an image was taken using a digital camera (Olympus EPL3) connected to the microscope. The images obtained were analysed using Magnus Pro Image software which enabled measurements to be made using different tools. Both mean trabecular and marrow width was highest in human males followed by females. Goat bone specimen simulates human bone specimen where variation was not much significant. Chicken bone shows plexiform arrangement of trabeculae thus narrow marrow width is appreciated. Although number of osteocytes are equal in almost all the species taken into consideration in the study, but there is no osteon formation in the chicken bone specimen (Table 1) (Figure 1 and Graph 1).

Table 1. Comparison of Mean trabecular width, mean marrow width, Ratio (Mean trabecular width/Mean marrow width), No. of osteocytes, No. of osteons in different species

		N	Mean	Std. Deviation	P value
Mean trabecular width	Human (Male)	5	67.29	6.52	0.00 (HIGHLY SIGNIFICANT)
	Human (Female)	5	51.33	5.12	
	Chicken	5	41.00	4.48	
	Goat	5	57.40	2.40	
Mean marrow width	Human (Male)	5	80.58	6.21	0.00 (HIGHLY SIGNIFICANT)
	Human (Female)	5	70.84	5.17	
	Chicken	5	10.60	4.33	
	Goat	5	63.40	5.77	
Ratio (Mean trabecular width /Mean marrow width)	Human (Male)	5	.8720	.19	0.01
	Human (Female)	5	.7240	.10	
	Chicken	5	4.49	2.04	
	Goat	5	.9054	.06	
No. of osteocytes	Human (Male)	5	33.60	5.22	0.07
	Human (Female)	5	40.20	4.38	
	Chicken	5	39.00	.12	
	Goat	5	37.40	2.70	
No. of osteons	Human (Male)	5	7.40	2.07	0.05
	Human (Female)	5	7.20	1.64	
	Chicken	5	.00	.000	
	Goat	5	7.20	.837	

Table 2. Correlation of gender to Mean trabecular width, mean marrow width, Ratio (Mean trabecular width/ Mean marrow width), No. of osteocytes, No. of osteons

		Mean trabecular width	Mean marrow width	Ratio	Number of osteocytes	Number of osteons
Male	Pearson Correlation	.556	-.704	.746	-.934[*]	.979[**]
	Sig. (2-tailed)	.331	.185	.148	.020	.004
Female	Pearson Correlation	-.373	-.743	.133	-.978[**]	.783
	Sig. (2-tailed)	.536	.150	.831	.004	.117

Gender Determination

Mean Haversian canal diameter and mean vessel density are significantly high in human females as compared to human males. (Graph 2) Correlation coefficient was statistically significant for number of osteocytes in both the genders (Table 2) (Figure 2).

