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RESEARCH ARTICLE

Seasonal Immunohistochemical Expression of Androgen Receptor (AR) in The Harderian Gland (HG) of Male Rabbit

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Abstract

A total number of (24) adult male rabbit with an average age ranged from (5-6) months were collected in different seasons (6 animal for each season). The animals were slaughtered and the harderian glands (HG) were dissected out, cleaned and fixed in 10% neutral buffered formalin solution. Then, paraffin sections were obtained and stained with: Delafields iron Haematoxylin and Eosin (H&E) to verify histological details, Periodic acid- Schiff (PAS) stain for demonstration of glycoprotein. Other paraffin sections were prepared and stained immunohistochemically for demonstration of androgen receptors (AR). The HG was bilobed with (large pink lobe and small white one). It was a tubuloalveolar gland. It was surrounded with connective tissue capsule sending interlobular connective tissue. The secretory end pieces were lined with columnar cells in white lobe and cuboidal cells in pink lobe. In autumn season, the secretory activity was high and immunohistochemical results revealed a high immune expression for (AR) in cytoplasm and cytoplasmic blebs like protrusions. However, it showed moderate immunoreactivity in the winter season. Image analysis showed significant increase in the brown colour was observed in the autumn season with a decrease in the winter season. While, the cytoplasm of epithelial cells revealed negative immune reaction in both spring and summer seasons.

INTRODUCTION

The HG was well developed in rodents (rat, mouse, golden hamster, mongolian gerbil, guinea pig), lagomorphs (rabbit, pika) and cetaceans **(Yousuke and Yasunobu, 2007)** and might have been lost in some types of mammals such as cows, horses and terrestrial carnivores (**Payne, 1994)** but present in camel **(Abou-Elmagd 1992).**

Numerous functions had been attributed to the HG, including lubrication of the eye and nictitating membrane, thermoregulation **(Thiessen, 1988; Shanas and Terkel, 1996)** and photoprotection **(Hugo et al., 1987; Spike et al., 1990).** Also, it was part of the retinal–pineal axis **(Hoffman et al., 1985)** as well as a source of either pheromones or growth factors **(Seyama et al., 1992; Shanas et al., 1996).**

The HG was an orbital gland which found in most terrestrial vertebrate. Its anatomical location, size and colour varied among different species. In rabbit, **Sohair Eltony (2009)** reported that, the HG was the largest orbital structure.

The secretory units of the HG showed a wide variety of the epithelial cell types. **Johnston et al. (1985) and Payne (1994)** mentioned that, the number of the epithelial cell types within the gland varied from one to three types and there were invariably intracellular specialization which made the gland in each species different from the other.

Nagata et al. (1980) revealed myoepihtelial cells around the tubule system of the exocrine glands to form a contractile meshwork which function in expelling their secretory product.

The HG secretory activity was affected by different endogenous factors (such as prolactin, thyroid hormones and steroid hormones), which varied among different species **(Rossella et al., 2007).**
Some of the gonadal hormone receptors, which were found in the HG were androgen and estrogen receptors.
The main function of the androgen receptor was as a DNA-binding transcription factor that regulated gene expression **(Mooradian et al., 1987)**. Androgen regulated genes were critical for the development and maintenance of the male sexual phenotype.
Receptors for androgens were first reported in the HG of the male rat **(Gustafsson and Pousette, 1975)** and subsequently in both sexes of the golden hamster ***(*Vilchis et al., 1987; Vilchis and Perez- Palacios, 1989). Vilchis et al. (1992)** reported that the androgen receptor in the harderian glands of rats, guinea-pigs and mice shared common binding characteristics with that from the hamster; they also reported androgen receptors in ducks, chickens, marine turtles and lizards. The size and appearance of lipid droplets were controlled by androgens **(Buzzell et al., 1995).**The primary endocrine control of 1-alkyl-2,3-diacylglycerol (ADG) in the HG appeared to be due to androgens **(Yousuke et al., 2007).**
As far as we are aware, there are no references dealing with the seasonal changes in the histology and immunohistochemistry of the harderian gland of male rabbit.This guide the principle objectives of the present investigation.

Materials and methods:

Histological procedures:
Samples from approximately (48) harderian glands (HG) obtained from a total number of (24) adult male rabbit with an average age ranged from (5-6) months were collected in different seasons (6 animal for each season). The animals were obtained from the animal house of the Faculty of Veterinary Medicine, Cairo University. They were housed in metal cages with food and water ad libitum. They were maintained at 12 hrs. Light and dark cycle. The animals were slaughtered. Then the HG was dissected out, cleaned rapidly of any adherent connective tissue and fixed immediately in 10% neutral buffered formalin solution. Then, the specimens were dehydrated in ascending grades of ethyl alcohol, cleared in xylol and embedded in paraffin wax. Sections of 6-7 µ thick were obtained and mounted on clean glass slides and stained with: Delafields iron Haematoxylin and Eosin (H&E) to verify general histological structure details, Periodic acid- Schiff (PAS) stain for demonstration of glycoprotein. Methods were adopted according to **(Drury and Wallington, 1980).** Additional sections were prepared for Immunohistochemistery for demonstration of androgen receptor (Avidin Biotin peroxidase complex) **(Ramos-Vara, 2005).**
Immunohistochemical examination for detection of androgen receptor (Avidin Biotin peroxidase complex):
Immunohistochemistry was performed on paraffin sections, and mounted on positively charged glass slides. Antigen was retrieved in citrate buffer (pH 6.0) microwave digestion (2 cycles of 12 minute each). Endogenous peroxidase was blocked with 0.05%hydrogen peroxide for 30 min. After incubation with a1:20 dilution of normal horse serum, the slides were incubated over night at 4˚C with primary antibodies **(Dako, 1:50)**. Secondary antibodies associated with astreptavidin-biotin-peroxidase method were applied **(Dako A/S)**. Diaminobenzidine was used as chromogen. All sections were counter-stained with haematoxylin. The sections were washed with phosphate buffered saline after each step. Negative controls were used using non-immune serum instead of the primary or secondary antibodies. The method used was outlined according to **(Ramos-Vara 2005)**.
Evaluation of immunostains:
Immunohistochemically stained sections were examined using Leica Quin 500 analyser computer system (Leica Microsystems, Switzerland) in Faculty of Dentistry, Cairo University. The image analyser was calibrated automatically to convert the measurement units (pixels) produced by the image analyser program into actual micrometre units. Androgen receptors immunostaining was measured as area percent (area %) in a standard measuring frame in six fields in each group using magnification (x400) by light microscopy transferred to the screen. The areas showing androgen receptors positive brown immunostaining were chosen for evaluation, regardless the intensity of staining. These areas were masked by a blue binary colour to be measured by the computer system **(Fig. 1)**. Mean value and standard deviation were obtained for each specimen.
Statistical analysis:
Data related to the area % of androgen receptors immunoreactivity were presented as mean and standard deviation (SD) values. Analysis of variance (ANOVA) test was used to detect statistical significance of the difference between all seasons. The significance level was set at $p<0.05$. Calculations were made using Statistical Package for Social Sciences (SPSS) software version 15.0 for Windows.

