A Histomorphometric Study on the Neurohypophysis of STZ-induced Diabetic Albino Rats

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ABSTRACT

AIM: To assess the hyperglycemia-induced microscopic changes in the posterior pituitary of streptozotocin (STZ) - induced diabetic adult albino rats.

MATERIAL AND METHODS: After clearance from the Institutional Animal Ethical Committee, 36 animals were divided into six groups having six rats each: control, two weeks, one month, two months, four months and six months. Diabetes was induced with a single dose of streptozotocin administered through intraperitoneal route (60 mg/kg). Body weight and blood sugar were monitored at the biweekly interval. At the end of each experimental period, animals were euthanized by deep ether anesthesia and blood samples were collected by direct puncture of heart for biochemical analysis. Tissues were fixed in Karnovsky fixative and processed for paraffin sectioning. Routine and special stained sections were studied under the light microscope and relevant findings were recorded.

RESULTS: Data obtained from biochemical analyses and histomorphometry along with the histopathological features revealed that with increasing duration of hyperglycemia was associated with increased serum creatinine and reduction in serum total protein; increased mean percentage of darkly stained large sized pituicytes and notable thickening of perisinusoidal collagen.

CONCLUSION: It is therefore concluded that long-standing hyperglycemia which is associated with increased occurrence of predominantly large and darkly stained pituicytes and remarkably increased deposition of perisinusoidal collagen appear to be the important contributing factors somehow responsible for the derangement of the function of the posterior pituitary in chronic diabetes.

Key words: Collagen; Diabetes; Herring bodies; Pituicytes; Posterior pituitary; Sinusoids

INTRODUCTION

Diabetes mellitus is a group of metabolic diseases of multiple etiologies characterized by chronic hyperglycemia with disturbances of carbohydrates, fats and protein metabolism resulting from defects in insulin secretion, insulin action, or both[5]. Hyperglycemia is believed to be associated with increased systemic and cellular oxidative stress combined with decreased antioxidant status initiating mitochondrial alteration[5] and chronic low-grade inflammation[5]. These factors induce cellular injuries leading to progression of diabetes and its associated complications in terms of long-term damage, dysfunction, and failure various organs, especially the eyes, kidneys, nerves, heart, and blood vessels[1]. Diabetes-associated changes of different kinds and grades have been reported quite...
extensively both in the central and peripheral nervous system like encephalopathy, neuropathy, and retinopathy[6, 9] but very few studies have reported diabetes-linked changes in the posterior pituitary which is essentially an extension of hypothalamic part of the central nervous system consisting of axonal processes and modified neuroglial cells called pituicytes. Hyperglycemia-induced neurotoxicity, increased angiogenesis and fibrosis in various organ systems have also been reported by different workers[10, 11]. In another study[12] it has been suggested that increased neuronal glucose, intracellular glucose and associated multiple biochemical alterations, endothelial dysfunction, altered Na+/K+ ATPase pump function, dyslipidemia[13] are found in diabetes. All these changes generally induce accumulation of high levels of intracellular Ca2+ concentration and glucose neurotoxicity[14] as well as glucose cytotoxicity[15] possibly responsible for a variety of functional and structural disorders in nervous systems and other organs[10, 11, 12]. Thus injuring cell membrane integrity and inducing apoptosis in the pituicyte glial cells of the posterior pituitary gland[16]. Neuronal axonal projections of the magnocellular neurosecretory cell from the pars nervosa store and release neurohypophysial hormones oxytocin and vasopressin into the systemic circulation via hypothyphalo-portal circulation[17]. Hpothalamo-hypophyseal axonal nerve fibers have specialized glial cells called Pituicyte and herring bodies (neurosecretory vesicles) which store hormones in the nerve terminal[18]. Recently few researchers[19] have tried to demonstrate micro-anatomical aging changes in three lobes of the pituitary. Therefore, it appears logical to expect some changes due to neuronal damage in the hypothalamus and thus affecting the hypothalamo-hypophyseal nerve fiber and microvascular changes in the posterior pituitary sinusoidal capillaries, pituicyte population, and their morphology. Therefore, the current study is aimed at demonstrating these and possibly other changes in the arrangement of peri-vascular connective tissue, nonmyelinated nerve fibers, pituicytes and herring bodies in the posterior pituitary by using special staining for collagen, glial cells in conjunction with histopathological, histomorphological and biochemical parameters in experimentally induced diabetic rats after 2W, 1M, 2M, 4M and 6M periods of experiment.

