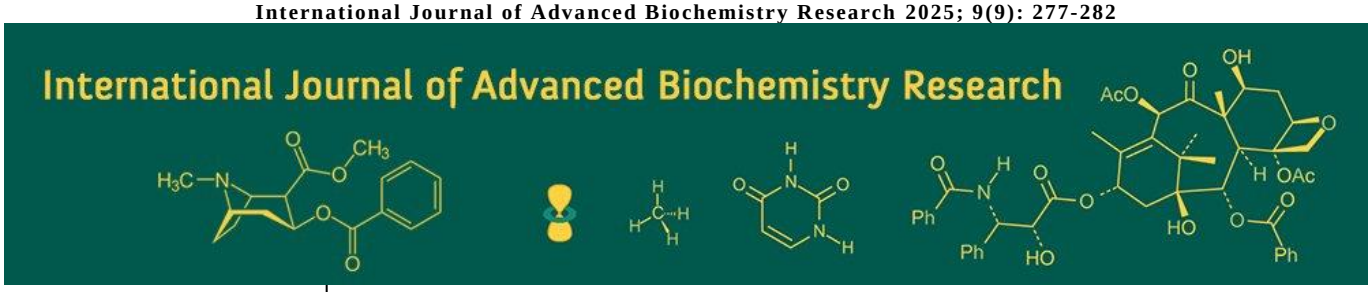

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Gross, histological and histochemical studies on the pineal gland of sheep (*Ovis aries*)

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Abstract
Samples of pineal gland were collected from pre and postnatal groups of sheep. Pineal gland in sheep was a small white, round shaped structure located between the thalamus and corpora quadrigemina of the brain. In group I, it consisted of pinealocytes. In group II, pinealocytes and a few glial cells were observed. In group III and IV outer cortex and inner medulla regions were observed. From group V and VI, cells of the cortex and medulla were reduced and vacuolated spaces increased. A moderate PAS positive and alkaline phosphatase positive activities were observed in pinealocytes of all groups. A weak Millon's, reaction and acid phosphatase positive reaction were identified in the pinealocytes. A strong positive alpha naphthyl acetate esterase reactions were noticed in the pinealocytes of all groups studied.

Keywords: Pinealocytes, development, histology, pineal gland, sheep

Introduction
Studying embryology is important in understanding clinical issues such as pregnancy loss, origins of congenital anomalies and developmental origins of adult disease, as well as fundamental insights into human biology.
It provides a logical basis for understanding the overall organization of the human body. Developmental anatomy is a powerful adjunct to an in depth understanding of gross anatomical pattern. Recognition of timing of embryological events is of crucial importance in the analysis of birth defects and various medico legal contexts (Carlson, 2019) [5].
Pineal gland modulates wake, sleep process in higher vertebrates. In some vertebrates it regulates circadian rhythm. In several species pineal hormones influence sexual development, hibernation and seasonal breeding (Pal *et al*., 2013) [18]. Sheep serves as an excellent model for the study of endocrine systems (Nathanielsz, 1976) [16]. Literature pertaining to the histogenesis of pineal glands in sheep is very limited.

Materials and Methods
The pineal glands of sheep from both the prenatal and postnatal age groups were collected from slaughter houses in Andhra Pradesh and Tamil Nadu. The gross and histological studies on these samples were conducted at the Department of Veterinary Anatomy, Veterinary College and Research Institute, Namakkal. The approximate age in prenatal age groups was calculated by obtaining the CRL and substituting in the formula given by Richardson, 1980 (Noakes *et al*., 2009) [17].

$$X = 2.1(Y+17)$$

Where 'X' is the age of fetus in days and 'Y' denotes crown rump length of fetus in centimeters. The crown rump length was measured from the crown to the base of the tail (Rao and Ramayya 2013) [20].
In postnatal age groups, the estimation of age is done basing on the dentition as described by Dyce *et al*. (1996) [8]. With 6 animals in each group, samples were collected from six different age groups.

Prenatal age groups (days of gestation)			Postnatal age groups		
Group I	Group II	Group III	Group IV (Prepubertal)	Group V (Pubertal)	Group VI (Adult)
1-50	51-100	101-150	Birth to 3 months	7-9 months	2 years and above

In prenatal groups from fetuses pineal gland was approached by making an incision on the head region of foetus. The gland was carefully located, collected, rinsed in normal saline. The gross morphological features like colour, shape, location and topography were recorded and were fixed in neutral buffered formalin and Bouin's fluid. In embryos several nicks were given deeply all over and were placed in the fixative.