Results:

In the present research, the histology, histochemistry and immunohistochemistry of the HG were described in detail during the autumn season of the year to establish a standard against which the subsequent seasons of the year (winter, spring and summer) would be compared.

By gross inspection, the HG of the male rabbit was bilobed. It was formed of small white lobe and large pink one **(Fig. 2)**. The HG was surrounded by a vascular collagenous connective tissue capsule. The latter gave interlobular connective tissue dividing the gland into lobules **(Fig. 3)**. A thin network of reticular fibers surrounded the secretory end pieces within each lobule. The HG was a compound tubuloalveolar gland. The secretory end-pieces of the pink lobe appeared lighter in staining ability than that of the white one. The secretory end pieces in both lobes were surrounded with myoepithelial cells. The acini in the pink lobe were lined with cuboidal epithelial cells with spherical nucleus. Cytoplasm was filled with large intracytoplasmic vacuoles **(Fig. 4)**. While, those of the white lobe were lined with columnar epithelial cells with rounded basally situated nucleus. Cytoplasm appeared acidophilic with small intracytoplasmic vacuoles **(Fig. 5)**. Cytoplasmic blebs like protrusions at the luminal surface of the epithelial cells of the white lobe were numerous than that of the pink lobe. Furthermore, this season characterized by numerous secretory activity. Also, the epithelial cells in both lobes showed variation in height. Histochemical studies with PAS stain revealed scarce reaction in the epithelial cells cytoplasm of both lobes but the apical cytoplasmic blebs like protrusions, the basement membrane and the surrounding connective tissue gave positive reaction **(Fig. 6)**. The intralobular duct was lined with stratified cuboidal epithelial cell layer with its apical part showed strong PAS positive granules **(Fig. 7)**. The latter converged into the interlobular connective tissue to join the main excretory duct which opened in the inner surface of the third eyelid. The stratified columnar epithelial cells of the main duct possessed goblet cells with strong PAS positive reaction. In winter season, the HG showed a decrease in the luminal secretion in the pink lobe **(Fig. 8)** and less cytoplasmic protrusion in the white lobe **(Fig. 9)**. With PAS stain the columnar cells of the white lobe showed scarce PAS positive reaction at their luminal surface **(Fig. 10).** In spring and summer seasons, the columnar cells of the white lobe showed some cytoplasmic protrusions and few secretory material in the lumen **(Fig. 11&12)**.

Concerning the immunohistochemical expression of androgen receptors, the two lobes of the HG of male rabbit did not differ in their hormone receptors immunoreactivity.

Immunohistochemical expression with androgen receptors (AR) of the male rabbit HG, in autumn season, revealed high immune expression in the cytoplasmic blebs and cytoplasm of all epithelial cells. Also, luminal secretory materials showed an intense reaction **(Fig. 13)**. The reaction was less in the apical part of the epithelial cells of the duct **(Fig. 14)**. While, in winter season there was a moderate immune reaction in the cytoplasm of the epithelial cells with (AR) **(Fig. 15)**. In both spring and summer seasons, there were a negative immune expression in the cytoplasm of the epithelial cells of both lobes **(Fig. 16)**. However, the interstitial areas showed positive reaction.

Image analysis:

Table (1) showed the statistical difference between mean values of area % of (AR) and standard deviation among different seasons.

On measuring the area % of (AR), the autumn season showed a high value when compared to the subsequent seasons **(Fig. 17).**

Significant increase in the brown colour was observed in the autumn season. This was obvious when compared with the subsequent seasons in **(Table 1)**.

