Age-Dependent Mapping of Somatotropic Hormone in Rat Pituitary Gland; Histological, Histochemical and Immunohistochemical Studies

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Abstract
The current study was performed to investigate age-dependent immunoexpression and localization of somatotropin in the pituitary gland. Twenty adult Wistar rats (fifteen females and five males) were housed; four animals per cage with observation of pregnancy. Samples from pituitary gland were collected from different ages of male offsprings. Tissue specimens were processed, and stained with Hematoxylin and Eosin (H&E), special stain and immunohistochemical technique. Sections were divided into four stages. H&E results showed that at stage I, acidophils were the most distinguished cells; but basophils were difficult to be seen then appeared at stage II. Special stain results showed increasing in somatotrophs number from stage I to stage III then decreased at the last stage. Immunoexpression of somatotropin in pars distalis showed that somatotrophs were numerous and uniformly distributed at the first three stages then they were restricted in clusters at the last stage.

Key words: somatotropin- immunoexpression- pituitary gland- rat.

Introduction

Somatotrophic hormone has functional and biological effects as one of the chief anabolic hormones whose functional role is vital for growth and metabolism throughout prenatal and postnatal development ***(Campbell, 1997, Lecomte et al., 2018,Zhang et al., 2018)***. It is a polypeptide hormone produced by the pituitary somatotrophs, under the control of two hypothalamic hormones, a stimulatory growth hormone releasing hormone (GHRH), and an inhibitory somatostatin hormone (SST)***(Tannenbaum et al., 2003*)**. The immunostained somatotrophs showing a granular cytoplasmic pattern and distributed homogeneously in the rat pars distalis of different postnatal ages ***(Jurado et al., 1998*)**. Thus, the aim of this work is to study the

immunohistochemical localization of somatotropin in the pituitary in male rat and to study the relationship between the immunoexpression and the age-dependent changes of the immunoreactive cells.

Materials and Methods

I-Animals and housing

Twenty adult Wistar rats weighting 200-250 g (fifteen females and five males) were housed under standard conditions. All animals received professional care in accordance with "The guide for the care use of laboratory animals" published by The National Institute of Health. This was carried out in accordance with guidelines of the animal care and use committee at Faculty of Veterinary Medicine, Suez Canal University (approval no. 2019006).

II-Experimental design

Animals were separated into four animals per cage (three females and one male) with observation of pregnancy. Each suspected pregnant female was isolated in a sporadic breeding cage. Recording of offspring's ages was performed in controlled documents.Samples of pituitary gland were collected from male offsprings at the following different ages (30, 40,50,70,80,150,160,170, and 180 days).Five male rats from each age were sacrificed through cervical dislocation under ketamine anesthesia (80 mg\Kg I.p). Then, samples were immersed in 4 % paraformaldehyde (pH 7.4) for 7 days.

III- Histological procedures

Dehydration of samples were conducted by using ascending ethanol series, cleared in xylene and then embedded in paraffin wax. Paraffin sections were cut at 5-7 um thick. Routine staining procedures were conducted by Harri's Hematoxylin and Eosin (H&E) stain; slides were mounted in DPX.

IV- Histochemical procedures

Histochemical procedure aimed to differentiate between chromophilic cells; this was done by using Peracetic acid -Alcian blue –PAS Orang G stain (PAA- AB-PAS Orange G stain) according to ***(Adams and Swettenham, 1958, El-Sakhawy et al., 2012)***.

V- Immunohistochemical procedures

Tissue samples were fixed in 4 % paraformaldehyde at pH (7.4) for 2 days; and then processed and mounted on positively charged slides for immunohistochemical detection of somatotrophic hormone **(Carson, 1997)**. Tissue slides were deparaffinized in xylene, rehydrated and incubated in citrate buffer (ph. 6) in a microwave oven.Then, vectastain rabbit blocking reagent was used. After that, polyclonal rabbit anti-HGH (cat# AR707-SR, Thermo Fisher Scientific, Co., Manor Park, UK) was used as primary antibody; it was dilated in PBS (1:300), then added to the slides and incubated for 30 min.Sections were washed in PBS

three times for 5 min. each; biotinylated polyvalent secondary antibody (Thermo scientific co., UK) was applied and incubated for 30 min.After rinsing with PBS two times for 3 min. each, they were incubated with Avidin-biotin complex (ABC Kit, Vector Laboratories). Then, slides were incubated in 3,3´-diaminobenzidine peroxidase enzyme substrate (DAB) for 30 min. and counterstained with Mayer´s hematoxylin. Finally, slides were dehydrated, cleared and mounted with DPX. For negative control, Sections were treated with similar steps with the exception of the primary antibody.All procedures were done according to Vectastain Elite ABC kit (Vector laboratories. California, USA).

