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Anxiety- and depression-like behavior are correlated with leptin and leptin receptor expression in prefrontal cortex of streptozotocin-induced diabetic rats

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Abstract

Anxiety and depression are common in diabetics. Diabetes also may cause reduced leptin levels in the blood. We investigated the relation between diabetes induced anxiety- and depression-like behavior, and leptin and leptin receptor expression levels in diabetic rats. The anxiety- and depression-like behaviors of rats were assessed 4 weeks after intraperitoneal injection of streptozotocin. Diabetic rats exhibited greater anxiety-like behavior; they spent more time in closed branches of the elevated plus maze test and less time in the center cells of the open field arena. Increased depression-like behavior was observed in diabetic rats using the Porsolt swim test. Prefrontal cortex (PFC), blood leptin levels and PFC neuron numbers were decreased, and leptin receptor expression and apoptosis were increased in diabetic rats. Blood corticosterone levels also were increased in diabetic rats. These results indicate that reduction of leptin up-regulates leptin receptor expression and may affect PFC neurons, which eventually triggers anxiety- and depression-like behaviors in diabetic rats.

Key words: anxiety, depression, diabetes mellitus, leptin, prefrontal cortex, receptors

Diabetes mellitus is the most common chronic endocrine metabolic disorder worldwide (Shaw et al. 2010). Diabetes causes various dysfunctions in organs including pancreas, retina, and the peripheral and central nervous systems (Gurpinar et al. 2012, Uysal et al. 2005). Neuropsychiatric sequelae of diabetes such as anxiety and depression are common in diabetics (Aksu et al. 2012, Collins et al. 2009). Diabetic subjects perform poorly on attention processing tasks, which are controlled by the prefrontal cortex (PFC) (Yan et al. 2011). The PFC is important in the pathophysiology of depression and anxiety (Aksu et al. 2012, Kim et al. 2009, Wisłowska-Stanek et al. 2013). Depression- and anxiety-like behaviors increase following lesions of PFC (Klein et al. 2010, Lacroix et al. 1998).

The hypothalamus pituitary adrenal (HPA) axis is activated in response to stressors to maintain or restore homeostasis (Roubos et al. 2012). The main output of the axis is the adrenal release of corticosterone in rats or cortisol in humans. Diabetes mellitus causes hyperactivity of the HPA axis; diabetic blood glucocorticoid levels are high at rest and glucocorticoid negative feedback is impaired in both humans and animals (Aksu et al. 2012, Plotsky et al. 1992, Roy et al. 1993).

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A protein product of the obesity gene, leptin, is expressed in adipose tissue and the central nervous system (Ahima et al. 2000). Leptin enters the brain from the peripheral circulation by active transport. It has been shown that leptin is carried by cerebrospinal fluid and enters the brain through the blood brain barrier (Schwartz et al. 1996). Leptin enters the brain independent of insulin (Banks et al. 1996). In humans and rodents, leptin receptor mRNA is expressed in the hypothalamus, hippocampus, amygdala and PFC (Couce et al. 1997, Dayi et al. 2012, Elmquist et al. 1998). Leptin reduces the activity of the HPA axis (Bell et al. 2000, Roubos et al. 2012). Serum leptin levels are decreased in diabetes (Ahmed et al. 2012, Barber et al. 2003); however, levels of leptin and leptin receptor in the PFC of diabetic rats are unknown.

We investigated whether there is a relation between anxiety- and depression-like behavior, and leptin and leptin receptor levels in the PFC of diabetic rats.

Materials and methods

Animals

Twenty-four adult male Wistar albino rats (Dokuz Eylul University, Experimental Animal Laboratory, Izmir, Turkey) were housed in individually in cages with free access to water and laboratory chow. Rats were maintained in a 12 h light/12 h dark cycle at constant room temperature ($22 \pm 1^\circ$ C) and humidity (60%). All experimental procedures were performed after approval by the Animal Care and Use Committee of the Dokuz Eylul University, School of Medicine.

Rats were assigned to either a control group ($n = 10$) or a diabetes mellitus group ($n = 10$). Diabetes was induced by a single intraperitoneal injection of 45 mg/kg streptozotocin (Sigma, St. Louis, MO). Twenty-four hours after streptozotocin treatment, induction of diabetes in the experimental group was confirmed by blood glucose levels greater than 250 mg/dl (Aksu et al. 2012, Gurpinar et al. 2012, Uysal et al. 2005).

Four weeks after streptozotocin injection, anxiety-like behavior of both groups was evaluated using the open field arena and elevated plus maze apparatus. Depression-like behavior was assessed using the Porsolt swimming test. We used the Noldus Ethovision XT video tracking system to record and analyze the behavioral tests.

Open field test

The open field apparatus consisted of 1 m^2 area surrounded by a 50 cm high wall. Each rat was placed in the center of the open field, then locomotor activity (ambulation) was measured for 5 min in a soundproof observation room illuminated with controlled light (100 lx).

