Original Article

Chronic unpredictable environmental stress impair biochemical and physiological homeostasis: Role in diabetes mellitus

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A B S T R A C T

Aims: Chronic unpredictable environmental stress (CUES) may induce predisposition to diabetes mellitus. This study investigates the role of CUES on impaired homeostasis.
Material and methods: Stressed group mice (n = 20) were exposed to CUES for 16 weeks. Weekly body weight, feed consumption, feed efficiency ratio, fasting blood glucose were monitored. Plasma HbA1c, plasma cortisol, plasma epinephrine and plasma insulin, serum lipids, antioxidants and carbohydrate metabolizing enzymes activity were assessed along with DNA damage and histopathological examination of liver, kidney, pancreas, spleen and skeletal muscles.
Results and conclusion: s: Fasting blood glucose levels & HbA1c in the stressed were significantly higher compared to control (p < 0.001). Serum lipids were found insignificantly higher in stressed mice compared to control. Body weights of the stressed mice and feed efficiency ratio were found significant (p < 0.001). Plasma corticosterone, plasma epinephrine, HOMA-IR was found to be significantly higher in the stressed group (p < 0.001). Plasma insulin level was found to be significantly lower in the stressed group (p < 0.001). Significant changes were observed in antioxidants level, carbohydrate metabolizing enzymes activity, peripheral tissues and DNA integrity. CUES initiates pathogenesis of diabetes.

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1. Introduction

Stress is explained as the state of the brain that interprets the stimulation quantity as excessive and threatening [1]. Exposure to mechanical stressors results in an event of adaptive responses that help an organism to cope up with the dynamic environment [2]. Adequate acute stress may prove beneficial but if chronic, leads to complications and may be fatal [3]. Chronic environmental unpredictable stress (CUES) in human is the most frequent type of stress that evokes drastic changes in blood circulation, hormone release, glucose metabolism along with behavioural alterations. Mechanistic hormonal pathway activated under chronic stress works via the release of catecholamines and corticosteroids from the adrenal medulla and adrenal cortex respectively along with the release of adrenocorticotropin from the anterior pituitary [2].

In humans, studies have demonstrated the onset of diabetes mellitus with chronic stress [4] and hyperglycemia [5]. The effect of CUES on glucose metabolism is mediated by the counter-regulatory release of hormones resulting in hyperglycemia and impaired insulin action. Lipolysis equally contributes to the progression of stress-related complications via its lipotoxic effects on peripheral organs like muscles, liver, pancreas and kidney [6]. Stress-induced ROS is the predominant cause of DNA damage [7]. A recent study showed the role of stress in the development of insulin resistance in different tissues [8]. Physiological significances of carbohydrate metabolizing enzymes are unforgettable in CUES. The activity of hexokinase, glucokinase, fructose 1, 6 bis phosphatase and glucose -6-phosphatase can be exploited as a marker for assessing the glucose metabolism in CUES. Stress besides causing impairment in carbohydrate metabolism also fosters the free radicals [9]. Although regulation of glycolytic flux is shared by all enzymes of the pathways but stress permits certain enzymes to perform its lead role. To maintain the homeostasis between production and removal of these ROS, there are antioxidants defence that includes superoxide dismutase, catalase, glutathione-S-transferase, glutathione peroxidase, glutathione (GSH) reduce [10]. Certain cell types including

neurons [11] and beta cells [12] of the pancreas are very susceptible to ROS induced damage due to inherent low levels of free radical scavengers [13]. With the increase in CUES, the inherent free radical scavenging ability of the body further decreases [14] and this results in severe damage leading to the possible cause of diabetes. A recent literature showed the role of chronic stress on tissue histology and DNA damage due to ROS and stress proteins [15].

The present study relates the effect of CUES on impairment of biochemical and histological homeostasis and their possible role in diabetic complications. Near genomic resemblance of mice with humans enable this study results to extrapolate with the humans. Therefore, this study aimed to implicate CUES of day to day human life to be tested in an animal model.

2. Material and methods

2.1. Ethical approval & experimental animals

Healthy 6–8 weeks old female Swiss albino mice (20 ± 5 g) were obtained from central animal facility (401/RO/C/2001/CPCSEA) J.N Medical College, Aligarh Muslim University, India as per guideline laid down by CPCSEA (Ministry of Environment & Forests, Govt. of India) and Institutional animal ethics committee, Aligarh Muslim University, India. The Institutional animal ethics committee, Faculty of Medicine, Aligarh Muslim University, India has approved the experimental procedure protocols. All experiments were performed in accordance with the relevant guidelines and regulations laid down by the above committee.

2.2. Housing and husbandry

Animals were maintained under standard laboratory conditions in the pathogen-free environment under the controlling factor of 12 h dark/light except on day 5 of protocol schedule. Housing room was maintained with an ambient temperature of 22 ± 2 °C with a relative humidity of 50–60%, 130–325 lux diffuse light except on day 5. Sterilized corncob bedding material was used to spread in the cages. During execution of protocol schedule, animals were fed on aqua guard filtered potable water and sterilized diet ad libitum to control and stressed mice (except day 7 of the protocol schedule to stress mice). Mice were sacrificed with sedation followed by cervical dislocation as per guideline of CPCSEA (Ministry of Environment & Forests, Govt. of India) and Institutional animal ethics committee.

2.3. Stressors

The stressors used in the present study were as follows: (a) wire meshed cages attached to wooden board of individual animal size (b) tarsons animal cage 43 × 27 × 15 (l × b × h mm) with its bin 43 × 27 × 15 (l × b × h cm) (c) cylindrical tank 50 cm height x 20 cm (d) restrainer (e) compact fluorescent lamp.

2.4. Study design

Baseline control data for HbA1c and lipid profile were obtained from randomly selected mice considered to be initial control. Mice were randomly divided into two groups and stressed protocol of 16 weeks was carried out as represented in Table 1.

Group I (Control = 20): Mice were allowed to ad libitum feed and water without performing any chronic unpredictable environmental stress procedure.
Group II (Stressed = 20): Mice were subjected to chronic unpredictable environmental stress procedure for the period of 16 weeks.