VI- Quantitative parameters used for histological, histochemical and immunohistochemical investigations:

All images were analyzed using image J software developed by The National Institute of Health (Betheda, Maryland. USA). They were performed as following: -

A- Measurement of somatotroph´s size using immunohistochemical sections.

Measurement of somatotroph size was performed on the immunostained cross sections of pituitary gland; somatotrophs boundaries were demonstrated by their affinity to the brown color of chromogen. Ten immunostained sections of each age with scale bar 20µm.

B- Counting of somatotrophs using pituitary sections stained with special stain.

Pituitary gland sections stained with PAA- AB- PAS -Orange G stain were examined for somatotrophs counting; somatotrophs were demonstrated by their yellow color. This was performed using ten sections of each age with scale bar 20µm.

C- Measurement of immunostaining intensity of somatotropin.

For the detection of the percentage of the immunostained area of the Somatotrophic hormone in pituitary gland, liver, lung, and testis; Images of selected parts were analyzed using the image J software at scale bar 50µm.

VII- Statistical analysis

Data were collected as mean ± SE for statistical analysis, one way ANOVA followed by Duncan test was applied. Data analysis was performed employing the statistical package for social science, version 20 (SPSS software, SPSS Inc. Chicago, USA). The level of significance was set at P value <0.05.

Results

I- **Histological, histochemical, and immunohistochemicalfindings.**

A- **General Hematoxylin & Eosin staining results:**

The pituitary gland was made up of the three common regions; Pars distalis, Pars intermedia and Pars nervosa (Fig. 1 A) from the earliest (30 days old) examined rats. In this study, we focused on the cell components of the pars distalis had the somatotrophs features. Sections were divided into four stages according to the histological findings of the pituitary gland.**Stages I (30 - 40 days),** the distinguished cells at this stage were chromophobes and acidophils; it was difficult to identify basophils (Fig. 1 B). Chromophobes had unstainable or faintly acidophilic cytoplasm and large, spherical, euchromatic nuclei (Fig. 1 B). Acidophils were sporadically distributed or arranged in small clumps; they had small, dark and polymorphic nuclei which were surrounded with a homogenous eosinophilic cytoplasm (Fig. 1 B). **Stage II (50 days),** Pars distalis was formed from chromophobes, acidophils and basophils (Fig 1 C). There was increasing in acidophils number whose nuclei were small and spherical with condensed chromatin at the periphery (Fig. 1 D). Furthermore, basophils could be seen at this stage, they were slightly small in size with spherical, vesicular nuclei and finely granular basophilic cytoplasm (Fig. 1 C, D). **Stage III (70-80 days),** two different sizes of acidophil's nuclei were observed; the first one, the medium-sized vesicular nuclei with condensed edges of chromatin and central nucleolus. The second one, was larger in size, lighter and more vesicular (Fig. 1E). Basophils were obviously increased in their number and size than the last stage. They contained spherical or oval nuclei with granular basophilic cytoplasm (Fig. 1 E). **Stage IV (150-180 days)**, there was an increase in acidophil's number (Fig. 1 F). The granulated appearance of the basophil's cytoplasm was more prominent (Fig. 1 F).

B- **Histochemical staining properties of chromophiles using per acetic acid alcian blue – PAS-Orange G (PAA- AB- PAS-Orange G) special stain:**

Staining of the Pars distalis with PAA- AB- PAS- Orange G stain had displayed three types based on the staining affinity of their cytoplasmic granules as follows; cells tinged with yellow color were representing somatotrophs, orange-reddish color were representing lactotrophs, and finally the magenta red colored cells were representing basophils (Fig.2 A). At the first three stages (stage I, II & III), somatotrophs were arranged into clusters while lactotrophs were distributed sporadically (Fig. 2 B, C & D). Basophils were difficult to be distinguished at stage I (Fig. 2 B) but they were seen in a few number at stage II (Fig. 2 C) and somewhat increased at stage III (Fig. 2 D). At last stage (IV), somatotrophs and lactotrophs were tended to be arranged in cords; lactotrophs were

mainly localized around the blood capillaries (Fig. 2 E).

C- **Immunohistochemical expression of somatotropin in somatotrophs:**

Section of Pars distalis showed negative somatotropin immunoreactivity; when primary antibody was replaced by normal goat serum (Fig. 3 A). At the first three stages (stage I, II & III), somatotrophs were numerous and uniformly distributed (Fig. 3 B, C & D). At stage IV, they were reduced in number and restricted into clusters (Fig. 3 E).

II- **Quantitative analysis of pituitary gland :**

A- Morphometric analysis of somatotroph´s size using immunostained sections :

Morphometric analysis of somatotroph's size at different stages showed a significant increase between stages I and stage IV (Fig. 4); but there was no significance between the early two stages (I & II) (Fig. 4).

