Effect of Induced Hypoglycemia on Postnatal Development of The Adrenal Gland in Albino Rats

Asmaa Monir, Abdelmonem Hegazy and Dalia Mandour

Department of Human Anatomy and Embryology, Faculty of Medicine, Zagazig University, Egypt

Corresponding author: Asmaa Monir Eltaweel
E-mail: asmaaeltaweel30@gmail.com Phone: 002-01157188450

ABSTRACT

BACKGROUND: Hypoglycemia is a life threatening stressor that is usually encountered in the neonatal period due to congenital hyperinsulinism, inborn error of carbohydrate metabolism or intensive insulin therapy for juvenile onset diabetes mellitus. Recurrent hypoglycemia usually affects the function of the adrenal gland. This evoked a question in this study; does this disaster stressor also affect the postnatal development of this gland?.

OBJECTIVES: This study was designated to elucidate the effect of experimentally-induced recurrent episodes of hypoglycemia on the postnatal development of the adrenal gland in the albino rat.

MATERIAL AND METHODS: Offspring rats of two weeks age were randomly divided into three equal groups, of 24 each. Group 1 (Negative control group): the offspring were not given any medication. Group 2 (Positive control group) were injected with 0.3ml normal saline subcutaneously (SC) three times weekly. Group 3 (Hypoglycemic group) were exposed to hypoglycemic episodes via SC injection with 3 IU/kg of human regular insulin three times weekly. Each of the above-mentioned groups was further subdivided (according to the week of sacrifice) into four equal subgroups. 3 weeks (Neonatal), 7 weeks (Pubertal), 11 weeks (Young adult) and 16 weeks (Adult) subgroups. Adrenal gland specimens were processed for light and electron microscopic studies and morphometric measurements including the thickness and cell count of each zone of the gland were performed. Also, body weight and serum cortisol level were measured.

RESULTS: In both control groups, the adrenal gland of 3 weeks old rats revealed ill-demarcation between the cortex and medulla. With a stepwise age progress, at the 7th week there was an apparent differentiation of the cortex into three zones; zona glomerulosa (ZG), zona fasiculata (ZF) and zona reticularis (ZR). At the 11th week, a differentiation of ZG into outer small and inner large cells was noticed. Ultrastructurally, at 3,7 and 11 weeks, the cortical cells exhibited the normal steroid synthesis criteria of euchromatic nuclei, many mitochondria, smooth endoplasmic reticulum (sER) and many lipid droplets. Also, the chromaffin cells of the medulla displayed their characteristic secretory granules with nerve fibers inbetween. Interestingly, at the 16th week; nearly similar histological features like those of the previous age subgroup were encountered together with further differentiation of ZF into outer large and inner small cells. On the other hand, hypoglycemia led to detrimental effects on the normal histological architecture of the adrenal gland, where at the 3rd week, focal hyperplastic areas in the cortex and mild congestion in the medulla were noticed, while at the 7th week, the cortical zones exhibited more hyperplasia with foci of lipid depletion. Moreover, at 11th week, partial degenerative changes were displayed, especially in the ZG in the form of irregular small pyknotic nuclei and distorted mitochondria. At 16th week, the sings of degeneration became prominent in all zones together with marked congestion, cellular infiltration and a decrease in the secretory granules of the medulla. Compared to the both control groups, the hypoglycemic rats exhibited a mild change in the thickness and the cell count of the cortical zones and the medulla. Also, hypoglycemia led to a relative decrease in the body weight and the serum cortisol level at the 16th week subgroup.

CONCLUSION: It could be concluded that hypoglycemia had age-dependent detrimental drawbacks on the postnatal development of the adrenal gland in a zone-specific manner.

KEY WORDS: Experimentally Induced Hypoglycemia, Postnatal Development, Adrenal Gland, Albino Rats
INTRODUCTION

In humans, the adrenal gland develops from two distinct embryological tissues: a mesodermal tissue, the intermediate mesoderm, which differentiates into the adrenal cortex and a neuroectodermal tissue, the neural crest, which differentiates into the adrenal medulla (Hammer et al. 2005). Actually, the development of the adrenal cortex begins at about the 4th week of gestation, in the notch between the dorsal mesentery of the gut medially and the urogenital ridge laterally, in the form of a wave of proliferating mesothelial cells of coelomic epithelium that invade the overlying mesenchyme of the intermediate mesoderm to form the adrenocortical primordium (the primitive adrenal cortex). By the 5th week, these primitive cells begin to migrate laterally and cranially to be accumulated eventually at the cranial pole of the mesonephric blastema forming "the adrenal blastema" (Ishimoto and Jaffe, 2011).

On the other hand, the development of the adrenal medulla begins at about the 6th week of gestation in the form of medullary primordium which commences as an assembling of sympathogonia from the sympathetic ganglia that are derived from the neural crest. These cells are differentiated into pheochromoblasts that migrate and invade the adrenal blastema from its medial aspect to be settled in its core (Ishimoto and Jaffe, 2011).

The development of the adrenal gland is reported to be continued postnatally and the age of complete maturation with maximal functional capacity is not yet settled, however some researchers have declared that full development of the adrenal is established nearly at adulthood (Wahdan, 2005). Interestingly, the postnatal development of the adrenal gland is reported to be affected by many endogenous factors like corticotrophin releasing hormone (CRH) of the hypothalamus, adrenocorticotropic hormone (ACTH) of the anterior pituitary gland, neurotransmitters, cytokines, and some growth factors. In addition, the development is affected by some exogenous factors including drugs, toxins and various stressors (Hammer et al., 2005; and Karpe et al., 2005).

Neonatal hypoglycemia is one of the most life threatening stressors that usually affects the function of the adrenal gland of human newborns and infants (Cryer et al., 2003). The common causes of hypoglycemia during these life periods are congenital hyperinsulinism, inborn error of carbohydrate metabolism (McGowan, 2006) and intensive insulin therapy for juvenile onset (type I) diabetes mellitus (Jones and Davis, 2003). The neonatal hypoglycemia may be asymptomatic or symptomatic with episodes of sweating, pallor, hypothermia, tremors, lethargy, stupor and/or convulsions. If this hypoglycemia is not recognized and promptly treated, it mostly leads to deleterious neurologic sequelae (Jain et al., 2010).