2.5. Experimental procedure

CUES procedure was performed according to slightly modified previously described protocol [16]. The sequence of stressors was used for seven days continuously up to 16 weeks outlined in Table 1. Detailed CUES events are as follows: for restraint stress, all mice were kept in restrainer for a period of 3 h with no movement allowed. Wet bedding was performed by filling 500 ml tap water in their home cages. Forced swim along with cold swim was carried out in a cylindrical tank filled with tap water up to 25 cm at 25 °C and 4 °C respectively. Crowding stress was performed by placing an iron sheet in their home cages to provide them minimum space to move. Illumination stress was achieved by placing a CFL lamp overnight above the cage. Mice were kept in separate sterilized autoclaved cages after each stress procedure. One baseline and one post-restraint sample were taken from stressed mice during the experimental protocol. Two days after baseline blood sampling, the mice of stressed group were started giving stress. Weekly fasting glucose (from the second week) was recorded by tail prick method along with feed consumption & body weight.

2.6. Standard kits and reagent

Serum lipid profile was done with the standard biochemical kit (Avantor performance materials. Deventer, The Netherlands). HbA1c was estimated with the standard kit (Recipe chemicals, Dessauerster 3, Gmbh, Germany). Fructose 1,6 bis phosphatase, glucose-6-phosphatse, ATP, NADPH, sulphosalicyclic acid, gultathione reduced (GSH), choloro-dinitro benzene (CDNB), folins reagent, bovine serum albumin (BSA), cysteine HCl and RPMI was purchased from Sigmachemical company (St. Louis, USA). Pyrogallol, H_2O_2, mannitol, succinic acid, sodium chloride, KH_2PO_4, K_2HPO_4, EDTA, DTNB (Ellman's reagent), tris HCl, Na_2CO_3, NaOH, ferrous sulphate, ammonium molybdate, Mgcl2, D-glucose was purchased from SRL Chemicals (India). All other chemicals and reagents used were of the highest analytical grade available.

Table 1
Chronic unpredictable environmental weekly stress schedule implemented on Swiss albino mice.

Days	Stress time	Stress type	Duration	Conditions
Day 1	10:00 h	Restraint	3 h	No
Day 2	11:00 h	Wet bedding (25 °C)	2 h	No
Day 3	15:00 h	Forced swim (25 °C)	30 min	No
Day 4	13:00 h	Crowding	2 h	No
Day 5	19:00 h	Lights on	Overnight	No
Day 6	09:00 h	Cold force swim (4 °C)	15 min	22:00 h, crowding overnight
Day 7	10:00 h	Restraint	2 h	19:00 h, food deprivation overnight

2.7. Collection of samples

At the termination of the experiment, overnight fasted animals were sacrificed ethically; blood was collected in EDTA vials, centrifuged at 1500 g for 15 min and kept at −20 °C for estimation. Tissue samples were washed with ice-cold normal saline thrice. Homogenates of liver and pancreas were prepared freshly for analysis in chilled phosphate buffer saline, pH 7.4 with glass Teflon homogenizer (REMI-RQ126/D) at 4000 g for 20 min in ice-filled glass beaker under chilling environment. Kidney homogenate was prepared in tris mannitol buffer to maintain the osmolarity. Protein content in tissue and serum sample was quantified according to the previously described method [17]. Peripheral tissue for histopathology analysis was preserved in formal saline, while the tissue for comet assay was kept in RPMI media.

2.8. Feed consumption and body weight

Animal body weight and feed consumption were recorded once a week throughout the experiment duration of 16 weeks. The Feed efficiency ration was calculated as per equation is given below:

Feed Efficiency Ratio = Body weight gain (gms) /Feed consumed (gms) x 100

2.9. Glycemic control estimation

Estimation of glucose has been performed weekly in all mice of both groups by tail sniping method with a glucometer (my life Pura X, YPSOMED, Switzerland). Plasma samples of all animals were assessed for HbA1c measurement by standard kit (Recipe chemicals, Dessauerster 3, Gmbh, Germany) at the termination of the experiment.

2.10. Serum lipid profile

Serum samples overnight fasting animals of both groups were processed for estimation of total cholesterol, HDL, triglycerides, LDL, and VLDL by a colorimetric method (Avantor performance materials, Deventer, The Netherlands).

2.11. Carbohydrate metabolizing enzymes activity

Hexokinase (HK) activity was assayed in tissue samples of liver, pancreas and kidney to demonstrate glucose phosphorylation by previously described method [18]. Fructose 1,6 bis phophatase (F1, 6BPase) activity was estimated in liver, kidney and pancreas [19]. Glucose -6- phosphatase was assayed by a previously described method [20]. Briefly, incubation mixture was prepared in citrate buffer along with substrate and sample. The reaction mixture for 1 h was incubated at 37 0C. Termination of the reaction in the incubation mixture was performed with the addition of 10% TCA solution followed by centrifugation at $5000 \times g$ for 10 min. The phosphorous content was estimated in the suspension according to the previously described method [21].

2.12. Estimation of reactive oxygen species (ROS) scavengers

2.12.1. Superoxide dismutase (SOD)

SOD activity in tissue and serum was determined by measuring the inhibition of auto-oxidation of pyrogallol in 0.05 M Tris succinate buffer (pH 8.2) at a wavelength of 412 nm. SOD activity was estimated with a previously described method [22]. The results obtained were expressed as specific activity in units/mg protein.

2.12.2. Catalase (CAT)

Serum and tissue catalase activity was measured with 0.05 M phosphate buffer (pH 7.0) as previously explained method [23]. The decrease in the absorbance at 240 nm due to oxidation of 30 mM hydrogen peroxide (H_2O_2) was recorded for the estimation of activity. The results were expressed as specific activity in units/mg protein sample.

2.12.3. Glutathione reduced (GSH)

Serum and tissue homogenate reduced glutathione (GSH) activity was estimated by the previously described method [24]. A briefly equal amount of sulphosalicylic acid was added to the sample and then incubated for 1 h at 4 °C. The incubated sample was then centrifuged at $12000 \times g$ for 15 min at 40C. DTNB (Elman's reagent) was then added to the supernatant in potassium phosphate buffer (0.1 M, pH = 7.4) and yellow colour developed was read at 412 nm. The results obtained were expressed as nmol/mg protein.

2.12.4. Glutathione-s-transferase (GST)

GST activity in serum and tissue homogenate was assessed in 0.2 M phosphate buffer (pH 6.5) as previously described protocol [14]. Freshly prepared GSH was dissolved in potassium phosphate buffer followed by addition of CDNB (1 mM) (Chloro-dinitrobenzene) prepared in acetone. Samples were read at 340 nm and activity was calculated as units/mg of protein.

