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Microscopic evaluation of the fetal heart growth at various stages of gestation by histological and immunohistochemical studies.

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Abstract: ***Objective: To define the microscopic structure of the fetal heart at various stages of development.***

Methods: A total of 100 spontaneously aborted fetuses at various gestational ages were collected after ethical approval and the heart was dissected. A detailed microscopic evaluation at various stages was performed by histological and immunohistochemical studies.

Results: A relative growth was seen along with the gestational age of the fetuses. The atrium showed extensive pectinate muscle pattern in the wall until maturity while the extent and complexity of trabeculation in ventricles increased with age. The nuclear density per square millimeter increased from 2,78,400 cells to 5,85,600 cells. From first group to second there was a rise in the percentage of proliferation. The proliferating cell concentration was strong till lowest gestational week of third group and then slowly the proliferation declined.

Conclusion: This study could enhance further investigation of factors influencing the regulation of the cardiac myocyte cell cycle, which may better elucidate cardiac conditions, such as myocardial hypoplasia and cardiac response to stress and injury.

Keywords: Fetal heart growth, Proliferation, trabeculation

1. Introduction

Through the 20th century, knowledge of the events occurring during cardiac development was clouded by conflicting descriptions, coupled with use of notably different terminologies. Furthermore, not all accounts were based on direct study of embryonic material, instead being constructed on the basis of interpretations of previous reports, supported by inferences made from the structure of the congenitally malformed heart. Such processes, in themselves, are understandable, since it is axiomatic that proper appreciation of the events occurring during formation of the heart will aid in the analysis of the morphogenesis of cardiac malformations, this being a desirable prerequisite in the search for optimal treatment. Over the past decade, there has been a change. There has been an explosion of work, anatomical and molecular, devoted to cardiac development. Advances in technology, coupled with the use of suitable animal models, now enable us to provide a more accurate account of the steps involved in formation and septation of the cardiac chambers [1].

The heart in higher vertebrates develops from a simple tube into a complex organ with four chambers specialized for efficient pumping at pressure. During this period, there is a concomitant change in the level of myocardial organization. One important event is the emergence of trabeculations in the luminal layers of the ventricles, a feature which enables the myocardium to increase its mass in the absence of any discrete coronary circulation. In subsequent development, this trabecular layer becomes solidified in its deeper part, thus increasing the compact component of the ventricular myocardium. The remaining layer adjacent to the ventricular lumen retains its trabeculations, with patterns which are both ventricle- and species-specific. During ontogenesis, the compact layer is initially only a few cells thick, but gradually develops multilayered spiral architecture. A similar process can be charted in the atrial myocardium, where the luminal trabeculations become the pectinate muscles.

Although reasonable knowledge about intrauterine cardiovascular development currently exists, certain aspects of this process are still causing much scientific discussion. Some details of intrauterine cardiovascular development in the human and other species have been clarified.

recently[2]-[12], but these still have yet to be confirmed. It is also important to assess interspecific differences to avoid rough interpretational errors in the results obtained [11]-[14]. Even though the idea that differences exist between myocardial

structures in the same individual during development is controversial, few studies have focused on this subject [15]-

Grading Criteria
0= Negative
1= Mild positivity in <25% of cells
2= Moderate Positivity 25-50% of cells
3= Moderate > 75% of cells
4= Strong in >75% of cells

[18]. A significant benefit of the cardiovascular assessment of anatomico-pathological studies is that the cause of death can be more rapidly clarified during the perinatal period when death is due to gross malformations. However, difficulties increase when the events are mainly related to microscopic structures.[17] For example, even though myocardial changes during sudden infant death have been described; only incipient knowledge in this area is available [17],[18].

Thus, the present study was conducted to establish the structural changes of fetal heart so as to find out the development of fetal heart in size during various gestational ages using histological and immunohistological techniques.

