



## RESEARCH ARTICLE

### Gross Morphology and Localization of Adenohypophyseal Cells in Camel (*Camelus dromedarius*) Using A New Combination of Stains

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#### ABSTRACT

Thirty normal camels (*Camelus dromedarius*) were selected for gross morphological and modified staining of anterior pituitary. Camels were divided in three age groups viz 2-4, 5-10 and above 10 years. Pituitary weight, length, width and circumference were recorded before preservation and at midseittal cutting. Pituitary weight increased significantly as these animals grew older. Male had heavier pituitary as compared to female. Higher pituitary weight was observed in old as compared to young camel. Sections (4 $\mu$ m) of camel pituitary gland were stained with "Phosphotungstic acid haematoxylin-Orange G-Acid fuchsin-Light green" combination of dyes. This combination of acidic and basic dyes showed affinity to their respective adenohypophyseal cells and proved a suitable combination for differentiation of adenohypophyseal cells and architectural pattern of pituitary gland. Use of Lugol's Iodine and sodium thiosulphate solution caused mercury fixation which ultimately enhanced the staining of camel adenohypophysis. The whole pituitary presented a brilliant appearance of clarity, enabling cell counts to be performed easily, purely with reference to the colors of adenohypophyseal cell types. This method can be applied for differential staining of adenohypophysis and with good cytology results to the hypophysis of many mammals. The method also provides a sharp contrast between cellular and connective tissue components. With this staining technique, the quantitative and qualitative characteristics of different adenohypophyseal cell types at various functional and hormonal stages, under certain physiological and pathological conditions can also be studied.

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#### INTRODUCTION

The adenohypophysis is comprised of nests and cords within interlocking network of small capillaries. Adenohypophyseal cells produce growth hormone (GH), prolactin (PRL), adrenocorticotrophic hormone (ACTH), follicle stimulating hormone (FSH), luteinizing hormone (LH) and thyroid stimulating hormone (TSH). The secretion of these hormones is regulated by "releasing" and "inhibiting" factors produced in the hypothalamus and transported to the adenohypophysis through the hypophyseal portal vein. A negative feed back controls the secretion of the releasing or inhibiting factors (DeLellis, 1989).

The adenohypophyseal cell types (acidophils, basophils and chromophobes) can be identified by using acidic and basic stains for thin sections, due to the

affinities of secretory granules for acidic and basic dyes (Ross *et al.*, 1989; Pernicone *et al.*, 1997). Some special histochemical stains give improved cellular differentiation (McKeever and Spicer, 1987; Pernicone *et al.*, 1997). Acidophils can be divided into GH producing cell and PRL producing cells (Kovacs *et al.*, 1981) using selective staining technique. Cells secreting GH and PRL stain with orange or light green, whereas basophils (Gonadotrophos and Adrenocorticotrophic cells) stains with basic dyes and some cells which stain poorly are termed as chromophobes. Acid fuchsin and Light Green belong to the same chromophoric group i.e. the chromophore of each of the two dyes is compatible with each other. Staining of the tissue with Acid fuchsin and Light green produces polychromic effect and various tissue elements become red, mauve, violet and blue (MacConaill *et al.*, 1960).

Considering the lack of details in the literature and research references on histology of camel pituitary, the present work was conducted to undertake the detailed light microscopic studies of adenohypophysis with differential staining procedures.

## MATERIALS AND METHODS

Thirty normal male and female camels were divided according to their age from 2-4 years (group I), 5-10 years (group II) and above 11 years (group III) slaughtered at Kot Kamboh slaughterhouse Lahore and Zainth Associates (Pvt.) Abattoir Lahore, Pakistan. Their pituitary glands were extirpated using the technique described by Gilmore *et al.* (1941) within three to four hours after slaughtering to avoid the diffusion and loss of hormones. The age of animals were determined by teeth examination (Schwartz and Doili, 1992) and from the history provided by the camel owners. Pituitary weight was taken using an electric balance while using Vernier Caliper, length and width were also taken to calculate the circumference of pituitary gland.

