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RESEARCH ARTICLE

Age-Related Gross and Histomorphometrical Dynamics of Pineal Gland (*Epiphysis cereberi*) Associated with Melatonin Profile in Beetal Goat *(Capra aegagrus hircus)* of Pakistan

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A B S T R A C T

Mammalian pineal gland, an integral part of brain, is a photo-neuroendocrine gland which controls the circadian rhythms and reproductive activity in seasonal breeders. This study was designed to evaluate the gross morphological and histological changes in pineal gland and plasma melatonin profile of Beetal goat in relation to different age groups. For this purpose, a total of 24 healthy female Beetal goats were selected from the local abattoir of Faisalabad and were divided into three age groups naming, prepubertal, pubertal and postpubertal (n=8). Before slaughtering, 10 ml blood was collected in EDTA coated tubes for determination of plasma melatonin level using ELISA kit. After slaughtering, heads of the animals were dissected, pineal gland was enucleated and gross morphometric measurements of the gland were taken. For light microscopic studies, the pineal tissues were fixed in a solution of 10% neutral buffered formalin and processed by the routine tissue paraffin sectioning technique. The tissue sections were stained with Hematoxylin and Eosin, modified von Kossa stain and toluidine blue. The results revealed that age of the animals has a significant effect ($P \leq 0.05$) on gross anatomical parameters of pineal gland (weight, length, width and volume) as well as histomorphometric parameters including number and volume of pinealocytes, relative area of parenchyma, brain sand, connective tissue and blood vessels of the pineal gland. Age also affected the plasma melatonin level and was found significantly highest (5.63±0.20 pg/ml) in the pubertal group. In conclusion, the present study revealed that age influences the gross and histomorphology along with plasma melatonin level of the Beetal goat.

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INTRODUCTION

Mammalian pineal gland (*Epiphysis cerebri*) is a major component of photo neuroendocrine system which acts a neuroendocrine transducer and receives the external stimuli from the retina of the eye and circadian rhythms from supra chiasmatic nuclei (SCN) and transforming both of them into hormonal response in the form of melatonin (Li and Zhou, 2015; Busolini and Rosales, 2017). Therefore, in mammals, pineal gland respond to daily and annual photoperiodic changes by altering its physiological state (Kalinina, 2019). Such type of photoperiodic dependent physiological activity plays a vital role in stimulation and inactivation of the reproductive phenomena in seasonally breeding animals (Dardente *et al.*, 2019).

Goat is a seasonally breeding species manifesting two distinct annual reproductive seasons i.e. breeding and non-breeding seasons. In goat, breeding season is characterized by repetition of a regular estrus and ovulatory activity after about every 21-22 days while non-breeding season is characterized by the anestrus and the complete absence of sexual activity. This seasonality is controlled by the pineal gland which plays a crucial role in determining the time of breeding in seasonally breeding species (Gómez-Brunet *et al.*, 2012).

Anatomically, pineal gland is situated in the medial depression formed between the two thalamai anteriorly and colliculi of corpora quardrigemina posteriorly (Bruno *et al.*, 2019). Histological architecture of the pineal gland is composed of lobules and islands of different types of cells such as pinealocytes, perivascular phagocytes, neurons and interstitial cells. Pinealocytes are predominant cells of the

gland and are committed for the production of melatonin (Gheban *et al.*, 2023). Contrary to the other cells of mammalian pineal gland, these cells have larger eukaryotic nuclei having prominent nucleoli (Redondo *et al*., 2010) and synthesize melatonin (Bolat *et al*., 2018). Melatonin is a neuroendocrine hormone synthesized from an amino acid, tryptophan, which is its precursor. In mammalian vertebrates the major source of melatonin is pineal gland (Gorman, 2020).

Various environmental factors as well as age of the animals affect the morphology and histology of the pineal gland (Tan *et al.*, 2018; Kalinina, 2019). With advancing age and age-related some pathological conditions are connected with the involution of the pineal gland. As result of these involutionary changes, the morphological, functional and molecular changes occur in the pineal gland (Khavinson and Linkova, 2012). Therefore, functional status of pineal gland can be determined by the number of pinealocytes and their size as well as the degree of calcification sites in the form of brain sand along with the neuroendocrine hormone, the melatonin.