2. Methods

A total of 100 spontaneously aborted fetuses were collected after ethical approval from various health science centers. The cause of the abortion was taken into notice. Since normal structure was required, all fetuses were selected on the basis of absence of maternal history of diabetes mellitus, chemotherapy, or viral infections and included fetuses with no congenital heart diseases or chromosomal anomalies. Fetuses with abnormal karyotypes were excluded to avoid bias in the results. A written informed consent was obtained from each parent with an aborted fetus included in the present study. Depending upon the gestational age of the fetus, the samples were divided into three groups' i.e.1-16 weeks, 16-24 weeks and 24-40 weeks of gestation.

2.1. Histological study

The fetuses with minimum and maximum gestational age in each group were processed for histological and immunohistochemical staining. Depending on the size of the heart, either full heart or part of left ventricle were sectioned and embedded with paraffin wax for histological study. The features of the cardiac area were noted. The thickness of each section was 5 µ and the section was taken obliquely for mounting entire heart. The procedure included staining with Haematoxylin and Eosin stains. The oblique section showing all chambers of fetal heart was then mounted.

2.1.1. Slide Observation: Fetal heart tissues were observed microscopically for the cardiac musculature and connective tissue present in between including the nuclear density present at different gestational age groups. The selection of the physical disector pairs was made as described by Sterio (Sterio, 1984) [19]. Based on this study, pairs from every 5th section were chosen randomly, and in this way, approximately 15–20 section pairs were obtained and evaluated on a digital Stereologer. The dimension of counting frame at PC screen used in this study was 10 cm x 10 cm, and the real dimension of this counting frame (6.250×10^{-5} cm^2) was estimated by following formula:

Real dimension = ¼ Screen size of frame / total magnification of microscope (1)

The mean numerical density of myocytes [$Nv_{(myocyte)}$] in per mm^2 was estimated using the following formula (Gundersen, 1986)[22].

$$Nv_{(myocyte)} = \sum Q^-_{(myocyte)}/t.\ A \quad (2)$$

Where $\sum Q^-_{(myocyte)}$ is the total number of nuclei (disector particles) counted in the reference section; t is the mean section thickness (5 µm), and A is the area of the unbiased counting frame.

2.2. Histochemical study

The percentages of proliferating myocytes in fetuses of different gestational ages were determined by this technique.

The procedure was referred from Yve Huttenbach et al [20]. Positive control slides included samples of normal tonsil in which nuclear staining was prominent in germinal center regions, observed as a brown reaction product. Negative control slides consisted of identically prepared slide sections with PBS (Biogenex) added in place of the primary antibody.

2.2.1. Interpretation of MIB-1 staining

The sections were examined by light microscopy (fig.1) and an approximation of the percentage of positively staining myocyte nuclei was expressed in four grades according to the following staining index (SI) (Table-1).

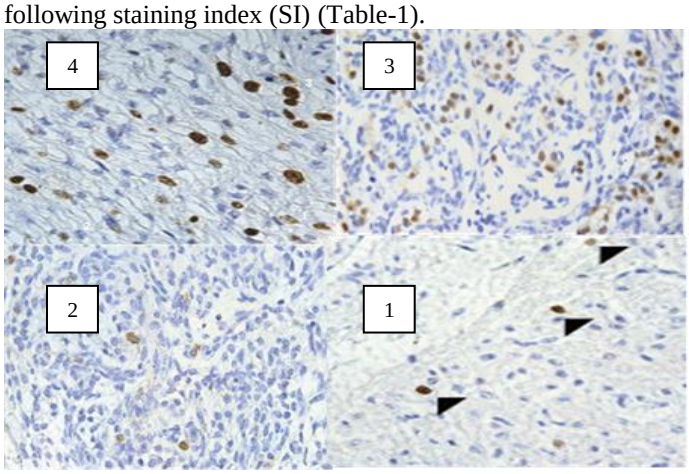

Fig. 1: Sections of left ventricular wall immunostained with MIB-1 monoclonal antibody. Grade-4: Intensity of staining is strong, Grade-3: Intensity of staining is moderate (About-50%), Grade-2: Intensity of staining is moderately positive and Grade-1: Intensity of staining is mild.