Pituitaries were fixed in Bouin's Holland solution to avoid decomposition of cortex and to maintain cellular distinction for 24 hours (Romeis, 1948), then washed in running water (8 hours), fixed in 50% ethanol followed by 70% ethanol (Humason, 1972) and onward processing to prepare paraffin blocks (Drury and Wallington, 1980).

Tissue was processed while passing through the series of ethanol (70%, 90%, 2X absolute), cleared in xylene (two changes), impregnated in paraffin wax at 60°C (two changes each for one hour duration). Tissue were embedded in mould with freshly melted paraffin wax (Culling, 1974). Keeping the cut surface down, wax was solidified using Tissue Tek (SKURF, Japan).

### Reagents preparation

The actual preparation of each solution and/or dyes is given below for staining of adenohypophysis of camel:

**Lugol's iodine:** In 5ml of distilled water 2g of potassium iodide was dissolved and then mixed with 1g of iodine crystal until dissolved completely. Final volume was adjusted to 100ml with distilled water.

**Bouin's Holland solution:** Aqueous picric acid (75ml) was dissolved in 25ml of commercial (38% V/V) formalin. Just before use, 5ml (1% V/V) of glacial acetic acid was added in 100 ml of stock solution.

**Coating of slides with Poly-L-Lysine:** Poly-L-Lysine (10 mg) was dissolved in 10ml of distilled water. Already cleaned slides were immersed in this solution for 30min and then dried in air for placement of tissue on these slides.

**Phosphotungstic acid haematoxylin:** Separately, 0.5g of haematoxylin powder was dissolved in distilled water and 5g of phosphotungstic acid was dissolved in 250ml of distilled water. Both solutions were mixed and heated until color was stable. After cooling at room temperature, 0.177g of potassium permanganate was added, stored in tight stopper bottle and used after 24hrs.

**Orange G solution:** Orange G (0.5mg) was dissolved in 95ml of absolute alcohol and 2g of phosphotungstic acid was dissolved in 5ml distilled water separately. Both solutions were mixed after preparation.

**Acid fuchsin solution:** Acid fuchsin (0.5g) was dissolved in 99.5ml of absolute ethanol and then mixed 0.5ml glacial acetic acid.

**Light green solution:** Light green (1.5mg) was dissolved in 98ml of distilled water and then 2ml glacial acetic acid was added into it.

**Phosphotungstic acid solution:** A one percent solution was prepared by measuring 1g of phosphotungstic acid and mixing it in 100ml of distilled water.

### Section preparation and staining

For more informative study of adenohypophysis, blocks were clamped in horizontal position (Drury and Wallington, 1980) to avoid the folding of section. Tissue was cut in 4µm thickness using rotary microtome. Properly flattened and wrinkle-free sections were picked up on clean Poly-L-Lysine pre-coated slides (Huang *et al.*, 1983).

After dewaxing and dehydration with ethanol, Lugol's iodine was used to remove the mercury pigment. The nuclei were stained with phosphotungstic acid haematoxylin for 10min, and slides were washed in water and for differentiation of the nuclei slides were treated with 1% hydrochloric acid in 70% ethanol. Slides were rinsed in 95% ethanol followed by one dip in absolute ethanol. Sections were stained with Orange G solution for 5min and rinsed in distilled water. Slides were dipped gradually in Acid fuchsin stain till the basophils became prominent and then rinsed in distilled water. Slides were treated with phosphotungstic acid for 5min and then rinsed in distilled water. After staining with light green for 2min, slides were rinsed in distilled water. Finally, stained slides were dehydrated in absolute ethanol for 2min, cleared in xylene for 2min and mounted with distyrene plasticiser xylene (DPX).

### Statistical analysis

The data on pituitary weights were analyzed statistically by analysis of variance (ANOVA) to obtain the difference between age and gender (Steel *et al.*, 1997). In case of significant difference, Duncan Multiple Range test (Duncan, 1955) was applied.

## RESULTS

Pituitary was found to be pear shaped, located at the base of the brain, lodged in bony cavity of sphenoid bone and attached with the hypothalamas by a stalk. Overall, a dense irregular connective tissue was observed surrounding the pituitary gland of camel. Capsule was thickest at the region of adenohypophysis.