Actually, sparse studies have been found in the literature regarding the effect of the neonatal hypoglycemia on the development of various body organs particularly the brain (Ennisa et al., 2008 and Rao et al., 2010). Despite this stressor has also detrimental effects beyond the brain, yet little attention has been paid to its effect on the development of the adrenal gland which is an "essential to life" gland. This was a motive to accomplish this study in which the impact of experimentally-induced hypoglycemia on the postnatal development of this gland was histomorphometrically assessed in the albino rat.

MATERIAL AND METHODS

Animals:

In this study, 12 pregnant albino Wister rats were used. These rats were obtained from the animal house unit in the Faculty of Medicine, Zagazig University. The rats were housed in plastic cages at a controlled temperature (25±2°C), with a 12 hour dark/light cycle and were supplemented with a standard laboratory pellet diet and water ad libitum. The experimental procedures were performed in accordance to the internationally accepted guidelines for ethical care and use of laboratory animals and were approved from the committee of Institutional Research Board (IRB) of Faculty of Medicine, Zagazig University, Egypt.

Experimental design:

After delivery, all offspring were left with their dams till the age of weaning (21st day postnataley), after which the offspring were housed in separate cages and were allowed the standard pellet diet and water ad libitum.

Experimental groups:
Offspring rats of two weeks age (equivalent to that of full term human newborns, *Avishai-Eliner et al. 2002*) were randomly divided into three equal groups, of 24 each.

**Group 1 (Negative control group):**  
The offspring of this group were not given any medication.

**Group 2 (Positive control group):**  
The offspring were injected with 0.3ml normal saline subcutaneously (SC), three times weekly.

**Group 3 (Hypoglycemic group):**  
The offspring were subjected to hypoglycemic episodes via SC injection with 3 IU/kg of human regular insulin (Novo Nordisk Inc., A/S Denmark) (*Fujino and Fujii, 2000*) dissolved in 0.3ml normal saline three times weekly.

Each of the above-mentioned groups of the offspring was further subdivided (according to the week of sacriﬁction) into four equal subgroups, of 6 rats each as follows:

- **3 weeks (Neonatal) subgroup:** In this subgroup, the rats were sacrificed at the age of 3 weeks.
- **7 weeks (Pubertal) subgroup:** These rats were sacrificed at the age of 7 weeks.
- **11 weeks (Young adult) subgroup:** These rats were sacrificed at the age of 11 weeks.
- **16 weeks (Adult) subgroup:** These rats were sacrificed at the age of 16 weeks.

**Experimental procedures:**  
**Induction of experimental hypoglycemia:**  
Insulin-induced hypoglycemic episode was encountered in the offspring by injecting each rat SC with 3 IU/kg of regular insulin dissolved in 0.3ml normal saline (*Fujino and Fujii, 2000*). Two hours after insulin injection, tail vein blood samples were obtained and the glucose level was measured using a blood glucometer (One touch pulse, Accu-cheq Performa, Roche Diagnostics, Germany) that was standardized periodically every week. The rats with blood glucose level of 40-55 mg/dl were considered hypoglycemic. This level is conventionally used to match the hypoglycemia in human newborns (*Cornblath et al. 2000 and Burns et al. 2008*).

**Dissection and isolation of the adrenal glands:**  
At the end of the experiment, the rats were weighted and anesthetized by 40mg/kg BW intraperitoneal sodium pentobarbital then, blood samples were withdrawn from the retro-orbital venous plexus and the serum was separated and stored at -20°C for further measurement of the serum cortisol. Afterwards, a midline laparotomy was done then the adrenal glands were dissected free from the adherent fats and connective tissue (CT) and rapidly excised out of the body to be processed for light and electron microscopic studies.

**Light microscopic (LM) study:**  
One of the excised adrenal glands was fixed in 10% neutral-buffered formalin for 24 hours, then it was dehydrated in ascending grades of ethanol and cleared in xylene then embedded in paraffin blocks from which 5 μm thick sections were cut and stained with Hematoxylin and Eosin (H&E) stain to study the general histological architecture of the adrenal tissue (*Bancroft and Lyton 2013*) and with a silver stain to reveal the reticular ﬁbers among the cells of the cortex and those of the medulla of the adrenal gland that appear as dark brown in colour (*Bradbury and Gordon 1990*). Finally, the stained LM sections were examined using a Leica light microscope (Leica DM LS2, Wetzlar, Germany) and were photographed with a digital camera (Leica Qwin standard, CH-9435 DFC 290, Wetzlar, Germany) in the department of Histology, Faculty of Medicine, Zagazig University.

**Transmission electron microscopic (TEM) study:**  
Adrenal specimens of about one mm³ were cut from one of the obtained adrenal gland and fixed in 2.5% glutaraldehyde buffered with 0.1M phosphate buffer saline (PBS, pH 7.4) for 24 hours at 4°C. Thereafter, the specimens were postfixed in 1% osmium tetroxide in PBS at 4°C for one hour followed by their dehydration in ascending grades of ethanol then embedded in epoxy resin forming gelatin capsules. Semithin sections (1μm) were stained with toluidine blue and examined using the Leica light microscope to detect a field of interest for the next ultrathin sections. Ultrathin sections of 50nm were cut, mounted on copper grids and stained with uranyl acetate and lead citrate (*Bozzola and Russel, 1999*). Finally, the grids were examined and photographed using a JEOL JEM-100 SX transmission electron microscope (JEOL Ltd, Tokyo, Japan) in the EM unit of Faculty of Medicine, Tanta University, Egypt.
Histomorphometric study:

H&E stained sections at magnification of x100 were morphometrically analyzed using the image analyzer computer system (Leica Qwin 500, Leica Ltd, Cambridge, UK) in the Image Analysis Unit of the Pathology Department, Faculty of dentistry, Cairo University, Egypt. The mean thickness in microns and the mean cell count of each of the three cortical zones; zona glomerulosa (ZG), zona fasciculata (ZF) and zona reticularis (ZR) and those of the medulla were measured from the 7th to the 16th week of age.

Measurement of the body weight (in grams):

This was performed for all age subgroups of rats at the day of sacrifice.