MATERIAL AND METHODS

Study approval, Animal Care, and Housing preparation
Untreated albino rats of either sex (young, weight ~ 250g, number-36 rats) obtained from the central animal house, AMU, Aligarh were used for the current study. All animals were housed in new environmental condition for a period of one week in clean polypropylene cages and maintained under standard laboratory environmental conditions (12/12 h light/dark cycle) with free access to the standard pellet diet and water ad libitum. The Institutional Animal Ethical Committee of Aligarh Muslim University, Aligarh approved the protocol of the present study (Ref. No.9025/2014).

Experimental Design
Animals were divided into following six groups having six rats in each group: (1) Non-diabetic healthy Control, age-matched (did not receive any active compound); (2) Diabetic Experimental groups: Two week, (3) One month (4) Two month (5) Four-month and (6) Six month.

Induction of Diabetes and Experimental method
After 12-hour fasting, experimental diabetic model rats received the single dose of streptozotocin (STZ) (Sigma-Aldrich Canada, Oakville, Ontario, Canada) (60 mg/kg, aqueous sol., I.P.). Blood sugar level was monitored with Glucometer (Dr. Morepen Gluco One BG03 Blood Glucose Meter) in the blood obtained from lateral tail vein before the beginning of the experiment and after 2nd day streptozotocin injection for checking induction of diabetes. Animals with fasting blood sugar level 250 mg/dl and above were considered as diabetic. Both body weight and blood glucose levels of all animals in each group were monitored biweekly. After assigned periods all experimental and their corresponding controls were sedated and euthanized with an overdose of ether general anesthesia and thereafter the rats were rapidly perfusion-fixed with Karnovsky fixative.

Histopathology and Histomorphometry
After proper fixation, pituitary gland was exposed and excised from hypophysial fossa en-bloc and then subjected to the standard histological procedures of dehydration, clearing and paraffin embedding (58-60°C melting point). Five µm thick sections were stained with Hematoxyline Eosin (H & E) for general histological features, Cresyl Violet (CV) for pituicytes, fibrocytes and endothelial cells; while PicroSirus Red (PSR) was used for collagen. Only H & E and CV-stained sections were used for counting pituicytes. Random photomicrographs were recorded under oil immersion (>1000 magnification) of trinocular light cum fluorescent microscope (Olympus, BX40, Japan) with the digital camera (Son y 18.2 MP, Japan) and counted all types of pituicytes from the total area of 3.5×103 µm². Only well-defined pituicytes were used for the histomorphometry. Data achieved from these were used to calculate the mean number, and ratio between dark and light pituicytes in different groups.

Biochemical Estimation and Analysis
Blood glucose levels were measured from lateral tail vein blood at the biweekly interval with the help of Glucometer (Dr. Morepen GlucoOne BG03 Blood Glucose Meter). At the end of each study period, blood samples were obtained from direct puncture of heart and collected into sterilized plastic vials. Samples were allowed to clot, centrifuged at 2500 rpm for 30 minutes, the serum was separated and stored in sterile plastic vials and subsequently assayed for serum total protein and serum creatinine level by using Avantor Benesphera™ clinical chemistry Analyzer C61.

Statistical Analysis
The data related to counting of pituicytes, serum total proteins, and serum creatinine level were statistically analyzed and the significance calculated using one-way ‘ANOVA’ followed by Tukey’s test. All numerical values were expressed as Mean ± SD and the value of p < 0.05 was considered as statistically significant.