Pituitary weight increased significantly both in male and female camel, as they grew older. The highest pituitary weight was observed in male camel at the age of above 11 years and lowest in young female at the age of 2-4 years. Overall, significantly higher pituitary gland weight was observed in male ( $P \leq 0.01$ ) as compared to female irrespective to their age group. Moreover, overall highest pituitary gland weight was observed in above 11 years old and lowest in 2-4 years old animals irrespective of their gender (Table 1). With age, a non significant increase in the length, width and circumference of pituitary was also observed (Table 2) and may be indicative of either proliferation and/or hyperplasia of certain types of cells.

**Table 1:** Mean weight (g $\pm$ SE) of pituitary gland of male and female camel (*Camelus dromedarius*) of different ages.

Sex	Age (Years)			Overall Mean
	2-4	5-10	Above 11	
Male	1.22 $\pm$ 0.10 <sup>c</sup>	1.65 $\pm$ 0.04 <sup>b</sup>	1.93 $\pm$ 0.05 <sup>a</sup>	1.60 $\pm$ 0.09 <sup>A</sup>
Female	0.72 $\pm$ 0.06 <sup>d</sup>	1.62 $\pm$ 0.17 <sup>b</sup>	1.86 $\pm$ 0.02 <sup>ab</sup>	1.40 $\pm$ 0.14 <sup>B</sup>
Overall Mean	0.97 $\pm$ 0.10 <sup>c</sup>	1.63 $\pm$ 0.08 <sup>b</sup>	1.90 $\pm$ 0.03 <sup>a</sup>	1.50 $\pm$ 0.08

a-d, Mean values with different alphabets differ significantly at P $\leq$ 0.05; ABC: Overall mean values with different alphabets differ significantly at P $\leq$ 0.01.

**Table 2:** Mean pituitary length, width and circumference ( $\mu\text{m}\pm\text{SE}$ ) of male and female camel (*Camelus dromedarius*) at different ages.

Age (Years)	Length		Width		Circumference	
	Male	Female	Male	Female	Male	Female
2-4	1.14 $\pm$ 0.03	1.35 $\pm$ 0.10	1.06 $\pm$ 0.10	1.04 $\pm$ 0.09	4.50 $\pm$ 0.16	4.08 $\pm$ 0.15
5-10	1.73 $\pm$ 0.06	1.76 $\pm$ 0.09	1.52 $\pm$ 0.10	1.50 $\pm$ 0.08	4.82 $\pm$ 0.18	4.94 $\pm$ 0.26
Above 11	1.79 $\pm$ 0.06	1.80 $\pm$ 0.06	1.58 $\pm$ 0.04	1.68 $\pm$ 0.04	5.14 $\pm$ 0.08	5.22 $\pm$ 0.10
Overall mean	1.55 $\pm$ 0.08	1.63 $\pm$ 0.07	1.39 $\pm$ 0.08	1.41 $\pm$ 0.08	4.82 $\pm$ 0.10	4.72 $\pm$ 0.17

The difference in length, width and circumference among various groups were statistically non significant.

The grossmorphological study showed that pituitary gland of camel (*Camelus dromedarius*) were subdivided into adenohypophysis and neurohypophysis (Fig. 1). The adenohypophysis consisted of pars distalis, pars tuberalis and pars intermedia. Pars distalis was largest part, composed of glandular cells arranged in nests and cords. Pars intermedia was partly separated from the adenohypophysis by a remnant of the hypophyseal cleft. A profused development of pars intermedia completely surrounded the infundibular process with a very thin layer of lobulation. Pars distalis formed a collar around the infundibular stem, a small part of hypophysis. It was separated from the stalk by a thin layer of connective tissue, continuous with the pars intermedia. Neurohypophysis was composed of the pars neurosia (infundibular process), the infundibulum (infundibular stalk) and median eminence of the cinereum. Both the lobes (anterior and posterior) and adenohypophyseal cleft could be easily seen and were also distinguished by mid sagittal cutting of the pituitary.