Biochemical study:

Measurement of serum cortisol level (in µg/dl) using ELISA kits (Jameel et al., 2014) of all age subgroups of rats at the day of sacrifice.

Statistical analysis:

The obtained quantitative results (morphometric, body weight and serum cortisol) were statistically analyzed using the program of Statistical Package for Social Science (SPSS, version 19, Inc, Chicago, IL, USA). One way analysis of variance (ANOVA) was used to detect the statistical significance among the different age subgroups and was followed by the post hoc test; least significant difference (LSD) for inter-subgroup comparison. Also, unpaired Student t-test was used to determine the significant differences between each two groups. A value of P ≤ 0.05 was considered statistically significant (Petrie and Sabin, 2005).

RESULTS

In this study, no marked difference in the histological results were displayed between the negative and the positive control rats except mild histological changes that encountered in the positive control group at the 16th week compared to the age-matched negative control group.

LM results

H&E stained sections in the adrenal gland of the negative control group at 3 weeks postnatally showed that the whole gland was surrounded by a thin capsule with an ill-differentiation between the cortex and the medulla and among the three cortical zones (Fig. 1A). The cells of the outer part of the cortex, beneath the capsule, that represented the prospective ZG (PZG) were irregularly arranged with rounded basophilic nuclei and faintly stained acidophilic vacuolated cytoplasm together with mitotic figures (Fig. 1B). The middle part of the cortex that represented the prospective ZF (PZF) showed cells that were arranged in columns of two to three cell wide and had rounded basophilic nuclei with pale acidophilic vacuolated cytoplasm and were separated by connective tissue (CT) strands containing fibroblasts and blood sinusoids (BS) (Fig. 1B). The silver-stained sections of 3 weeks old negative subgroup revealed scanty reticular fibers among the cells of the cortex and the medulla (Fig. 1C).

On the contrary, H&E stained sections in the adrenal gland of the hypoglycemic group at 3 weeks old showed mild morphorological changes compared the age-matched negative control subgroup. There was congestion of BS in the prospective zona reticularis (PZR) and the medulla (Fig. 1D). In addition, there were focal hyperplastic areas having increased number of closely packed small cells with deep basophilic nuclei in PZG. Also, some cells of PZF exhibit little lipid depletion with less vacuolated deeply acidophilic cytoplasm (Fig. 1E). The silver stained sections of this age subgroup showed little reticular fibers in the cortex and medulla compared with the age-matched negative control subgroup (Fig. 1F).

Examination of H & E stained sections in the adrenal gland of the negative control group at 7 weeks old revealed a relatively thicker capsule with apparent demarcation between the cortex and medulla, and differentiation between ZG, ZF and ZR compared to the 3 weeks old subgroup. Also, a narrow band of zona intermedia (ZI) was noticed between ZG and ZF (Fig.2A). The ZG showed regularly arranged ovoid clusters of polyhedral cells with vacuolated acidophilic cytoplasm with basophilic nuclei. The cells of ZI were small with acidophilic cytoplasm and central basophilic nuclei. Some ZG and ZI cells showed mitotic figures (Fig.2B). The cells of ZF were large in size, having rounded vesicular nuclei, vacuolated acidophilic cytoplasm and arranged in narrower cell columns (one or two cells wide) separated by CT strands and BS (Fig.2B). In the silver stained sections of 7 weeks old negative control rats, the amount of
reticular fibers in the cortex and medulla increased slightly in comparison with the previous 3 weeks old negative control subgroup.

On the other hand, H&E stained sections in the adrenal gland of the hypoglycemic group at 7 weeks old showed diffuse dense basophilic areas in ZF, ZR and the medulla together with congested medullar BS (Fig. 2C). Some cells of ZG having more vacuolated cytoplasm and pyknotic nuclei. Moreover, some areas of ZF displayed foci of hyperplasia with increased nuclear density and others exhibited lipid depletion with less vacuolated deeply acidophilic cytoplasm (Fig. 2D). The silver stained sections of the adrenal gland of 7 weeks old hypoglycemic rats revealed defined reticular fibers among the cells of the cortex and those of the medulla compared with the age-matched control subgroup.

Interestingly, H & E stained sections in the adrenal gland of the negative control group at 11 weeks postnatally revealed that the cells of ZG were differentiated into outer small cells with little lipid-loaded cytoplasm (little vacuolation) and inner large cells with more lipid-loaded cytoplasm (more vacuolation) (Fig. 3A). The cells of ZF were polyhydral with pale acidophilic lipid-loaded (vacuolated) cytoplasm and rounded vesicular nuclei. The cells were arranged in narrower (one cell wide) fascicles separated by CT strands (Fig. 3A). The cells of ZR possessed deep basophilic nuclei and acidophilic cytoplasm and were arranged in network of anastomosing short cords. The medullary cells were large with vesicular nuclei and basophilic cytoplasm and were arranged in clusters. The cords of ZR and clusters of the medulla were separated by much CT strands and many wide BS (Fig. 3B). In the silver stained sections of the 11th week negative control subgroup, the reticular fibers were regularly arranged and increased in amount (Fig. 3C).

Comparatively, light microscopic examination of the adrenal gland in 11 weeks old hypoglycemic rats displayed areas of disarranged cells of the ZG and ZF with marked vacuolation and pyknotic nuclei and hyperplastic foci with increased density of CT strands (Fig.3D). Also, ZR and medulla had disarranged cells and focal areas of hyperplasia and many dilated BS (Fig.3E). The silver stained sections of adrenal gland of hypoglycemic 11th week old rats revealed much more reticular fibers with increased density that were irregularly distributed compared with the age-matched negative control subgroup (Fig.3F).

Obviously, H & E stained sections of the negative control group at 16 weeks postnatal period revealed somewhat similar histological pictures with the previous 11 weeks age subgroup except some more developmental features, where the capsule became thicker, the demarcation between the cortex and medulla become more sharp with obvious differentiation among the cortical zones with distinct ZI. In addition, the cells of ZG displayed glomeruli-like appearance. Also, the cells of ZF have got differentiated into outer ZF with vacuolated cytoplasm and inner ZF with little vacuolated cytoplasm (Fig. 4A). The cells of ZR showed more acidophilic cytoplasm with more deeply-stained basophilic nuclei. The medullary cells were more arranged with more CT strands inbetween its clusters (Fig. 4B). The silver stained sections of 16 weeks old negative control rats revealed no apparent increase of the reticular fibers in the cortex and medulla compared with the previous 11 weeks old negative control subgroup.