RESULTS
Common typical clinical manifestations of diabetes such as polyphagia, polydipsia, and polyuria were observed in all diabetic groups after induction of diabetes. The mean values of body weight in diabetic groups were reduced at all experimental stages as compared to control groups and after 2 days of STZ administration, blood glucose levels in experimental groups reached 250 mg/dl, and animals were then considered diabetic. The blood sugar level in all diabetic groups showed the hyperglycemic state (> 500 mg/dl) throughout experimental periods. Data exhibit our previous studies[19].

Anatomy, Organization, and Histopathology of the Pituitary gland
The pituitary gland is a small reddish color structure located midline,
below the brain in the depression of sphenoid bone- called sella turcica. The routine and special stained sections of the pituitary gland from both control and diabetic group revealed similar basic cellular architecture, having three parts- pars anterior (PA) or adenohypophysis, pars intermedia (PI) and pars nervosa (PN) or neurohypophysis. PA shows clumps of secretory cells (chromophobe and chromophil) separated by capillary sinusoids (Figure 1). PI located between PA and PN having primarily small, basophilic cell clusters. Occasional eosinophilic material filled small spaces were also observed in all groups (Figure 1). PN consisted of thin nonmyelinated axonal nerve fibers (hypothalmo-hypophyseal nerve fiber) of magnocellular neurons of the hypothalamus, both dark stained and light stained pituicytes and occasional herring bodies. Endothelial cells of hypophysial sinusoids were also observed. In control group, thin collagen fiber surrounded the sinusoids and nerve fibers of PN but in 4M and 6M diabetic group similar finding was also noticed however they revealed slight increment in the collagen fibers condensation (Figure 2). A thin layer of connective tissue separated the PI from the PN (Figure 1).

**Histomorphometry**

The pituitary glands were histomorphometrically assessed in both the control and diabetic groups. Randomly selected images from the total area of $3.5 \times 10^4 \ \mu m^2$ were used for measurements and quantification of pituicytes. It revealed significant ($p < 0.05$) increase in the mean number in general and especially darkly stained pituicytes of 4M and 6M diabetic groups as compared with age-matched control group (Figure 5 and 6). Mean number of darkly stained pituicytes significantly ($p < 0.05$) increases in 6M diabetic groups simultaneously lightly stained pituicytes decreases with the advancement of hyperglycemia.

From the total number of pituicytes light and dark stained were separated and then the mean of the percentage was calculated for each group and compared with the age-matched control diabetic groups. Results showed that the increase in the mean percentage of darkly stained pituicytes are directly correlated with the duration of diabetes in 4M and 6M diabetic groups which interestingly coincides with the decrease of lightly stained pituicytes ($p > 0.05$) as compared with age-matched control groups (Figure 6). Based on parameters of the intensity of staining and diameter, the pituicytes constitute a mixed population. However, the ratio of dark: light stain pituicytes which is ~ 0.39 in control group goes up to ~1.19 in the 6M diabetic group. The size of pituicytes in the present study ranged from 2.5 to 9.0 $\mu m$ and they could be classified into small-sized (< 4 $\mu m$), medium-sized (4-6 $\mu m$) and large-sized (> 6 $\mu m$).

**Biochemical analysis**

The serum creatinine levels were significantly ($p < 0.05$) increased in all diabetic group except 2W diabetic group as compared to age-matched control group. However serum total protein levels significantly ($p < 0.05$) decreases in all diabetic groups as compared to age-matched control group as presented earlier[16] (Figure 7).