Age Estimation

Two age groups were taken into account, i.e., 10–20 years and 20–40 years. As the age progressed number of osteons increased. On visualising under special stains such as Picrosirus and Azan stain, differential immature areas were appreciated in 10–20 years age group whereas
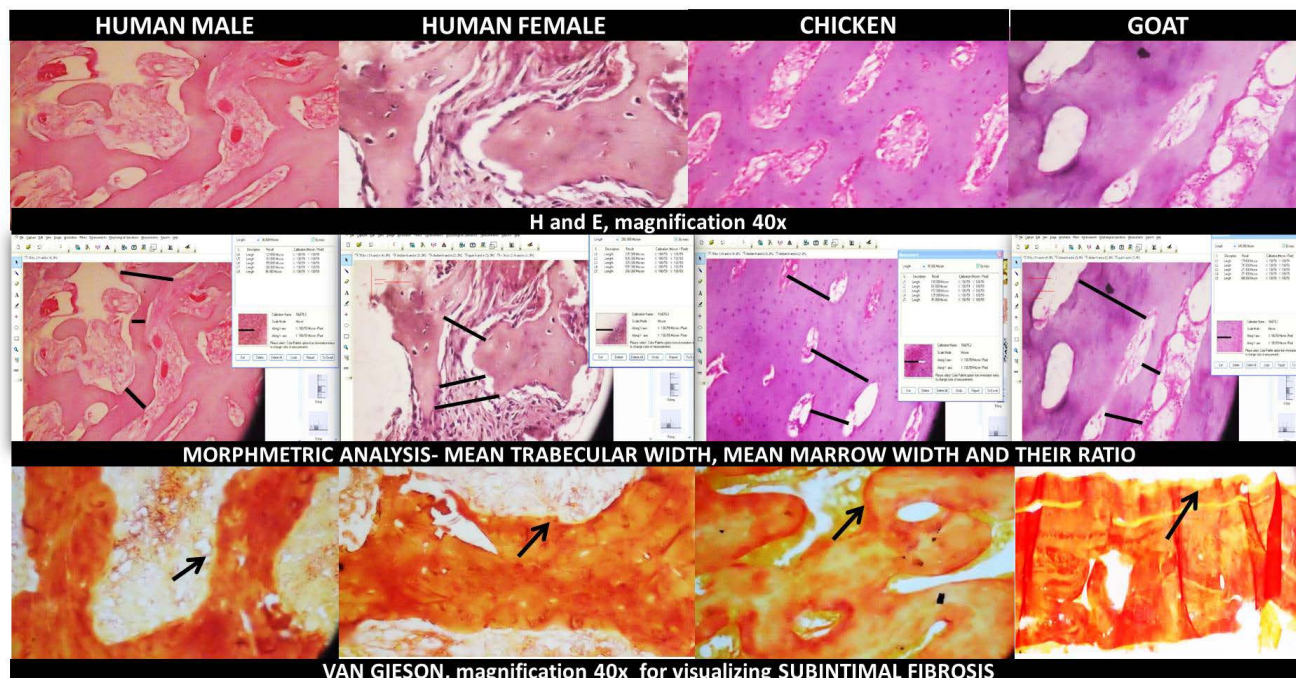
Figure 1. (Row 1) Hematoxylin and Eosin-stained section showing bony trabeculae in a A) Human male B) Human Female C) Chicken and D) Goat. (Row 2) Comparative analysis of trabecular and marrow width using Magnus Pro software in A) Human male B) Human Female C) Chicken and D) Goat. (Row 3) Van Gieson staining to highlight sub intimal fibrosis present chiefly in goat specimen as compared to human and chicken bone specimen.
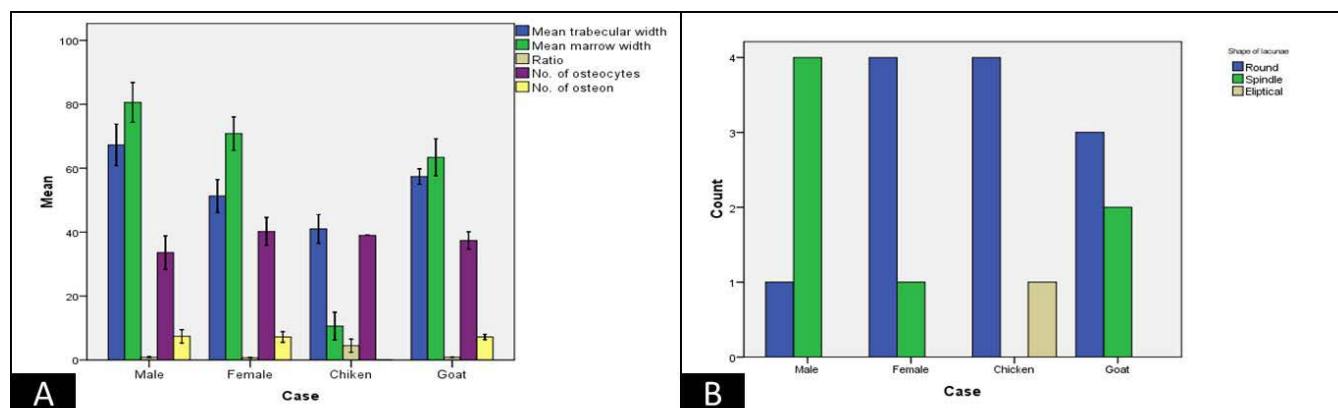
Graph 1. **A)** Comparison of Mean trabecular width, mean marrow width, Ratio of trabecular width to marrow width, Number of osteocytes and number of osteons in various species **B)** Distribution of round, spindle and elliptical shape of osteocytes in study species.

completely mature lamellated bone was seen in age group 20–40 years. Picrosirus showed yellow green and orange red birefringence in polarizing microscope for younger age group whereas completely red lamellated bone was seen in 20–40 years of age group. Azan stain showed intermixed magenta areas with focal blue areas in 10–20

years age group whereas it showed completely dark blue component in 20–40 years age group (Table 3, Figure 3).