On the contrary, light microscopic examination of the adrenal gland at 16 weeks old hypoglycemic rats revealed lipid depletion, hyperplasia and focal areas of degeneration in the cortical tissue (Fig. 4C). The cells of ZR and medulla were disarranged, displayed pathologically vacuolated cytoplasm, pyknotic nuclei, diffuse hemorrhage and diffuse hyperplasia (Fig. 4D). The silver stained sections of 16 weeks old hypoglycemic rat showed much increase in the density of the reticular fibers among the cortical zones and in the medulla compared with the age-matched negative control subgroup.

**TEM results**

TEM examination of the ultrathin adrenal sections of the negative control group at 3 weeks old revealed the cells of ZG having pyramidal shape with euchromatic nuclei and prominent nucleoli. The cytoplasm contains many mitochondria, smooth ER (sER), rough ER (rER) and many electron-lucent lipid droplets (Fig 5A). At this age subgroup, the cells of the ZF and ZR had also euchromatic nuclei, many mitochondria, sER and lipid droplets. The medulla contained the chromaffin cells with euchromatic nuclei, rER, and secretory granules of variable electron
density. Unmyelinated and myelinated nerve fibers were observed between the medullary cells (Fig. 5B). Comparatively, TEM examination of the adrenal cortex of 3 weeks old hypoglycemic rats displayed focal areas of hyperplasia (increased number and decreased size of cells), small elongated hyperchromatic nuclei and accumulated lipid droplets that had variable electron density and size (Fig 5C and 5D).

Ultrathin sections in the adrenal gland of the negative control rats at 7 weeks old showed somewhat similar ultrastructural features like those of 3 weeks subgroup with more apparent steroid synthesis criteria, viz., many mitochondria, sER, much lipid droplets, and peroxisomes (Fig. 6A and 6B). On the other hand, ultrathin sections of the adrenal gland of 7 weeks old hypoglycemic rats showed the cells of ZG with dark irregular pyknotic nuclei, swollen mitochondria with ruptured cristae and condensed electron-dense lipid droplets (Fig. 6C). Furthermore, the cells of ZF were hyperplastic with hyperchromatic nuclei with little electron dense lipid droplets (lipid depletion) (Fig. 6D). Congested BS were observed in-between the cells of ZF, ZR and those of medulla.

Ultrastructurally, the sections in the adrenal gland of the negative control rats at 11 weeks old showed ZF exhibiting light and dark cells with euchromatic nuclei, many mitochondria and lipid droplets (Fig.7A). Moreover, the cells of ZR had small rounded nuclei, few lipid droplets, many mitochondria and lysosomes. Wide BS were observed in-between the ZR cells (Fig.7B). On the contrary, TEM examination of the adrenal gland of 11 weeks old hypoglycemic rats revealed the cells of ZF having euchromatic nuclei, large number of swollen mitochondria with ruptured cristae, numerous dilated sER, hypertrophied Golgi apparatus and scanty lipid droplets (Fig. 7C). Moreover, the cells of ZR and medulla displayed pyknotic and irregular nuclei and distorted mitochondria. The medulla displayed a decrease in the secretory granules of its light and dark chromaffin cells with infiltration by lymphocytes. Also, congested BS were observed in-between ZR cells and in medulla (Fig. 7D).

Ultrathin sections in the adrenal gland of the negative control group at 16 weeks old showed somewhat similar ultrastructural features like those of the 11 weeks negative subgroup, except that the ZF was characteristically differentiated into outer large fasciculata cells with more lipid droplets and inner small fasciculata cells with little lipid droplets (Fig. 8A and 8B). Comparatively, the ultrathin sections of the adrenal gland of 16 weeks old hypoglycemic rats displayed sings of cellular degeneration that were more prominent in the ZG. Moreover, the ZF cells showed hypertrophied nuclei, swollen mitochondria with ruptured cristae and dilated sER (Fig. 8C). The medulla revealed irregular nuclei, distorted mitochondria, multiple vacuoles and much decrease in the secretory granules of its chromaffin cells (Fig. 8D).

Histomorphometric results

In the negative control group, there was a significant progressive increase in the thickness of ZG, ZF, ZR and adrenal medulla till the age of 11 weeks, after which there was no significant difference in the measurement of this thickness till the 16th week. On the other hand, in the hypoglycemic group, there was a significant progressive increase in the thickness of both ZG and ZR till the age of 11 weeks; however, there was a significant progressive increase in the thickness of ZF till the age of 16 weeks. The medulla showed a progressive increase in its thickness till the age of 11 weeks after which it decreased till the age of 16 weeks (Table 1).

Regarding the cell count of three cortical zones, in the negative control group, there was no significant increase in the cell count of ZG and ZF till the age of 16 weeks, while there was a significant progressive increase in the cell count of ZR and the medulla till the age of 16 weeks. However, in the hypoglycemic group, there was no significant increase in the cell count of ZG till the age of 16 weeks together with a significant progressive increase of ZF till 11th week and ZR and medulla till 16th week (Table 2).

Results of the body weight

In the present study, no significant difference in the body weight was encountered between the negative and hypoglycemic groups at the age of 3 and 7 weeks; however, there was a significant decrease in the body weight in the hypoglycemic rats at the age of 11 and 16 weeks compared to the age-matched negative control group (Table 3).

Results of the serum cortisol

In the present study, no significant difference in the serum cortisol was displayed
between the negative control and hypoglycemic groups at the age of 3 and 7 weeks. However, there was a significant increase in its level at the age of 11 weeks and a significant decrease at 16 weeks in the hypoglycemic rats compared to the age-matched negative control group. Also, in the hypoglycemic group, the serum cortisol progressively increased till the age of 11 weeks after which it decreased till the age of 16 weeks (Table 3).