**DISCUSSION**

With the onset of diabetes, persistent and chronic hyperglycemia causes increased production of free radicals through auto-oxidation of glucose, via non-enzymatic protein glycation and enhanced the flux of glucose through the polyol pathway[17]. The generation of free radicals beyond the scavenging abilities of endogenous antioxidant defenses results in macro- and microvascular dysfunction[18] and associated low-grade inflammatory metabolic disorder[19].
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Figure 2 Photomicrograph showing the PN of Control, and all diabetic groups. Note almost uniformly distributed light blue nuclei of pituicytes (>) in all groups. Light yellow areas interspersed represents thin non-myelinated nerve fibers (↑). Sinusoids (*) are seen as unstained empty spaces of varying size and shapes surrounded by red-stained collagen fibers. PSR & CV stains; initial magnification × 1000.

Figure 3 Photomicrograph showing the pituicytes in PN (A) Control, (B) 6M diabetic groups. Note that the pituicytes in 6M (↑) are predominantly large and darkly stained. CV stain, initial magnification × 1000.
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Figure 4 Photomicrograph of the different diabetic groups showing light («) and dark (↑) stained pituicytes. Note that the density of pituicytes and their intensity of staining appear to increase with increasing duration of hyperglycemia. CV stain: under combination of blue fluorescents plus visible light. Initial magnification × 1000.

Figure 5 The mean ± SD number of pituicytes from the total area of $3.5 \times 10^4 \, \mu m^2$ significantly ($p < 0.05$) increased in all diabetic groups as compared with age-matched control group.
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Figure 6 6A shows that the number of lightly stained pituicytes decreases and 6B shows that the number of darkly stained pituicytes increases with advancement of duration of hyperglycemia.

In the current study, the mean body weight at the end of the experimental period in all diabetic groups was reduced and weight losses were maintained throughout the experimental period. These findings are in agreement with related studies[20,21] concluding that diabetic group had lower body weight than control group due to lack or reduced anabolic insulin hormone[22] followed by loss of tissue proteins during release of amino acids for gluconeogenesis[23] thus initiating progressive loss of body weight.

In general, hyperglycemia is a risk factor for muscle loss and subsequent weakness. Serum creatinine level is an indicator of muscle mass which helps to clear progression of diabetes. Creatinine is an amino acid derivative waste product that comes from the wear and tear on muscles of the body and it is cleared by tubular secretion[24]. However elevated serum creatinine considered as biomarkers of diabetic nephropathy. An increased urea concentration in diabetic rats is also associated with greater protein catabolisni[25]. In our study, the serum creatinine levels were increased as well as total serum protein decreases, due to low-grade inflammatory process[26] in all diabetic groups parallel to the severity of hyperglycemia. Similar findings have been shown in another similar study[21].

The unmyelinated nerve fibers in the pars nervosa of pituitary gland arise from the supraoptic and paraventricular nuclei of the hypothalamus are in close contact with the pituicytes and basal lamina of sinusoidal capillaries[27]. In the present study, structure and orientation of lobes of the pituitary gland in both control and experimental groups were similar to those reported earlier[28,15]. Sinusoidal capillaries in the pituitary gland generally have many intercellular gaps and fenestrations. These very large openings and slow blood flow allow for the passage of the hormones to the bloodstream[27]. The electron microscopic study of sinusoids in hyperglycemia has shown numerous and thick collagen bundles underlying the endothelial cells, peri-sinusoidal cells, and sinusoidal