Elevated plus maze test

The elevated plus maze apparatus consisted of a central platform (5×5 cm) with two open arms (50 cm long, 10 cm wide, 0.5 cm high borders) and two closed arms (50 cm long, 10 cm wide, 40 cm high walls) that were elevated 50 cm above the ground. Rats were placed on the platform facing the open arm and observed for 5 min.

Porsolt swim test

For the Porsolt swim test, the rats were placed individually in a cylinder (height 50 cm, diameter 30 cm) that contained water at a level that did not allow the animal to touch the floor or jump over the wall; a video camera was placed lateral to and 50 cm away from the cylinder. The temperature of the water was maintained at 25° C. Animals were placed in the Porsolt test apparatus and observed for 6 min. The total duration and frequency of immobility and mobility during the last 5 min were measured.

Five days after the behavioral tests, a light ether anesthesia was administered and blood was drawn by cardiac puncture for serum corticosterone and leptin measurements. The brain was removed and the PFC region was isolated. The thymus and adrenal glands also were removed.

Serum corticosterone levels

Serum corticosterone levels were measured by radioimmunoassay using a double antibody kit (ImmuChem, MP Biomedicals, Orangeburg, NY) according to the manufacturer's instructions.

Hippocampal leptin receptor expression

RNA isolation and cDNA synthesis

For half of each group, PFC tissues from individual animals were homogenized with Qiagen TissueLyser-II homogenizer in 1ml QIAzol lysis reagent, and total RNA was isolated using the RNeasy lipid tissue mini kit (Qiagen, GmbH, Hilden, Germany) according to the manufacturer's

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instructions. DNA contamination was removed by on-column DNase digestion using the RNase-Free DNase Set (Qiagen). Total RNA was measured using the Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). The integrity of total RNA was analyzed in a MOPS-buffered 1.2% agarose gel that contained formaldehyde and was confirmed by visualization with ethidium bromide staining and UV illumination. The absence of contaminating genomic DNA was confirmed also by the reverse transcriptase minus (RT-) negative control reaction. cDNA synthesis was performed using a RevertAid First Strand cDNA synthesis kit (Thermo Fisher Scientific Inc., Waltham, MA) using 2 μg of total RNA according to the manufacturer's instructions.

Real time polymerase chain reaction (RT-PCR)

Specific rat primer pairs and probes for reference and target genes for PCR analysis were designed using the ProbeFinder Software, which is available online (www.universalprobelibrary.com). β-Actin was used as the reference gene. The universal probe library (UPL) probe number for the leptin receptor gene is #118 and the primer sequences were as follows: forward: 5′-TGTCAGAAATTCTATGTGGTTTTGT–3′, reverse: 5′-TTGGATAGGCCAGGTTAAGTG(76 bp). The UPL probe number for the β-actin gene was #115 and the primer sequences were as follows: forward: 5′-CTAAGGCCAACCGTGAAAAG-3′, reverse: 5′-GCCTGGATGGCTACGTACA-3′(79 bp). All PCR reactions were performed using LightCycler®480 (Roche Diagnostics, Indianapolis, IN).

Probes Master Mix (Roche Diagnostics) in a 20 μl reaction mixture (2 μl cDNA; 10 μl LightCycler®480 Probes Master; 2 μl (10 mM) each specific gene primer pairs and upl probes) in a LightCycler®480 Instrument,96-well format (Roche Diagnostics). The PCR program was initiated at 95° C for 10 min followed by 40 cycles, each for 10 sec at 95° C, 30 sec at 60° C, 1 sec at 70° C and 30 sec at 40° C for cooling. Negative controls consisted of real time RT-PCR reaction mixture using sterile distilled water instead of template RNA and were included in each batch of PCR. In addition, repeatability and reproducibility of the assays were assessed by performing three trials. Each amplification cycle was analyzed using the LightCycler®480 software.

Biochemical analysis

Serum and PFC leptin levels were determined using a radio-immunoassay method (sensitivity limits: 0.63 ng/ml, 50 ng/ml, 2.0–4.6%, 3.0–5.7%) (RL-83K, Millipore, Billerica, MA). Tissue protein contents were determined using a total protein kit (Randox Labs, Crumlin, UK).

Western blot analysis

For western blot analysis, protein concentration was measured using a fluorometric assay according to the manufacturer's instructions (Qubit, Invitrogen, Paisley, UK). Tissue samples were lysed in NuPAGE LDS Sample Buffer (4 X, NP0007, Invitrogen). Subsequently, the sample was heated to 70° C for 10 min, then cooled on ice before loading onto NuPAGE 10% Bis–Tris Gel (4–12%, NP0301; Invitrogen) with NuPAGE MOPS SDS running buffer (NP0001; Invitrogen). Proteins then were electro-blotted on microporous polyvinylidene difluoride (PVDF) membranes. Immunodetection of the leptin protein then was achieved using the WesternBreeze Chromogenic Western Blot Immunodetection Kit (WB7105; Invitrogen). We used the polyclonal mouse anti-leptin antibody (OB, sc-842; Santa Cruz Biotech., Santa Cruz, CA).

