Saudi Journal of Biological Sciences 28 (2021) 566–571

Contents lists available at ScienceDirect

Saudi Journal of Biological Sciences

journal homepage: www.sciencedirect.com

Original article

Diazepam ameliorates altered proinflammatory and cardiac markers in stress exposed rats

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ARTICLE INFO

Article history:
Received 29 April 2020
Revised 9 July 2020
Accepted 20 October 2020
Available online 29 October 2020

Keywords:
Stress
Diazepam
Cardiac marker
Inflammatory markers
C-reactive protein

ABSTRACT

Regular exposure to stress causes alteration in biochemical parameter but till date no specific medicine prescribed for controlling it. Current study aimed to determine the effect of Diazepam on proinflammatory and cardiac markers in stress exposed rats. Male Wistar rats were divided into four groups with six animals in each group for 90 days study. Group-1 served as a Normal Control (NC), Groups-2, as a Disease Control (DC), Group-3 as a Diazepam Control (DMC) and Group-4 as a Disease + Diazepam Treatment (DT). DMC and DT animals exposed to regular stress by forced swimming exercise method for 90 days. DMC and DT received 5 mg/kg, p.o the daily dose of Diazepam. At the end of the protocol, animals were sacrificed. The level of serum proinflammatory marker interleukin-6 in DC increased significantly ($p < 0.001$) while restored significantly ($p < 0.001$) in DT. Level of interleukin-10 in DC decreased significantly ($p < 0.001$) while restored significantly ($p < 0.001$) in DT. Level of fibrinogen was also increased by stress, which was restored significantly ($p < 0.05$) by diazepam. Increased level of Creatine kinase-MB (CK-MB) by stress was restored significantly ($p < 0.05$) by diazepam. The level of cortisol was increased also significantly ($p < 0.001$) and restored to normal by diazepam. The level of C-reactive protein (CRP) and cholesterol was increased significantly ($p < 0.01$; $p < 0.001$) by stress while restored significantly ($p < 0.01$; $p < 0.001$) by diazepam. Findings from results suggest that diazepam ameliorates altered proinflammatory and cardiac markers in stress exposed rats.

1. Introduction

Stress plays precipitating and predisposing role in the onset of affective disease and associated biochemical fluctuations (Jia et al., 2020). Regular exposure to stress shows effects on biochemical parameters, immunity, reproduction and growth (Hou et al., 2019). Alteration in the homeostasis and physiology of the living being by external stress or factors has been the topic of interest since 1938 with the work of Selye (Selye, 1938). This stress concept is also defined as General Adaptation Syndrome (Delahanty et al., 2019). In different phases of stress fluctuation of different biochemical parameters can be utilized as markers for determination of physiological changes in the body in response to exposure of stress (Buselli et al., 2019). These markers, can be analyzed by researcher examination of different biochemical parameters of blood serum, urine and organs (Schiavone and Trabace, 2017). Response of stress in living being can be observed into three phases such as primary, secondary and tertiary (Filgueiras et al., 2019). Primary response begins in the hypothalamus of the brain. With continuous stress, this primary response is exaggerated by pituitary renal axis stimulation which leads to rise in catecholamine hormone secretion and cortisol secretion (Kumar and Joy, 2019). Extra secretion of these hormones enhances immunological, hematological and metabolic response, which is cumulatively considered as the secondary response in stress (Li et al., 2019). Exposure to continuous stress leads the last response stage, which is regarded as a disease or physical alteration of the body (Miller et al., 2019).

Brain plays major role in stress and associated complications. From a biochemistry point of view, adrenoceptors and 5-HT 2 receptors are involved in stress and depression (Nachtigall et al., 2019). The tricyclic anti-depressant drugs act thought these receptors to stem by decreasing expression of these receptors. Diazepam, a benzodiazepine act by modifying GABA receptors (Sharma et al., 2019), commonly used for a number of neurological

Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

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https://doi.org/10.1016/j.sjbs.2020.10.042
1319-562X/

disorders like seizures, anxiety, benzodiazepine withdrawal syndrome, alcohol withdrawal syndrome, sleeping disorder, restless legs syndrome and spasm of muscle (Calcaterra and Barrow, 2014; Soyka, 2017). It is also used for purposely loss of memory during various medical surgeries.