Statistical Analysis

Statistical analysis was performed using SPSS software (Version 20.0). Data have been expressed as mean and

Table 3. Histochemical characterization in different age groups of human species

|	Age	N	Mean	Std. Deviation	p value
No of Osteons	<20 years	5	6.80	1.304	0.017
	>20years	5	7.40	2.702	
Picrosirus	<20 years	5	2.20	.447	0.029
	>20years	5	3.00	.000	
Azan	<20 years	5	2.40	.548	1.09
	>20years	5	2.40	.548	
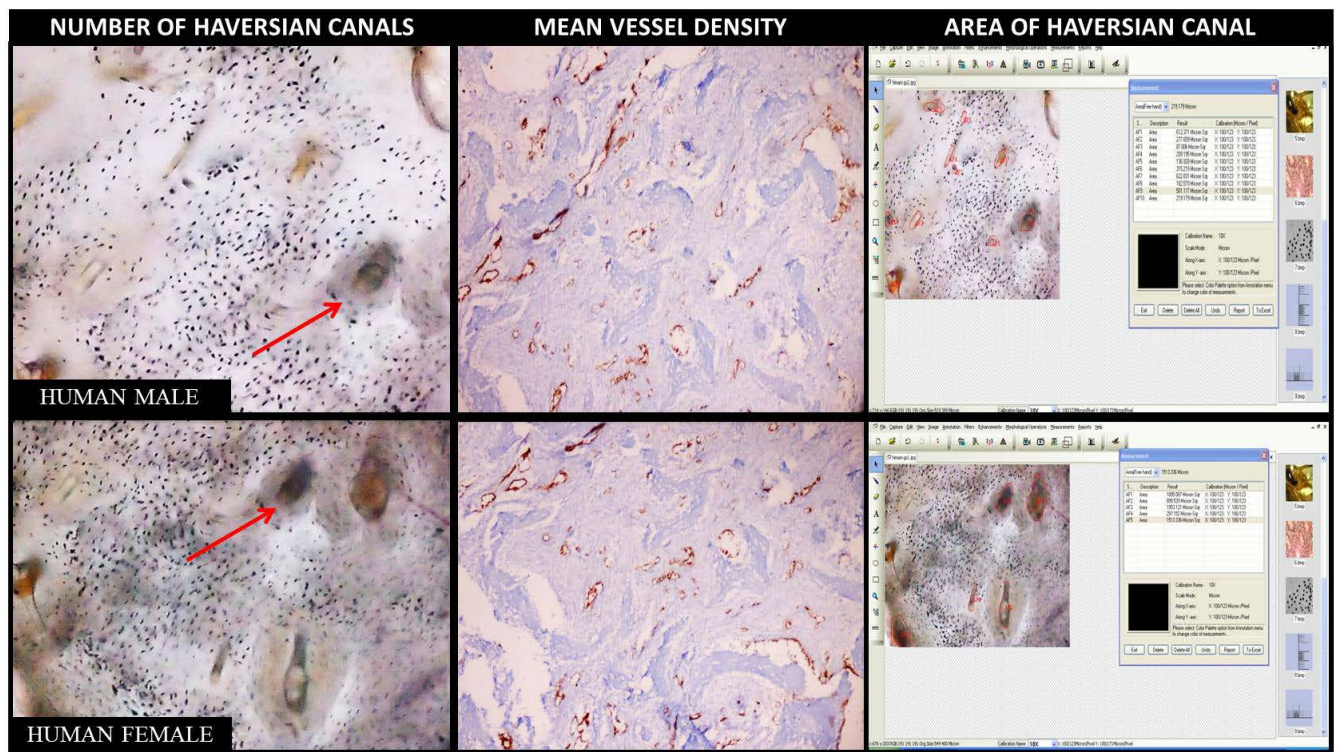
Figure 2. (Row 1) Number of haversian canals-counted by preparing ground section, Mean vessel density using Factor VIII immunohistochemistry, Area of Haversian canal calculated using Magnus pro morphometric software in Human male. (Row 2) Number of haversian canals-counted by preparing ground section, Mean vessel density using Factor VIII immunohistochemistry, Area of Haversian canal calculated using Magnus pro morphometric software in Human females.

standard deviation. Pearson's Chisquare test was carried out to determine the level of correlation or association between the groups under study. P<0.05 was considered statistically significant.