- Table-1 Staining Index

Nonmyocyte nuclei (endothelial cells, fibroblasts, nerves, and intravascular lymphocytes) were not included in the determination of the SI. The myocyte nuclei can be distinguished from those of interstitial cells, which are more slender and located at the periphery of groups of myocardial bands. The location of cell nuclei also helps identify endothelial cells in the myocardium when the vessels can be traced in the section. Nerve nuclei often gather as a closely set cluster.

3. Results:

The lowest and highest gestational week fetuses of individual age group were selected and processed for histological and Immunohistochemical studies. Until maturity, the atrium showed extensive pectinate muscles while the extent and complexity of trabeculation increased in ventricular wall with the gestational age. There was an outer compact and inner

trabeculated myocardium seen. The trabeculated myocardium could be subdivided into outer basal portion adjacent to compact layer and the central luminal part. The outer basal layer could be distinguished from the inner luminal by shorter and finer trabeculae with small, round intertrabecular spaces. The trabeculae increase with age. The trabeculae were initially radially arranged but later adopted a spiral course, which persists in simplified form in adulthood.

Myocardium was initially comprised of radially oriented cells with large intercellular spaces gradually becoming more tightly packed. Intercellular spaces decrease and the cells assume a circumferential orientation. Fetal hearts of early age group had thin walls and included erythrocytes and the heart lumens were large. As age increased, the heart walls extended into the lumen. Later samples showed histologically, scant and short myocytes occupying the hearts. Endocardium and pericardium were obviously seen. In youngest fetuses, myocytes were irregularly polygonal in cross section and occurred in small clumps interspersed with capillaries (fig: 2(1)). The myocytes displayed a prominent rounded nucleus. As age increased, connective tissue was more definitively seen, the staining property of nuclei gradually increased (fig:3). The myocyte cell size and cell boundaries were definitively seen as the age increased. Myocyte fiber organization was clearly seen as the gestational age increased (fig: 4).

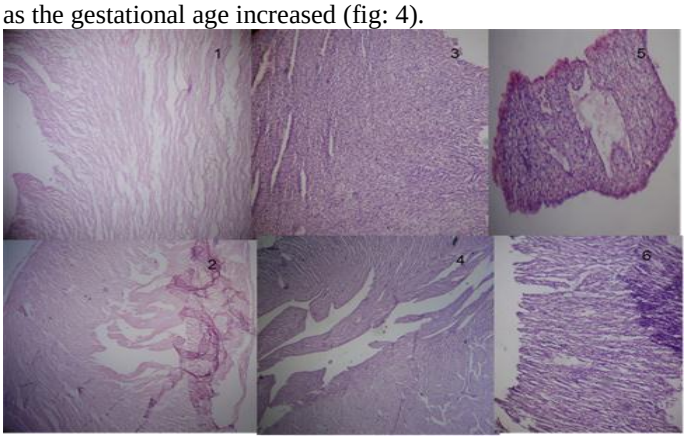

Figure. 2: Low power (10x) view of Haematoxylin and Eosin stained slides. 1. G.A -8.5 weeks, 2. G.A -14.3 weeks, 3. G.A - 16.4 weeks, 4. G.A-23.2 weeks , 5. G.A - 25 weeks, 6. G.A - 32 weeks (G.A- Gestational Age)

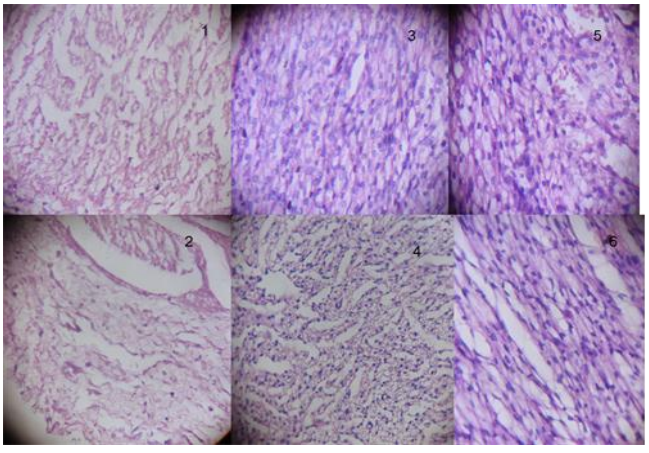

Figure.3 : High power(40x) view of Haematoxylin and Eosin stained slides. 1. G.A -8.5 weeks, 2. G.A -14.3 weeks, 3. G.A - 16.4 weeks, 4. G.A-23.2 weeks , 5. G.A - 25 weeks, 6. G.A - 32 weeks (G.A- Gestational Age)