Till date, no study is available on the effect of diazepam in fluctuating biochemical parameters like inflammatory and cardiac markers in stress exposed animals. The role of diazepam on these fluctuating biochemical parameters and is yet not clear, how it shows the effect on these marker proteins and whether it is efficient in restoring these fluctuating parameters or not. Hence, to evaluate the positive or negative diazepam effect in stress exposed rats, the present protocol was performed for investigation of effects of diazepam in fluctuating cardiac and inflammatory markers in stress-exposed rats.

2. Materials and methods

2.1. Drugs and chemicals

Diazepam was procured from Sigma Aldrich Chemical, USA. Chemicals used in this study were procured from Himgiri Traders, Dehradun, Uttarakhand, India. Chemicals utilized in the experiment were from a commercial source and of analytical grade quality.

2.2. Animals

24 albino Wistar male rats approximate weight 140–160 g were procured from the Animal House facility of Department of Biochemistry, Science Faculty, King Abdulaziz University, Kingdom of Saudi Arabia. Animals were kept under appropriate climatic conditions, 24–27 °C with 12:12 cycle of light and dark, and fed with a good quality pellet diet. The experiment was approved and permitted for conduction by the Institutional Committee of Animal Ethics, Faculty of Science, King Abdulaziz University (approval no. 347–17). Experimental procedures were performed with strict adherence to ethical guidelines and principles given by OECD guidelines (OECD 452, 2008; OECD 471, 2008B; ICH S2A 2008; ICH S2B, 1997).

2.3. Induction of disorder

For induction of disorder induced by stress, Disease Control animals and Disease + Diazepam Treatment animals exposed to daily regular stress for half an hour by forced swimming exercise method for 90 days (Hejazi et al., 2018).

2.4. Experimental design

Male Wistar Albino rats were divided into 4 groups with 6 animals in each group for 90 days of the experimental protocol. Group I, was Normal Control (NC), Group 2 was Disease Control (DC), Group 3, Diazepam Control (DMC) and Group 4, Disease + Diazepam Treatment (DT). For induction of disorder, DC and DT animals exposed to daily regular stress for half an hour after feeding by forced swimming exercise method for 90 days. DMC and DT received 5 mg/kg, p.o the daily dose of diazepam. At the end of the protocol, 5 ml blood was collected from each rat by the tail vein for biochemical estimation. Before collection, the site cleansed with alcohol (70%), kept under control, and then blood is withdrawn by using a needle of 21–22 gauge from the lateral vein of the tail. Quick after collection, the flow of blood was stopped with the application of pressure with sterile gauze for stopping blood flow (Zou et al., 2017). Collected blood was centrifuged serum separated and processed for further biochemical study. After blood collection, animals were sacrificed.

2.5. Biochemical estimation

The serum levels of interleukin-6 (IL-6), interleukin-10 (IL-10), fibrinogen, creatine kinase-MB (CPK-MB), cortisol, C-reactive protein (CRP) and cholesterol analyzed by utilizing a standard auto analyzer kit (DimensionR RXL MAXTM, Siemens, Malvern, USA).

2.6. Statistical analysis

Data expressed as Mean Standard error of mean. The significance value among different groups was calculated by one way analysis of variance and then student's *t*-test was also used with Graph Pad Prism-5 software. The differences of $p < 0.05$ were considered as statistically significant. Significant $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with Control.

3. Results

3.1. Interleukin-6 (IL-6)

The level of serum inflammatory marker interleukin-6 was 1.71 ± 0.21 pg/ml in NC animals. In DC animals, this level was increased significantly ($p < 0.001$) to 2.92 ± 0.32 pg/ml in comparison to NC. In DMC group animals, level ofIL-6 was was decreased significantly ($p < 0.001$) up to 1.24 ± 0.23 pg/ml in comparison to DC group. This increased level was restored up to 1.39 ± 0.23 pg/ml significantly ($p < 0.001$) in DT in comparison to DC (Fig. 1).