In postnatal groups the pineal glands were collected from slaughtered animals, rinsed in normal saline, weight, length and width were measured. For histological studies, tissue pieces of 5mm thickness were cut and were placed in neutral buffered formalin and Bouin's fluid. Gross biometrical parameters such as weight, length and width of the glands were measured. The length and width of the glands of different age groups were measured by using the digital vernier caliper. The weight of the glands of different age groups was taken with the help of electronic balance.

For histological study, the tissue pieces were fixed in neutral buffered formalene and Bouin's fluid. The fixed tissues were processed as per the methods described by Luna (1968) [14]. Harri's Haematoxylin and Eosin (H&E) method for the routine histological study (Bancroft and Stevens, 1996) [2].

Staining methods

- For collagen fibres Van Gieson's method (Singh and Sulochana, 1996) [2].
- For reticular fibres Gomori's reticulin method (Bancroft and Stevens, 1996) [2].

For histochemical studies tissue pieces collected were fixed in neutral buffered formaldehyde and Carnoy's fluid. The fixed tissues were processed as per the methods described by Singh and Sulochana (1996) [2]. Paraffin sections of 4-6 µm thickness were cut for the following staining techniques:

- McManus's method for glycogen (PAS), (Singh and Sulochana, 1996) [2].
- Combined Alcian blue-PAS technique for acid and neutral mucins (Bancroft and Stevens, 1996) [2].
- Millon's reaction for tyrosine (Bancroft and Stevens, 1996) [2].

For histochemistry of lipids and enzymes, frozen sections of 10-20 µm thickness were cut using Leica CM 1510 Cryostat Microtome.

The following staining methods were used

- Oil red O method for lipids (routine) (Bancroft and Gamble, 1996) [2].
- Naphthol AS-BI method (substitution naphthol) for alkaline phosphatase activity (Bancroft and Stevens, 1996) [2].
- For acid phosphatase activity Azo dye coupling method (Bancroft and Stevens, 1996) [2].
- Alpha naphthyl acetate method for nonspecific esterase (Bancroft and Stevens, 1996) [2].

The histometrical parameters were recorded with the help of Lieca trinocular microscope (DM 1000) with image analyzer. Various parameters like the width of the capsule, length of the cortex and medulla, diameter of the glands and cells were recorded. The morphometrical and histometrical features of samples of the six groups were recorded irrespective of the sex. These observations were analysed statistically as per the Snedecor and Cochran method (1992) [24] and the mean of different parameters was compared.

Results and Discussion

In all the groups studied, pineal gland was a small white, round shaped structure which was in accordance with observation of de Carvalho *et al.* (2009) [7] in buffaloes. On contrary, Nawal *et al.* (2012) [28], Beheiry and Moselhy (2016) [3] and Soliman *et al.* (2019) [25] described that the pineal gland was pear or cone shaped in humans, reddish grey coloured in camel, pine cone shaped in younger age group while slightly elongated in older camels, light to dark brown coloured, fusiform shaped in rabbits respectively. It was located between the thalami and corpora quadrigemina of the brain (Figure 1). This was in accordance with Kumar *et al.* (2007) [13] in horse, Beheiry and Moselhy (2016) [3] in camel and Sharma *et al.* (2019) [23] in Jaffarbadi buffaloes. The mean weight and diameter of the gland increased with the age (Table 1) on contrary with the findings of Burkitt *et al.* (1993) [4] in humans who observed that the size of pineal gland decreased from childhood to puberty.

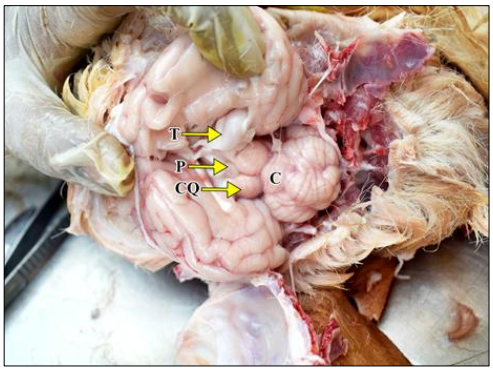

Fig 1: Photograph showing pineal gland in 2 months old sheep P-Pinealgland, CQ-Corpora Quadrigemina, C-Cerebellum, T-Thalamus