2.12.5. Glutathione peroxidase (GPx)

Glutathione peroxidase (GPx) activity in tissue homogenate and serum was determined [25]. Tissue homogenate was centrifuged at 4000 rpm for 5 min and washed gently with 0.1 M potassium phosphate buffer (pH 7.4) having EDTA (1 mM). GPx activity was calculated using an extinction coefficient of 6.22 mM extinction coefficient of β-NADPH at 340 nm.

2.13. Tissue histopathology

The mice were sacrificed by cervical dislocation and liver, kidney, pancreas, and glycolytic skeletal muscles were taken for histopathology. Tissues were fixed in 10% formalin in PBS, embedded in paraffin, and cut into 3−5-μm thick sections with a rotary microtome and stained with hematoxylin and eosin stain. Stained slides were observed under a trinocular light microscope (Olympus BX40, Japan).

2.14. Comet assay

The comet assay of liver, pancreas and kidney was done as previously described protocol with few modifications [26]. Pre-coated and frosted slides with 1%normal melting agarose (as a base layer) were prepared a day before sacrificing the mice. About 10,000 cells isolated from each organ cell suspension were mixed with 100 ml 1% of low melting point agarose to form the working cell suspension. This suspension was pipetted over the base layer at 37 °C followed by covering with coverslips immediately. After solidification, a third layer of 0.5% LMPA (100 ml) was placed over followed by covering with coverslips and kept on ice packs again. Slides were dipped for 3 h in cold lysing solution (2.5 M NaCl+100 mM EDTA+10 mM tris-base+1% Triton X-100, pH = 10). Later, slides were allowed to unwind for 30 min in alkaline electrophoretic running buffer (300 mM NaOH + 1 mM EDTA, pH = 13). Then, electrophoresis was performed for 35 min at 40 C

with the constant current strength of 300 mA. Slides were placed in neutralizing buffer (0.4 M Tris-base) of pH 7.5 followed by washing with cold saline thrice. The process of neutralization followed by washing was repeated thrice. Staining of slides was performed with 80 ml ethidium bromide (20 mg/ml). EtBr stained slides were scored with a fluorescent microscope (Olympus, Japan) coupled with an image analysis system (Komet 5.5, Kinetic imaging, Liverpool, U.K.) Comet tail-length (migration of DNA from its nucleus in mm) was measured to assess the level of DNA damage.

2.15. Estimation of stress hormones, insulin and HOMA-IR

Blood was extracted from mice during early morning timings for estimation of stress hormones. Plasma epinephrine quantity was estimated using mouse epinephrine ELISA kit (procured from Abnova, KA1882, Taiwan). The protocol manual procedure supplied by the kit manufacturer was followed. Plasma corticosterone was determined using mouse corticosterone ELISA kit (procured from Abnova, KA0468, Taiwan). Furthermore, the protocol supplied in the manual was followed. The plasma insulin was measured by mouse enzyme-linked immunosorbent assay (ELISA) kit (Mercodia, Sweden) (Intra- and inter-assay coefficient of variations were 3.40% and 2.20% respectively). The Homeostatic model assessment-Insulin resistance was calculated for each group with the given equation below:

HOMA-IR = fasting glucose level (mmol/L) x fasting insulin level (μU/mL)/ 22.5

2.16. Statistical analysis

All statistical analysis of results was performed with the Statistical Program of Social Sciences (SPSS) for Windows 7 version. Results of the analysis were expressed as mean ± SD and the significant difference between control and the stressed group was determined by using unpaired student's t-test. Repeated measure analysis of variance (RMANOVA) was used to evaluate the statistical significance among body weight, fasting plasma glucose and feed consumption of stressed and control mice. A p value of <0.05 was considered to be significant.

3. Results

3.1. Effect of CUES on body weight and latency to consume feed

One way repeated ANOVA (RMANOVA) statistical analysis of animal body weight showed that chronic exposure to environmental unpredictable stressed group resulted in a decrease in body weight compared to control group (Fig. 1a) Table 2. Significant changes were not found in the feed consumption attributed of all stressed mice compared to control (Fig. 1b) Table 2. The latency to consume feed in the stressed group was found less as compared to control. The feed efficiency of the control group was found to be higher than the treatment group (p < 0.001) Table 2.

3.2. Glycemic control assessment

The fasting blood glucose level in stressed mice was found significantly higher than control group. Moreover, an increase in fasting blood glucose was observed from the second week of initiation of an experiment in the stressed group (Fig. 1c) Table 2. HbA1c results showed significant increase in stressed group

(7.1 ± 0.13%) compared to control mice (6.15 ± 0.31%) at 16th week with a p-value of 0.0002 (F = 3.369, df = 8).

3.3. Serum lipid profile

No significant change in lipid and lipoproteins were observed in stressed group as compared to control mice. Serum cholesterol, HDL, LDL, VLDL and triglycerides in stressed mice after 16 week stress protocol were 161 + 63.98 mg/dL (p = 0.6260) (F = 5.68,df = 8), 40.72 + 16.30 mg/dL (p = 0.5516) (F = 13.67, df = 8), 86.29 + 48.52 mg/dL (p = 0.8332) (F = 3.689, df = 8), 34 + 7.12 mg/dL (p = 0.2088) and 170 + 35.61 mg/dL (p = 0.2088) respectively, insignificantly higher than serum cholesterol, HDL, LDL, VLDL and triglycerides 145 + 29.82 mg/dL,35.74 + 7.43 mg/dL,81.10 + 22.32 mg/dL, 28.16 + 6.36 mg/dL and 140.8 + 31.82 mg/dL respectively in the control group.

3.4. Carbohydrate enzymes activity

The significant changes were found in the activity of hexokinase (HK), and glucose-6-phosphatase in stressed mice compared to controls. The activity of F1, 6 BPase decreased in stressed mice compared to control but is not significant statistically. The results obtained showed that CUES alters the carbohydrate metabolism in liver, kidney and pancreas homogenate as shown in Table 3.

3.5. Antioxidants activity

Serum SOD, catalase, GSH, GST& Gpx activity in stressed mice were significantly lower than of control mice as shown in Table 3. Furthermore, antioxidants activities in liver, kidney and pancreas demonstrated significant decrease in activity in stressed mice (Table 3). Results of this study clearly reflect the relation of CUES in the alteration of ROS scavenger expression.

3.6. Histopathology

Histopathological studies of the liver, kidney, pancreas and glycolytic skeletal muscles for the elucidation of chronic stress severity have been reported as one of the pivotal approaches. Chronic stress severely affects the histology of these peripheral tissues as shown in Fig. 2.

