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Altered Markers of Oxidative Stress and Biochemical Parameters and Their Modulation by Diazepam In Anxious Rat.

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ABSTRACT

Study was undertaken to investigate the various oxidative stress markers and biochemical parameters in anxious rat. Rats were subjected for short (21 days) and long term (up to 84 days) social isolation; the rats displayed an increase in anxiety on elevated plus maze and open field test relative to control. Various markers of oxidative stress like lipid peroxidation (LPO), reduced glutathione (GSH), Supers oxide dismutase (SOD), catalase (CAT) and biochemical parameters like SGOT, SGPT, blood sugar etc. were determined. There was significant increase in the level of LPO and decrease in the levels of GSH, SOD and CAT long term anxiety. Increased oxidative stress in anxiety leads to alteration of biochemical parameters. Treatment with Diazepam an anxiolytic agent significantly decreases in level of LPO, SGOT, SGPT and increase in the levels of GSH, SOD and CAT in long term anxiety.

Keywords- Diazepam, Anxiety, Adrenal gland, Hyperglycemia, Oxidative stress.

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INTRODUCTION

Social anxiety disorder (SAD, or social phobia) is an important mental health issue. Conservative estimates give a lifetime prevalence of approximately 7% [1], whereas some epidemiologic studies raise this to 13.3%, the third highest of all psychiatric disorders [2]. It is associated with almost all emotional disorder and frequently with physical illnesses. Amygdala is responsible for expression of anxiety or fear and prefrontal cortex plays a role in fear extinction by regulating by the amygdala - mediated expression of fear. [3] Anxiety is a pervasive phenomenon the common denominator in most forms of mental disorder. [4] The vast majority of individuals suffering from mental health problems like Anxiety [5]. A variety of physiologic and pathophysiologic procedures are believed that reactive oxygen species play an important production of disease. Oxidative stress is defined as the deleterious impact in cell function as a consequence of the loss in homeostatic balance between reactive oxygen species (ROS) and antioxidants in the cellular milieu. ROS are formed continuously as a result of normal cellular respiration, enzymatic metabolism, and exogenous insults. [6] At low or moderate concentrations, ROS exert beneficial effects on cellular responses and immune function but at high levels, free radicals and oxidants generate oxidative stress, a deleterious process that can damage cell structures, including lipids, proteins, and DNA are well-known outcomes of oxygen-derived free radicals, leading to cellular pathology and ultimately to cell death. [7]

Cellular antioxidant enzymes and the free-radical scavengers normally protect a cell from toxic effects of the ROS. However, when generation of the ROS overtakes the antioxidant defense of the cells, oxidative damage of the cellular macromolecules (lipids, proteins, and nucleic acids) occurs, leading finally to various pathological conditions.

There is significant evidence that psychological stress is associated with increased oxidative stress in both animal and human models. [8]

The generation of reactive oxygen species (ROS) plays an important role in producing liver damage, other hepatic alterations and progression liver disease (initiating hepatic fibrogenesis). [9 and 10]. OS disrupts lipids, proteins and DNA, induces necrosis and apoptosis of hepatocytes and amplifies the inflammatory response. ROS also stimulate the production of profibrogenic mediators from Kupffer cells and circulating inflammatory cells and directly activate hepatic stellate cells, resulting in the initiation of fibrosis. Advances in understanding the mechanisms involved in fibrosis have identified new molecular targets with therapeutic potential for more targeted and personalized control of this disease. [10]

Females may be more vulnerable to events affecting their close emotional ties and more likely to develop anxiety in response to them. [11]

In this study, we investigated the effect of anxiety treatment on oxidative stress and biochemical parameters present in anxiety induced by social isolation and it characterizes by behavioral changes in female rat.

Diazepam belongs to a class of medicines called benzodiazepines. Diazepam is commonly used to treat anxiety and panic attacks. Benzodiazepines also have a relatively low toxicity in overdose.[12]

In this study, we investigated the various oxidative stress markers and biochemical parameters and their modulation by diazepam treatment.

MATERIAL AND METHOD

Chemical

Vitamin E, Diazepam, Ethylene diamine tetra acetic acid (EDTA), Hydrogen peroxide H_2O_2 , Metaphosphoric acid, Pyrogallol, Thiobarbituric acid (TBA), Tris buffer, Trichloroacetic acid (TCA), 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB), Phosphate buffer, Phosphate buffer saline, Drafin's Reagent, SGOT determination kit, SGPT determination kit.

Experimental animals

Healthy young female Sprague–Dawley rats (200-250 gm) were used. The animals were housed in polypropylene cages and maintained under standard environmental conditions ($25 \pm 2^\circ C$, relative humidity $60 \pm 5 \%$, light- dark cycle of 12 hours each) and fed with standard pellet diet (Trimurti feeds, Nagpur) and water *ad libitum*, were used for the entire animal study. The rats were housed and treated according to the rules and regulations of CPCSEA and IAEC. The protocol for all the animal study was approved by the Institutional Animal Ethics Committee (IAEC).

Experimental design

The animals were grouped in to 4 groups each group possesses 6 animals as follows: -

Group I (Normal Control group): Animals were not subjected for social Isolation and any treatment.