Table 1: Mean (± SE) values of gross morphometrical parameters of pineal gland in pre and postnatal age groups of sheep

Group	Mean weight of pineal gland (mg)	Mean diameter of pineal gland (mm)
I	-	-
II	4.93±0.18	2.13±0.03
III	6.43±0.20	2.69±0.02
IV	91.46±0.33	4.63±0.051
V	128.83±2.06	5.09±0.07
VI	175.167±1.94	6.281±0.424

In group I at 44 days of gestation, pineal gland was surrounded by delicate connective tissue capsule consisting predominantly of reticular fibres. Parenchyma of the pineal gland consisted of pinealocytes with centrally placed vesicular nucleus.

In group II at 69 days of gestation, the pineal gland was surrounded by a piamater which formed the capsule. The

capsule consisted of reticular fibres, few collagen fibres, connective tissue cells and capillaries (Figure 2) as reported by Regodon *et al*. (1998) [21] in ovine fetuses between 29 and 69 days of development. The gland stroma was made up of reticular fibres as reported by Regodon and Roncero (2005) [22] in bovine fetuses. The parenchyma had majority of freely distributed pinealocytes and a few oligodendrocytes and microglial cells (Figure 3) which was in agreement with the findings of Regodon and Roncero (2005) [22] who stated that the pinealoblasts and interstitial cells which began to differentiate at 70 days of embryonic development in bovine fetuses. Whereas, Regodon *et al*. (1998) [21] stated that in ovine fetuses between 29 and 69 days of prenatal development the pinealoblasts were the only cells observed and were distributed throughout the parenchyma. It was also in contrast with Redondo *et al*. (1996) [29] in sheep fetuses from 54 to 92 days of gestation, the pineal gland was composed entirely of pinealoblasts. The appearance of pinealocytes and glial cells indicates the progress in development of pineal histoarchiteture. Pinealocytes were polymorphic cells with numerous processes, the cytoplasm was eosinophilic and had large round to oval vesicular nucleus which was similar to the findings of Regodon *et al*. (1998) [21] in ovine fetuses between 29 and 69 days of prenatal development and Regodon *et al*. (1998) [21] in ovine fetuses between 70 and 97 days of prenatal development.

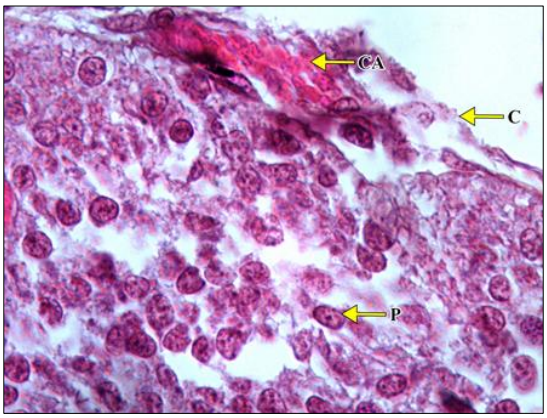

Fig 2: Photomicrograph showing pineal gland of sheep fetus at 69 days of gestation
H&EX1000, C-Capsule, P-Pinealocyte, O-oligodendrocyte, M-Microglial cell

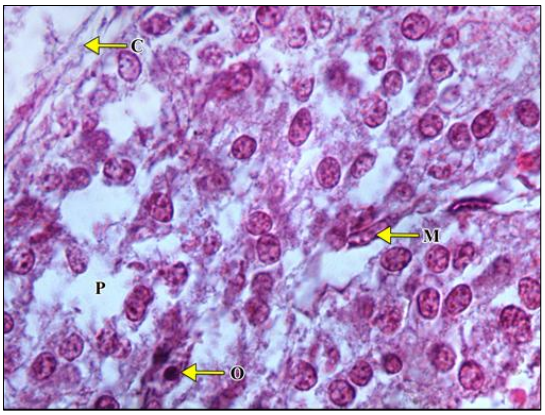

Fig 3: Photomicrograph of pineal gland in sheep fetus at 69 days of gestation
H&EX1000, C- Capsule, P-Pinealocyte, O- oligodendrocyte, M-Microglial cell

At 77 days of gestation, the capsule consisted of connective tissue fibres, cells, capillaries. Gland parenchyma consisted of greater number of pinealocytes compared to 69 days of gestation. Trabeculae entered from the capsule into the gland parenchyma and extended in between the developing clumps of cells (Figure 4) as reported by Regodon *et al*. (1998) [21] in ovine fetuses between 70 and 97 days of prenatal development.