3.7. Comet assay

Chronic stress exposure in stressed mice caused prominent DNA damage in the kidney, liver and pancreas as evidenced by tail length elongation with respect to control mice. A significant change in tail length was observed in stressed mice compared to control as shown in Fig. 3 (Table 4).

3.8. Estimation of the plasma stress hormone, insulin and HOMA-IR

The mean values of plasma epinephrine were found significantly higher in mice of stressed group (21.66 ± 1.99 pg/ml) compared to control mice (13.15 ± 2.11 pg/mL) P > 0.05. Moreover, plasma cortisol levels in stressed mice are found to be 46.62 ± 1.99 ng/mL, which is significantly higher than the control mice 16.11 ± 2.19 ng/mL respectively (P > 0.05). Plasma fasting insulin and HOMA-IR was demonstrated in Table 5.

4. Discussion

The present study demonstrated the adverse effect of 16 weeks CUES on carbohydrate metabolism, lipids profile, glycemic control
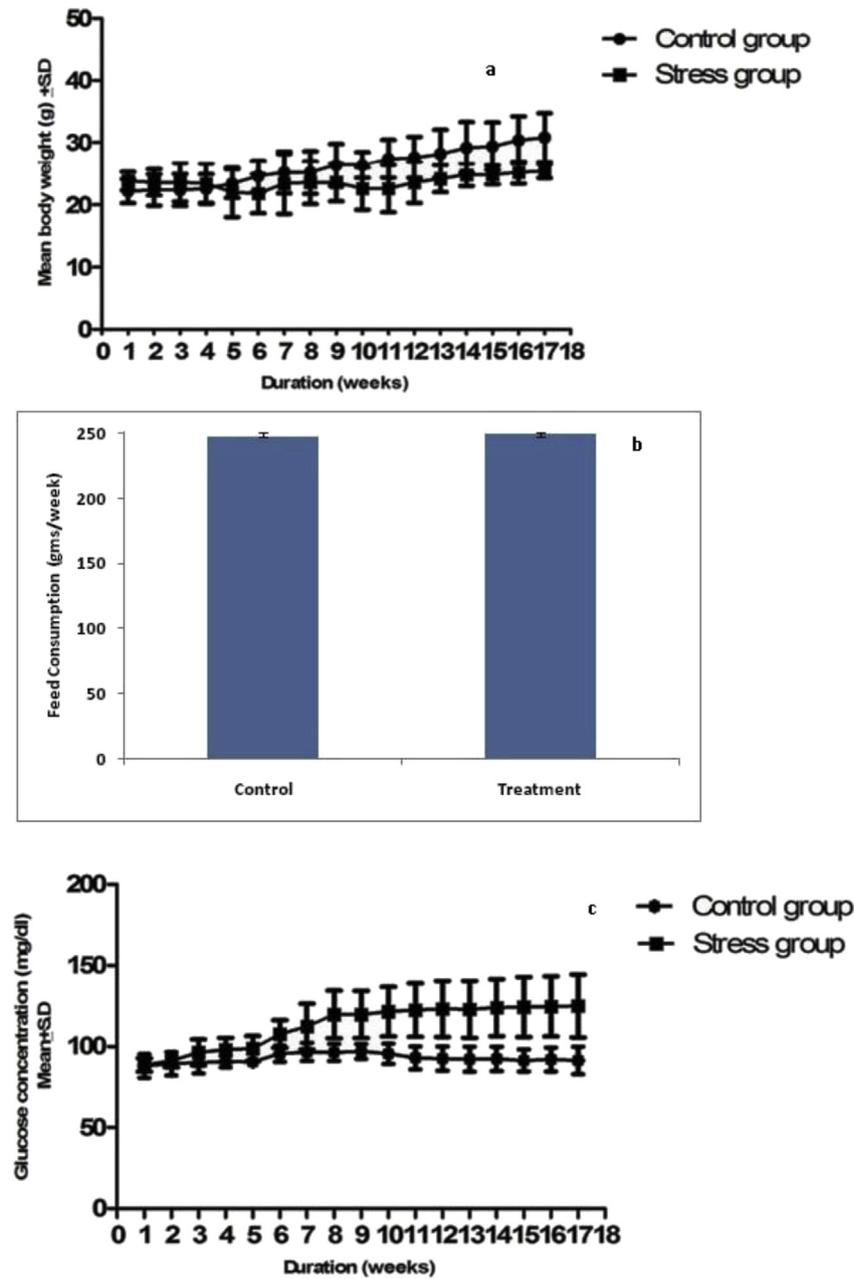
Fig. 1. (a): Evaluation of chronic unpredictable environmental stress on mean body weight in gms. Stressed mice were showing significant decrease in mean body weight compared to control (p < 0.05). Data are presented as mean + S.D. **(1b)** Effect of chronic unpredictable environmental stress on feed consumption. Stressed mice were showing no significant changes in feed consumption compared to control mice. Feed consumption recorded in grams consumed per week. **(1c)** Evaluation of chronic unpredictable environmental stress on weekly plasma fasting glucose (mg/dl). Stressed mice were showing significant increase in fasting plasma blood glucose compared to control. Data are presented as mean + SD. A p value < 0.05 were considered as significant (n = 20 in each group).

Table 2
Repeated measure analysis of variance (RMANOVA) was performed to demonstrate the statistical changes among control and stressed mice. All values are represented as mean ± S.D. A p value (<0.05) were considered to be significant. Non significant p values were shown by *.

Groups (n = 20)	Fasting blood glucose (mg/dL)	Body weight (Gms)	Feed Weight (Gms)	Feed Efficiency Ratio
Control	93.187 ± 2.37	26.35 ± 2.821	49.72 ± 0.86	54.26
Stressed	116.10 ± 10.62	23.63 ± 1.06	49.91 ± 1.95	47.90
F Value	74.89	23.408	0.160	0.236
P Value	<0.001	<0.001	0.695*	<0.001

Table 3
Effect of chronic unpredictable environmental stress on antioxidants activities in serum, liver, kidney and pancreas along with carbohydrate enzymes activity in liver, kidney and pancreas. Statistical differences were evaluated by student unpaired *t*-test. Data are presented as mean ± SD. A p value < 0.05 were considered as significant. Non-significant p value were designated with *. CAT= Catalase, SOD=Superoxide dismutase, GSH = Glutathione, GST = Glutathione-S-transferase, Gpx = Glutathione peroxidase, HK=Hexokinase.