Discussion

Bone is an active, three-dimensional, and dynamic tissue that changes its structure for the entire life.[11] It is often difficult to distinguish between mammalian and poultry bones, which is usually done by examining various characteristics such as colour, shape, density of the bone fragments, shape and macroscopic features. In order to distinguish remnants of the bone accurately histological evidence provides better data. Trabecular variation was measured using morphometric software analysis by measuring Average of trabecular width, marrow width and their ratio. The metabolic rate and bone remodelling rate in goats are considered to be similar to that of humans. The mean trabecular width and mean marrow width is
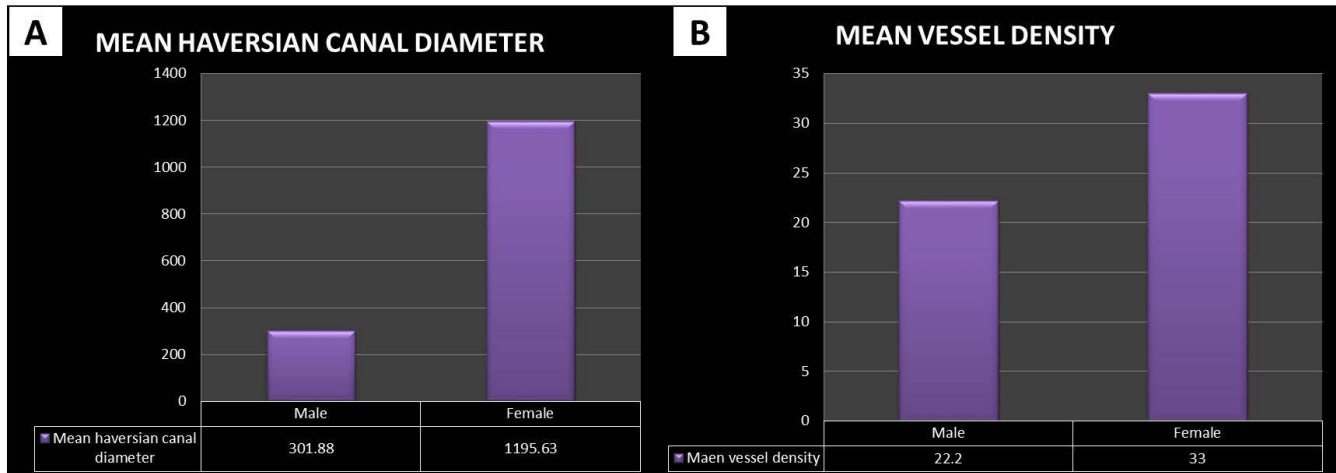
Graph 2. **A)** Comparison of mean Haversian canal diameter in human males and females **B)** Comparison of mean vessel density in human males and females.
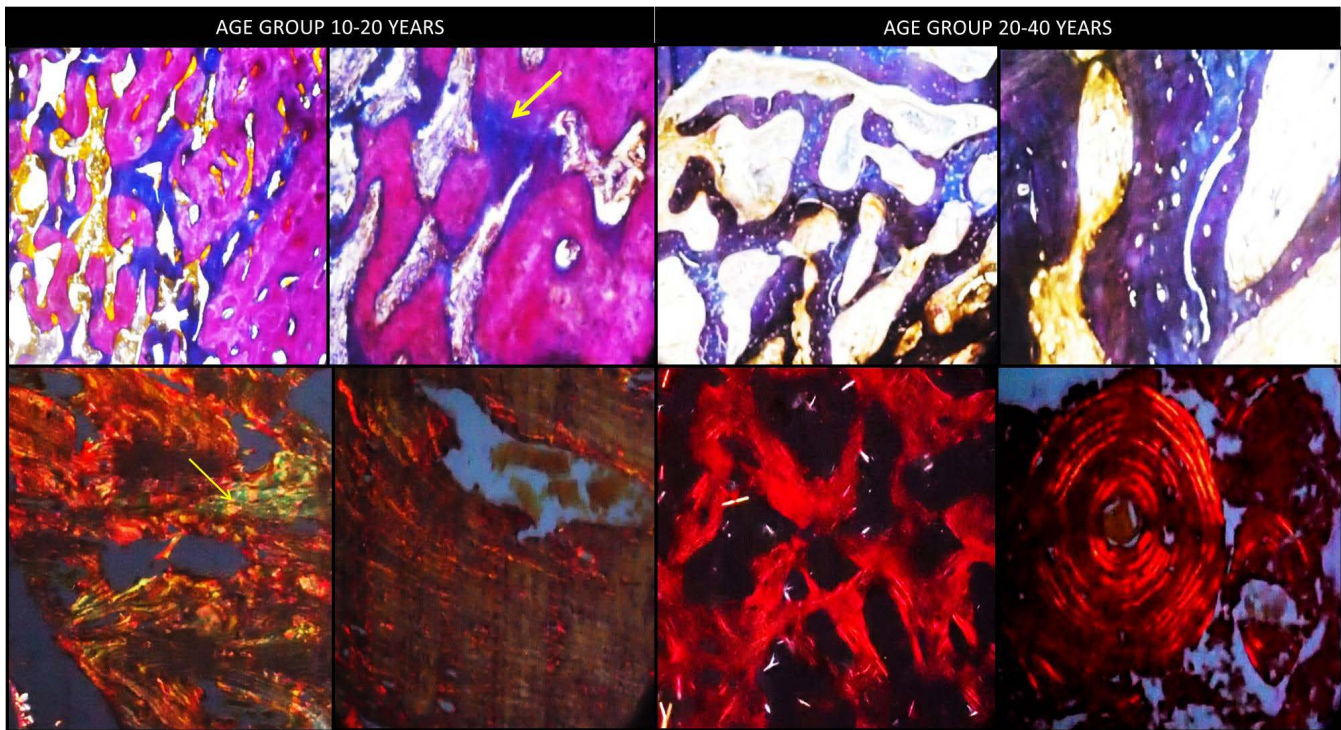