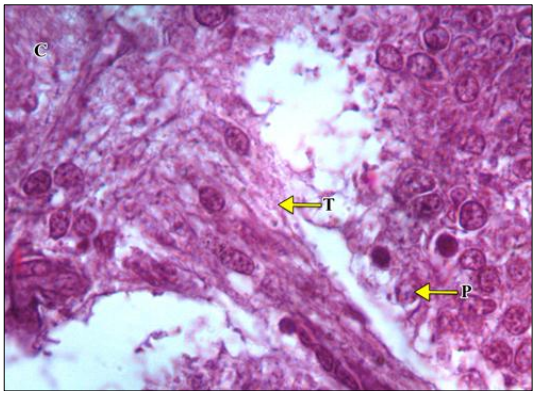

Fig 4: Photomicrograph showing pineal gland of sheep fetus at 77days of gestation
C- Capsule, T-Trabeculae, H&EX1000, P- Pinealocyte

In group III, at 110 days of gestation the parenchyma of the pineal gland showed outer cortex made up of closely arranged pinealocytes and glial cells and inner medulla comprised of freely distributed pinealocytes and glial cells (Figure 5) as observed by Regodon *et al*. (1998) [21] in ovine fetuses between 98 and 116 days and Redondo *et al*. (1996) [29] at 98 days of prenatal development. Connective tissue trabeculae from the capsule extended into the outer cortical region of the gland and divided it into irregular compartments while the medulla was undivided as observed by Regodon *et al*. (1998) [21] in ovine fetuses between 98 and 116 days of prenatal development.

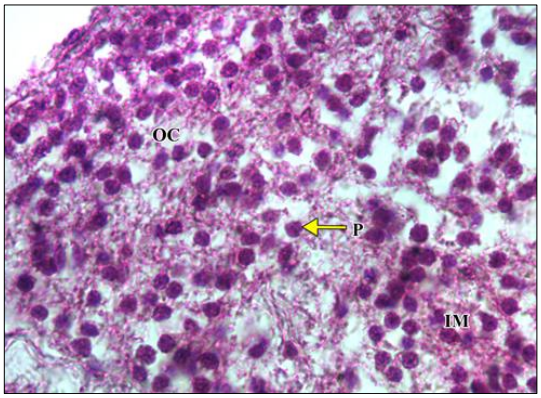

Fig 5: Photomicrograph showing pineal gland of sheep fetus at 110 days of gestation
H&EX1000, OC- Outer Cortex, IM-Inner Medulla, P- Pinealocyte

In group IV, pineal gland in sheep upto three months of age was surrounded by a thin capsule made up of reticular fibres, collagen fibres, connective tissue cells and few capillaries as observed by Kumar *et al*. (2007) [13] in horse, Babu and Ramayya (2014) [1] in adult pig and Beheiry *et al*. (2016) [3] in camel. The gland parenchyma consisted of outer cortex with closely arranged stratified layers of cells and inner medulla with less population of cells which was

similar to the reports of Prabhavathi *et al.* (2010) [19] in sheep, Kumar *et al.* (2007) [13] in horse, Babu and Ramayya (2014) [1] in pig and Soliman *et al.* (2019) [25] in rabbits. On contrary, Cozzi (1986) reported that the pinealocytes were diffused throughout the gland, Burkitt *et al.* (1993) [4] found that in humans, pinealocytes were arranged in clumps and cords, Matsunaga *et al*. (2011) reported that the pineal gland of mice was divided into incomplete lobules of different sizes and Beheiry *et al*. (2016) [3] observed that the pineal gland of camel was divided into indistinct lobules. This might be attributed to species variation.

Cortical pinealocytes were round in shape with round compact nucleus and basophilic cytoplasm. In the medulla, the pinealocytes had pale staining cytoplasm and round vesicular nucleus as observed by Beheiry *et al*. (2016) [3] in camel. On contrary de Carvalho *et al.* (2009) [7] found that the pinealocytes were round in shape with acidophilic cytoplasm and large round nucleus in buffaloes. Babu and Ramayya (2014) [1] described that in adult pig the pinealocytes were oval with dark cytoplasm and euchromatic nucleus. The variation in the cytoplasmic staining of cells implies to its higher activity in younger age compare to adult.