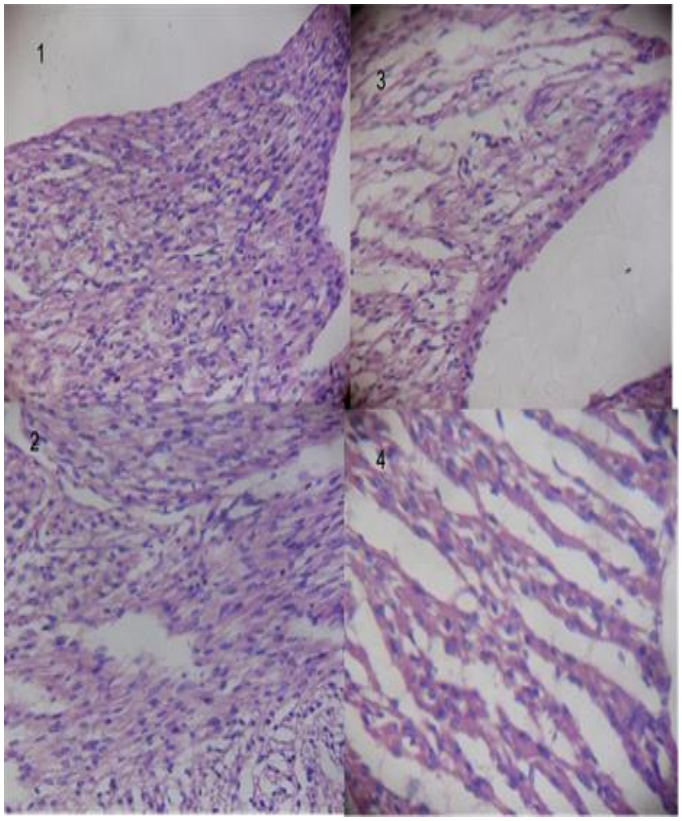

Figure:4. Sequential growth of luminal trabeculae in ventricles from 1 (lowest age group) to 4 (highest age group).

3.1. Histological features

Absolute numbers of myocyte nuclei per square millimeter are summarized in Table 2. The nuclear density per square millimeter increased from 278400 cells to 585600 cells. Over this period, the increase in number was linear and conformed to the equation (graph-3), N= (9375.2 x Age) + 256785 (fig.4), where gestational age is expressed in weeks. The slope and predicted intercept were both different from zero ($p<0.001$ in both cases).

3.2. Immunostaining

The staining index is expressed as grades at different gestational ages. The values are presented in the Table-2. Grading of the nuclei was done depending on the percentage of the nuclei stained with mib-1 antibody staining. The results (fig.5) showed an increase in number of proliferating cells from lowest gestational age in Group-I to highest in the same group. From first group to second there was a rise in the percentage of proliferation. The proliferating cell concentration was strong till lowest gestational week of third group and then slowly the proliferation declined. Comparison with the morphological parameters, the heart size had shown increase in total dimensions during later gestational ages of group-3. By this study it is clear that during the end stages of gestation, the proliferation of cardiac myocytes reduces and the size of the heart is growing due to increase in the size of individual cardiac myocyte and not by increase in number.