Histological examination

PFC, adrenal and thymus tissues were fixed in 10% formalin in phosphate buffer for 24 h. All tissues were cut into sequential 5 μm sections using a microtome; the PFC tissues were sectioned coronally. Neuron density was measured by taking three coronal sections through the PFC that corresponded approximately to plates 9 and 11 in the rat atlas of Paxinos and Watson (1982). All sections were stained with cresyl violet (Baykara et al. 2013, Uysal et al. 2012). The images were analyzed using a computer assisted image analyzer consisting of a microscope (Olympus CX-41 Tokyo, Japan) equipped with a high resolution video camera (Olympus DP71, Japan). The neurons in these regions were counted at 40 X magnification using a 16800 μm^2 counting frame. The counting frame was placed randomly three times on the image analyzer system monitor, the neurons were counted and the average PFC neuron density was calculated. Adrenal and thymus tissue sections were stained with hematoxylin and eosin (H & E) and examined by light microscopy (Aksu et al. 2012, 2013).

To detect DNA fragmentation in cell nuclei, the terminal deoxynucleotidyl-transferase-mediated dUTP-nick end-labeling (TUNEL) reaction was applied to the paraffin sections using an apoptosis kit (Roche, Mannheim, Germany).

Immunohistochemical staining was performed using the streptavidin/biotin method (85-9043, Invitrogen). Immunohistochemistry was performed

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using anti-leptin (OB, sc-842; Santa Cruz Biotech.) and anti-leptin receptor (OB-R, sc-1834-R; Santa Cruz Biotech.) antibodies.

Statistical analysis

Statistical analysis was performed using SPSS 15.0. Differences between groups were calculated using the nonparametric Mann Whitney U test. Correlations among parameters were calculated using Pearson correlation analysis. Results are presented as mean ± SEM; $p \leq 0.05$ was considered statistically significant.

Results

In the open field test, total movment time was increased and the number of entries into the center cells were reduced in diabetic rats compared to controls (total movment time, $p < 0.008$; number of entries into the center cells, $p < 0.001$) (Fig. 1A–C). The diabetic group spent more time in the closed