Figure 7 Showing quantitative changes in biochemical parameter from the control and diabetic groups. Note: In all diabetic groups the serum total protein levels significantly (p < 0.05) decreased compared to age matched control group. Serum creatinine levels were significantly (p < 0.05) increased in all diabetic groups except 2W (p > 0.05).
membrane\textsuperscript{[29,30]}. One researcher considered the progression of fibrosis in a diabetic heart by PKC-β and p38 mitogen-activated protein kinase expression in redox reaction\textsuperscript{[31]} and also due to AGE and RAGE interaction and increased expression of TGF-β which contributes to the development of sub-mesothelial fibrosis and neoangiogenesis\textsuperscript{[32]}. In the current study of 4M and 6M diabetic groups, special stain revealed that there was thickening of collagen around the sinusoidal capillaries and also along the non-myelinated nerve bundles. One of the studies observed the reduction of the sinusoidal fenestrations in hyperglycemia suggesting the reason for the vascular pathology in diabetes\textsuperscript{[33]}. These results indicate that the hyperglycemia seems to promote fibrosis in terms of the amount of collagen and the thickness of individual collagen fibers as well as the vascular pathology which is in agreement with previous observations regarding link between hyperglycemia and fibrosis\textsuperscript{[33]}. Interestingly one study on adult rat suggested that posterior pituitary gland is not well developed because there was the low density of glial cells and low vascularization\textsuperscript{[30]}. In addition, they also noticed that the sinuses and sinusoid were also devoid of blood. Chronic hyperglycemia has also been shown to change the normal arrangement of collagen fibers in the trigeminal ganglion and dorsal root ganglion\textsuperscript{[16,34]}. During fibrosis, collagen fibrils are considered to be produced by fusion of short and thin fibrils with tapered ends. However in the current study PSR- special stain for collagen revealed well-developed numerous sinusoidal capillaries found throughout the PN in both control and diabetic groups.

Pituicytes are commonly described as modified astroglial glial cells quite uniformly interspersed and lying in contact with the unmyelinated axons and Hering bodies. However, as many as four morphological forms under the light microscope\textsuperscript{[35]} and five type based on ultrastructure\textsuperscript{[36]} have been described. These morphological forms have been linked to their sources of development, the density of cytoplasm and functional status. Though the majority of them resemble protoplasmic astrocytes\textsuperscript{[36]} quite many of them have also been shown to be derived from bipotential oligodendrocyte precursor cell in the developing neurohypophysis\textsuperscript{[27]} and thus also imparting the functions of oligodendrocyte in the neurohypophysis. The main role of pituicytes is to assist in the storage and release of neurohypophysial hormones\textsuperscript{[37]} and it is the part of neuromuscular component\textsuperscript{[39]} Pituicytes are believed to have a tropic and supportive function and to maintain the appropriate ionic composition of the extracellular fluid compartment under normal and pathological conditions\textsuperscript{[27,40]} through glutamate uptake and potassium buffering\textsuperscript{[41]}. It has also been shown that diabetes exacerbates cerebral ischemia which enhanced acidosis followed by astrocytes functional involvement\textsuperscript{[42]} which alters Kir4.1 potassium channel expression and homeostatic functions of astrocytes\textsuperscript{[41]}. In the current study revealed that in the 6M group most of the pituicytes were darkly stained and quite many of them fell in the range of large-sized pituicytes. One of the possible explanations for the hypertrophy of pituicytes could be the increased production of hydrogen peroxide in glia\textsuperscript{[43]} following lipid peroxidation in all parts of the cell and thus affecting the cell membrane permeability in cytoplasmic organelles and nuclei\textsuperscript{[44]} thus allowing water into cytoplasmic organelles and nuclei which probably initiates hypertrophy of glia during diabetes.

**CONCLUSION**

Based on histopathological, histomorphological and biochemical findings it is concluded that STZ-induced prolonged hyperglycemic state leads to increased serum creatinine and reduced serum total protein levels. There is an overall increase in the darkly stained pituicytes and thickening of collagen fibers around sinusoidal capillaries in the posterior pituitary gland. Therefore, it seems that one of the important contributing factors in the development of posterior pituitary gland dysfunction in chronic diabetes might be the hyperglycemia-induced neuronal cytotoxicity, pituicyte hyperplasia and hypertrophy and altered microvascular environment of the posterior pituitary in chronic diabetes.

**ACKNOWLEDGMENTS**

The authors would like to gratefully acknowledge all kinds of support and co-operation received from Department of Anatomy, and Neuroanatomy laboratory, JN Medical College, Aligarh Muslim University, Aligarh.

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