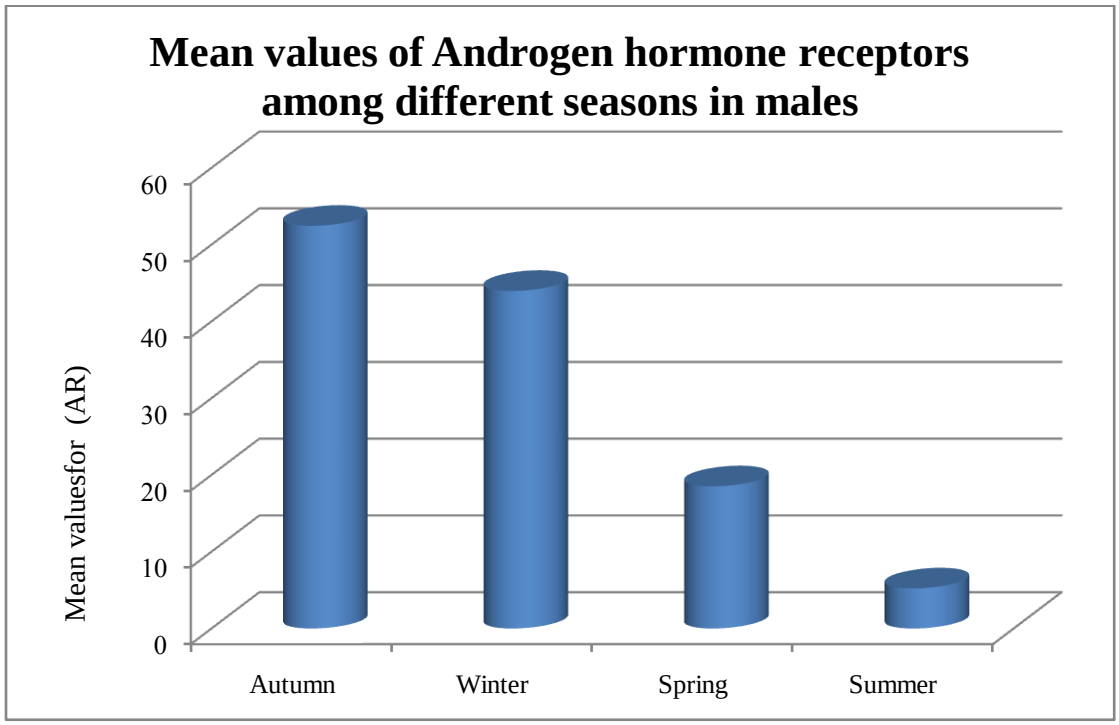
(Table 1): Statistical difference between mean values of Androgen receptors among four seasons in males:

Seasons	Mean±SD	F value	p-value
Autumn	52.45± 3.93	214.8	0.000**
Winter	43.97±4.26		
Spring	18.52±2.9		
Summer	5.22± 1.49		

F value by Analysis of Variance Test **(ANOVA)**

At confidence interval 95%, p value <0.05 is considered significant*

P value <0.001 is considered highly significant**



(Fig. 17): Bar chart illustrating mean values of Androgen hormone receptor among four seasons in males

Fig. 1: A photomicrograph of a paraffin section of an adult male rabbit HG in the autumn season showing the area % of the androgen receptors (AR) in the white lobe represented by the blue color.
Area % X 400
Fig. 2: A photomicrograph of a paraffin section of an adult male rabbit HG in the autumn season showing the white and pink lobes.
H&E X 40
Fig. 3: A photomicrograph of a paraffin section in the white lobe of an adult male rabbit HG in the summer season, showing the presence of interlobular C.T.
H&E X 100
Fig. 4: A photomicrograph of a paraffin section in the pink lobe of an adult male rabbit HG in the autumn season, showing cuboidal cells with spherical nucleus, large intracytoplasmic vacuoles and secretory material in the lumen.
H&E X 400
Fig. 5: A photomicrograph of a paraffin section in the white lobe of an adult male rabbit HG in the autumn season, showing columnar cells with rounded nucleus, acidophilic cytoplasm, cytoplasmic blebs and small intracytoplasmic vacuoles.
H&E X 400
Fig. 6: A photomicrograph of a paraffin section in the white lobe of an adult male rabbit HG in the autumn season, showing positive PAS reaction in the cytoplasmic blebs.
PAS X 1000
Fig. 7: A photomicrograph of a paraffin section of an adult male rabbit HG in the autumn season, showing interlobular duct lined with stratified cuboidal cells. PAS positive granules at the apical part of the cells could be observed.
PAS X 1000
Fig. 8: A photomicrograph of a paraffin section in the pink lobe of an adult male rabbit HG in the winter season, showing large intracytoplasmic vacuoles.
H&E X 400
Fig. 9: A photomicrograph of a paraffin section in the white lobe of an adult male rabbit HG in the winter season, showing few luminal secretory material

H&E X 400

Fig. 10: A photomicrograph of a paraffin section in the white lobe of an adult male rabbit HG in the winter season, showing PAS positive reaction in the basement membrane and surrounding C.T.

PAS X 1000

Fig. 11: A photomicrograph of a paraffin section in the white lobe of an adult male rabbit HG in the spring season, showing small intracytoplasmic vacuoles.

H&E X 400

Fig. 12: A photomicrograph of a paraffin section in the white lobe of an adult male rabbit HG in the summer season, showing luminal secretory material.

H&E X 400

Fig. 13: A photomicrograph of a paraffin section in the white lobe of an adult male rabbit HG in the autumn season, showing a high immune expression in the cytoplasm and cytoplasmic blebs.

AR X 400

Fig. 14: A photomicrograph of a paraffin section in the HG of an adult male rabbit in the autumn season, showing interlobular duct with few immune expression at the apical surface of the cells and positive immune expression in the luminal materials.

AR X 400

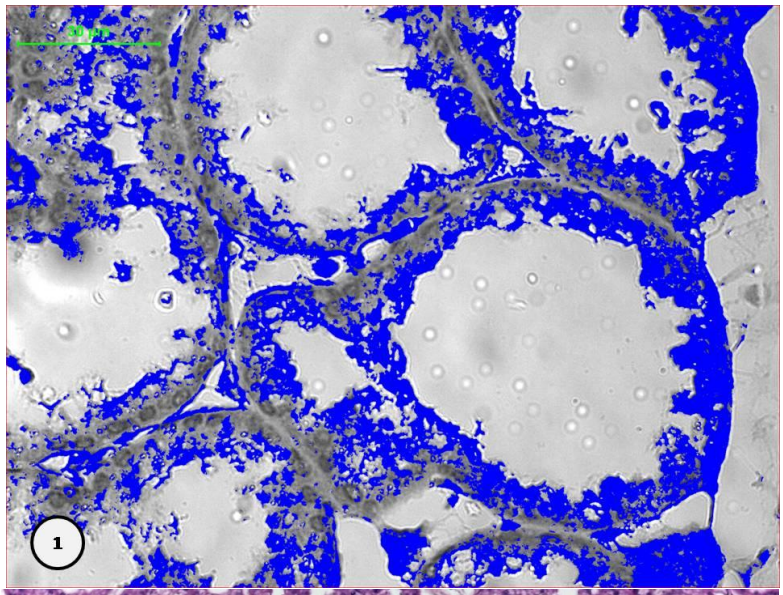
Fig. 15: A photomicrograph of a paraffin section in the pink lobe of an adult male rabbit HG in the winter season, showing a moderate immune expression in the cytoplasm and cytoplasmic blebs.