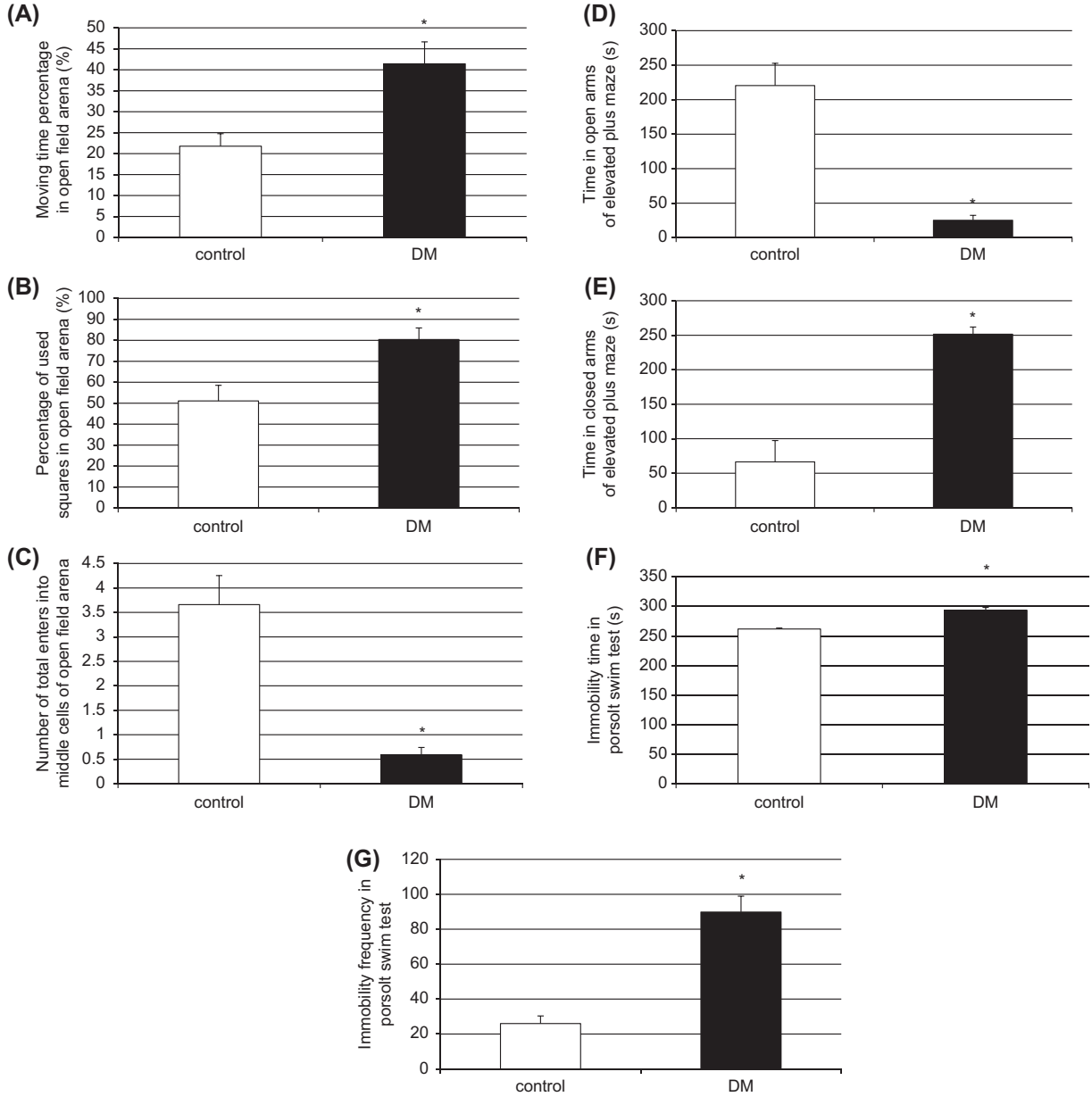


Fig. 1. Behavioral tests results. A) Percentage of moving time in open field test. B) Percentage of used squares in open field arena. C) Number of entries into the middle cells of open field arena. D) Time in open arms of elevated plus maze. E) Time in closed arms of elevated plus maze. (F) Immobility time in Porsolt swim test. G) Immobility frequency in Porsolt swim test. *$p < 0.05$ compared to control group. DM, diabetes mellitus.

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branches and less time in the open branches of the elevated plus maze device, and their anxiety levels appeared greater than the control group (time spent in closed arms, $p < 0.003$; time spent in open arms, $p < 0.003$) (Fig. 1D, E). Diabetes also increased the immobility time and frequency in Porsolt swim test (immobility time, $p < 0.001$; immobilty frequency, $p < 0.0001$) (Fig. 1F, G).

Histological analysis of the PFC sections of diabetic rats revealed that many neurons were shrunken, picnotic and stained darkly, and apoptosis was greater than controls (Fig. 2A–D). There were fewer neurons in the PFC of the diabetic rats than in controls ($p < 0.001$) (Fig. 2J). TUNEL positive neurons were detected more frequently in the PFC of diabetic rats than in controls ($p < 0.001$) (Fig. 2C, D, K).

Diabetes was associated with decreased PFC and blood leptin levels ($p < 0.001$, $p < 0.05$, respectively) (Fig. 3A, B). PFC leptin receptor expression was significantly greater in the diabetes group compared to controls ($p < 0.003$) (Fig. 3C). PFC leptin expression also decreased compared to controls according to western blot analysis (Fig. 2I). By immunohistological examination, leptin reactivity was reduced (Fig. 2E, F) and leptin receptor activity was increased in the diabetic rats compared to control rats (Fig. 2G, H).

Basal resting blood corticosterone levels were increased in diabetic rats ($p < 0.001$) (Fig. 4A). Adrenal weight and relative adrenal weight (adrenal weight/body weight ratio) increased in diabetic animals compared to control animals ($p < 0.001$, $p < 0.05$, respectively) (Fig. 4B). H & E staining of adrenal glands showed an enlarged zona fasciculata and disorganized cell arrangement in diabetic rats compared to controls. Also, the characteristic columnar cell arrangement of the zona fasciculata was replaced by disorganized cells with more eosinophilic cytoplasm in diabetic rats compared to controls (Fig. 4C, D). The histologic examination of thymus revealed reduced numbers of lymphoid cells in the cortex and medulla of diabetic rats compared to controls. In addition, there were abundant large vacuoles in the cytoplasm of the diabetic thymus (Fig. 4E, F).

We found a strong negative correlation between elevated plus maze test results and PFC leptin receptor expression levels (the time spent in the open arms and PFC leptin receptor expression; $r = -0.760$, $p < 0.0001$). Moreover, we found a strong positive correlation between Porsolt swim test results and PFC leptin receptor expression levels (immobility frequency and PFC leptin receptor expression; $r = 0.756$, $p = 0.0001$) and a strong positive correlation between PFC leptin receptor expression levels and blood corticosterone levels ($r = 0.672$, $p < 0.003$). In addition, serum and PFC tissue leptin levels were correlated negatively with the time spent in the open arms of elevated plus maze (serum leptin levels: $r = -0.699$, $p = 0.001$; PFC leptin levels: $r = -0.790$, $p = 0.0001$).