3.2. Interleukin-10 (IL-10)

The level of anti-inflammatory marker interleukin-10 in NC group was 8.63 ± 0.42 pg/ml. In DC animals. This level was decreased significantly ($p < 0.001$) to 3.00 ± 0.25 pg/ml in comparison to NC. In DMC group level was also decreased up to 5.9 ± 0.41 pg/ml significantly ($p < 0.001$) as compare to DC animals. This

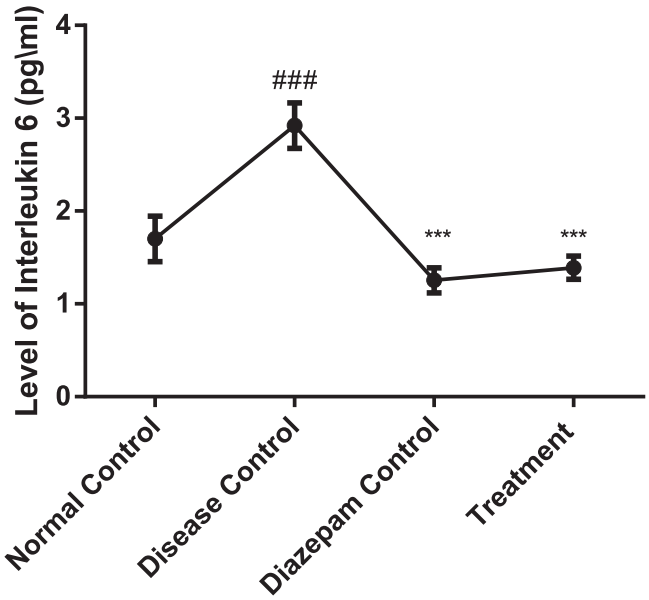

Fig. 1. Effect of diazepam on level of interleukin-6 in stress exposed rats. Values are the mean S.E.M. of 6 rat/treatment. Significant $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with Control.

decreased level was significantly ($p < 0.001$) restored up to 8.68 ± 0.46 pg/ml in DT group in comparison to DC (Fig. 2).

3.3. Fibrinogen

Level of fibrinogen was 2.5 ± 0.38 g/l in NC animals. This level was increased up to 5.6 ± 0.46 g/l significantly ($p < 0.001$) in the DC group as compared to NC group. In DMC group, the level of fibrinogen was 2.4 ± 0.28 g/l, almost similar to NC group value. Increased level was decreased up to 4.3 ± 0.16 g/l significantly ($p < 0.05$) as compare to DC animals (Fig. 3).

3.4. Creatine kinase-MB (CK-MB)

Level of CK-MB was 145.5 ± 15.95 u\l increased in NC animals. This level was increased significantly ($p < 0.001$) up to 350.5 ± 16. 02 u\l in DC as compare to NC group animals. CK-MB level was also increased in prophylactic group DMC up to 188.2 ± 13.02 u\l as compare to DC animals. The level was restored up to, which was restored significantly ($p < 0.05$) up to 140.2 ± 12.56 u\l in DT as compare to DC (Fig. 4).

3.5. Cortisol

The level of cortisol was 14.4 ± 0.95 µg/dl in NC group. The level was increased to 41 ± 3.45 µg/dl significantly ($p < 0.001$) in comparison to NC animals. IN DMC group, the level of cortisol was slightly increased to 18.6 ± 1.06 µg/dl in comparison to DC group. Increased level was significantly ($p < 0.001$) restored to 13.5 ± 0. 93 µg/dl in DT group in comparison to DC (Fig. 5).

3.6. C-reactive protein (CRP)

Level of CRP was 8.5 ± 0.48 mg/dl in NC group animals. This level was increased significantly ($p < 0.001$) up to 18.1 ± 1.15 mg/dl in DC group as compared to NC group. The level was also increased in the DMC group to 12.4 ± 0.83 mg/dl in comparison to DC animals. CRP level significantly ($p < 0.001$) decreased to 8.4 ± 0.91 mg/dl in DT animals as compared to DC group (Fig. 6).