H&E X 40.

Fig. 16: A photomicrograph of a paraffin section in the pink lobe of an adult male rabbit HG in the spring season, showing negative immune expression in the cytoplasm of the cells lining the acini.

H&E X 400

Fig. 17: Comparison between mean values of area % of (AR) of the autumn season and the subsequent seasons.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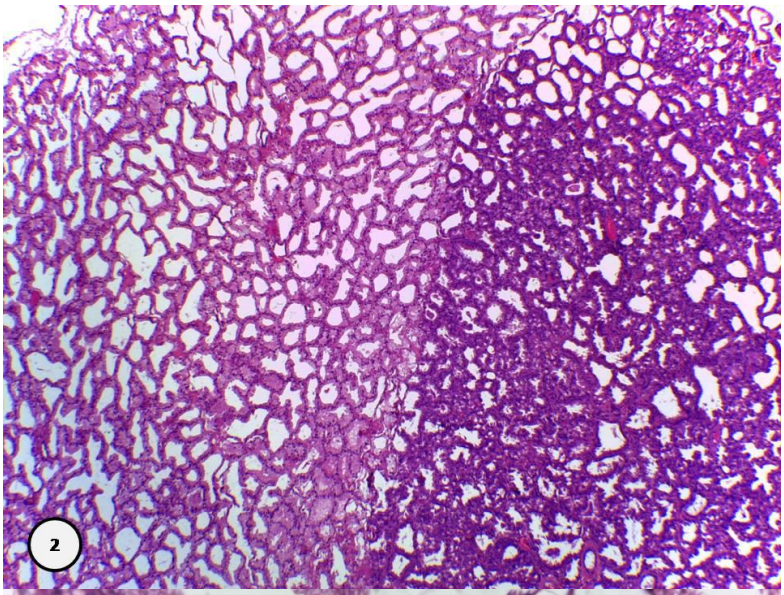
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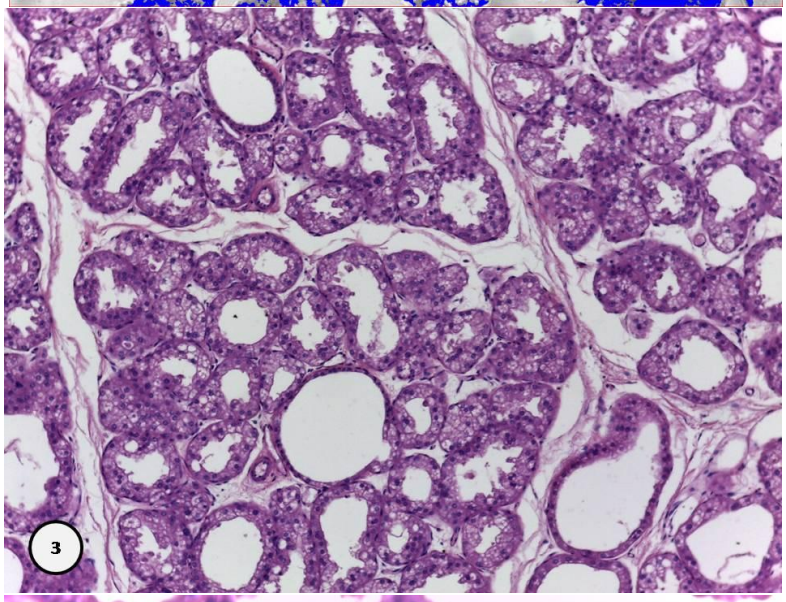