Thus, the present study was planned to measure the gross anatomical and histomorphometrical dynamics with relation to hormonal changes in pineal gland of prepubertal, pubertal and postpubertal female Beetal goat.

MATERIALS AND METHODS

The whole protocol of this study was got approved from Ethical Committee of the University of Agriculture Faisalabad, Pakistan. A total of 24 healthy female Beetal goats (*Capra aegagrus hircus*) were selected from the local abattoir of Faisalabad and were subdivided into three age groups: prepubertal (<1.5 year), pubertal (<5 year) and postpubertal (>5 year) in such a way that each group consisted of eight animals (Archana *et al.*, 2014). The age of the animals was estimated by following the dentition method as described by Vigal and Machordom, (1985). Before slaughtering, 10 ml blood was collected to determine the plasma melatonin level. After slaughtering, heads of the animals were collected, brought to the gross anatomy lab, opened the skulls to collect the brain and pineal glands were enucleated from the brain.

Gross morphometrical and histological studies: Gross morphometric measurements i.e. weight of pineal gland, length, thickness and volume were done. With the help of Vernier caliper, the length of the gland and its thickness were determined. The weight of the gland was measured by an electric analytical weighing balance. To calculate the volume of the gland, a stereological procedure called Cavalieri's direct estimator method as described by Bolat *et al*. (2018) was adapted.

For light microscopic studies, the tissues of pineal gland were fixed in the solution of 10% neutral buffered formalin and were further processed by the routine tissue paraffin technique (Suvarna *et al.*, 2018). The sections of 5 µm thickness were taken with the help of rotary semi-automatic microtome and staining was done with hematoxylin and eosin for histological studies, toluidine blue for morphometrical studies and Von Kossa for determination of calcium deposits in the form of brain sand. Slides were scanned at 400X with the help of a camera fitted microscope connected to computer and histomorphometry was done by using stereological technique for image analysis, the Image J® software. By using this technique, different histomorphometric parameters such as number and volume of pinealocytes, relative percentage area of parenchyma, connective tissue, blood vessels and brain sand were determined as described by Mironov (2017).

Hormonal analysis: Blood samples, collected at the time of slaughtering in the EDTA coated test tubes, were centrifuged at 500 g for 10 minutes to collect plasma. Hormonal analysis was done by using the commercially available goat melatonin ELISA kit (Cat. # Y-85191-48T, Zokeyo, China).

Statistical analysis: Analysis of variance was done by using the one-way analysis of variance (ANOVA) under factorial design using the descriptive stat software, Statistix 8.1. After the ANOVA, Tukey's Honestly Significant test was applied as the post ANOVA test at $P<0.05$ level of significance (Montgomery, 2019).

RESULTS

Gross anatomy and morphometry: The pineal gland of the Beetal goat (*Capra aegagrus hircus)* was oval to roughly triangular pine cone shaped organ located in the medial depression formed between the two thalamai anteriorly and superior colliculi of corpora quardrigemina posteriorly as shown in the Fig. 1. The color of the gland was pink to white depending upon the age. In prepubertal and pubertal groups, it was pink in color; while in postpubertal animals, it was light pink to white as shown in the Fig. 2.

The mean (±SEM) weights (mg) of pineal gland in prepubertal, pubertal and postpubertal group were 42.62±1.49, 52.00±1.46 and 48.88±2.04 respectively. Significant difference ($P\leq0.05$) was found between prepubertal, pubertal and postpubertal groups as shown in the Table 1. The mean (±SEM) length (mm) of prepubertal, pubertal and postpubertal group were 5.58±0.11, 5.69±0.12 and 5.75±0.09 respectively. The mean (±SEM) length was found highest in postpubertal group, lowest in the prepubertal group but this difference was non-significant ($P\geq 0.05$) among all the groups. Mean (±SEM) thickness (mm) of the pineal gland of prepubertal, pubertal and postpubertal group were 4.70±0.14, 4.90±0.10, and 4.63±0.11 respectively.