Discussion

We have shown that diabetic rats had greater anxiety- and depression-like behavior. We also showed that leptin levels were lower and leptin receptor levels were higher in the PFC of diabetic rats and that the number of neurons in the PFC were decreased. We showed that diabetes related anxiety- and depression-like behavior is correlated with structural changes in the brain of streptozotocin-induced diabetic rats. Serum leptin levels decreased while corticosterone levels increased in the diabetic rats. To our knowledge, ours is the first report that relates anxiety-like behavior, depression-like behavior, and PFC leptin and leptin receptor levels to diabetes.

It is known that the HPA axis is activated in response to physical or psychological stress and that it is important for maintaining or restoring homeostasis (Roubos et al. 2012). Diabetes mellitus causes blood glucocorticoid levels to be high at rest and in response to stress (Roy et al. 1993). HPA axis alteration contributes to the neurological complications of diabetes. Plasma corticosterone levels increased in different animal diabetic models. Plasma corticosterone increased and the circadian corticosterone cycle changed in response to streptozotocin-induced diabetes (Nicola et al. 1977). Similarly, obese Zucker diabetic rats exhibited elevated basal levels of corticosterone (Plotsky et al. 1992).

In our study, the basal corticosteroid levels were high in streptozotocin-induced diabetic rats. Activation of the HPA axis results in decreased thymus weight (thymus atrophy), increased adrenal weight (adrenal hypertrophy) and decreased body weight (Tramullas et al. 2012). Similarly, in our study, streptozotocin-induced diabetes caused adrenal hypertrophy, which indicated activation of the HPA axis. The fact that we did not observe a decrease in thymus weight may be due to the technical difficulties of removing the whole thymus owing to its small size. Histologic examination of thymic tissue, however, revealed signs of atrophy. The hyperactivity of the HPA axis in diabetes could be due to activation of hypothalamic neurons that release corticotrophin, changes in ACTH release from pituitary cells, or alterations in

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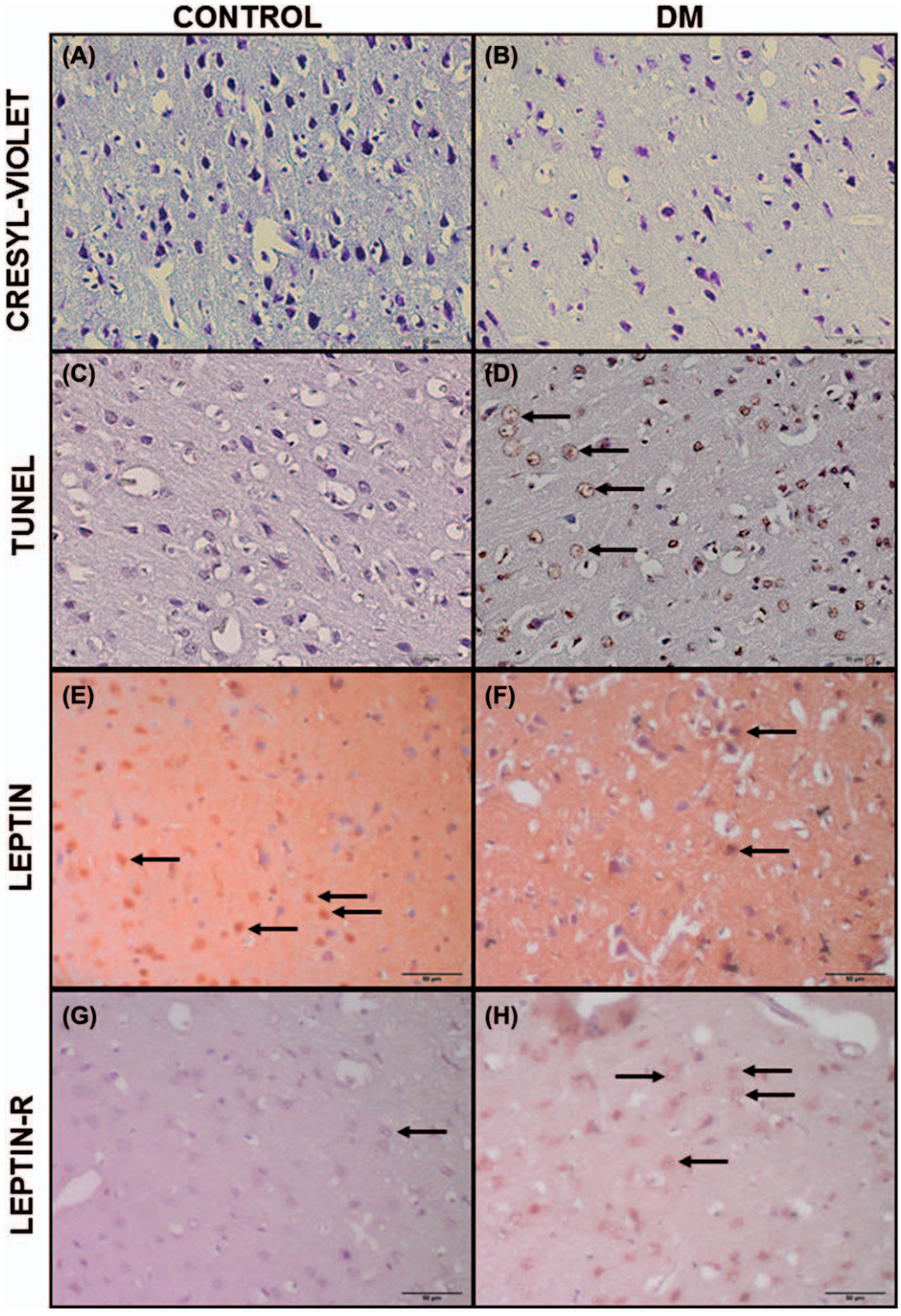