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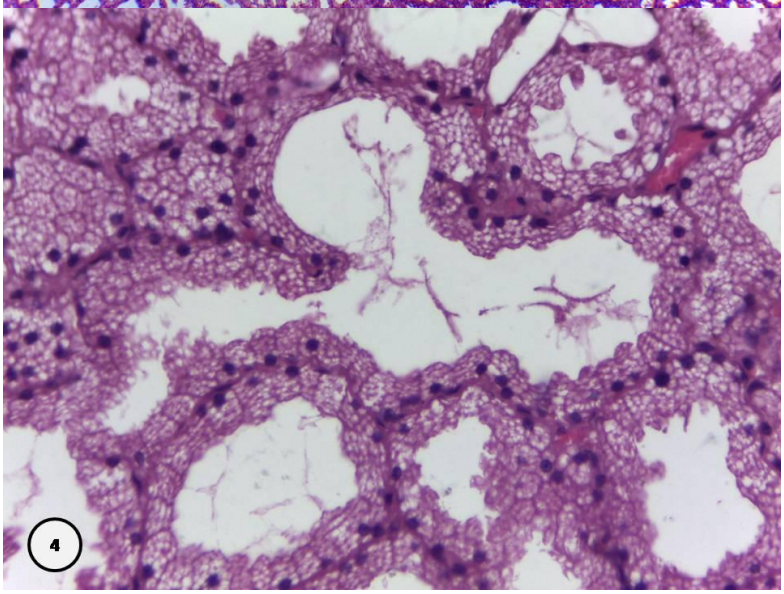
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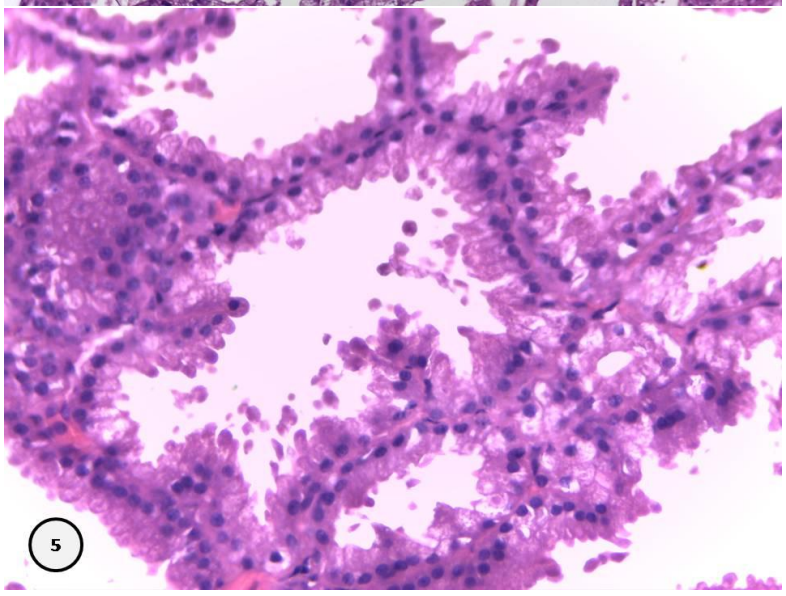
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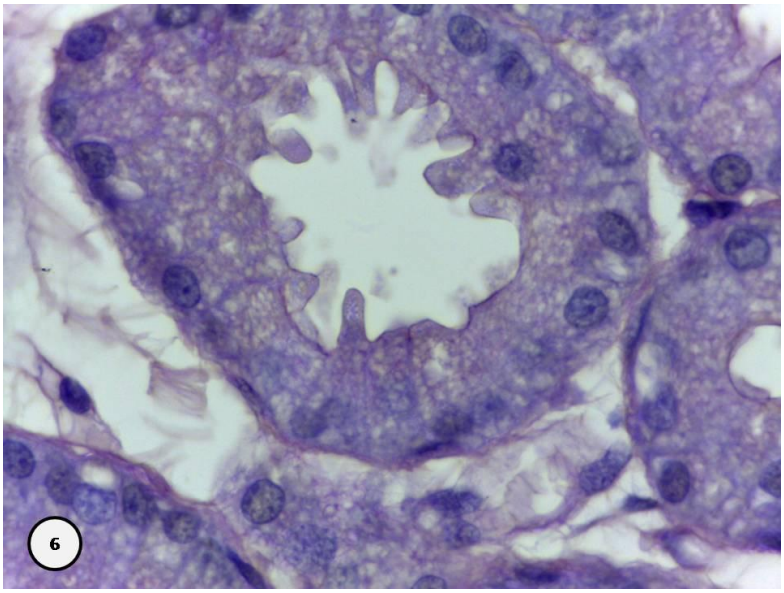
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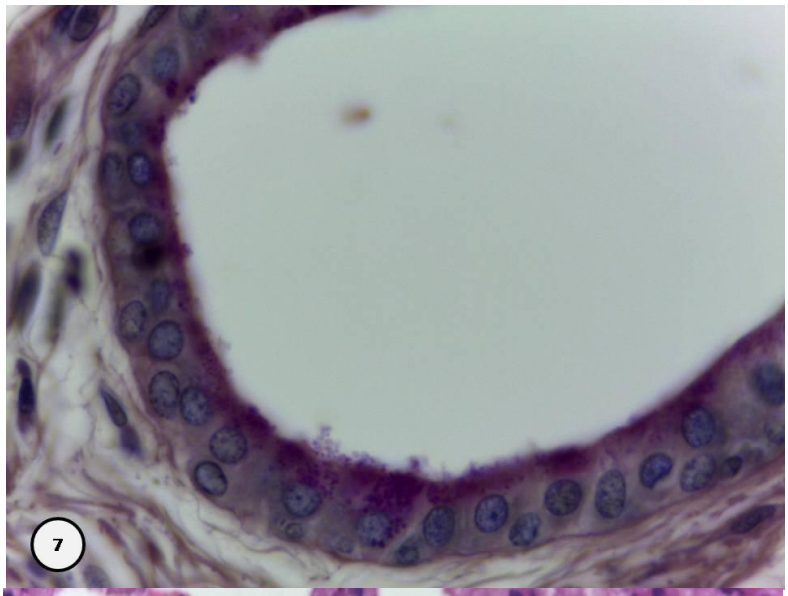
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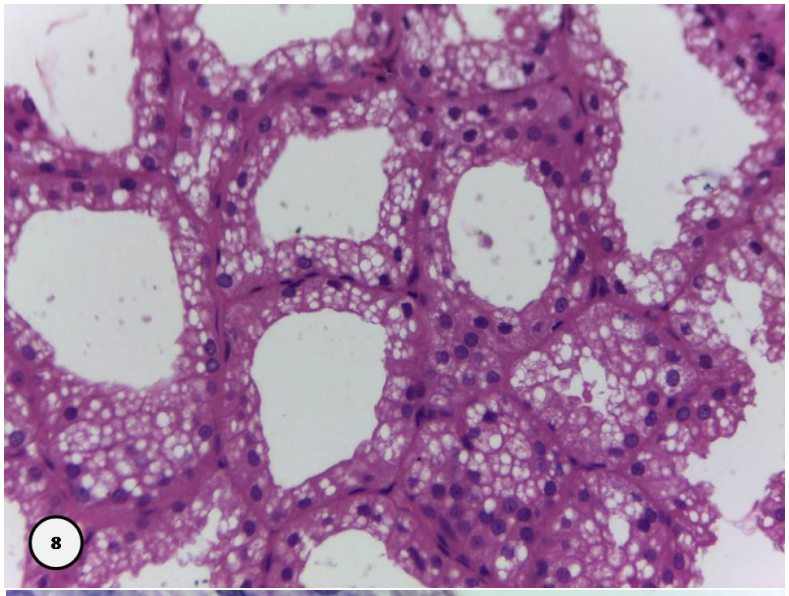