B- Counting of somatotrophs:

somatotrophs cell count at different stages revealed significant increase at early stage I until the stage III then significant dropat stage IV (Fig. 5).

C- Mean % area of Somatotropin immunoreactivity in pars distalis:

Mean % area of somatotropin immunoreactivity showed that the most reduced intensity was at stage I (Fig. 6) while the highest intensity was at stage III (Fig. 6).

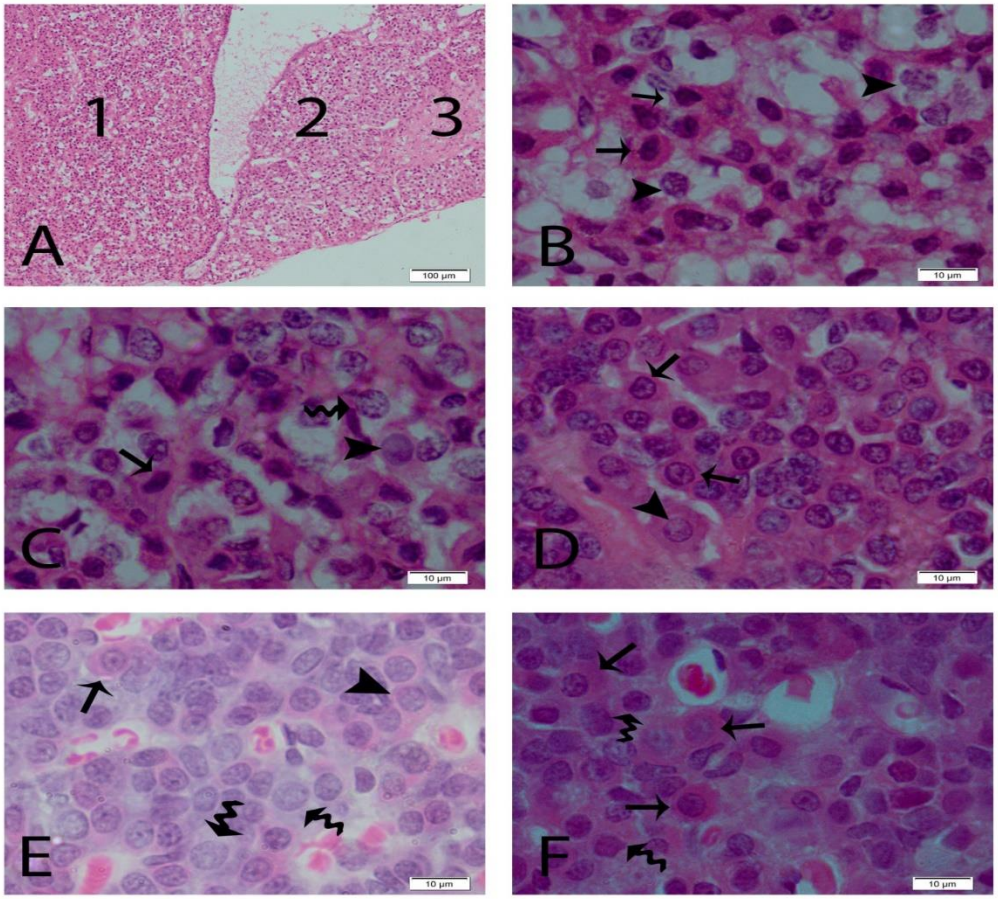

Fig (1): Photomicrographs of pituitary gland showing histomorphological changes at different stages. (A) Pituitary gland showing three well-developed parts on day 30, Pars distalis (1), Pars intermedia (2) and Pars nervosa (3). Scale bar 100µm. (B) At stage I, pars distalis showed faint acidophilic cytoplasm of chromophobes (arrow head) and homogenous acidophilic cytoplasm of acidophils (arrow). (C) At stage II, Pars distalis consisted o acidophils (arrow), basophils (arrow head) and chromophobes (curved arrow). (D) At stage II, Condensed chromatin at the periphery of acidophil´s nuclei (arrow) and euchromatic nuclei of basophils (curved arrow). (E) At stageIII, basophil´s nuclei (curved arrow) were larger than those of acidophils. Note two sizes of acidophil´s nuclei; medium sized one (arrow) and large sized nuclei (arrow head). (F) At stage IV, notice increasing in number of acidophils (arrow) and the granulated cytoplasm of basophils (curved arrow). H&E stain, Scale bar 10µm.