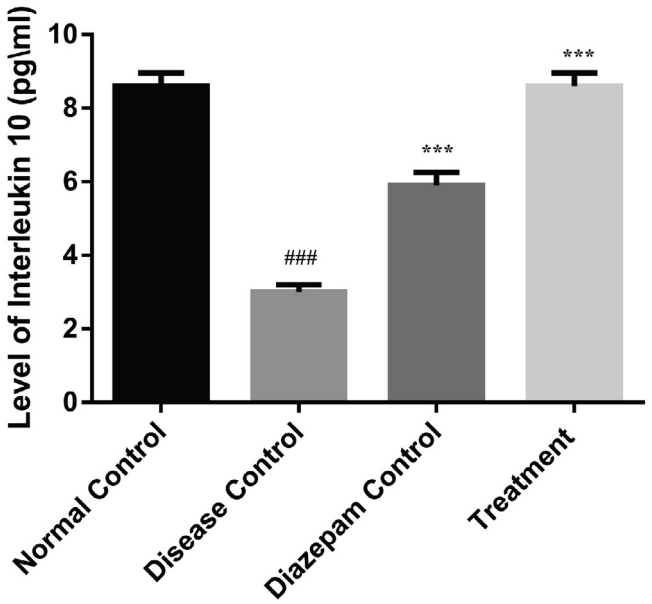

Fig. 2. Effect of diazepam on level of interleukin-10 in stress exposed rats. Values are the mean S.E.M. of 6 rat/treatment. Significant $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with Control.

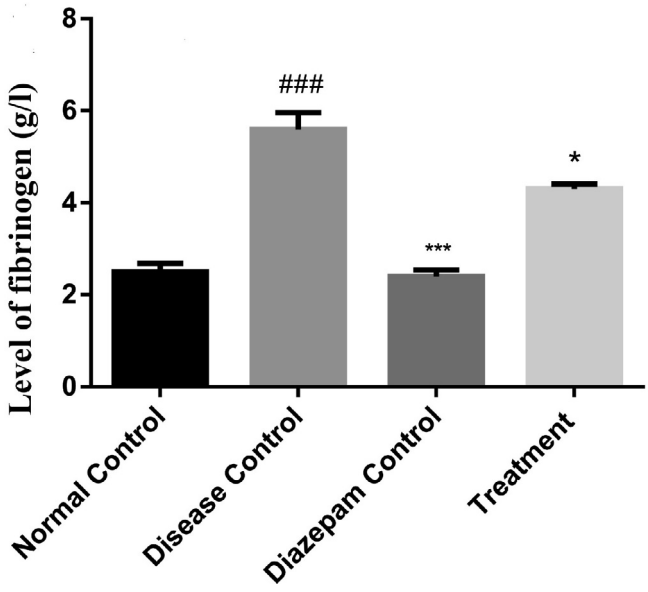

Fig. 3. Effect of diazepam on level of fibrinogen in stress exposed rats. Values are the mean S.E.M. of 6 rat/treatment. Significant $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with Control.

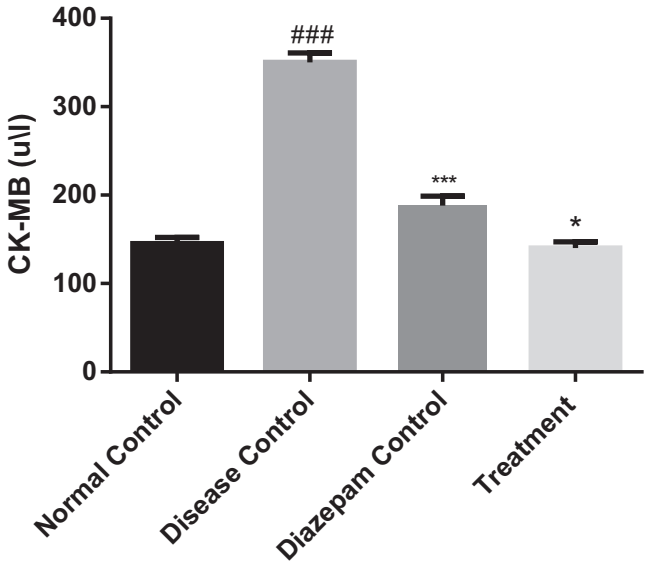

Fig. 4. Effect of diazepam on level of CK-MB in stress exposed rats. Values are the mean S.E.M. of 6 rat/treatment. Significant $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with Control.