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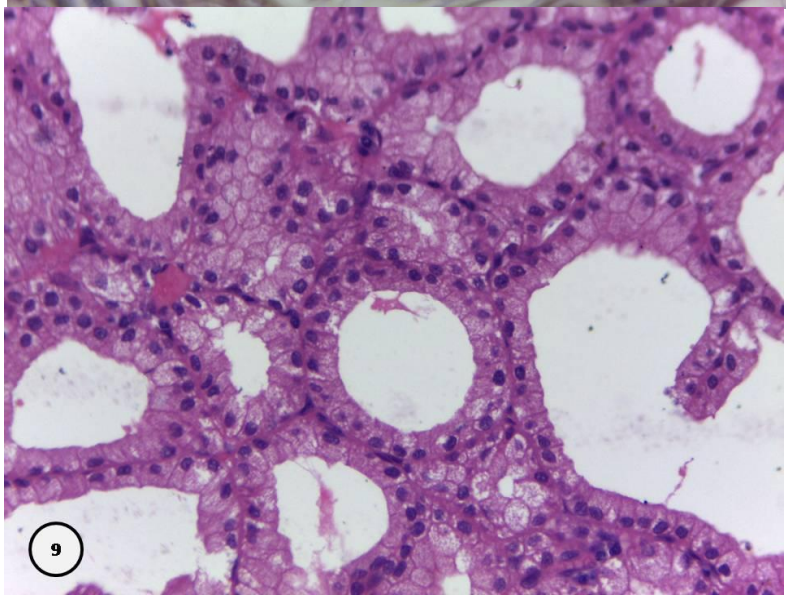
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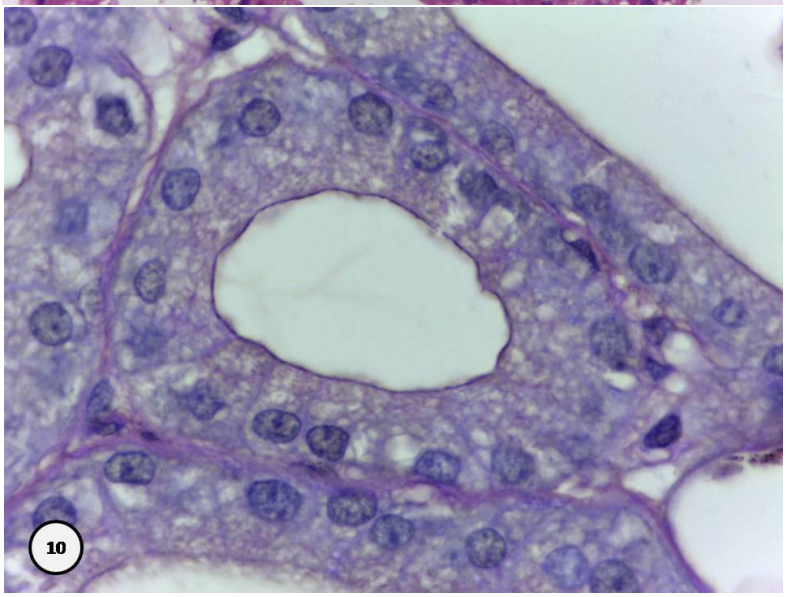
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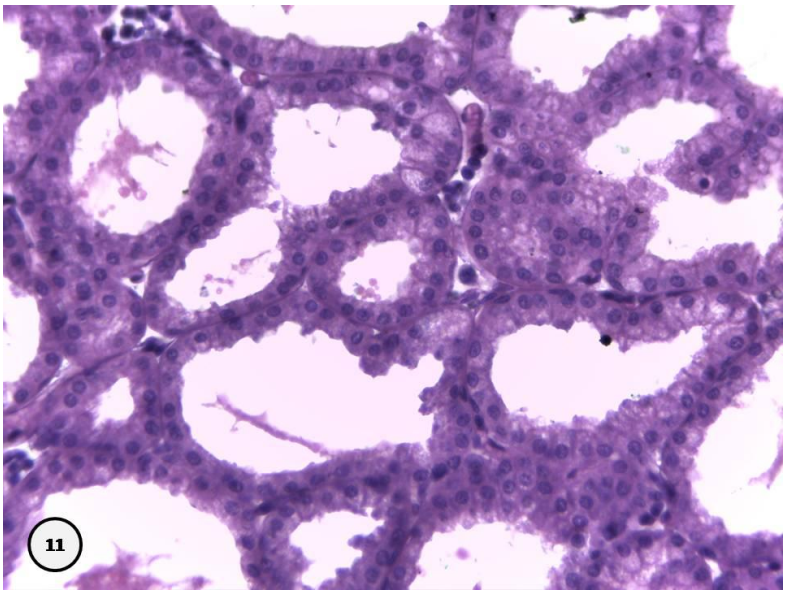
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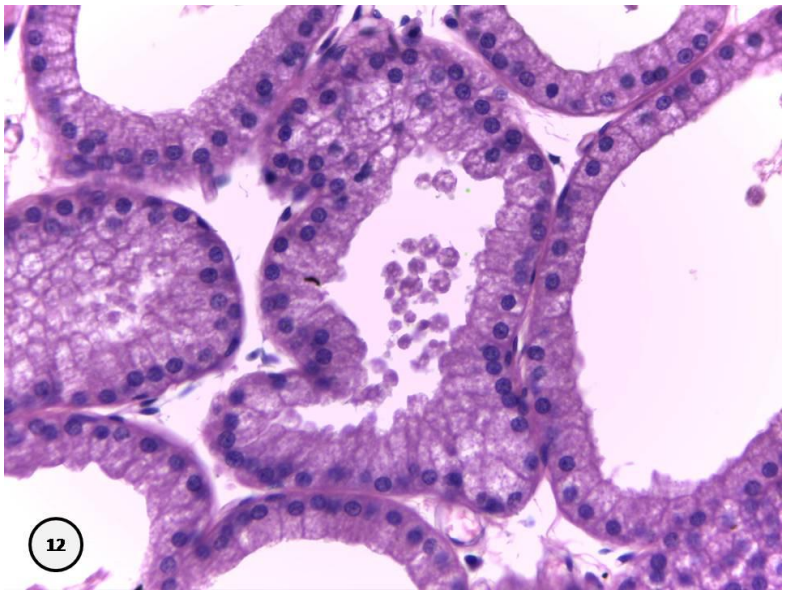
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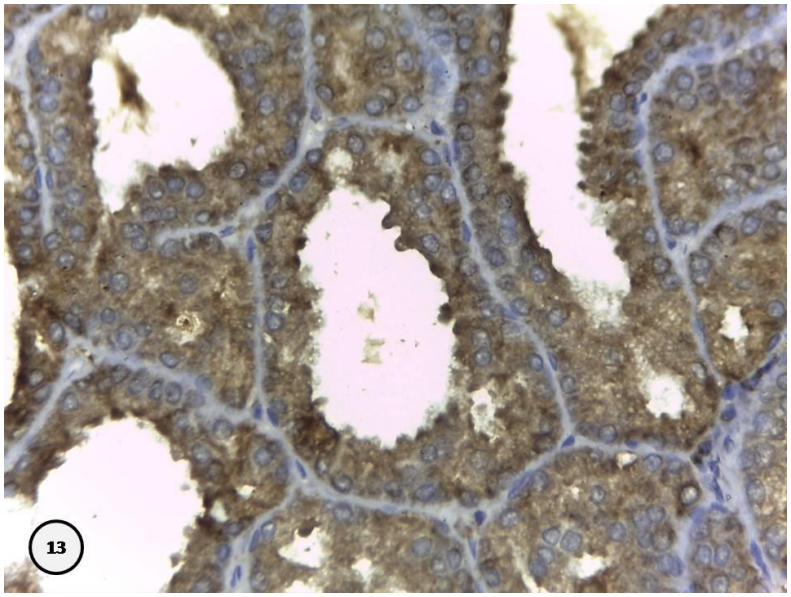