Fig. (1): Photomicrographs of the adrenal gland of 3 week old negative control (A,B&C) and hypoglycemic (D,E&F) rats
A: A thin capsule (cap), ill-differentiation among the cortical zones and between the cortex (C) and medulla (M) with a central medullary vein (CV) (H&E X100).
B: Irregularly arranged cells of prospective zona glomerulosa (PZG) just beneath the capsule (cap). Few mitotic figures (arrow head) are seen. The cells of prospective zona fasciculata (PZF) are arranged in wide columns that are separated by connective tissue strands with fibroblasts (arrow) and blood sinusoids (BS) (H&E X400).
C: Ill-defined scanty brown reticular fibers (arrows) in the cortex (C) below the capsule (cap) (Silver stain X400).
D: Mild congested BS (arrow head) in the PZR and in the medulla (M) (H&E X100).
E: Focal hyperplastic area (H) in PZG having increased number of closely packed small cells with deep basophilic nuclei. Some cells of PZF exhibit lipid depletion having less vacuolated deeply acidophilic cytoplasm (arrows) (H&E X400).
F: Few reticular fibers (arrows) in the cortex (C) below the capsule (cap) (Silver stain X400).
Fig. (2): Photomicrographs of the adrenal gland of 7week old negative control (A&B) and hypoglycemic (C&D) rats

A: Apparent demarcation between the cortex and medulla (M) with differentiation between ZG, ZF and ZR. A narrow band of zona intermedia (ZI) was noticed between ZG and ZF (H&E X100).

B: Relatively thick capsule (Cap), the cells of ZG are arranged in clusters separated by CT strands (arrow). Cells of ZI appear small and crowded with acidophilic cytoplasm and central rounded nuclei. Some ZG and ZI cells showed mitotic figures (arrow head). The cells of ZF are arranged in narrower cell columns separated by CT strands (arrows) and BS (H&E X400).

C: There are diffuse dense basophilic areas (arrows) in ZF, ZR and in medulla (M). Moderate congested medullary BS (arrow head) (H&E X100).

D: Some cells of ZG with more vacuolated cytoplasm and pyknotic nuclei (arrow head). Some areas of ZF display foci of hyperplasia (H) with increased nuclear density and others exhibit lipid depletion with less vacuolated deeply acidophilic cytoplasm (curved arrows) (H&E X400).

Fig. (3): Photomicrographs of the adrenal gland of 11week old negative control (A,B&C) and hypoglycemic (D,E&F) rats

A: A thicker capsule (cap). The cells of ZG are differentiated into smaller cells (SG) with little vacuolation and large cells (LG) with more vacuolation. The cells of ZF are polyhedral with pale acidophilic, vacuolated cytoplasm and rounded vesicular nuclei. The cells were arranged in one cell wide fascicles separated by CT strands (arrows) (H&E X400).

B: The cells of ZR appear small with deep basophilic nuclei and pale acidophilic cytoplasm. The cells are arranged in network of anastomosing short cords. The medullary cells are large with vesicular nuclei and basophilic cytoplasm. The cells are distributed in clusters. The cords of ZR and clusters of medulla (M) are separated by much CT strands (arrows) and many wide BS (H&E X400).

C: Increased reticular fibers (arrows) in the capsule (cap) and between the cell clusters of ZG and the cords of ZF (Silver stain X400).

D: Areas of disarranged cells of ZG and ZF with marked vacuolation and pyknotic nuclei (arrow head) and other areas with hyperplastic foci (H). Increased density of CT strands (arrows) is noticed (H&E X400).

E: ZR and medulla (M) with disarranged cells and focal areas of hyperplasia (H) and dilated BS inbetween (H&E X400).

F: Much more and irregularly distributed reticular fibers (arrows) in the capsule (cap), ZG and ZF (Silver stain X400).
Fig. (4): Photomicrographs of the adrenal gland of 16 week old negative control (A&B) and hypoglycemic (C&D) rats

A: Thick capsule (Cap) and sharp differentiation between ZG, ZF, ZR and medulla (M) with distinct ZI. Also, cells of ZF are differentiated into outer fasciculata (OFZ) with vacuolated cytoplasm and inner fasciculata (IFZ) with little vacuolated cytoplasm (H&E X100).

B: The cells of ZR appear small with deep basophilic nuclei and acidophilic cytoplasm. The cells are arranged in network of short cords and BS in-between. The medullary cells (M) are large with vesicular nuclei and basophilic cytoplasm. The cells are arranged in clusters that are separated by much CT strands (arrows) and BS (H&E X400).

C: Areas of degeneration in ZG and ZF (arrow heads) and focal basophilic areas in ZF and ZR (arrows). Also, lipid depletion (curved arrows) is noticed in ZF and ZR (H&E X100).

D: Disarranged cells of ZR and medulla (M) with diffuse hemorrhage (hg) and hyperplastic (H) areas. Some areas showed marked vacuolation with pyknotic nuclei (arrow head) (H&E X400).

Fig. (5): TEM micrographs of the adrenal gland of 3 week old negative control (A&B) and hypoglycemic (C&D) rats

A: The cells of ZG appear pyramidal in shape with euchromatic nucleus (N) and prominent nucleolus (no). The cytoplasm contains many mitochondria (m), sER (arrow head), rER and electron-lucent lipid droplets (L) (TEM X7500).

B: The medulla shows the chromaffin cells (ch) having euchromatic nuclei (N), rER, and granules of variable electron density (G). BS, unmyelinated (um), myelinated (my) nerve fibers with Schwann cell nucleus (Sc) are seen between the cells (TEM X7500).

C: The cells of ZG have focal areas of hyperplasia (increased number & decreased size) with small elongated irregular nuclei (N) and accumulation of lipid droplets (L) of variable electron density and size (TEM X5000).