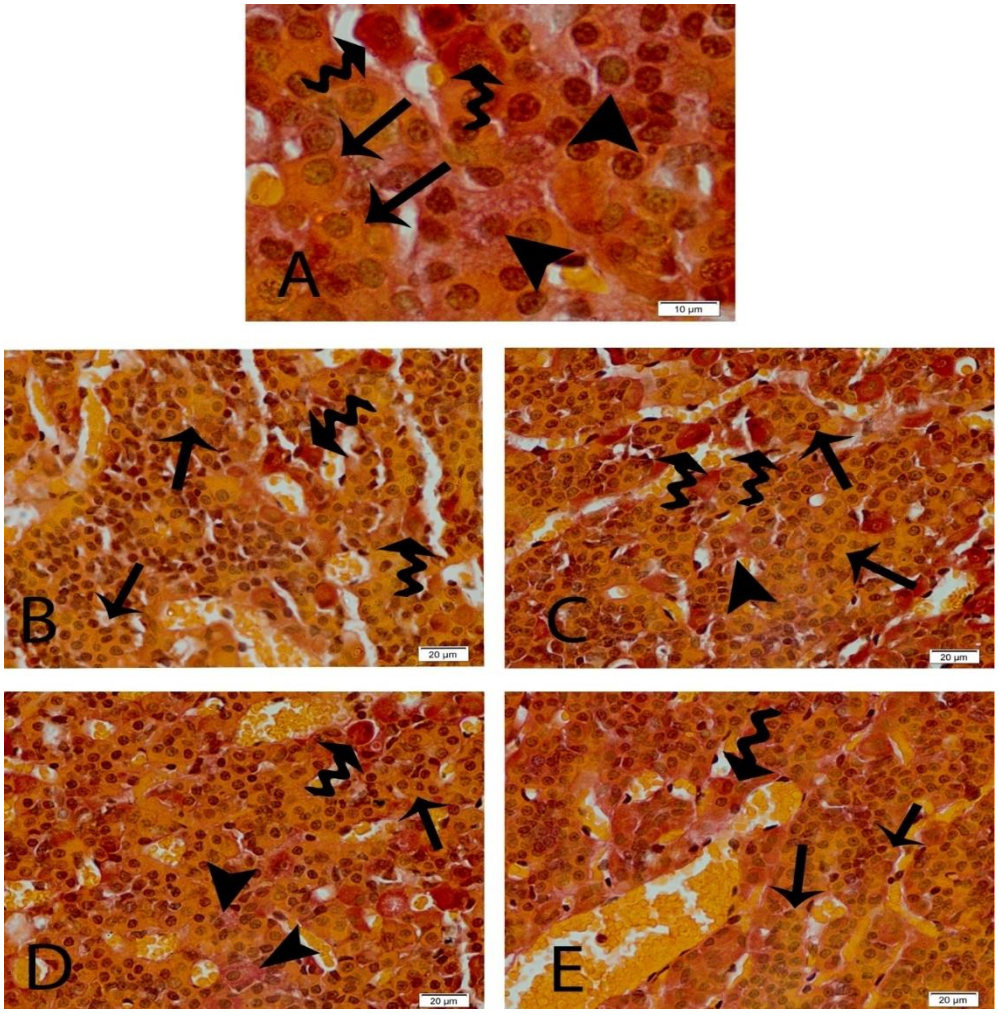

Fig (2): Photomicrographs of Pars distalis at different stages. (A)Pars distalis on day 70 showing the staining affinity of somatotroph to yellow color (arrow), lactotrophs to orange red color (curved arrow) and basophils to magenta red color (arrow head).Scale bar 10µm. (B) stage I, (c) Stage II and (D) Stage III showing the distribution of somatotrophs in clusters (arrow) and lactotrophs were distributed sporadically (curved arrow). Note few number of basophils (arrow head) at stage II and slight increase in basophils number (arrow head) at stage III. (E) Stage IV showing decreasing of somatotrophs (arrow) and distribution of lactotrophs around the blood sinusoids (curved arrow). PAA-AB-PAS-Orange G stain, Scale bar 20µm.