3.7. Cholesterol

The level of cholesterol was 194.2 ± 19.45 mg/dl in NC group animals. This level was increased significantly ($p < 0.01$) to 280 ± 22.58 mg/dl as compared to NC group. In DMC group, it was also slightly increase up to 202.4 ± 20.53 mg/dl in comparison to the DC group. Increased cholesterol was significantly ($p < 0.01$) restored to 189.3 ± 19.46 mg/dl in DT group as compare to DC group (Fig. 7).

4. Discussion

It has been established that many diseases like diabetes, heart disorder, gastric disorders are linked with stress (Vance et al.,

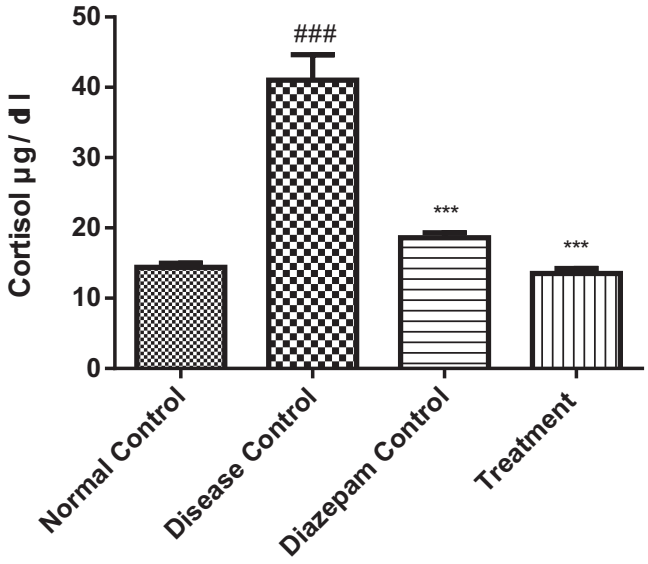

Fig. 5. Effect of diazepam on level of cortisol in stress exposed rats. Values are the mean S.E.M. of 6 rat/treatment. Significant *P < 0.05, **P < 0.01 and ***P < 0.001 compared with Control.

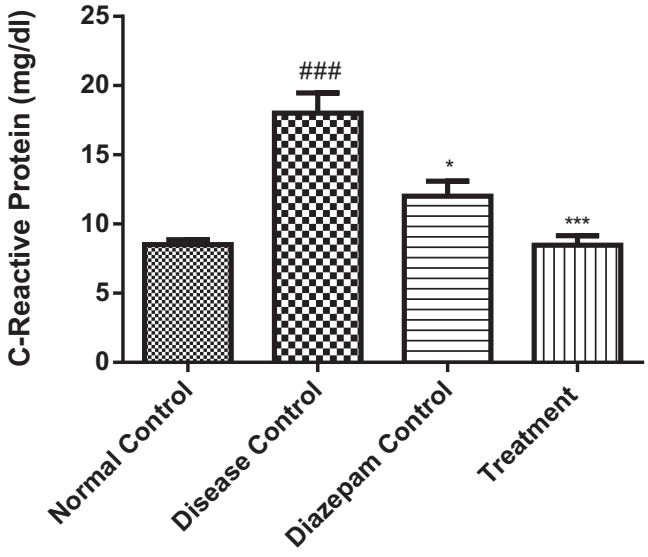

Fig. 6. Effect of diazepam on level of C-reactive protein in stress exposed rats. Values are the mean S.E.M. of 6 rat/treatment. Significant *P < 0.05, **P < 0.01 and ***P < 0.001 compared with Control.

2019). The current trend of medication is only based on treatment of symptoms of disease without treating the root cause (Peng, 2016). It has been reported from various literature that regular stress may be root cause different types of disease and fluctuation in the biochemical parameters of the body. Biochemical parameters of the body vary according to low and high stress response (Kaur et al., 2019). The level of serum ions, protein and enzymes may be a marker and an indicator of the health problem of the body (Argalasova et al., 2019). Mechanism of induction of disorder from stress is mediated by hypothalamus-pituitary-gland adrenal-axis, which is known as the stress axis (Kircanski et al., 2019). This axis play key role in reaction to the applied stress. Main elements linked with this stress axis are CRP, interleukins, CK-MB, tumor necrotic factor-α, cortisol, corticotrophin releasing hormone, fibrinogen, to name a few (Thomas et al., 2019).