The parenchymal cells were numerous compared to group III. Few capillaries were also noticed in between the pinealocytes. In between the pinealocytes, two different types of glial cells namely microglial cells and oligodendrocytes were observed. Microglial cells were oval in shape with elongated compact nucleus and oligodendrocytes appeared round in shape with centrally placed compact nucleus (Figure 6). On contrary, Burkitt *et al.* (1993) [4] reported that in pineal gland of humans, neuroglial cells similar to astrocytes were seen in between the clumps of pinealocytes and Prabhavathi *et al.* (2010) [19] studied that the pineal gland of sheep had three types of neuroglial cells.

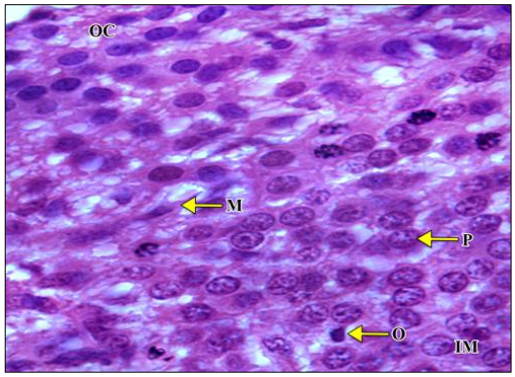

Fig 6: Photomicrograph showing pineal gland of 2 months old sheep
H&EX1000, OC- Outer Cortex, IM-Inner Medulla, P- Pinealocyte, M-Microglial cell

In group V, the pineal gland of sheep between seven and nine months of age, cells of the cortex and medulla were less numerous and loosely distributed compared to group IV. Vacuolated spaces appeared within the pinealocytes. (Figure 7). Average diameter of pinealocytes were increased compared to group IV (Table 2). These observations were in concurrence with the reports of Kassab and Hasan (2005) [11] in pineal gland of dogs between five months and two years of age.

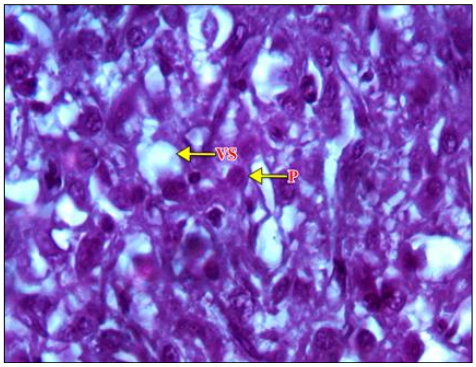

Fig 7: Photomicrograph showing pineal gland of 9 months old sheep
H&EX1000, P- Pinealocyte, VS-Vacuolated Space

In group VI, the pineal gland of sheep above two years of age, number of pinealocytes were reduced while average diameter was increased compared to group V (Table 2). Interstitial space was also increased within the parenchyma of the gland. The cells of the cortex had vesicular nucleus and basophilic cytoplasm whereas the cells of medulla were similar to that of group IV (Figure 8). These observations were in uniformity with the findings of Kassab and Hasan (2005) [11] in pineal gland of dogs between two to four years of age.

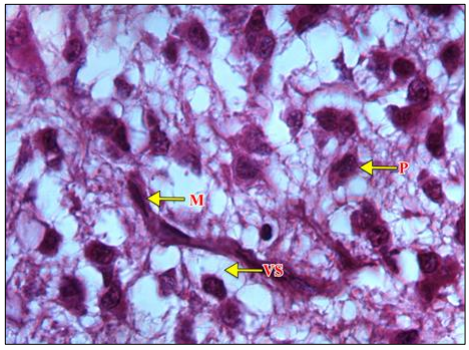

Fig 8: Photomicrograph showing pineal gland of 3 years old sheep
H&EX1000, P- Pinealocyte, M-Microglial cell, VS- Vacuolated Space

Table 2: Mean (± SE) values of histometrical parameters of pineal gland in postnatal age groups of sheep

Group	Mean diameter of pinealocytes (µm)
IV	8.895±0.41
V	11.286±0.79
VI	10.08±0.59

In the pineal gland of sheep, a moderate PAS positive reaction was observed in the pinealocytes of all the groups studied. This was in accordance with Pawar *et al.* (2001) in Indian donkey, Kumar *et al.* (2007) [13] in horse and Prabhavathi *et al.* (2010) [19] in adult sheep. Upon treatment with alcian blue, a positive reaction was not observed indicating the absence of acid mucopolysaccharides. In all the age groups, a weak positive reaction for the presence of tyrosine was observed in pinealocytes. A few lipid deposits were found in the pineal gland of sheep in all the age groups studied whereas, Kappers and Schade (1965) [10] reported that the parenchymal cells of pineal gland in new born rabbit contained large amount of lipid droplets and this content increased up to 3 weeks of age and then after decreased with increase in age.