In different age groups, the mean (±SEM) volume (mm^3) of pineal gland in Beetal goat is given in the Table 1. The mean (±SEM) volume (mm^3) of prepubertal, pubertal and postpubertal group were 26.46±1.40, 48.54±1.73 and 62.01±1.27 respectively. The mean (±SEM) volume (mm^3) of pubertal group was significantly ($P\leq0.05$) greater than that of prepubertal group but significantly lower than that of postpubertal group.

Histology and histo-morphometry: Histologically pineal gland was covered by the thin tunic of fibrous connective capsule which penetrates into the parenchyma of the gland and divides it partially into the lobules. Parenchyma of the gland consisted of various types of cells such as the

Table 1: Gross anatomical and histomorphometric values (Mean±SEM) of pineal gland along with plasma melatonin level in different age groups of Beetal goat

Age Group/ Parameter	Prepubertal	Pubertal	Postpubertal
Weight (mg)	42.62±1.49^{c}	52.00±1.46^{a}	48.88±2.04^{b}
Length (mm)	5.58±0.11^{a}	5.69±0.12^{a}	5.75±0.09^{a}
Thickness (mm)	4.70±0.14^{ab}	4.90±0.10^{a}	4.63±0.11^{b}
Volume (mm^3)	26.46±1.40^{c}	48.54±1.73^{b}	62.01±1.27^{a}
Number of pinealocytes ($\times 10^6$)	4.88±0.26^{b}	7.82±0.28^{a}	7.28±0.32^{a}
Volume of pinealocytes ($10^3/\mu m^3$)	5.3±0.21^{b}	5.57±0.16^{a}	7.68±0.16^{a}
Melatonin (pg/ml)	1.74±0.16^{c}	5.63±0.20^{a}	5.26±0.15^{b}

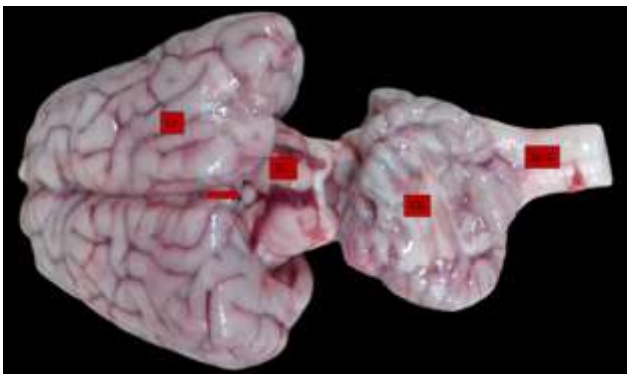

Fig. 1: Brain and the anatomical location of the pineal gland of Beetal goat. Pineal gland (arrow), (Superior colliculus (SC), (Cerebral hemisphere (Cr), cerebellum (Cb) and spinal cord (Sp.c).

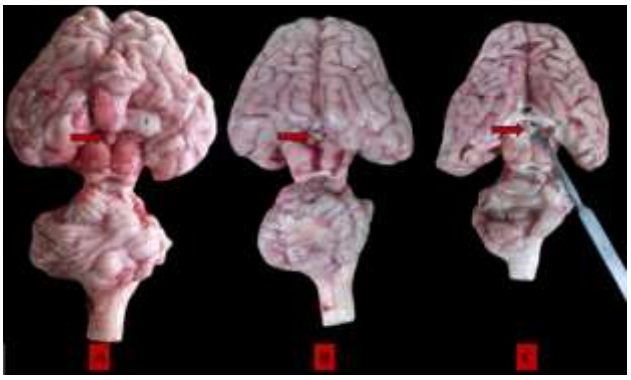

Fig. 2: Brain and pineal gland of the three different age groups female Beetal goat. Arrows showing the pineal gland, (A) Prepubertal group, (B) pubertal group and (C) postpubertal group.

pinealocytes and supportive glial cells which are also called astrocytes. The former was abundant while the latter were scarce in number as shown in Fig.3. The brain sand, also called as carpora arenaecea, is the deposits of the calcium in the blood vessels which was also found scattered among the parenchymal cells of the gland as shown in Fig. 4.