D: The cells of ZR have small nuclei (N), some distorted mitochondria (m) and accumulated electron dense lipid droplets (L) of variable size. Also, monocytes (mo) inside the BS and tissue macrophage (mg) in-between the cells are seen (TEM X5000).
Fig. (6): TEM micrographs of the adrenal gland of 7 week old negative control (A&B) and hypoglycemic (C&D) rats
A: A cell of ZG with an euchromatic nucleus (N), intact mitochondria (m), electron-lucent lipid droplets (L), sER (arrow head), rER and peroxisomes (arrow) (TEM X10000).
B: An electron micrograph of 7 weeks old negative control rat showing the cells of ZF having euchromatic nuclei (N), many mitochondria (m), electron-dense lipid droplets (L), sER (arrow head), rER and peroxisomes (arrow) (TEM X5000).
C: cells of ZG with dark irregular pyknotic nucleus (N), swollen mitochondria (m) with ruptured cristae and condensed electron-dense lipid droplets (L). Also, phagosomes (arrows) are seen (TEM X10000).
D: Cells of ZF showing a focal area of hyperplasia (increased cell number) with hyperchromatic nuclei (N). Little electron dense lipid droplets (L) (lipid depletion) are observed. Congested BS are seen (TEM X5000).

Fig. (7): TEM micrographs of the adrenal gland of 11 week old negative control (A&B) and hypoglycemic (C&D) rats
A: ZF show light (*) and dark cells (**) with large rounded euchromatic nuclei (N), many mitochondria (m), electron-dense lipid droplets (L) and small dense bodies (arrow). Also, BS is seen. (TEM X5000).
B: Cells of ZR with small rounded nuclei (N), few lipid droplets (L), many mitochondria (m), lysosomes (arrow). BS appear between the cells (TEM X5000).
C: A cell of ZF having euchromatic nucleus (N), large number of swollen mitochondria (m) with ruptured cristae, numerous dilated sER (arrow head), hypertrophied Golgi apparatus (Ga) and little lipid droplets (L) (TEM X10000).
**D:** Corticomedullary junction showing cells of ZR with pyknotic and irregular nuclei (N), distorted mitochondria (m) and accumulation of lipid droplets (L). The medulla (M) shows light (🔹) and dark (🔹🔹) chromaffin cells with vacuoles (arrow head) in their cytoplasm and a decrease in the secretory granules (G). Congested BS with a lymphocyte (arrow) are noticed (TEM X5000).

**Fig. (8):** TEM micrographs of the adrenal gland of 16 week old negative control (A&B) and hypoglycemic (C&D) rats

**A:** Cells of outer ZF show large rounded euchromatic nuclei (N) and packed cytoplasm with many lipid droplets (L). BS with eosinophil (eo) and macrophage (mg) are noticed (TEM X5000).

**B:** Cells of inner ZF with small euchromatic nuclei (N) and little lipid droplets (L). Some cells have pyknotic (P) nuclei. Also, BS is noticed (TEM X5000).

**C:** Cells of ZF with hypertrophied nuclei (N), peripheral nucleolus (n) and perinuclear space (arrow). Also, swollen mitochondria (m) with ruptured cristae and dilated sER (arrow head) are noticed (TEM X10000).

**D:** The medulla shows the chromaffin cells with apparent decrease in the secretory granules (G). The nuclei (N) of these cells are irregular and the cytoplasm exhibit distorted mitochondria (m) and multiple vacuoles (arrows) (TEM X7500).
### Table (1): Statistical comparison of the thickness of ZG, ZF, ZR and the medulla between the negative control and hypoglycemic groups at 7, 11 & 16 weeks of age (Mean±SD)

<table>
<thead>
<tr>
<th>Thickness of cortical zones</th>
<th>Negative control group</th>
<th>Hypoglycemic group</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZG</td>
<td>ZF</td>
<td>ZR</td>
</tr>
<tr>
<td>7W</td>
<td>111.9±15.0</td>
<td>1053.3±112</td>
</tr>
<tr>
<td>11W</td>
<td>144.4±24.2</td>
<td>1255.3±89.6</td>
</tr>
<tr>
<td>16W</td>
<td>142.7±6.9</td>
<td>1284.0±88.5</td>
</tr>
<tr>
<td>P value of ANOVA</td>
<td>0.000*</td>
<td>0.000*</td>
</tr>
</tbody>
</table>

### Table (2): Statistical comparison of the cell count of ZG, ZF, ZR and the medulla between the negative control and hypoglycemic groups at 7, 11 & 16 weeks of age (Mean±SD)

<table>
<thead>
<tr>
<th>Cell count of cortical zones</th>
<th>Negative control group</th>
<th>Hypoglycemic group</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZG</td>
<td>ZF</td>
<td>ZR</td>
</tr>
<tr>
<td>7W</td>
<td>322.0±22.7</td>
<td>313.0±10.4</td>
</tr>
<tr>
<td>11W</td>
<td>330.0±15.7</td>
<td>315.25±9.7</td>
</tr>
<tr>
<td>16W</td>
<td>334.0±18.2</td>
<td>322.1±15.0</td>
</tr>
<tr>
<td>P value of ANOVA</td>
<td>0.22**</td>
<td>0.315**</td>
</tr>
</tbody>
</table>

### Table (3): Statistical comparison of the mean values of the body weight and serum cortisol between the negative control and hypoglycemic groups at 3, 7, 11 & 16 weeks of age (Mean±SD)

<table>
<thead>
<tr>
<th>Negative control group</th>
<th>Hypoglycemic group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (gm)</td>
<td>Serum cortisol (ug/dl)</td>
</tr>
<tr>
<td>3W</td>
<td>47.4 ± 2.9</td>
</tr>
<tr>
<td>7W</td>
<td>109.3±5.4</td>
</tr>
<tr>
<td>11W</td>
<td>184.8±3.4</td>
</tr>
<tr>
<td>16W</td>
<td>270.0±5.1</td>
</tr>
<tr>
<td>P value of ANOVA</td>
<td>0.000*</td>
</tr>
</tbody>
</table>

In table 1, 2 & 3, one way ANOVA is used to compare the means among the subgroups. Also, unpaired Student t-tests (P1, P2, P3 and P4 values) are used in comparing the means between the negative control and hypoglycemic groups at 3, 7, 11 and 16 weeks of age, respectively.

* : p value is significant (<0.05)  
NS: p value is non-significant (>0.05).
DISCUSSION

In this study, in an attempt to explore the impact of recurrent hypoglycemia on the postnatal development of the adrenal gland, an experimental work was settled in which a histomorphometry of this gland was assessed in a negative control, a positive control and a hypoglycemic group of rats, from the newborn till the adulthood period. The obtained results revealed a non-significant difference in the histological features between the positive and the negative control of rats except mild histological changes that encountered in the positive control group at the 16th week compared to the age-matched negative control group.