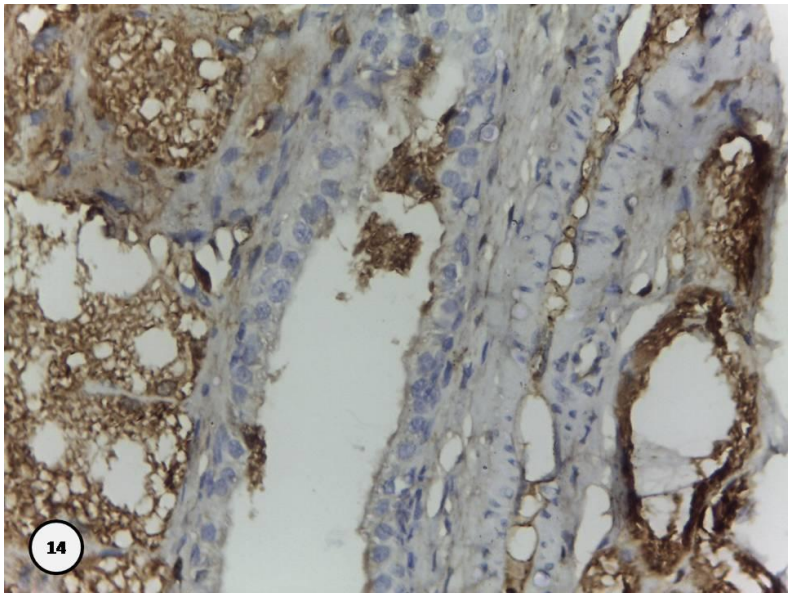

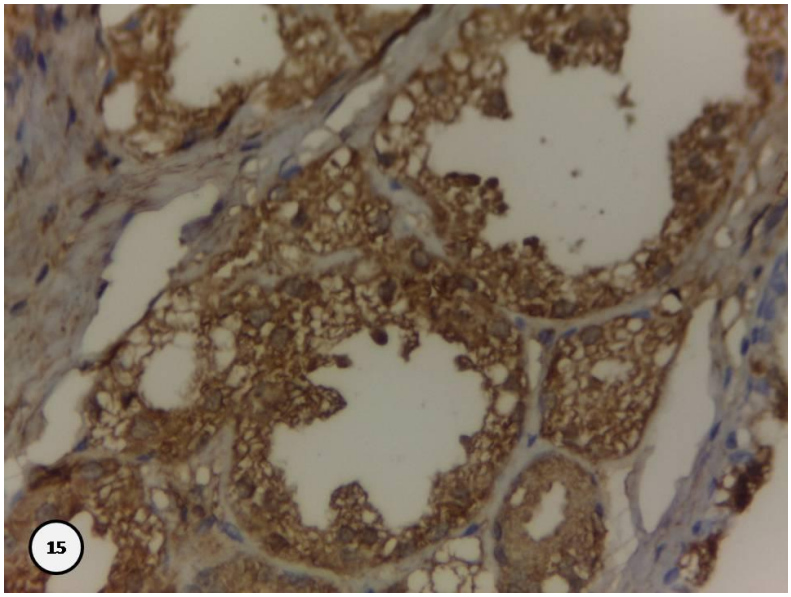

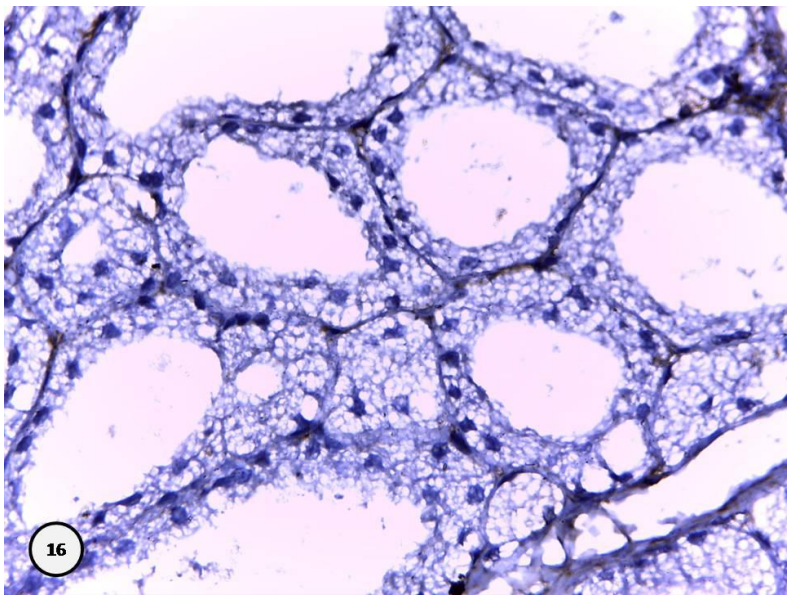

Discussion:

The gross inspection of the HG of male rabbit revealed that it was the largest structure in the orbital cavity. It was bilobed with small white lobe and large pink one. This finding was in agreement with that of **(Björkman et al., 1960; Sohair Eltony, 2009).**

The histological studies of the gland revealed that it was surrounded with a collagenous connective tissue capsule which sending interlobular connective tissue dividing the gland into lobules. In the same concern, **Wight et al. (1971)** in domestic fowl, **Djeridane (1992 and 1994)** in the desert rodent and wistar rat, **Murat and Mehmet (2009)** in domestic geese and **Sohair Eltony (2009)** in rabbit.

The HG was described as a compound tubuloalveolar gland. This observation simulated that of **Davis (1929) and Sohair Eltony (2009)** in rabbit, **Wight et al., (1971)** in domestic fowl, **Khan et al., (2007)** in broiler and native chicken, **Murat et al., (2009)** in domestic geese.

Our light finding revealed that staining of the pink lobe was lighter than that of the white one. This observation was in agreement with that of **(Sohair Eltony, 2009).**

This finding returned to the presence of two epithelial cell types. One type possessed small intracytoplasmic vacuoles and the other possessed large ones. This finding agreed with that of **Bucana and Nadakavukaren, (1973); Payne, (1977)** in male golden hamster; **Björkman et al., (1960)** and **Sohair Eltony, (2009)** in rabbit**.**

In rabbit, these two epithelial cell types were found to be segregated. Each lobe possessed one type of cells. However, this finding disagreed with **Shirama et al., (1997)** who mentioned that the HG of rabbit divided to three parts. One part composed of one type of cells with small lipid vacuoles (white lobe) and the other consisted of cells with large vacuoles (pink lobe) and the last possessed both types of cells (pink and white mixed portion). In our research, the secretory end pieces of the pink lobe were lined with cuboidal epithelial cells with spherical nucleus and large intracytoplasmic vacuoles. While, the white one was lined with columnar epithelial cells with rounded basal nucleus. Cytoplasm was acidophilic with small intracytoplasmic vacuoles. These observation simulated that of **Björkman et al., (1960)** and **Sohair Eltony, (2009)** in rabbit.

Our results revealed that there were cytoplasmic blebs like protrusions at the luminal surface of the epithelial cells of the white lobe, suggesting that the cells performed an apocrine secretion. This was in accordance to the finding of **Sohair Eltony, (2009)** in rabbit and not with the finding of **(Björkman et al., 1960).**