Group (n = 20)	CAT (U/mg protein)	SOD (U/mg protein)	GSH (nmol/mg protein)	GST (U/mg of protein)	Gpx (nmol/min/mg protein)	HK (moles of glucose phosphorylated/g/h)	F 1, 6 bis phosphates (moles of pi liberated /min/mg)	Glucose-6-phosphatase (moles of pi liberated /min/mg)
Liver (control group)	105.12 + 6.79	169.17 + 6.79	6.91 + 0.36	66.02 + 3.75	121.29 + 3.66	124.08 + 17.00	0.29 + 0.07	0.18 + 0.011
Liver (stress group)	90.20 + 11.81	140.62 + 5.34	5.23 + 0.54	58.04 + 1.75	73.44 + 1.75	102.77 + 7.68	0.32 + 0.05	0.22 + 0.023
P-value (Liver)	0.0278	<0.0001	0.0004	0.0026	0.0001	0.0340	0.4912*	0.0049
Kidney (control group)	101.19 + 3.87	156.28 + 6.35	7.37 + 0.23	40.96 + 3.48	83.51 + 5.80	106.62 + 8.8	0.19 + 0.05	0.20 + 0.00
Kidney (stress group)	92.47 + 6.34	122.85 + 9.22	5.30 + 0.50	32.89 + 6.56	65.06 + 3.60	94.28 + 5.76	0.12 + 0.04	0.18 + 0.15
P-value (kidney)	0.0218	0.0002	0.0001	0.0413	0.0003	0.0312	1.0000*	0.0374
Pancreas (control group)	97.55 + 7.23	87.58 + 6.69	6.70 + 0.79	22.63 + 2.35	70.70 + 2.60	115.64 + 17.14	0.13 + 0.048	0.21 + 0.01
Pancreas (stress group)	85.92 + 6.95	61.45 + 5.17	4.71 + 0.49	17.50 + 1.64	58.28 + 7.19	98.13 + 4.93	0.10 + 0.006	0.199 + 0.00
P-value (Pancreas)	0.0320	0.0001	0.0015	0.0040	0.0067	0.0594	0.1901*	0.1242*
Serum (control group)	132.1 + 5.41	150.5 + 4.0	8.19 + 0.86	1.27 + 0.40	81.83 + 2.42	–	–	–
Serum (stressed group)	111.2 + 6.20	123.5 + 6.60	4.94 + 0.51	0.96 + 0.14	61.86 + 5.0	–	–	–
P-value (Serum)	0.0005	<0.0001	<0.0001	0.0017	<0.0001	–	–	–

and antioxidants activity. A significant decrease in body weight was observed in stressed mice as compared to control group. Feed intake was also found lower in the stressed group. The significant decrease in feed efficiency ratio (FER) was found in the stressed group compared to control mice. The increased FER in the control group is the suggestive of body weight gain occurred due to normal glucose metabolism. A significant increase in fasting blood glucose and HbA1c at 16th week was found in stressed mice compared to the control group. An insignificant change in lipid profile was observed in the stressed group as compared to the control group. The increase in serum lipids and lipid proteins were insignificant probable due to less number of animals in each group that could not reach at statistical significance. The present study also deciphers the alterations in antioxidants activities as a resultant of CUES. In a stressed group, decrease activities of CAT, SOD, GST, Gpx and GSH were found compared to control mice. Hexokinase activity in stressed mice was significantly higher than control mice. However, an insignificant increase in activities of F 1, 6BPase and glucose-6-phosphatase were found in stressed mice compared to control animals. It is well known, that chronic psychological stress decreases biological resilience, impairs homoeostasis of the living system and induces changes in various physiological functions leading to pathological complications [27]. Moreover, several published literature reflects the adverse effect of chronic stress on hereditary unit integrity [28]. These changes would be due to a significant increase in the ROS and deleterious effect on beta cells resulting in alterations in carbohydrate metabolism. A previous study reported that stress-induced the release of corticotrophin-releasing hormone (anorexigenic neuropeptide) thereby leads to the decrease in the latency of feed consumption and body weight,

producing a "negative energy balance" and increase in corticosterone release [29].

Impairment of glycemic control could be the combo of two mechanistic pathways, the hyperglycaemic environment of the release of glucocorticoids and catecholamines upon activation of sympatho-adreno-medullary and pituitary-adreno-cortical scheme respectively [2,3]. Chronic stress results in elevated plasma catecholamine along with corticosterone that constitutes fight or flight response thereby stimulating glycogenolysis resulting in increased metabolic rate and glucose concentration in the body. This hyperglycaemic environment enables haemoglobin susceptible to glycation [30].