Fig. 2. Light microscopic images of rat brain PFC sections. A, C, E, G) Controls. B, D, F, H) diabetic group. A, B) Cresyl violet stained sections of rat PFC. The morphology of neurons in the control group was normal. In the diabetic group, more dark blue cells with shrunken morphology and neuronal loss can be seen. C, D) Representative photomicrographs of TUNEL positive cells (arrows). TUNEL positive cells were increased in the diabetic group. E, F) Leptin immunohistochemical stained section. Leptin positive cells decreased in the diabetic group. G, H) Immunohistochemical stained section for Leptin receptor. Leptin receptor immunopositive cells were increased in the diabetic group. I) Western blotting analysis. J) The percentage of neuron numbers. K) The percentage of TUNEL positive neurons. *$p < 0.05$ compared to control group. DM, diabetes mellitus.

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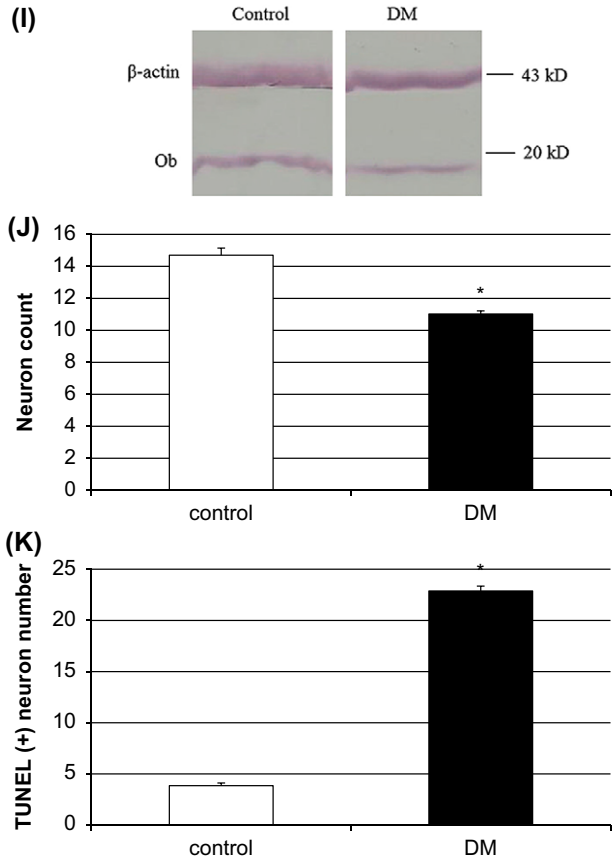


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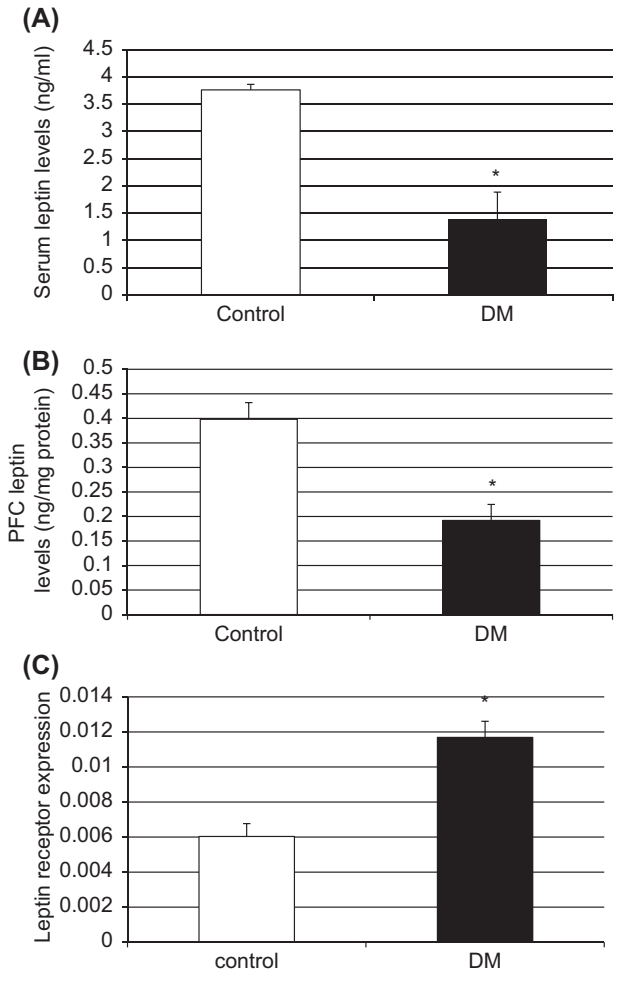