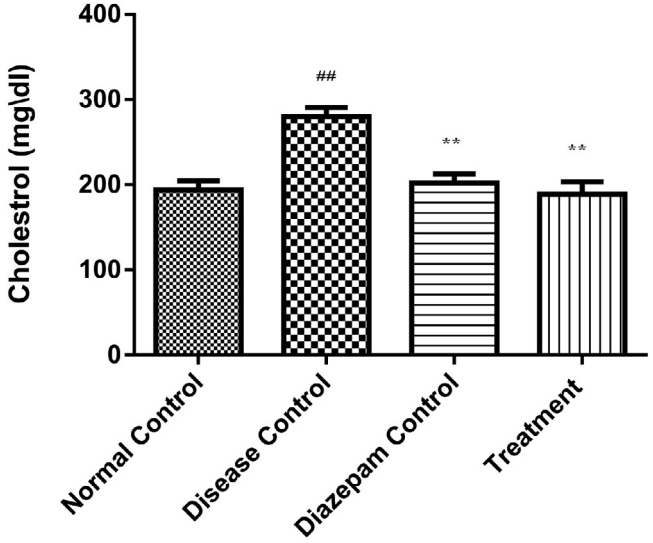

Fig. 7. Effect of diazepam on level of cholestrol protein in stress exposed rats. Values are the mean S.E.M. of 6 rat/treatment. Significant *P < 0.05, **P < 0.01 and ***P < 0.001 compared with Control.

IL-6 is an inflammatory marker cytokine used in the analysis for many pathological changes (Zhang et al., 2019). IL-6 exhibit response in the mediation of adaptive and innate immunological response (Hunter and Jones, 2015). Various innate immunological cells, such as macrophages, monocytes and neutrophils produce IL-6 protein, which leads to the increase in inflammation. Regular increased production of IL-6 lead to a chronic inflammatory state (Gomez-Lopez et al., 2019). In our study, the level of IL-6 was increased significantly ($p < 0.001$) to 2.92 ± 0.32 pg/ml in comparison to level of NC group animals (1.71 ± 0.21 pg/ml). This increase of level of IL-6 protein marker indicates the presence of inflammation, injury or disorder in the body induced by stress (Nadeem et al., 2020). Further, the level of IL-6 was restored upto 1.39 ± 0.23 pg/ml significantly ($p < 0.001$) in DT in comparison to DC. This restoration of IL-6 level indicates that diazepam is effective in reducing inflammation and disorder of the body induced by stress.

IL-10 is an anti-inflammatory marker protein, which reduce inflammatory reactions in both adaptive as well as innate immunological cells. Decrease in level of IL-10 in blood indicate presence of inflammation and disorder in the body (Mazer et al., 2019). In DC animals, level of IL-10 was decreased significantly ($p < 0.001$) to 3.00 ± 0.25 pg/ml in comparison to NC group animals (8.63 ± 0.42 pg/ml). This decrease of IL-10 level indicates the presence of inflammation and disorder induced by stress. Surprisingly, in the DMC group animals, level was also decreased up to 5.9 ± 0. 41 pg/ml significantly ($p < 0.001$) as compared to DC animals without use of any kind of stress. In the DMC group, this decrease in IL-10 levels was associated with toxicity of the drug when administered prophylactically (Kern, 2019). This decreased level was further significantly ($p < 0.001$) restored up to 8.68 ± 0.46 pg/ml in the DT group in comparison to DC. Restoration of IL-10 level indicates that diazepam is effective in reducing inflammatory disorder in stress exposed animals.

Fibrinogen, is the thrombin substrate, gives the main network in arterial thrombosis (Yang et al., 2019). The level of fibrinogen increases in inflammatory disorder a response of the acute phase. Increased level of fibrinogen is associated with cardiovascular disorders. Further, decreased level of fibrinogen in the body may lead to hemorrhage inside the body (Stokes et al., 2019). In this study, the level of fibrinogen was increased in DC animals up to 5.6 ± 0.

46 g/l significantly ($p < 0.001$) from 2.5 ± 0.38 g/l. Since fibrinogen is a marker of inflammation and cardiac disorder, increased level indicates that this is due to regular stress. Further, level of fibrinogen in the prophylactic group (DMC group) was almost unchanged, indicates diazepam does not show any effect on fibrinogen level. Increased level was decreased up to 4.3 ± 0.16 g/l by administration of diazepam.