Table-2: Percentage of proliferating cells appearing at each gestational age group					
Age Group	**Order of gestational age**	**Intensity of staining**	**Percentage of positive cells**	**Grade**	**Localization**
Group-I	Lowest	Moderate	50%	2	Nuclear
	Highest	Strong	75%	3	Nuclear
Group-II	Lowest	Strong	more than 75%	4	Nuclear
	Highest	Strong	more than 75%	4	Nuclear
Group-III	Lowest	Strong	more than 75%	4	Nuclear
	Middle	Moderate positivity	25-50%	2	Nuclear
	Highest	Mild	Less than 25%	1	Nuclear

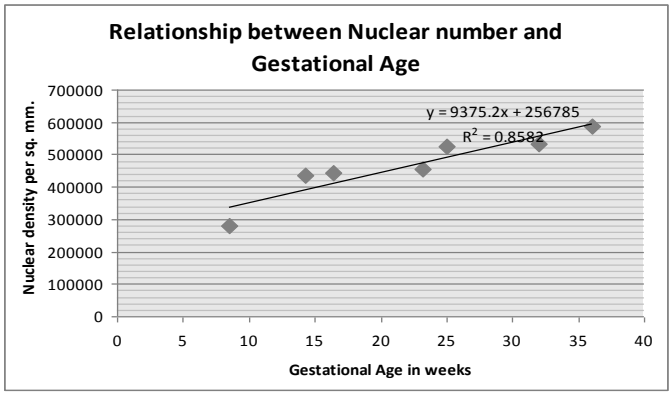

Fig. 5: A graph showing relationship between nuclear number and gestational age.

4. Discussion

Congenital heart disease (CHD) is a leading cause of infant mortality, with an estimated incidence of about 4–13 per 1000 live births [21]-[23]. Between 1950 and 1994, 42% of infant deaths reported to the World Health Organization were attributable to cardiac defects [24].

Although relatively few studies have been performed in the animal heart, , the developing heart differed in humans from other experimental animals in several aspects: 1) the proliferation of fetal myocytes; 2) the disposition of connective tissue and cellular arrangement of cardiac myocytes at different ages; and 3) quantitative differences in the of the two ventricles during fetal development.

Myogenesis and heart development has been investigated for many decades (Rumyantsev, 1977; Virchow, 1989). In these studies, generally laboratory animals were used to aid in the extrapolation to human assessment. The postnatal changes of heart morphology are substantially similar among the all mammalian species, but display differences in the timing of events. In small laboratory animals; such morphological changes happen faster than humans (Hew and Keller, 2003). Stereology is a set of simple and efficient methods that are used for quantification of two-dimensional microscopic sections, which provide quantitative data about morphology, such as volume, surface area and particle number. So, the methods allow to understand real three dimensional characteristics of these structures (Gundersen et al., 1988; Yazici et al., 2009). Until recently, it has been believed that myocyte growth in fetal period occurs largely by hyperplasia, but in the post-natal period, heart enlarges by hypertrophy of myocyte and interstitial tissue (Wulfsohn et al., 2004). So, the process of differentiation and morphogenesis of heart has been largely unexplained (Abdelwahid et al., 2002; Balsam et al., 2004; Spitkovsky et al., 2004). In such a case, it is essential to clearly understand and eventually control myogenic cell process for learning developmental process and also preventing many heart pathologies such as heart failure and post-ischaemic situations. In the literature, there are many studies about heart structure. Some of these articles are performed with clinical, histopathological or biochemical methods. However, the number of quantitative and/or embryological studies that were made with unbiased stereological methods is few. Also, none of the stereological and/or embryological studies evaluated histological structure of heart in comparison with Immunohistochemical methods. Albeit, previous stereological studies talking about developing heart did not evaluated at different gestational ages. When considering the results presented from the present study, it is possible that cell division of myocytes during post-natal life is more important than the cell division that occurs during prenatal life. The mean nuclear density increased during prenatal growth until mid of gestation rapidly, but the mean nuclear density then decreased in the very last prenatal ages. This result is not surprising because it is probably a reflection of reduced cellular activity of the older myocytes. Although the number of observed most nuclei was higher than previously described, we observed that the nuclei were smaller at the end stages of gestation periods compared to the nuclei in mid stages of gestation period myocytes. The possible reason for this observation is that the newly formed daughter myocytes had naturally smaller nuclei. According to morphometric results, size of whole heart reduced during mid-prenatal life and increased in last prenatal days. Unlike this, ventricle was increasingly thicker from youngest to oldest gestational age groups. When heart samples evaluating histologically heart of the 8-day-old embryo had thin wall, it included nucleated erythrocytes and lumen was large. Cardiac myocytes had one euchromatic nucleus with central position. The cells included large spaces which are glycogen deposits possible. Heart histology of later stages was also similar. In these samples, scant and short myocytes, endocardium, pericardium and mitotic figures were seen. Especially after mid gestational age, bundles showing striations were found. Also, cardiac muscle cells were longer. Our study presented a more detailed description than previous studies concerning the histological and morphological structure of the developing