Figure 3. (Row 1) Azan stain showing admixture of magenta and blue color in human age group 10–20years whereas showing completely mature blue trabeculae in human age group 20-40years. (10x,40x) (Row 2) Picrosirus red staining under polarizing microscope showing focal yellow green immature birefringent areas in human age group 10–20years whereas completely red mature lamellated arrangement in human age group 20–40years (10x,40x).

observed more in males.[12] This can be done by observing various characteristic features including osteocyte lacunae shape, number of osteocytes and number of osteons. According to the observations carried out in the different planes, it was found that chicken osteocytes, are similar to humans that is, it has a biconvex lens shape. This morphology seems to be maintained in all skeleton parts; however the different arrangement and some size variation of the osteocytes in the various bone segments influence the microscopic features of corresponding

lacunae. Chicken bone is made of a parallel-fibered tissue, without osteons[10]. The shape of lacunae in humans were elliptical to round, goat lacunae was mimicking like human, on the other hand the chicken lacunae were ranging from round to elliptical to spindle shaped due to major presence of compact bone. Absence of osteons was observed in chicken due to absence of spongy bone which leads to easy fracture and fast formation of bone as a compensatory method. Due to this effect of bone fracture and formation at a higher rate, the number of osteocytes in chicken was observationally higher as compared to the other two subgroups. It was observed that the number of osteons in males were higher as compared to females. Age-related increase in Haversian canal size have been recorded as contributing to the increasing cortical bone porosity[13]. Number of osteons increases with age because of rhythmic deposition of lamellated bone. Bone remodeling plays a crucial role in maintaining bone health which can be highlighted by studying the collagen characterization using a panel of special stains to distinguish mature and immature bone. The collagen fibers under the polarizing microscopy using picrosirus showed yellow green and orange red fibers in the age group of patients less age 20 years of age whereas more than 20 years of age the collagen fibers were red to orange in colour[14,15].

Azan staining revealed both blue and magenta component in patients less than 20 years of age and only blue component in more than 20 years of age. Old age and estrogen deficiency are the two most critical factors for the development of osteoporosis in both women and men[16,17]. The vascularity was higher in females due to the hormonal effect of estrogen which causes vasodilatation. When females enter puberty, periosteal apposition is inhibited, probably due to the inhibitory effect of estrogen on periosteal bone formation, whereas endocortical bone formation is stimulated, increasing cortical thickness and narrowing the medullary cavity. On the other hand, when the women enters the menopausal age due to absence of estrogen periosteal apposition continues, whereas endocortical bone formation is inhibited, decreasing cortical thickness and widening of the medullary cavity is observed[18]. Chicken bone is fragile without osteons and significantly high number of osteocytes whereas, goat bone had plexiform trabecular arrangement with osteon presence just like human bone.

Conclusion

The biological information encrypted in the histomorphology of bone, yields a wealth of information relating to skeletal structure and function useful in identification. Bone histomorphological assessment is an effective method for species identification, gender determination and age estimation by fragmentary osseous remains.

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