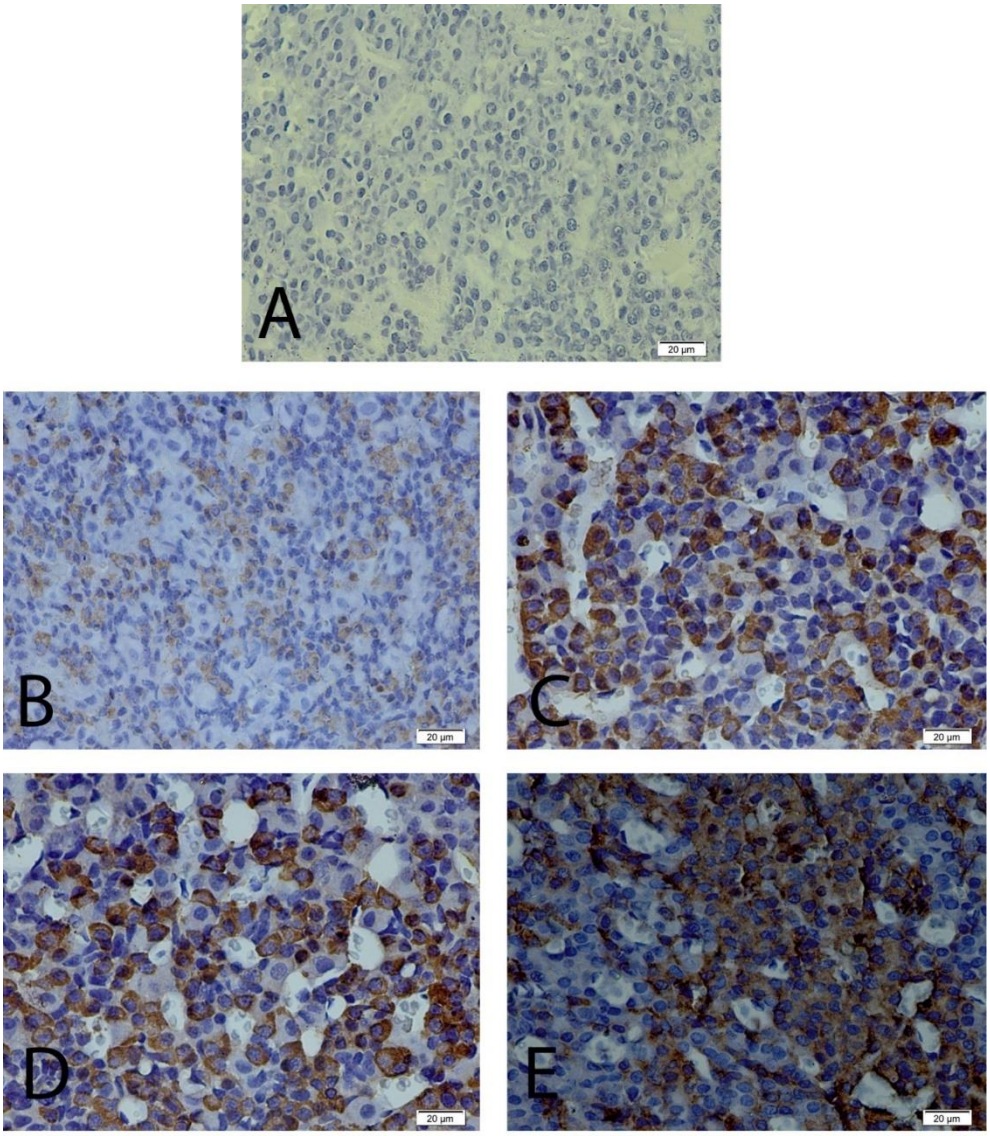

Fig (3): Photomicrographs of immunostained Pars distalis at different stages.(A) Pars distalis stained as negative control for somatotropin immunoreactivity showing negative reaction. (B) Stage I showing the lowest intensity of somatotropin. (C) Stage II showing increasing the intensity of somatotropin. (D) Stage III showing the highest intensity of somatotropin. (E) Stage IV showinglower staining affinity of cytoplasmic granules than stage III.ABC stain, Scale bar 50µm.

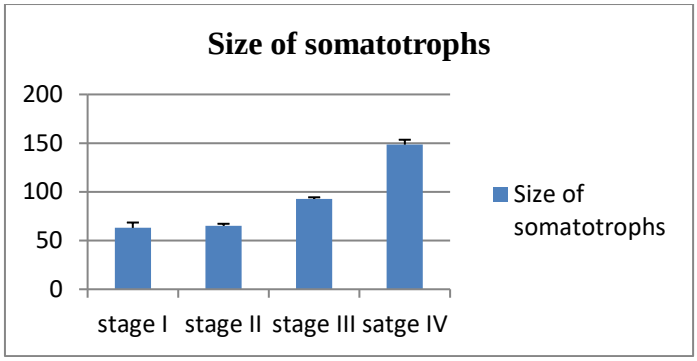

Fig (4): An illustration of the measurement of size of somatotrophs at different stages. Data were the mean ± SE ($p < 0.05$).