The mean parenchymal percentages in different age groups were found as 88.39±1.74, 80.82±2.25 and 71.03±1.64 in prepubertal, pubertal and postpubertal groups, respectively as shown in Fig. 7. The parenchymal cell percentage was significantly (P≤0.05) different among the age groups which was highest in prepubertal group and lowest in the postpubertal group. The Mean values for area of connective tissue (%) in pineal gland of prepubertal, pubertal and postpubertal group were 9.29±1.27, 17.53±1.27 and 28.179±1.27 respectively and are shown in the Fig. 7. The mean percentage of connective tissue (%) of pubertal group was significantly (P≤0.05) higher than that of prepubertal group and significantly (P≤0.05) lower than that of postpubertal group.

The blood vessels of the pineal gland included the small arterioles and capillaries which were predominantly located near the perivascular and trabecular region. The mean values of percentage area of blood vessels (%) in the gland of prepubertal, pubertal and postpubertal group were 2.62±0.47, 1.81±0.45 and 1.38±0.29 respectively as shown in the Fig. 7. Among the three age groups, percentage area of blood vessels (%) was found highest in prepubertal group and lowest in the postpubertal group. It was non-significantly (P≥0.05) higher than those of pubertal group and postpubertal group.

The pinealocytes in the sections were counted by the image analysis software Image J®. The mean (±SEM) × 10^6 number of pinealocytes in prepubertal, pubertal and postpubertal group were 4.88±0.26, 7.82±0.28 and 7.28±0.32 respectively as shown in Table 1. The mean number of pinealocytes of pubertal group were significantly (P≤0.05) higher than that of prepubertal and postpubertal group.

To determine the percentage area of brain sand in the pineal gland, the tissue sections were stained with a special type of stain called as modified von Kossa stain. Brain sand was predominantly found in the peri-vascular region of the gland. The mean percentage area of brain sand in the gland of prepubertal, pubertal and postpubertal group were 6.56±0.69, 9.51±0.72 and 11.68±0.51 respectively. The mean (±SEM) percentage of brain sand area (%) in postpubertal group was significantly higher (P≤0.05) than those of pubertal and prepubertal groups.

The mean volume (μm^3) of the pinealocytes in prepubertal, pubertal and postpubertal groups were 5.38±0.21, 5.57±0.16 and 7.68±0.16 respectively and are given in the Table 1.

Neuroendocrine hormone: The mean plasma melatonin level (pg/ml) of female Beetal goat in different age groups is given in the Table 1. The level of melatonin in pubertal group was significantly (P≤0.05) higher than that of prepubertal as well as that of postpubertal group but it was non-significantly (P≥ 0.05) higher in latter group.

DISCUSSION

This study provides first discernment about gross anatomical, histological and hormonal changes in pineal gland of Beetal goat in relation to the different age groups. It is an important neuroendocrine gland which plays a vital role in regulation of the circadian rhythms and seasonal productivity in different domestic as well as wild animals. In goat pineal gland was oval to roughly triangular pine cone shaped organ located in the medial depression formed between the two thalami anteriorly and superior colliculi of corpora quardrigemina posteriorly. In previous studies as reported by Busolini *et al*. (2017) in male viscacha the shape of the gland was oval with an elongated stalk attached to the brain. In contrast to our study, the pineal gland in buffalo is pea-shaped in young and somewhat elongated in old animals (Abou-easa *et al*., 2009), however, the anatomical location of pineal gland in buffalo was similar. The shape of the gland in camel as reported by Beheiry and Moselhy (2016) was fusiform. In prepubertal and pubertal groups, it was oval to cone shaped but in postpubertal animals it became elongated and triangular in shape.