human heart. These findings suggest that (1) myocyte number might increase during the first trimester of gestation and also during post-natal life; and (2) post-natal ventricular growth is probably hyperplasic rather than hypertrophic and/or interstitial.

Although a few previous studies have measured the proliferative activity of cardiac myocytes in the perinatal period in animal models [25]-[28], to date, no previous immunohistochemical studies have been performed on human tissue to determine if the proliferation pattern in comparison with morphometric parameters is, in fact, similar in humans. In the present study, we examined cardiac tissue from autopsies of aborted fetuses. The pattern of proliferation seen by MIB-1 staining was similar to the pattern seen in some of the previous animal studies [25]-[27]. The rate remained comparatively high from 16 to almost 28 weeks of gestation, with a statistically significant decrease in proliferation after 28 weeks of gestation. A biphasic pattern, previously reported in one animal study, was not seen [28]. Although the general trend was a decrease in proliferation with gestational age, variation was seen in individual cases, with some older hearts showing more proliferative activity than some of the younger hearts. The reason for the individual variation is difficult to specifically identify, given the incomplete understanding at the current time of all of the mechanisms controlling myocyte division. The influence of various hemodynamic and humoral factors is likely a complex process. Furthermore, while the hearts used in this study were grossly and microscopically unremarkable, underlying conditions in certain fetuses and infants could potentially have altered the myocardium in subtle ways undetectable by light microscopic examination.

Cellular proliferation is recognized as a primary means of cardiac growth during embryonic and fetal life. Indeed, the number of myocytes in the heart increased between group-1 and group-2 fetuses. However, proliferative growth was not responsible for the exponential cardiac growth during the last third of gestation. Rather, the contribution of proliferation was relatively constant, and consequently a proportion of daily free wall growth became smaller with advancing gestational age. Proliferation is but one outcome of myocyte cell cycle activity. As the fetuses grew older and matured, the outcome shifted from karyokinesis followed by cytokinesis (proliferation) to karyokinesis alone terminal differentiation) .These changes in myocyte numbers, driven by both proliferation and terminal differentiation, are reflected in the declining percentage of myocytes. The results of our study suggest that the decrease in myocyte cycle activity with increasing maturity is due to a declining proportion of myocytes capable of proliferation. During the last third of gestation, the myocyte mass of the heart increased by proliferation, enlargement of myocytes, and enlargement of myocytes as they underwent terminal differentiation. It is remarkable that these processes are so well coordinated that growth of the heart remained closely matched to growth of the body during this period. The contributions to growth of proliferation and enlargement myocytes were relatively constant and proportionately much larger earlier than later in gestation. Enlargement of differentiated cardiac myocytes contributed only modestly and only near term. Both the interval between nuclear divisions and the duration of cell cycle activity declined substantially during this same period. The duration of the cardiac myocyte cycle in the fetal large mammal warrants further investigation into earlier as well as later stages. These data on normal cardiac growth may enable a more detailed understanding of the consequences of experimental and pathological interventions in prenatal life.

5. Conclusions

The proliferative pattern of cardiac myocytes in the perinatal period in human tissue reflects that seen in most of the previous animal studies. Further investigation of factors influencing the regulation of the cardiac myocyte cell cycle may better elucidate cardiac conditions, such as myocardial hypoplasia and cardiac response to stress and injury.

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