Cholesterol synthesis occurs in the liver is governed by series of transcriptional factors primarily sterol regulatory element binding protein 2(SREBP2) which lead to increased expression of cholesterol [31]. Chronic stress mobilizes lipids in circulation to cope with flight and fight response [32]. The lipids can be oxidized to restore energy and used for gluconeogenesis [31]. Rodent trials have also been found to have higher cholesterol level among stressed group compared to control [33]. The dynamic mobilization of lipids due to stress increases catecholamines mediated expression of adipose triglycerides lipase and hormone-sensitive lipase [34]. Catecholamine also plays a subsidiary role in the phosphorylation and activation of hormone-sensitive lipase, as a results perilipin protein gets phosphorylated and a conformational change occurs that allows lipases to retain in contact with lipids [35]. In addition to this stress induces the activation of glucagon, cortisol and catecholamines that activates phosphatidate phosphohydrolase which in turn results in an increase of hepatic triglycerides synthesis. Similarly, stress-induced release of glucocorticoids and free fatty acid
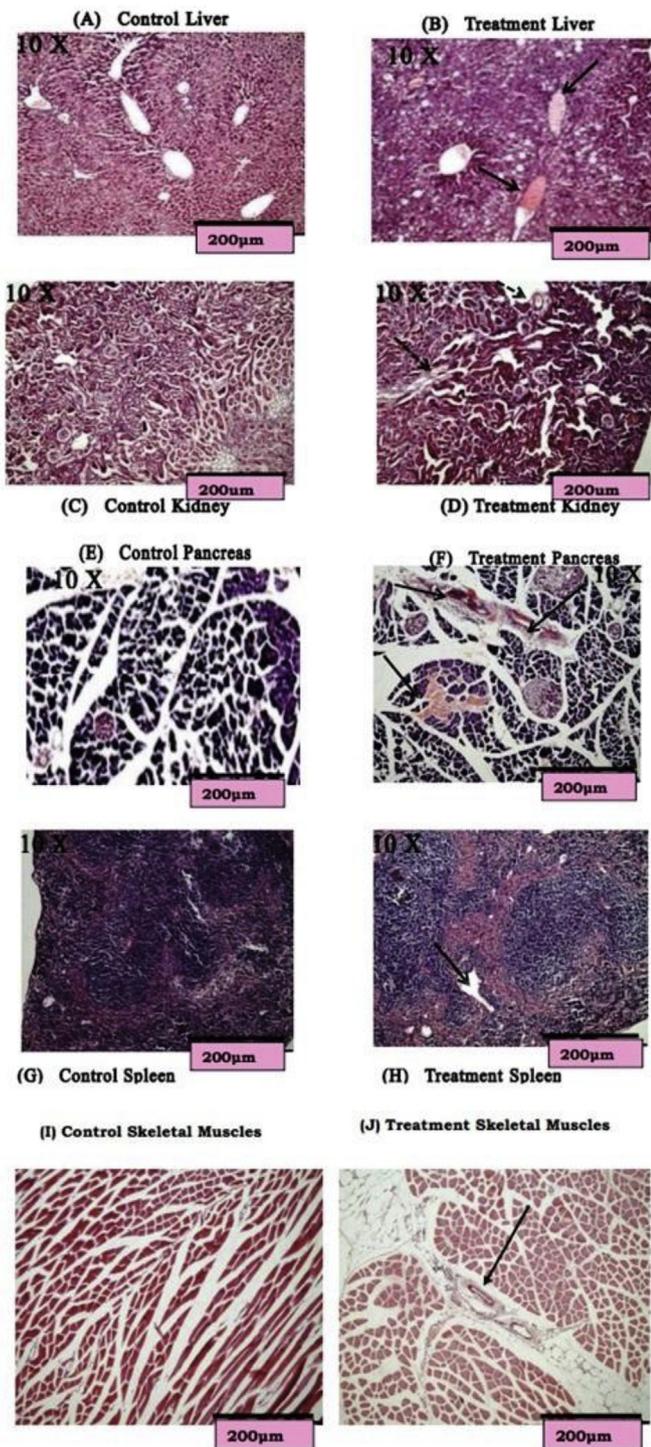
Fig. 2. Haemotoxylin and Eosin stained sections of tissues under bright field microscope with $10\times$ (A) Liver (control group) showing normal hepatocytes with normal hepatic venules surrounded by hepatocytes plates. It is showing no aggregation of nuclei. (B) Liver (stressed group) showing vacuolated spaces with clustered nuclei; as shown by single black solid arrow (C) kidney (control group) showing normal symmetry of glomeruli, vessels and tubule along with same thickness of vessels. (D) Kidney (stressed group) showing dilated and flattened bowmen's capsules and clustered nuclei in glomerulus as shown by single dashed arrow. (E) pancreas (control group) showing normal pancreatic acini, islets cells and blood vessels arrangement (F) pancreas (stressed group) showing lymphocytic infiltration in islets cells & moderate congestion of blood capillaries; shown by thick solid black arrow (G) spleen (control group) showing normal red pulp and white pulp surrounded by fibrous tissues. (H) Spleen (stressed group) showing vacuolated spaces & megakaryocytes in red pulp and white pulp region as shown by thick solid black arrow. (I) skeletal muscles (control group) showing normal epimysium in both vertical and horizontal fibre (J) skeletal muscles (stressed group) showing mild hyperplasia along with moderate congestion of blood capillaries indicated by thick solid black arrows.

stimulate the activation of HMG-CoA reductase of liver thereby synthesizing cholesterol [36]. Increase level of VLDL during stress was also found in this study, this may be due to the high supply of free fatty acids via lipolytic mechanism effect [34].

The antioxidant enzymes demonstrate the first line of defence against reactive oxygen species and their decrease activities may contribute to the oxidative stress. A published literature also reported exposure to 21 days stress decreases GSH activity [16]. GST activity was also found to decrease during chronic stress exposure due to altered glutathione metabolism and impaired antioxidant defence pathways. The overall impairment in the antioxidant defence system and biochemical parameters may be due to high depletion of both deep energy (stored) and superficial energy (ready to use) during the exhaustion phase of chronic stress. A recent study on chronic stress also demonstrates the adverse effect on carbohydrate metabolism in male albino rats [37]. The decrease activity of catalase during stress could be due to the combinatorial effect of depletion of NADPH, lipid peroxidation and excessive production of free radicals [38]. The decrease in GPx, GSH and GST activity might be because of depletion or inactivation of the enzyme by stress-induced ROS [38]. The reduction of catalase and SOD activity may also be due to either loss of substrate H_2O_2 or their less transcription due to overproduction of ROS.

CUES induced downfall in the activity of glucose-6-phopshatse dehydrogenase results in diminished operation of pentose phosphate pathway and thereby enhances the production of reducing molecules such as NADH and NADPH [39]. In the current study administration of the CUES, protocol decrease the glucose-6-phosphatase activity thereby causing activation of the pentose phosphate pathway and increase in NADPH due to inactivation of sorbitol pathway [40]. It could be noticed that in the stressed group, the glycogenolysis is enhancing the production of endogenous glucose and along with its peripheral utilization. Contrastingly, the HK activity was significantly reduced in stressed mice suggesting decrease phosphorylation of hepatic glucose after stress stressed. Gluconeogenic enzymes (G-6-Pase and F1,6 BPase) showed parallel decrease in its activities. As for experimental stress, our results showed that besides hyperglycemia there is also a decrease in glycogenolytic and glycolytic enzymes (G6-Pase, and HK) activity. The present study also shows a distinct pattern of enzyme activity expression. The decrease in hexokinase activity in stressed group is a marked evidence of consistent hyperglycemia as an insufficient amount of glucose is reaching to liver even in high glycemic control [41]. However, the stress group also showed decreased activity in G6Pase thereby giving an insight on glucose homeostasis [39].