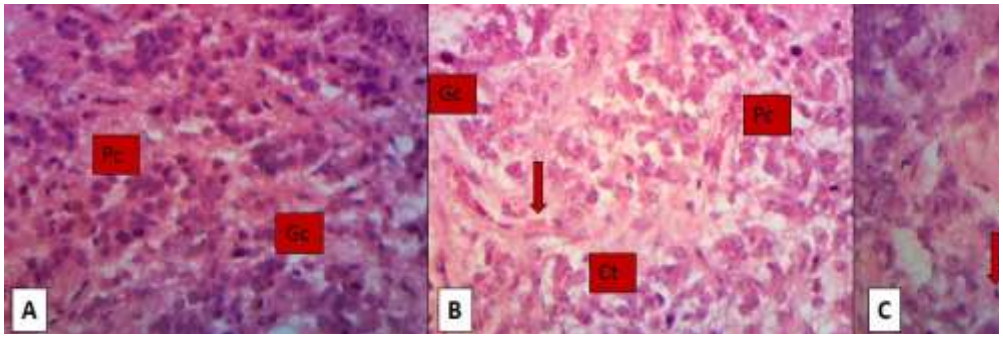

Fig. 3: Histomicrographs A, B and C (Hematoxylin and eosin) showing the light microscopic structure of pineal gland at 400x. A: Pinealocytes (Pc), Glial cells (Gc). B: Pinealocytes (Pc), Glial cells (Gc), Connective Tissue (Ct), C: Blood vessel (Bv), Connective tissue (Ct), Glial cell (Arrow).

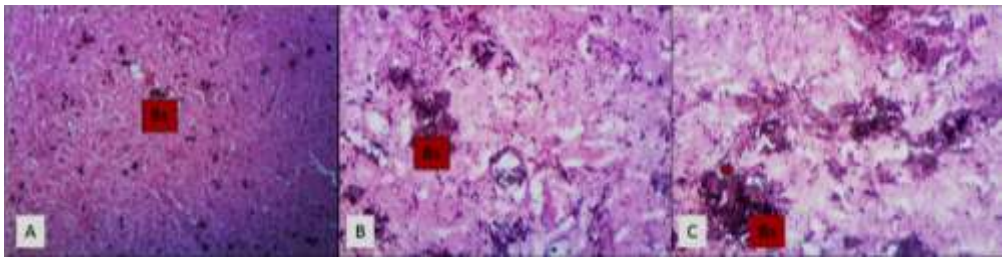

Fig. 4: Histomicrographs A, B and C (von Kossa stain) showing the calcification of the pineal gland in the form of brain sand under light microscope at 400x. (A) Prepubertal group), (B) Pubertal group, (C) Postpubertal group. Brain sand (Bs), blood vessel (Esteric).

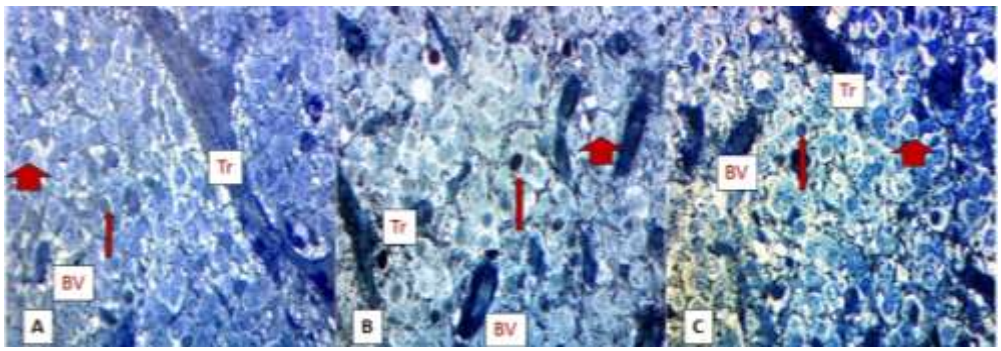

Fig. 5: Histomicrographs A, B and C (Toluidine blue) showing the light microscopic structure of pineal gland of goat at 400x. (A) Prepubertal group), (B) Pubertal group, (C) Postpubertal group. Blood vessels (BV), Trabeculae (Tr), Pinealocute (arrow head) and Glial cell (arrow).