The method provided a sharp contrast between cellular and connective tissue components. Camel adenohypophysis was found to be populated with acidophils (orange-yellow/yellow), basophils (magenta red/reddish-purple) chromophobes (unstained/ light yellow) and connective tissue (green) of young (2-4 years old), adult (5-10 years old) and old (above 11 years old) camels (Fig. 2).

About  $\frac{3}{4}$  of the gland comprised of adenohypophysis (anterior pituitary). The parenchyma of this lobe was composed of anastomosing cords of the cells separated from sinusoidal capillaries by only a meager amount of irregularly arranged connective tissue. Small masses of colloid occurred within cell cords only occasionally. Some cells showed strong affinity for acidic dyes which were more numerous in central and posterior half of the pars distalis and are described as orangeophils (Fig. 2). These cells can be described as acidophils. These cells stained readily and were easy to identify. These cells were larger than chromophobes. Acid fuchsin stained the basophils (round/ polyhedral or angular in shape) which were present peripherally and interlay in the pars distalis. Chromophobes appeared near the centre of cords. Their nuclei were surrounded by a small amount of diffused and lightly stained cytoplasm. Cell boundaries were not distinguishable.

## DISCUSSION

Increase in pituitary weight of camels with age may be due to the normal physiological phenomenon as body mass increases. It may also be due to increased number of cells for proper functioning of the body as it is evident in the present study by the different types as well as number of cells present (Fig. 2). Namboothripad and Luktuke (1978) also reported higher mean weights of pituitary glands collected from adult animals than those of heifers. Similar pattern was observed by Ganguli and Yadava (1975) in Indian buffaloes.

Pituitary glands were bisected midsegitally which enabled us to distinguish its anterior and posterior lobes and to see the adenohypophyseal cleft. Longitudinal bisections of the pituitary gland could put forth such a differentiation of the adenohypophysis. Therefore, our work suggests the midsagittal cutting of big sized pituitary gland from camel.

In the present study, Bouin's fluid proved a valuable fixative for camel pituitary gland as its penetration power is great. The pituitary gland was left in Bouin's fluid for 24 hours (Romeis, 1948). Washing of tissue (8 hours) in running water could not extract the whole yellow picric acid. We had to transfer the tissue in 70% ethanol until it removed picric acid from the tissue. Mercuric chloride is a powerful protein precipitant and penetrates hard tissue fairly and quickly. According to Baker (1958) some mercuric chloride-protein complexes are soluble in potassium iodide (an integrated of Lugol's iodine). Lugol's iodine was used to convert mercury by iodine into mercuric iodide which is soluble in alcohol. This treatment itself produced brown coloration of the section.

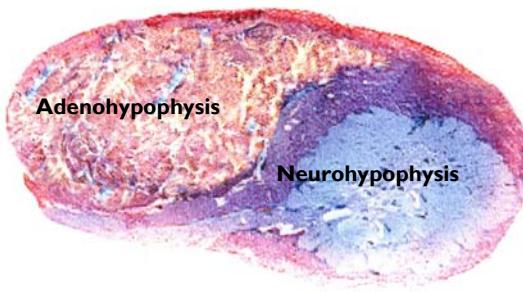
After Lugol's Iodine treatment, the slides were transferred to sodium thiosulphate solution until the sections regained their natural colors. Most of the sections remained yellow/pale which affected the brightness of colors of adenohypophyseal cells. To overcome this difficulty we treated the sections with 70% alcohol, followed by 2.5% sodium thiosulphate before immersing in basic dyes. It enabled us to avoid the precipitation of tissue.

Acid fuchsin and light green belong to the same chromophobic group and chromophore of each of the two dyes is compatible with each other. Light green united chemically with acid fuchsin to produce a blue dye,

triflagic acid. This blue dye caused partial ionization on its application to the tissue and as a result a proportion of triflagic acid splitted into monoflagic and diflagic acids (MacConaill *et al.*, 1960). These three acids were taken up by various types of tissue element to give a polychrome picture. Phosphotungstic acid solution removed the overstraining of acid fuchsin. Protective phosphotungstic acid bath restarted the decolorizing effect of light green solution.