Fig. 3. Leptin and leptin receptor levels. A) Serum leptin levels. B) PFC leptin levels. C) PFC leptin receptor expression. *$p < 0.05$ compared to control group. DM, diabetes mellitus.

adrenal sensitivity (Chan et al. 2002). In addition, glucocorticoids cause pancreatic beta cell dysfunction and contribute to hyperglycemia (van Raalte et al. 2010).

Dysregulation of HPA axis frequently is implicated in the pathophysiology of depression and anxiety disorders (De Kloet et al. 2005). Earlier studies have shown hyperactivity of the HPA axis in depressed patients and animals (Barden et al. 2004, De Kloet et al. 2005). Furthermore, response to glucocorticoid is reduced and glucocorticoid receptor function is impaired during major depression (Raison et al. 2003). Depression is twice as common among diabetics than non-diabetics (Egede et al. 2002). In addition, depressed diabetic patients often have poorer plasma glucose control and more frequent diabetes related complications (Collins et al. 2009). Depression-like behaviors frequently are observed in diabetic animals (Hirano et al. 2007). Similarly, increased fear-related behaviors and anxiety-like behaviors are observed in streptozotocin-induced diabetic rats (Aksu et al. 2012, Meehan et al. 1986, Sharma et al. 2010). We observed that anxiety-like and depression-like behavior increased in the diabetic group.

Several limbic forebrain structures regulate HPA axis activity (Barden 2004, Kim et al. 2009). The PFC is connected to regions related to emotional behavior including the amygdala and hypothalamus (Kim et al. 2009). A lesion in the PFC can cause depression- and anxiety-like behaviors in animal models (Klein et al. 2010, Lacroix et al. 1998). PFC lesions cause deterioration of the HPA axis (Diorio et al. 1993). The thickness of PFC gray matter decreased and morphological changes were observed in diabetic humans and animals (Ajilore et al. 2010, Aksu et al. 2012). The number of PFC neurons decreased in our diabetic rats.

The PFC regulates dopamine release from neurons of the mesocortical projection systems. The mesocortical dopaminergic neurons originate from the ventral tegmental area and project to the PFC. There is evidence that dopaminergic neurons may

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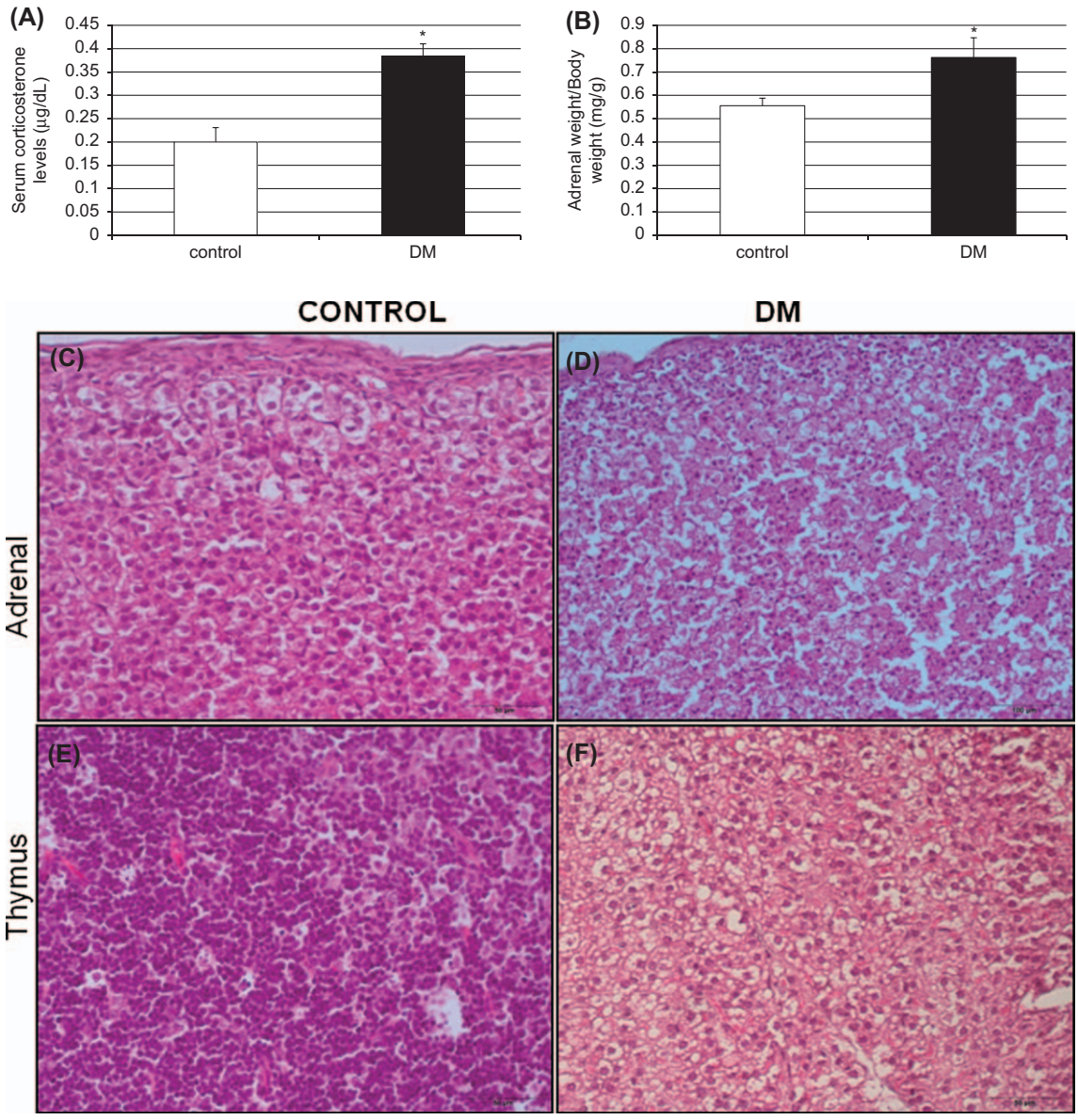