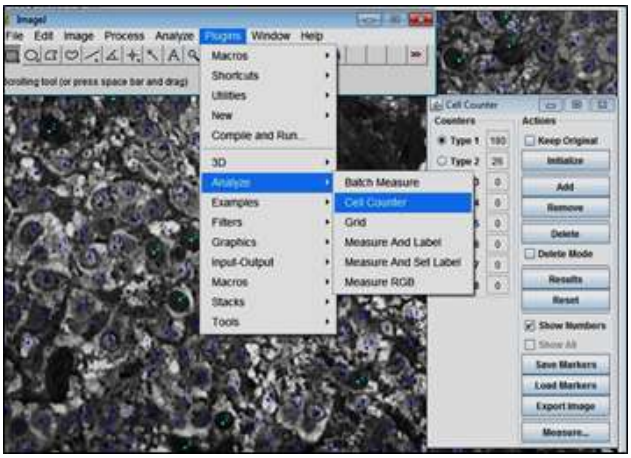

Fig. 6: The Screenshot of ImageJ® (drop down menu) showing the pineal tissue section stained with Toluidine Blue at 400 x magnification. Cell count by ImageJ® Type I cells (1) and Type 2 cells (2).

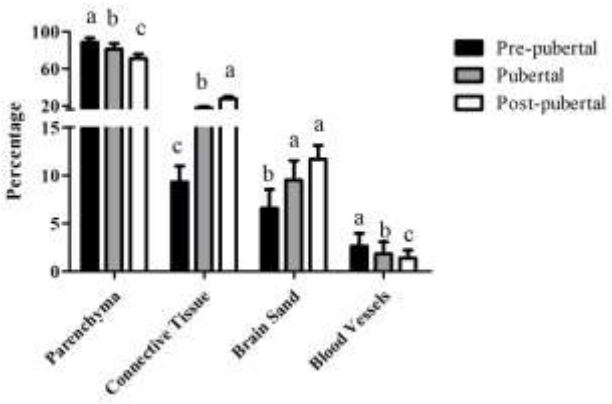

Fig. 7: Bar graph showing the relative percentage areas of parenchyma, connective tissue, brain sand and blood vessels in the pineal gland of different age groups of female Beetal goat.

The similar shape of the gland was reported by Kumar and Meshram (2020) and Gheban *et al*. (2019) in guinea fowl. The color of the gland in goat was found pink to white in different age groups. In prepubertal and pubertal groups, it was pink in color while in postpubertal animals it was light pink to white. The color of the pineal gland in camel was reported as dark brown by Beheiry and Moselhy (2016) while Gheban *et al*. (2019) reported the color of pineal gland in human as red-gray. These differences in the

color of pineal gland in three age groups might be due to the relative percentage area of blood vessels and blood supply to the gland. The pink color in pre-pubertal and pubertal groups might be due to more number of blood vessels as compared to those of post-pubertal groups.

In present study, gross morphometric study of pineal gland showed that mean±(SEM) weight (mg) of prepubertal, pubertal and postpubertal groups were 42.62±1.49, 52.00±1.46 and 48.88±2.04 respectively. The values of weight of pineal gland, found by Abou-easa *et al*. (2009) in buffalo, were 110, 337.5 and 160 mg in young, adult and old animals, respectively. In the same study, he also reported the values of weight of pineal gland in camel as 90, 300 and 130 mg in young, adult and old animals respectively. These values were higher in buffalo and camel. These differences might be due to higher body weights of buffalo and camel.

The mean (±SEM) length of the pineal gland in present study was found as 5.54 to 5.85 mm. Golan *et al*. (2002) measured the length of the pineal gland in human and reported it as 5 to 9 mm. The length of gland was reported as 2.5 mm in cat and 11.16 mm in penguin (Boya *et al*., 1995; Barcelos *et al*., 2015). In birds its length is much more due to its peculiar shape. Thickness (mm) of the gland in the present study was found as 4.70±0.14, 4.90±0.10, and 4.63±0.11 in prepubertal, pubertal and postpubertal groups. These values were in consistent with the values of thickness of pineal gland in donkey as reported by Ebada (2012). The values of thickness of pineal gland, as they reported, were 5 mm. The mean volume of pineal gland in goat, as recorded in present study, ranged between 25 to 62 mm^3. The volume of the pineal gland in goat was significantly higher than that of sheep reported by Bolat *et al*. (2018) in which he reported as ranging from 7.53 to 17.75 mm^3. The volume of pineal gland in horses and human was 70 mm^3 (Ebada, 2012) and 124 mm^3 (Golan *et al*., 2002) respectively.