A weak reaction for the presence of acid phosphatase was noticed in the pinealocytes in the pineal gland of sheep in all the age groups studied (Figure 9). As recorded by Wight and Mackenzie (1971) [27] in pineal gland of domestic fowl. In all the age groups studied, moderate alkaline phosphatase positive reaction was observed in the pinealocytes (Figure 10). Whereas, Kumar *et al.* (1995) [12] reported a non-significant reaction of alkaline phosphatase in goat pineal gland. The capsule covering the gland, pinealocytes and the interstitial tissue showed strong positive reaction for the presence of alpha naphthyl acetate esterase reaction in the pineal gland of all the age groups studied (Figure 11) as observed by Japha *et al.* (1977) [9] in the pineal gland of Mangolian gerbil.

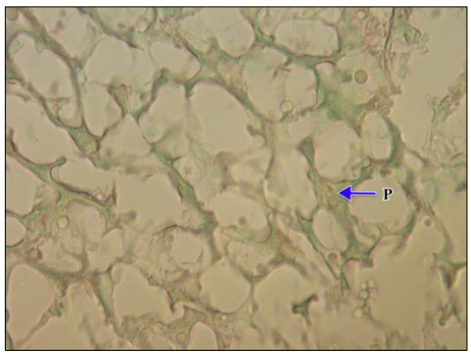

Fig 9: Photomicrograph showing acid phosphatase activity in pineal gland of sheep fetus at 85 days of gestation
Azo dye Coupling Method X 400, P- Pinealocyte

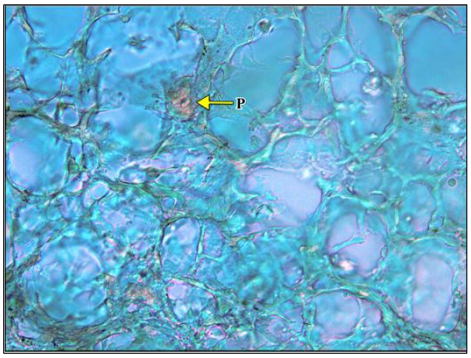

Fig 10: Photomicrograph showing alkaline phosphatase activity in pineal gland of sheep fetus at 110 days of gestation
Naphthol AS-BI method X 400, P-Pinealocyte

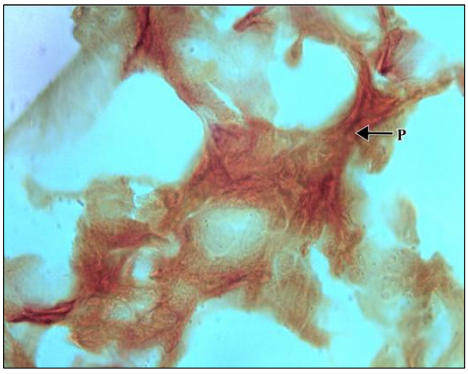

Fig 11: Photomicrograph showing alpha naphthyl acetate esterase activity in pineal gland of sheep fetus at 110 days of gestation
Alpha naphthyl acetate method X 1000, P- Pinealocyte

Conclusion

Pineal gland in sheep was a small white, round shaped structure located between the thalamus and corpora quadrigemina of the brain. In group I it consisted of pinealocytes. In group II, pinealocytes and a few oligodendrocytes and microglial cells were observed. Trabeculae from the capsule entered into the parenchyma and extended in between the developing clumps of cells. In group III, outer cortex and inner medulla regions were observed. In group IV, consisted of outer cortex with closely arranged stratified layers of cells and inner medulla with less population of cells. In group V, cells of the cortex and medulla showed vacuolated spaces and were less numerous. In group VI, number of pinealocytes were reduced. A moderate PAS positive reaction and weak positive reaction for the presence of tyrosine was observed in the pinealocytes. A weak acid phosphatase, moderate alkaline phosphatase activity and strong positive reaction for alpha naphthyl acetate esterase was noticed in the pinealocytes of all the age groups studied.

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