LM and TEM examination of the adrenal sections were performed in a chronological manner at 3, 7, 11 and 16 weeks postnatally. The results revealed full established differentiation among the cortical zones and obvious demarcation between the cortex and medulla at the 11th week (young adult) subgroup. However, at the 16th week (adult) subgroup, no much detectable changes was noticed in such demarcation. These findings were in line with Wagieh et al. (2009) who found that established demarcation between the cortex and medulla was reached at young adulthood in mice. This mostly indicated that the growth of the adrenal gland reach its maximum peak at the young adulthood in rodents. In this study, zona intermedia (ZI) appeared at 7 weeks between the ZG and ZF and persisted till the adulthood subgroup. Functionally, ZI is an inconstant narrow band of lipid-free cells with high mitotic figures serving as reserve cells that could be transformed into ZF cells (Mitani et al., 2003).

Characteristically, in the 3 weeks (neonatal) subgroup of the negative control rats, LM examination of the prospective ZG (PZG) revealed small irregularly arranged cells with mitotic figures, while at 7 weeks subgroup the cells of ZG became larger in size and arranged in ovoid clusters. At 11th week, these cells were differentiated into outer smaller cells with little lipid droplets and inner larger cells rich in these droplets. Additionally, on TEM examination from the newborns till the young adulthood of negative control rats, the cells of ZG displayed the steroid synthesis criteria, viz., many sER, mitochondria, Golgi apparatus and cytoplasmic lipid droplets together with small dense bodies, mostly peroxisomes. Nearly similar results were obtained by Wahdan, (2005) who also interpreted these dense bodies to be peroxisomes. Actually, together with mitochondria, the peroxisomes serve in cholesterol synthesis needed for steroidogenesis in the adrenal cortical cells (Midzak and Papadopoulos, 2015). With advancement of the development, at 16 weeks subgroup, no detectable morphometric differences could be noticed in ZG compared with the previous 11th week subgroup. These histological consecutive postnatal features of the ZG were confirmed by the concomitant morphometric measurements in the thickness of this zone that was progressively increased till the 11th week; after which no significant increase in the width till the age of 16 weeks. These morphometric results were consistent with Pingatelli et al. (1998) who found that ZG increased in width from the newborns till the postnatal day 75 (young adulthood) in rats; after which a stationary course of growth was established.

In this study, LM and TEM examination of the ZG of the hypoglycemic rats showed focal areas of hyperplasia in the form of smaller, more basophilic, and closely packed cells, especially at the age of 3 and 7 weeks subgroups. At 11 and 16 weeks hypoglycemic subgroups, more hyperplasia and some degenerative changes were encountered in the cells of ZG in the form of cellular disarrangement, pyknotic nuclei, highly vacuolated cytoplasm, destructed mitochondria, marked decrease in the cytoplasmic organelles and wide intercellular spaces compared to the normal histological features of age-matched negative control of rats. Being a serious stressor, chronic recurrent hypoglycemic episodes were able to induce the above-mentioned degenerative changes in the adrenal gland. These degenerative changes were also encountered following other types of chronic stress, including immobilization and noise stress in rats (Pellegrini et al., 1998 and Gannouni et al., 2014).

Regarding the normal postnatal development of ZF in the negative control rats in this study, LM examination displayed a chronological age-related changes, where the parenchymal cells of prospective ZF (PZF) at 3 weeks old were initially arranged in wide cords of 2-3 cell wide, while from 7th to 16th weeks, the cells became arranged in narrower 1-2 cell wide fascicles. This may indicated the occurrence of the normal autophagy with rearrangement of the ZF cells with the advancement of development. Particularly, at 16th week subgroup, the ZF was differentiated into 2 bands; outer ZF with relatively more lipid-loaded cytoplasm and hence appeared more vacuolated and inner ZF with less vacuolation. These results were in close conformity with Wahdan (2005) and Ulrich-Lai
et al. (2006) who found that ZF of the adult adrenal gland displayed 2 bands; an outer ZF which is more active secretory band than the inner one. Ultrastructurally, pyknotic nuclei were encountered in the inner ZF that mostly denoted gradual transmission from one zone into another. Also, the cells of ZF showed more prominent steroid secreting criteria. In addition, the morphometric study revealed that the width of ZF of the negative control group increased progressively and reached its maximal growth at the 11th week subgroup. This was consistent with the results of Pignatelli et al., 1998 who found that ZF increased progressively in width till the postnatal day 75 in rats.

On the other hand, in this study, LM examination of the ZF of the hypoglycemic rats displayed a progressive hyperplasia till the 11th week (young adult) subgroup. This indicated increased cell number of this zone with the progress of development as was confirmed by the significant increase in its cell count that reached its maximum at the 11th week. These LM results were associated with ultrastructural changes in the cells of ZF in the form of cellular hypertrophy with increased cell size, large number of swollen mitochondria, and hyperchromatic nuclei with clumped chromatin.

This hypoglycemic-induced hyperactivity of ZF cells was consistent with Rao (2015) who have reported that recurrent hypoglycemia led to activation of the hypothalamic-hypophyseal axis (HPA) that promotes the secretion of CRH and ACTH. The later hormone is known to have a proliferative effect on the whole adrenal gland with bilateral hyperplasia and hypertrophy of the three zones, particularly the ZF (Kobayashi et al., 2006 and Ferreira et al. 2007). Also, Ulch-Lai et al. (2006) have studied the effect of chronic variable stressors on the adrenal gland and they have found a significant increase of the adrenal weight and the DNA and RNA contents of the adrenal cells and they attributed these changes to the development of both cellular hyperplasia and hypertrophy following exposure to the stressors.

Also, chronic hypoglycemia in this work, led to lipid depletion with characteristic decrease in the cytoplasmic lipid droplets, particularly in the cells of ZF and ZR. The lipid depletion were mostly attributed to the increased secretory activity of these cells upon chronic stress of hypoglycemia (Monsefi et al., 2006). Functionally, ZF and ZR cells normally have a large store of lipids that are used as substrate for the steroidogensis process (Mahar et al., 2012).