Fig. 4. Stress markers results. A) Serum corticosterone levels. B) Relative adrenal weight expressed as tissue weight (mg)/body weight (g). C, D) Histology of adrenal cortex tissue. H & E staining of adrenal glands showing enlarged zona fasciculata and disorganized cell arrangement in diabetic rats. Also in zona fasciculata the characteristic columnar cell arrangement was replaced by disorganized cells with increased eosinophilic cytoplasm in diabetic rats. E, F) Histologic investigation of thymus showed reduction of lymphoid cells in cortex and medulla in diabetic rats. Abundant large cells showed vacuolic in cytoplasm of diabetic thymus. F, H, Control; G, I, diabetic group. *$p < 0.05$ compared to control group. DM, diabetes mellitus.

be related to fear, anxiety and depression (Dunlap and Nemeroff 2007, Pezze and Feldon 2004). Pezze and Feldon (2004) also showed that dopamine release from the PFC can be related to regulation of anxiety and fear. Decreased dopamine input to the nucleus accumbense from cortical structures and the amygdala causes an exacerbation of anxiety symptoms (Charney et al. 1993). Dopamine levels of cerebrospinal fluid decrease in depressed patients (Willner 1995). Also, dopaminergic activity decreases in the nucleus accumbens of "depressed" rats (Jahng et al. 2012). Decreased dopamine levels in the PFC are associated with increased anxiety in rats (Mizoguchi et al. 2010). Dopamine levels are significantly decreased in cortex, hypothalamus and cerebellum of diabetic rats (Kumar et al. 2010).

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Leptin is a hormone derived from adipose tissue that signals information about adiposity to the brain. Leptin increases energy expenditure and decreases food intake, which results in decreased body mass (Ahima 2000). Serum leptin levels decrease in diabetic patients and streptozotocin-induced diabetic animals (Ahmed et al. 2012, Barden 2004). We found that leptin levels decreased in the blood and PFC of diabetic rats. PFC leptin receptor expression may have been up-regulated in our diabetic rats owing to a negative feedback effect. Elevated plasma leptin levels reduce HPA axis activity (Bell et al. 2000). Also, leptin administration decreases blood corticosterone levels (Roubos et al. 2012). Leptin also plays a role in the function of mesolimbic dopaminergic neurons (Fulton et al. 2006) and increases dopamine flux in nucleus accumbens (Leinninger et al. 2009). Release of dopamine is reduced in the nucleus accumbens of leptin mutant mice (Fulton et al. 2006). In addition, it is known that leptin can modulate the GH-IGF-1 pathway (Nyomba et al. 1999). We have reported earlier that streptozotocin-induced diabetes associated anxiety was correlated with decreased blood and PFC IGF-1 levels (Aksu et al. 2012, 2013). Also, it is known that IGF-1 protects nigral dopaminergic neurons (Quesada et al. 2008). Decreased levels of IGF-1 may be due to decreased levels of leptin.

Leptin induced modification of dopamine turnover via IGF-1 may affect mood controllers in the PFC of diabetic rats. Further studies are needed to elucidate the leptin, IGF-1 and dopamine relation in anxiety and depression behaviors of diabetics.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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