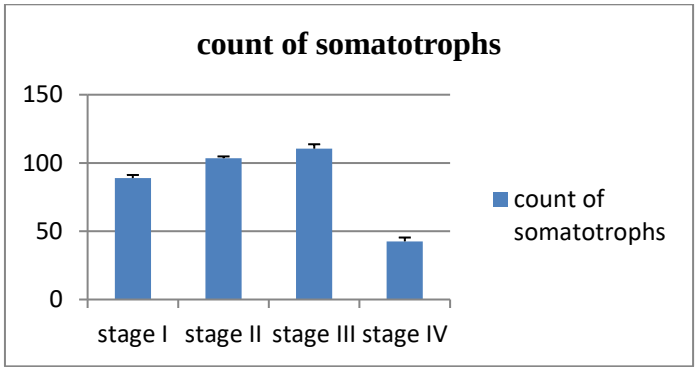

Fig (5): An illustration of the count of somatotrophs at different stages. Data were mean ± SE ($p < 0.05$).

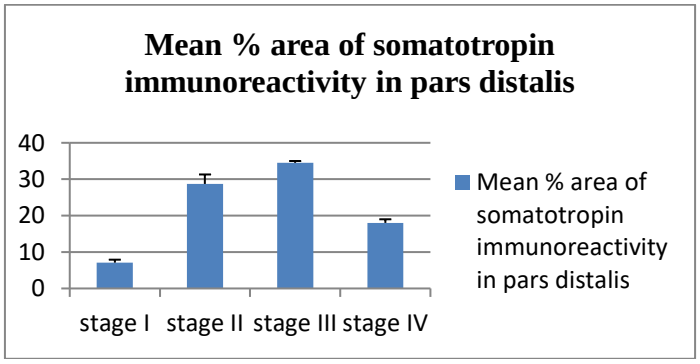

Fig (6): An illustration of the measurement of mean percentage area of somatotropin immunoreactivity at different stages. Data represented the mean ±SE ($p < 0.05$).

Discussion

In this study, H&E sections of the pituitary gland at stage I (30 days) showed a well-differentiated Pars distalis, Pars intermedia and Pars nervosa. This was supported by many authors who reported that the three parts of the pituitary gland were completely differentiated in rat fetus on day 19 of pregnancy ***(Manojlović-Stojanoski et al., 2007)***. At stage I, the distinguished cells in the Pars distalis were Chromophobes and acidophils; increased number of acidophils was observed at stage II. The predominance of acidophils in young rats was also recorded by the previous study **(*Ibrahim et al., 2002)***. It was speculated that the rapid growth rate of the young rats is correlated to a concomitant increase of the acidophil cell fraction which is responsible for the synthesis of specific hormones ***(Badway et al., 2004*)**. The present study has revealed that basophils were not clearly seen at the sage I, whereas they could be seen at stage II; they were comparatively small in size with spherical, vesicular nuclei and finely granular cytoplasm. A significant increase in the size and number of basophils was observed at stage III. Similar results were also recorded in previous studies ***(Ibrahim et al., 2002*)**. Our findings showed that at stage IV, chromophils were well-differentiated with a pronounced increase in the number and size of acidophils with their vesicular nuclei and homogenous acidophilic cytoplasm. Also, basophils were larger in size than acidophils with their granular basophilic cytoplasm and vesicular eccentric nuclei. Some studies on the age-related changes of the pituitary gland structure had supported these findings ***(Ibrahim et al., 2002, Badway et al., 2004)***.

Our study demonstrated three cell types forming the pars distalis; somatotrophs, lactotrophs, and basophils. This was based on the staining affinity of their cytoplasmic granules to PAA- AB-PAS- Orange G stain. Differentiation of basophils into two sub-types by using this specific stain was not noticed; a possible explanation for this point that the alcian blue was not reactive in rat pituitary gland. In a preceding study, researchers showed that alcian blue-PAS stain differentiated two types of basophils in the human pituitary gland, but it failed to do the same action when applied to the pituitary of rat ***(Swettenham, 1960*)**. Counting of Somatotrophs at different stages revealed a significant increase at the early three stages; the highest number was at stage III. But, a dramatic drop was recorded at stage IV. This observation might be corresponding to the previous study which stated that somatotrophs increased after 15 days of gestation in rat and occupy a greater area of Pars distalis in young stage (2-3 month) than in the other stages of growth **(Tauiguchi**