As shown in results that a significant decrease of gluconeogenesis enzymes was found hampered by CUES. The decrease in gluconeogenic enzymes may also hamper the enzymes of the TCA cycle. CUES allow the release of epinephrine and norepinephrine into the blood stream by adrenal glands that in turn stimulate the pancreatic α cells to secrete glucagon. This released hormone binds to β adrenergic receptors and stimulates adenylate cyclise and produces cAMP along with inositol-1,4,5-triphosphate(IP3), diacylglycerol (DAG) and Ca+2, also with the activation of α adrenergic receptors. The liver's response to chronic stress incorporates the participation of two secondary messenger systems. cAMP mediates the stimulation of glycogenolysis and inhibition of glycogen synthesis and thereby inactivate the glycogen synthase. This stimulation event hampers the glucose metabolism and allows hyperglycemia to persist.
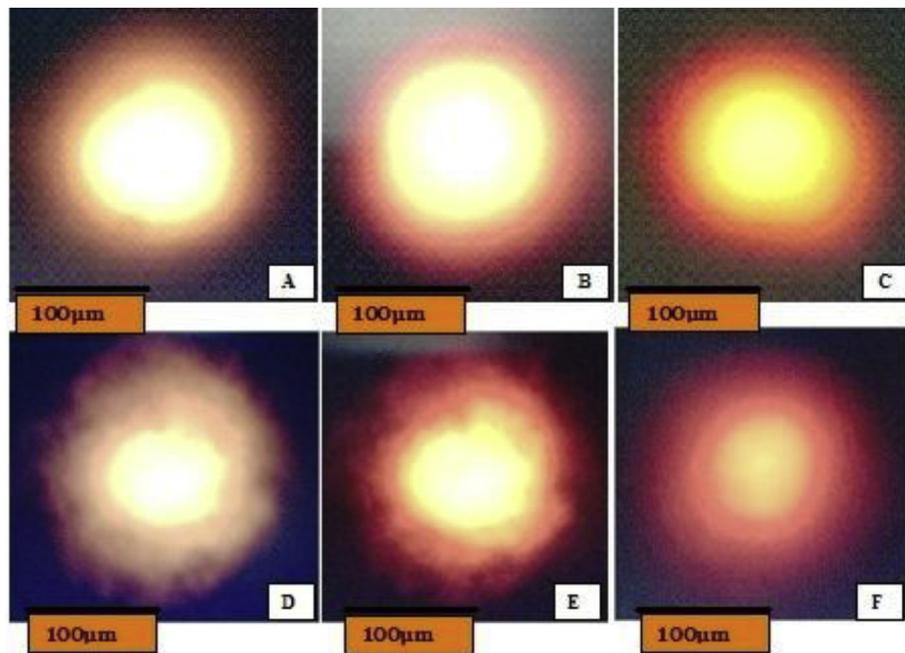
Fig. 3. Effect of chronic unpredictable environmental stress on average comet tail length of tissues (A) liver (control group) (B) kidney (control group) (C) pancreas (control group) (D) liver (stressed group) (E) kidney (stressed group) (F) pancreas (stressed group). The images of ethidium bromide stained slides were captured with fluorescent microscope. The comet tail length was measured with Komet 5.5 imaging system to acquire images.

Table 4
Effect of chronic unpredictable environmental stress on DNA damage assessed by comet tail length. Statistical differences were evaluated by student unpaired t-test. Data are presented as mean $\pm$ SD.

Group (n = 20)	Liver (μm)	Kidney (μm)	Pancreas (μm)
Control	4.50 ± 0.48	6.01 ± 0.10	5.88 ± 0.50
Stressed	24.21 ± 1.37	27.21 ± 1.59	26.59 ± 1.65
P-value	<0.0001	<0.0001	<0.0001

Table 5
Effect of chronic unpredictable environmental stress on plasma insulin and HOMA-IR. Statistical differences were evaluated by student unpaired t-test. Data are presented as mean $\pm$ SD.

Group	Fasting insulin (μg/L)	HOMA-IR Index
Control (n = 20)	2.06 ± 0.31	2.19 ± 0.54
Stressed (n = 20)	0.51 ± 0.06	5.13 ± 1.11
P-value	<0.0001	<0.0001

The changes in peripheral tissues that include liver, kidney, pancreas, spleen and skeletal muscles are also the remarkable finding of the present study. Liver of the control group of mice shows normal hepatic cells, hepatic veins and prominent central vein. While after administration of stress, degenerative changes were observed in hepatic cells, several vacuolated spaces were observed along with clustered nuclei suggestive of fatty liver (Fig. 2). Control group of mice shows normal glomerulus in kidney along with both proximal and distal convoluted tubules and bowmen's capsule. Stressed mice kidney shows clustered nuclei in glomerulus with frequent degeneration of cytoplasm along with dilated and flattened bowmen's capsules. The above changes in the liver and kidney histology may be due to the release of the stress proteins HSP25, HSP72, GRP-75 after chronic exposure to stress (Fig. 2) [1]. The pancreas of the control group shows the normal structure of islets cells, whereas, in the stress group, pancreatic islets cells show lymphocytic infiltration as shown in Fig. 2. Stress-

induced free radicals readily damage the beta cells of the islets, thereby causing insulinitis as reported previously in the literature [42]. Skeletal muscles in a stressed group show moderate congestion of blood capillaries along with mild hyperplasia compared to control mice probably due to chronic stress that alters the normal secretion of Insulin-like growth factor (IGF). IGF mediates the insulin metabolism and stimulates protein synthesis. Two types of IGF are found in skeletal muscles IGF-I and IGF-II that are responsible for proliferation and differentiation of satellite cells. In response to chronic stress, the IGF-I levels enhance and cause the hypertrophy and hyperplasia of skeletal muscles as reported earlier. The observed changes in splenic red pulp and white pulp in the stress group showed vacuolated spaces and megakaryocytes.

A significant quantity of DNA damage in terms of increased comet tail length was observed in the liver, pancreas and kidney of stressed mice [3]. A plausible and easy approach to understand this damage lies with the explanation of ROS production in high concentration due to chronic exposure of the stress in stressed mice. Deoxyribose backbone in the DNA is the accessible target for ROS thus initiating oxidative DNA lesions [15]. ROS primarily indirectly attack other cellular components generating secondary reactive intermediates that form DNA adduct irreversibly. Thus chronic stress lead to impairment in the homeostasis and ROS mediated damage to DNA as measured by comet tail lengths. The mechanism of environmental stress on ROS mediated DNA damage has been demonstrated in Fig. 4.