CK-MB is one of the most specific and indicator diagnoses of various heart disorders like myocardial infarction. The level of CK-MB isoenzyme generally increased by heart disease (Alkireidmi et al., 2018). In our study, level of CK-MB was significantly ($p < 0.001$) increased up to 350.5 ± 16.02 u\l in DC animals, which indicates cardiac injury and inflammation in rats by stress. This abnormal level was restored significantly ($p < 0.05$) to the normal value significantly ($p < 0.05$) up to 140.2 ± 12.56 u\l in DT group animals by diazepam, which reflects diazepam prevents cardiac injury and inflammation. Further, in the prophylactic group (DMC group) increased level of CK-MB indicates toxicity of diazepam administration without induction of disorder.

Cortisol is a stress hormone secreted from the adrenal gland of the body (Yu et al., 2019). Cortisol is an inflammatory marker hormone which regulates reserves of glucose for production of energy and also inflammation (Somvanshi et al., 2019). Cortisol also plays role in facilitation of fear based memory for survival in the future and danger avoidance (Hawiset, 2019). Stress for small duration are usually adaptive in nature. The body maintains and adjusts state of homeostatis according to changes. Prolonged exposure to stress leads to dysfunction of cortisol, severe pain and inflammation in the body (Hannibal and Bishop, 2014). In this study, the level of cortisol was increased up to 41 ± 3.45 μg/dl significantly ($p < 0.001$) in DC group animals from 14.4 ± 0.95 μg/dl. This increased level of cortisol indicates inflammation and abnormal pathology of the body. This increased level was significantly ($p < 0.001$) restored to 13.5 ± 0.93 μg/dl in DT group. Restoration of level of cortisol indicates anti-inflammatory recovery potential of diazepam in stress exposed animals.

CRP is a protein that increases in the serum with infection, inflammation, surgery, heart attack, other heart disease and trauma (Kamath et al., 2015). CRP shows elevation in expression in inflammation disorders like cardiovascular diseases infection and rheumatoid arthritis (Ntusi et al., 2018). In this study, in DC animals the level of CRP increased 18.1 ± 1.15 mg/dl from 8.5 ± 0. 48 mg/dl, which confirms cardiac disorder and inflammation induced by stress. The level was also increased in the DMC group to 12.4 ± 0.83 mg/dl in comparison to DC animals. Increase in CRP level in the DMC group indicates prophylactic toxicity of diazepam. Elevated level as successfully lowered to the normal value 8.4 ± 0.91 mg/dl by diazepam, confirms the ameliorative efficacy of diazepam.

Cholesterol is a fatty acid present in various food items and also synthesized by our body (Farias-Pereira et al., 2020). When cholesterol level is very high, the extra amount will be deposited in our arteries. Deposition of cholesterol in vessels affects the flow of blood to our heart and brain, which may cause heart attack or stroke (Baumer et al., 2017). It has been established from various previous researches that stress alters cholesterol profile of the body which ultimately many metabolic functions of the body (Assadi, 2017). In this study, in DC group animals, the level of cholesterol was increased up to 280 ± 22.58 mg/dl from 194.2 ± 1 9.45 mg/dl indicates stress alters lipid profile and creates more complications for the body while in the DT group altered level of cholesterol profile was significantly ($p < 0.01$) restored to 189.3 ± 19.46 mg/dl by diazepam. Restoration of level of cholesterol indicates that diazepam is efficient in controlling heart and metabolic disorders.

5. Conclusion

From the outcomes of results, it can be suggested that diazepam shows a protective effect against altered biochemical parameters in stress-exposed rats. It shows protective effects by the restoration of altered IL-6, IL-10, fibrinogen, CK-MB, CRP, cortisol and cholesterol level in regular stress-exposed rats. The further clinical study required to explore this finding in patients with stress induced disorders.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This project was funded by the Deanship of Scientific Research (DSR) at King Abdulaziz University, Jeddah, under grant no. G-296-130-1439. Authors, therefore, acknowledge with thanks DSR for their technical and financial support.

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