et al., 1989). The drop of the somatotrophs number might be attributed to the concomitant increase of the somatostatin receptor expression in the pituitary gland with progression of postnatal age **(*Reed et al., 1999)***. Growth hormone- releasing hormone receptor (GHRHR) mRNA levels were increased during the first 30 days after birth and then declined with age ***(Korytko et al., 1996*)**. The synthesis and secretion of the somatotrophic hormone is controlled by GHRH and somatostatin, which produced its actions through their receptors GHRHR and somatostatin receptor (SSTR) ***(Gunawardane et al., 2015*)**. Also, GHRHR mRNA was increased from Day 7 to Day 40 postnataly then decreased on Day 90 **(*Zhang et al., 2018*)**. In this study, the somatotropin immunoreactivity showed that the most reduced immunoreactive intensity was at stage I, while the highest intensity was at stage III. Then, it was reduced at the last stage (stage IV). Our findings were inconsistent with many data ***(Hemming et al., 1986, KUROSUMI et al., 1986, Takahashi, 1991, JURADO et al., 1998,Xiaojing et al., 2018*)**. Somatotrophs were distributed uniformly in the Pars distalis with the presence of some clusters located laterodorsally near the Pars intermedia in male rat **(Smets et al., 1987)**. They were uniformly and distributed densely in the lateral wings of the adenohypophysis of adult male rat **(*Wilson and Wyatt, 1988*)**. Regarding the highest intensity at stage III, this finding came a line with the previous study which reported that the immunoreactive somatotrophs were numerous in young rats suggesting a parallel increase in the synthesis and accumulation of somatotropin until immediate release **(*Smets et al., 1987*)**. The decreasing of GH mRNA and GH immunoreactivity with age occurred as a result of decreasing in GHRH neurons **(*Kuwahara et al., 2004*)**. The level of GH mRNA in the pituitary gland was increased with age in the male rat from day 7 postnataly then it decreased markedly by day 90 days ***(Zhang et al., 2018*)**. Morphometric analysis of somatotrophs size at different stages displayed a gradual increase from early to late stage; a previous study has shown an increase in somatotroph's size with age from suckling to adulthood on the female mice **(*Sasaki, 1988*)**.

References:

Adams, C. & Swettenham, K. 1958. The histochemical identification of two types of basophil cell in the normal human adenohypophysis. *The Journal of pathology and bacteriology*, 75**,** 95-103.

Badway, L., El-Anany, T., Abed Elkader, H. & Naser, S. 2004. *age- related changes in acidophils of pars distalis in albino rat's*

pituitary gland. Ph.D, Cairo university.

Campbell, G. S. 1997. Growth-hormone signal transduction. *The Journal of pediatrics,* 131**,** S42-S44.

Carson, F. 1997. Histotechnology: a self–instructional text Department of pathology. Baylor University Medical center Dallas, Texas,(ASCP) Press.

El-Sakhawy, M., Ghareeb, N., El-Bargeesy, G. & Rahman, A. 2012. Post-Hatching Development of the Pars Distalis of the Adenohypophysis of the Quail (Coturnix coturnix). *J. Vet. Anat. Vol,* 5**,** 1-15.

Gunawardane, K., Hansen, T. K., Christiansen, J. S. & Jorgensen, J. O. L. 2015. Normal physiology of growth hormone in adults. *Journal of Turkish Pediatric Endocrinology and Diabetes Society,* 29**,** 1-7.

Hemming, F., Dubois, M. & Dubois, P. 1986. Somatotrophs and lactotrophs in the anterior pituitary of fetal and neonatal rats. *Cell and tissue research,* 245**,** 457-460.

Ibrahim, S., El-Shawarby, A. & Soliman, A. 2002. Age related changes in thyrotrophs of the anterior pituitary gland and the thyroid gland of male albino rats ;Histological, Immuncytochemical and Biochemical study. *The Egyptian journal of Histology,* 25**,** 41-56.

Jurado, S., Cónsole, G. & Dumm, C. G. 1998. Sexually dimorphic effects of aging on rat somatotroph cells. An immunohistochemical and ultrastructural study. *Journal of veterinary medical science,* 60**,** 705-711.

Korytko, A. I., Zeitler, P. & Cuttler, L. 1996. Developmental regulation of pituitary growth hormone-releasing hormone receptor gene expression in the rat. *Endocrinology,* 137**,** 1326-1331.

Kurosumi, K., Koyama, T. & Tosaka, H. 1986. Three types of growth hormone cells of the rat anterior pituitary as revealed by immunoelectron microscopy using a colloidal gold-antibody method. *Archivum histologicum japonicum,* 49**,** 227-242.

Kuwahara, S., Sari, D. K., Tsukamoto, Y., Tanaka, S. & Sasaki, F. 2004. Age-related changes in growth hormone (GH) cells in the pituitary gland of male mice are mediated by GH-releasing hormone but not by somatostatin in the hypothalamus. *Brain research,* 998**,** 164-173.

Lecomte, M.-J., Bertolus, C., Ramanantsoa, N., Saurini, F., Callebert, J., Senamaud-Beaufort, C., Ringot, M., Bourgeois, T., Matrot, B. & Collet, C. 2018. Acetylcholine Modulates the Hormones of the Growth Hormone/Insulinlike Growth Factor-1 Axis During Development in Mice. *Endocrinology,* 159**,** 1844-1859.