The increase in the secretory activity of ZF and ZR cells following exposure to hypoglycemia was reflected upon the serum level of cortisol that synthesized and secreted mainly from these cells. Actually, serum cortisol was measured in this study to assess the function of adrenal cortex, and it was found to be progressively increased till the age of the 11th week. This progressive rise of serum cortisol was corresponding to the observation of Rao (2015) who related this response to the stimulatory effect of ACTH on the cells of ZF and ZR upon exposure to hypoglycemia.

In this work, in the negative control rats at 3 weeks subgroup, initially the prospective ZR cells were irregularly arranged, while at the 7th week (pubertal) subgroup, the cells were partially arranged in short cords. At 11th weeks, the cells become larger and were arranged in a network of short branching and anastomosing cords separated by more reticular fibers and wider BS. Also, the width of ZR was progressively increased till the age of 11th week and the cell count till the age of 16th week. These morphological results were supported by Hornsby (2002) who stated that ZR is an androgen secreting tissue that begins its activity at the embryonic life, however its secretory activity in the postnatal period is intermittent and reaches its maximum at the period of puberty, after which its activity remain quiescent.

Collectively, in this study, the hypoglycemic rats have displayed some degenerative changes in the cortical zones with congestion and infiltration by monocytes and lymphocytes. These changes started in the cells of ZG at the age of 11th week but they developed in the ZF and ZR cells at the age of 16th week. This may indicate that the ZG cells were more vulnerable to the detrimental effect of stress (Rao, 2015). The possible explanation of these degenerative changes is the released reactive oxygen species (ROS) upon exposure of the tissues to hypoglycemia. These ROS cause distortion of the cellular architecture with loss of cell membrane, mitochondrial and nuclear integrity (Anju et al., 2016).

These devastating effects of ROS may be the cause of decrease serum cortisol level at the 16th week old rats following a long period of
recurrent hypoglycemic episodes. This explanation was also advocated by Moslem and Arrak (2009) who attributed these results to the exhaustion and/or degeneration of the adrenal cortical cells. Moreover, Rao, 2015 has attributed this decline in serum cortisol to the development of “stress hyporesponsive period” with temporal HPA insensitivity of unknown mechanism. This condition is known as hypoglycemia-associated autonomic failure (HAAF) or hypoglycemia unawareness.

Interestingly, in this study, the adrenal medulla of the negative control group has reached its maximal growth at the age of 16th week (adulthood) that was confirmed by the histological and morphometric results. On light microscopic examination of the medulla of the 3 weeks old rats, it was composed of irregular small clusters of chromaffin cells. With advancement of development, at 7, 11, and 16 weeks, the chromaffin cells were gradually arranged in large clusters separated by wide BS and dense reticular fibers. Ultrastructurally, the cells of the medulla were differentiated especially at the young adult and the adult subgroups into dark cells with dark homogenous cytoplasm and light cells with pale granular cytoplasm. The dark cells "for adrenaline secretion" were presumed to be more active secretory cells than the light cells "for noradrenaline secretion" (Mughal et al., 2004). These results were in accordance with Wagieh et al. (2009) who found that the dark and light cells of the medulla have got maximal differentiation at around the 11th week postnatally in mice. These results were also in close proximity with Eranko and Raisanen, (2016) who found that the amount of catecholamines secreted from adrenal medulla steadily increased in the course of the development till the young adulthood. These normal stepwise developmental changes of the adrenal medulla were confirmed in this study by measuring its width and its cell count.

In this study, the effect of hypoglycemia on the postnatal development of the adrenal medulla was evident in the histological and morphometric studies. Initially, at 3 and 7 week subgroups, there were hyperplasia and congestion of BS and a decrease in the chromaffin cell granules at the 11th week. Obviously, at 16th week, degenerative changes of the medulla with a decrease in its thickness were encountered. Such sequence of stress-induced changes was termed "the general adaptation syndrome" of stress, that involves three stages, the first is the alarm stage in which the adrenal gland is stimulated but its function is still quiescent, second is the stage of adaptation in which adrenal hyperfunction was present with increasing the resistance to the stressor and eventually the third stage of adrenal exhaustion called adrenal fatigue or adrenal burn out (Goldstein, 2010).

Moreover, exposure of the rats to hypoglycemia led to a significant decrease in the body weight compared to the negative control group. This was in agreement with Nagaraja et al. (2006) and Bozzo et al. (2011) who reported a decrease of the body weight associated with a relative increase in the weight of the adrenal gland following chronic stress. The possible mechanism of the decline of the body weight is that hypoglycemia led to activation of the sympathetic-adrenomedullary axis with subsequent hyperplasia and hypertrophy of the chromaffin cells that in turns secrete increasing amounts of both adrenaline and noradrenaline (Senthilkumaran et al., 2016). These two hormones are known to induce protein catabolism. However, they have a unique role in counteracting the drop of blood glucose by stimulating gluconeogenesis and glycogenolysis and inhibiting the peripheral glucose utilization, thus the blood glucose level has been returned nearly back to normal and the detrimental effects of hypoglycemia were abolished (Cryer et al., 2003).

Finally, in this work, hypoglycemia led to a relative increase in the amount and density of the reticular fibers that support the secretory cells in the cortex and the medulla. The mechanism of such increase of reticular fibers was explained by Nagaraja et al. (2006) who stated that chronic stress regardless of its type is known to enhance the production of ROS that activate the fibroblasts leading to marked increase in the amount CT fibers in the adrenal gland.

**Conclusion:**

According to the obtained results of this study, it could be concluded that normally the adrenal cortex of rats grows gradually up to the age of 11th week (young adulthood) postnatally; afterwards its growth becomes somewhat stationary till the adulthood period. However, the growth of the medulla continued up to the 16th week (adulthood). Comparatively, experimentally-induced hypoglycemia led to age-dependent devastating drawbacks on the postnatal...
development of the adrenal gland in a zone-specific manner. These detrimental effects may have implications in the clinical field for the infants and children particularly the diabetic ones who are frequently susceptible to insulin-induced hypoglycemia during their treatment course.

REFERENCES