The aim to measure plasma cortisol and epinephrine in the present study is to evaluate the effect of CUES on body homeostasis. These hormones are secreted in an emergency situation and in stress response [43]. The presence of variable and chronic stress during the period of the experiment when exceeding the threshold may activate HPA (hypothalamus-pituitary axis) mediated by a central afferent cycle that contributes in the release of stress hormones [44]. Adrenal cortex region secretes two forms of glucocorticoids that includes (a) active and (b) inactive, both act as stress indicator [45]. Hence the estimation of cortisol and epinephrine in
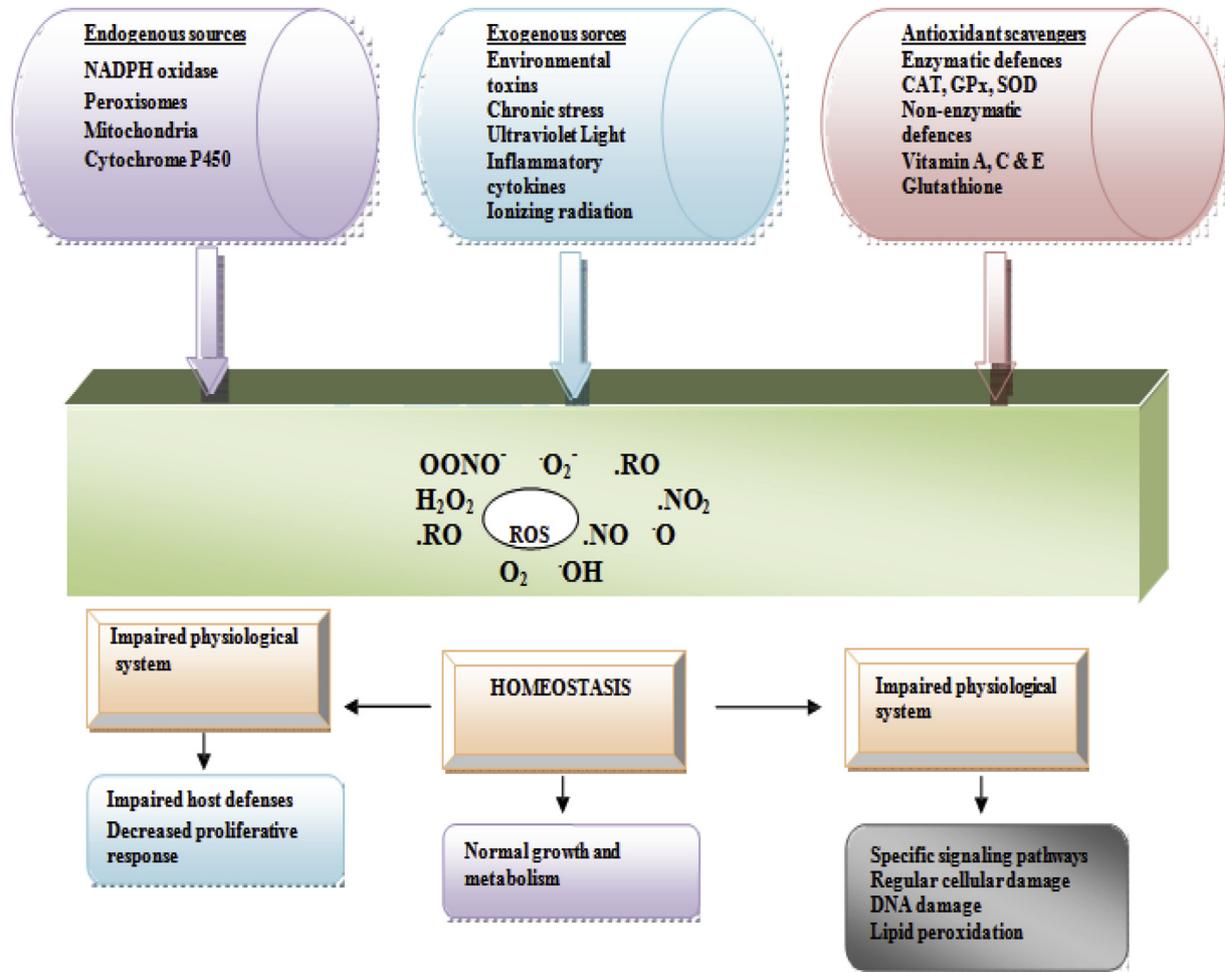
Fig. 4. The cellular response and sources of reactive oxygen species (ROS). Generation of ROS results from normal metabolism in mitochondria and peroxisomes along with cytosolic enzymes mechanisms. Numerous external agents can triggers ROS generation. Enzymatic and non-enzymatic antioxidants defence system counteracts these ROS to maintain normal physiological homeostasis. Decrease level of ROS below cutoff value of homeostasis interrupt the physiological role of ROS in host defence and cellular proliferation. Similarly, elevated ROS level accelerate the impairment of homeostasis and lead to detrimental effects on cells and other components of living system. Biomolecules like DNA, RNA, lipids, and proteins several becomes the target of ROS and get damaged. Further increase in ROS may initiate a stress signal that triggers the specific redox-sensitive signaling pathways.

stressed mice indicate the suffering from chronic stress that impairs the homeostasis of the system.

5. Conclusion

The present study concludes that CUES in female Swiss albino mice for a period of 16 weeks leads to a decrease in antioxidants activities and alterations in carbohydrate metabolizing enzyme expression. CUES induced hyperglycemia impairs the metabolism and free radicals scavenging significantly. Histopathology examination and comet assay reveal the fact that exposure to chronic stress is harmful at tissue level also.

Author's contribution

Conceptualization: AR, JA, IN.

Data curation: JA, IN.
Formal analysis: AR, JA, IN.
Investigation: AR.
Methodology: AR.
Project administration: JA, IN.
Resources: JA, IN.
Software: AR.
Supervision: JA, IN.
Validation: JA, IN.
Visualization: AR, JA.
Writing — original draft: AR.
Writing — review & editing: JA, IN.

Conflicts of interest

The authors have declared that there is no conflict of interest.

Abbreviations Used

CUES	Chronic unpredictable environmental stress
n	Number of animals in each group
HDL	High density lipoprotein
LDL	Low density lipoprotein
VLDL	very low density lipoprotein
CPCSEA	Committee for the Purpose of Control and Supervision of Experiments on Animals
$l \times b \times h$	length x breadth x height
mm	millimetre
cm	centimetre
$°C$	degree centigrade
CFL	Compact fluorescent lamp
Fig	Figure

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dsx.2019.01.020.

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