The apocrine secretion was not the only mode of secretion present in the HG of rabbit but merocrine and holocrine might also present. The holocrine mode of secretion was present in some rodents as mentioned by **(Johnston et al., 1983; Johnston et al., 1985; Djeridane 1994 and 1996).**

In agreement with **Sohair Eltony (2009)** the basement membrane and surrounding connective tissue gave positive reaction with PAS stain while the cells underlying the acini showed scarce reaction. Although, other investigators **Wight et al., (1971)** in domestic fowl, **Murat et al. (2009)** in domestic geese reported positive PAS reaction in the epithelial cells of the glandular secretory units.

In our investigation, there was variation in the epithelial cell height, the secretory activity and also in the immunostaining expression of the (AR). Both the secretory activity and the immunostaining expression were high in the autumn season. **Di Matteo et al., (1989)** in the green frog ***Rana asculenta*** reported that the cell height, secretory activity and nuclear staining ability were changes of a seasonal nature and might be controlled by hormonal and environmental stimuli.

Our study revealed that the male rabbit HG showed a high immune expression for (AR) at the autumn season. From the present investigation there was evidence that the seasonal changes in the HG of rabbit were controlled by androgen hormones.

Yousuke et al., (2007) stated that the lipid secreted from the HG was not a triacylglycerol (TG) but an 1-alkyl-2,3-diacylglycerol (ADG). The TG was used as a source of storage energy in the cell and that of ADG was to be used outside of the body after secretion from the gland. The primary endocrine control of the harderian gland ADG appeared to be due to androgens. They proposed a hypothesis that the lipid exudates from the HG served as pheromone to declare the golden hamster individual territory and to seek the mate with a good congeniality. The sexual difference of the (ADG) would be a signal of such pheromone and the profile of acyle and alkyl group of (ADG) would be used to differentiate their affinity.

Nagata et al., (1980) mentioned that the myoepithelial cells formed a contractile meshwork around the secretory units of the exocrine glands which function in expelling their secretory products. This finding simulated our results in the HG of rabbit.

In our study, the HG of rabbit showed a clearly distinct intralobular duct which was lined with stratified cuboidal cells. By PAS stain the apical part of these cells showed positive granules. The intralobular duct converged into interlobular connective tissue to join the main excretory duct which was lined with stratified columnar epithelium with strong PAS positive goblet cells in between. **Weaker (1981)** in armadillo reported the presence of intralobular and interlobular ducts. Although other investigators mentioned that there was no distinct duct in the gland substance **(Johnston et al., 1985 and Djeridane, 1994)**

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