Histologically pineal gland was covered by the capsule of fibrous connective, the part of piamatar, whose branches penetrate into the parenchyma of the gland and partially divide it into the lobes. Parenchyma of the gland comprised of two types of cells, the pinealocytes and glial cells also called as astrocytes. Pinealocytes constituted the major part of the parenchyma of the gland while glial cells were found predominantly at the periphery of the gland. Similar histological features were reported in donkey by Ebada (2012) and in camel by Beheiry and Moselhy (2016).

The brain sand or carpora arenaecea were the deposits of the calcium predominantly located in lumen of the blood vessels and were also scattered among cells of the parenchyma of the gland. The mean (±SEM) values of percentage-area (%) of brain sand in prepubertal, pubertal and postpubertal groups were 6.56±0.69, 9.51±0.72 and 11.68±0.51 respectively. Values of brain sand area in this study are not in agreement with those reported by Khavinson and Linkova, (2012) in sheep in which they reported that brain sand areas are not directly related to the age of the animals in sheep.

In present study, among the three age groups, mean (±SEM) number of pinealocytes were found highest in pubertal and postpubertal groups, and lowest in the prepubertal group. Our results are not in agreement with those found by Bolat *et al*. (2018) in pineal gland of different age groups of sheep in which the number of pinealocytes were highest in postpubertal age group and lowest in prepubertal group. As he reported it might be due to increase in volume in postpubertal group of sheep but in case of goat the number of pinealocytes remained same even after the slight increase in volume of the gland in postpubertal group. Relative percentage (%) area of parenchyma of the pineal gland was found highest in prepubertal group and lowest in the postpubertal group (Fig. 4 and 7). The parenchymal percentage area was highest in the prepubertal group and lowest in the postpubertal group. The findings of the current study are in accordance with the results of Bolat *et al*. (2018) in sheep but the relative percentage values of parenchyma in goat were relatively less.

The relative percentage of connective tissue (%) in present study was found maximum in postpubertal aged group of animals (Fig. 5 and 7). It was significantly ($P \leq 0.05$) higher than that of prepubertal group and pubertal group. These findings were in agreement with those reported by Bolat *et al.* (2018) in sheep where amount of connective tissue, in pubertal group, more as compared to the younger animal. The postpubertal group had significantly more amounts of connective tissue as compared with the prepubertal and pubertal animals. In addition to this, another study reported that amount of connective tissue increased with the aging of the animals like rats and rabbits (Redondo *et al.*, 2010; Kalinina, 2019).

The percentage area of blood vessels (%) was found maximum in prepubertal group and minimum in the postpubertal group. Similar findings were reported by Bolat *et al*. (2018) in different age groups of sheep. Redondo *et al.* (2010) also reported that number of blood vessels in the pineal gland are directly related with the age of the sheep. The degree of blood vascularization, in pubertal group, was much more and was prominent in the center of the pineal gland. In some other species, abundant number of sinusoids were reported in some cases as well (Beheiry and Moselhy, 2016). The variable percentage of blood vessels in the gland might be due the physiological need of the gland.

In goat, the mean values (±SEM) of plasma melatonin level (pg/ml) in prepubertal, pubertal and postpubertal groups were 1.74±0.16, 5.63±0.20 and 5.26±0.35 (pg/ml) respectively. These values were significantly higher ($P \leq 0.05$) in pubertal group as compared to those of prepubertal group and were non-significantly higher ($P \geq 0.05$) those of postpubertal group. The values of melatonin reported by Zarazaga *et al*. (2010) during day and night as 5.2 pg/ml and 55.8 pg/ml respectively. The nocturnal values of melatonin were much higher than the values found in the present study in Beetal goat. These differences of melatonin concentrations can be related to various factors such as age, species of animal and photoperiodicity (Redondo *et al.*, 2010).

Conclusions: From the present study, it may be concluded that age of the animals affects the gross and histo-morphometry of the pineal gland in Beetal goat and these changes related with the advancing age ultimately affects the physiology of the body especially the reproductive physiology in seasonally breeding species.

Authors contribution: This manuscript is from PhD thesis of MH. The idea and design of the study was given by RK and ASQ. The research work was done by MH under the supervision of RK. All the authors contributed in data analysis, drafting and approval of this manuscript.

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