Chromophobes were actually acidophils or basophils that have become degranulated following a secretary cycle. These cells lacked the staining affinity and were distinguished from chromophilic cells. Their boundaries were indistinct until their nuclei were washed in phosphotungstic acid. Similar observations were made by Chakravarthy and Mariappa (1975) in the adenohypophysis of buffalo.

Time required for staining of basophils to a prominent level created a problem. Our findings indicated that 4-6 minutes treatment of section with acid fuchsin was the stipulated duration. However, after 4 minutes it is necessary to examine under the microscope while preparation is wet. In case of poor staining of basophils, the prepared slides were returned to Acid fuchsin for 1-2 minutes extra to get desired results. Moreover, the treatment of 1% aqueous phosphotungstic acid bleached the over staining of Acid fuchsin.



**Fig. 1:** Gross morphology of camel pituitary (cranial section) after trichrome staining showing adenohypophysis and neurohypophysis

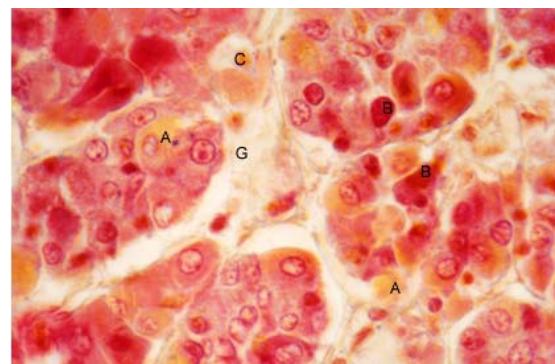
To overcome the folding of sections the blocks were clamped in horizontal position (Drury and Wallington, 1980) for more informative study. Distyrene plasticiser xylene (DPX), a colorless and neutral excellent synthetic mountant (Kirkpatrick and Lendum, 1939, 1941) was used to avoid the fading of stains and for good preservation of colors.

In conclusion, pituitary glands were successfully transected from clinically normal camel, preserved properly and were stained for the differentiation of basophils, acidophils and chromophobes based on trichrome staining procedure.

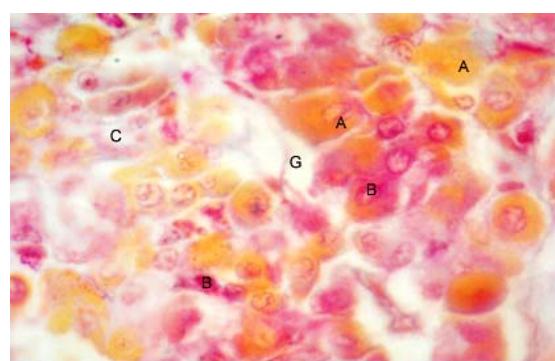
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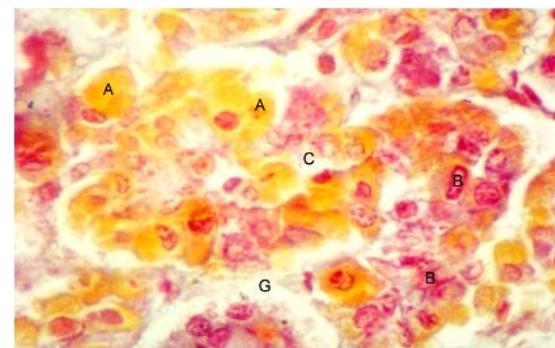
#### Group I (2-4 years)



#### Group II (5-10 years)



#### Group III (Above 11 years)



**Fig. 2:** Cells of the anterior pituitary gland of male camel at ages 2-4 (Group I), 5-10 (Group II) and above 11 years (Group III) stained by trichrome staining technique. Anterior pituitary cell were stained as acidophils (orangeophils) (A=orange yellow/yellow), basophils (B= red/red purple) and as chromophobes (C= unstained/ light yellow), while connective tissue (G) were stained as green.

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