Manojlović-Stojanoski, M., Nestorović, N., Negić, N., Trifunović, S., Sekulić, M. & Milošević, V. 2007. Development of pituitary ACTH and GH cells in

near term rat fetuses. *Archives of Biological Sciences,* 59**,** 37-44.

Reed, D., Korytko, A., Hipkin, R., Wehrenberg, W., Schonbrunn, A. & Cuttler, L. 1999. Pituitary somatostatin receptor (sst) 1–5 expression during rat development: age-dependent expression of sst2. *Endocrinology,* 140**,** 4739-4744.

Sasaki, F. 1988. Changes with age in the number and size of anterior pituitary cells in female mice from suckling to adulthood. *Journal of endocrinology,* 117**,** 5-NP.

Smets, G., Velkeniers, B., Finne, E., Baldys, A., Gepts, W. & Vanhaelst, L. 1987. Postnatal development of growth hormone and prolactin cells in male and female rat pituitary. An immunocytochemical light and electron microscopic study. *Journal of Histochemistry & Cytochemistry,* 35**,** 335-341.

Swettenham, K. 1960. The buffered performic-acid-alcian-blue-periodic-acid-Schiff method for the differentiation of basophils in the human and rat pituitary. *Journal of clinical pathology,* 13**,** 256-260.

Takahashi, S. 1991. Immunocytochemical and immuno-electron-microscopical study of growth hormone cells in male and female rats of various ages. *Cell and tissue research,* 266**,** 275-284.

Tannenbaum, G. S., Epelbaum, J. & Bowers, C. Y. 2003. Interrelationship between the novel peptide ghrelin and somatostatin/growth hormone-releasing hormone in regulation of pulsatile growth hormone secretion. *Endocrinology,* 144**,** 967-974.

T**auiguchi, Kanezaki & Mikami 1989.** Immunocytochemical and morphometric studies on the pars distalis of the golden hamster from perinatal to senile stage. *Nippon-Juigaku- Zasshi Oct,* 51(5)**,** 893-903.

Wilson, D. B. & Wyatt, D. P. 1988. Immunofluorescent analysis of somatotroph distribution in the adenohypophysis of developing lit/lit mice. *Journal of anatomy,* 156**,** 51.

Xiaojing, Z., Qiaoqiong, T., Jiazhe, Y., Jiabao, H., Xiaoge, G., Yajun, W., Shanliang, X. & Danli, W. 2018. Cloning and differential expression of the growth hormone in Pampus argenteus. *Journal of Applied Ichthyology,* 34**,** 954-963.

Zhang, H., Qi, Q., Chen, T., Luo, J., Xi, Q., Jiang, Q., Sun, J. & Zhang, Y. 2018. Age-Related Changes in MicroRNA in the Rat Pituitary and Potential Role in GH Regulation. *International journal of molecular sciences,* 19**,** 2058.

المسح المعتمد على العمر لهرمون النمو فى الغدة النخامية للفئران؛ دراسات هستولوجية وهيستوكيميائية وهيستوكميائية مناعية

الملخص العربى

تم إجراء الدراسة الحالية لدراسة التعبيرالمناعي المعتمد على العمر و توطين هرمون النمو في الغدة النخامية. تم إيواء عشرون من الفئران (خمسةعشراناث وخمس ذكور), بأربعة حيوانات لكل قفص مع ملاحظة الحمل. جمعت العينات من الغدة النخامية من مختلف الأعمار من ذرية الذكور. تم معالجة العينات النسيجية وصبغاتها بالهيماتوكسيلين والايوسين والصبغة الخاصة و تقنية النسيجوكيميائية المناعية. قسمت القطاعات النسيجية الى اربعة مراحل على حسب النتائج الهيستولوجية للغدة النخامية. أظهرت نتائج الهيماتوكسيلين والايوسين أن هناك تغيرات نسيجية في الفص الامامى للغدة النخامية في جميع المراحل. فى المرحلة الاولى كانت الخلايا الحامضية هي الخلايا المميزة لكن كان من الصعب رؤية الخلايا القاعدية، ثم ظهرت في المرحلة الثانية. و باستخدام الصبغة الخاصة لوحظ زيادة اعداد الخلايا المفرزة لهرمون النمو من المرحلة الاولى الى الثالثة ثم تنخفض فى المرحلة الاخيرة. أظهرالتعبيرالمناعي لهرمون النمو في الفص الامامى للغدة النخامية أنا لخلايا المفرزة لهرمون النمو كانت عديدة وموزعة بشكل موحد في المراحل الثلاث الأولى. ولكن، في المرحلة الأخيرة، انخفض عددها وتقيدت في مجموعات.