Group II (Negative control group): Animals were subjected for social Isolation and drug treatment was not given.

GroupIII (Social isolation+Diazepam): Animals were subjected for social Isolation and treated with Diazepam (2 mg/kg, i.p).

Group IV (Social isolation + Diazepam + Vit. E): Animals were subjected for social Isolation and treated with Diazepam and Vit. E

Behavioral and Oxidative stress parameters (in blood & tissue) were studied in Socially Isolated rats and normal rats {social isolation for 3 weeks (i.e. 21 days, short term) and 12 weeks (i.e. 84 days, long term)} [13 &14]. After short and long term isolation, rats were assessed for anxiety using various animal behavioral models like EPM and open field test.

Determination of behavioral parameter

Elevated Plus Maze

Apparatus consist of two open arms 50 x 10 cm for two closed arms 50 x 10 x 40 cm and an open roof with the entire maze elevated 50 cm from the floor. The animals were placed individually in the centre of the maze, head facing towards open arms and parameters like First preference of rat to open and closed arm, Number of entries in open and closed arms (an arm entry defined as the entry of four paws into the arm), Average time each animal spends in each arm (average time = total duration in the arm/number of entries) were noted for 5 min. [15]

Open field apparatus

Apparatus consisted of a wooden box (87.5 x 75 cm) consisting of a white floor with 42 evenly spaced squares (5.5 cm) outlined in black. The apparatus was cleaned with an unscented disinfectant solution during experiment. Rats were placed in a corner of the open-field apparatus at the beginning of the 5-min test. [16] Two main parameters studied were number of occurrences at center and time spent at center.

Determination of oxidative stress parameters in blood

Sample preparation

Suspension of RBC (red blood cells, 5%) was prepared by adding phosphate buffer saline (8 ml) to packed cells. 0.5 ml of 5% RBC was mixed with 5 ml of distilled water, shaken for 5 min. then kept at 4°C for 5min. Subsequently, 0.4ml of 3:5 chloroform ethanol mixtures was added, shaken vigorously to precipitate hemoglobin, and then 0.15 ml of distilled water was added. The mixture was centrifuged to get a clear erythrocyte lysate.

Determination of LPO

LPO (lipid peroxidation) was determined on the basis of the molar extinction coefficient of MDA (1.56/105) and expressed in terms of nanomoles of MDA/gHb.[17]

Determination of CAT

The activity of CAT enzyme was determined in the erythrocyte lysate as the decrease in absorbance was measured spectrophotometrically at 240 nm for 1 minute. [18]

Determination of SOD

The activity of SOD was determined in the erythrocyte lysate by monitoring spectrophotometrically the increase in the absorbance at 420 nm for 3 minutes. One unit of enzyme activity represents 50% inhibition of the rate of auto-oxidation of pyrogallol, as determined by change in absorbance/minute at 420nm [19].

Determination of GSH

Blood GSH (reduced glutathione) was measured by addition of 0.2 ml of whole blood to 1.8 ml of distilled water followed by 3.0 ml of precipitating mixture. It was centrifuged at 2000 rpm for 5 minutes and 1 ml of supernatant was added to 1.5 ml of phosphate solution, followed by addition of 0.5 ml of DTNB {Dithionitrobenzoic acid; 5, 5'-Dithiobis (2-nitrobenzoic acid)} reagent. The absorbance was measured at 412 nm. [20]

Determination of oxidative stress parameters in tissue

Tissue preparation

After receiving the treatments for 84 days, the rats were sacrificed using deep ether anesthesia. The liver was removed and thoroughly washed with ice-cooled 0.1 M phosphate buffer saline (PBS) containing 0.1 mmol/L phenyl methanesulfonyl fluoride. This tissue was blotted dry and homogenized in 0.1 M PBS in an ice bath to prepare a 10% suspension. This suspension was then centrifuged at 16000 × rpm for 1 h in a cooling centrifuge at 0°C. The supernatant was employed to assess the parameters of oxidative stress after estimating the protein content. [21]

Determination of LPO

According to the method of Esterbauer and Cheeseman (1990), the extent of lipid peroxidation in terms of thiobarbituric acid reactive substances (TBARS) formation was measured. As 99% TBARS are malondialdehyde (MDA), so TBARS concentrations of the samples were calculated on the basis of the molar extinction coefficient of MDA (1.56×10^5) and expressed in terms of nanomoles of MDA/mg tissue. [17]

Determination of CAT

The activity of CAT tissue homogenate, the decrease in absorbance was measured at 240 nm for 1 minute using spectrophotometer by calculating the rate of degradation of H₂O₂, the substrate of the enzyme. [18]

Determination of SOD

An increase in the absorbance was measured at 420 nm for 3 minutes using spectrophotometer. [19]

Determination of GSH

GSH was measured by addition of 0.2 ml of tissue homogenate to 1.8 ml of distilled water followed by 3.0 ml of precipitating mixture. It was centrifuged at 2000 rpm for 5 minutes and 1 ml of supernatant was added to 1.5 ml of phosphate solution, followed by addition of 0.5 ml of Dithionitrobenzoic acid; 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB) reagent. The absorbance was measured at 412 nm. [20]

Determination of Biochemical Parameters

Determination of Glucose

The serum glucose concentration was determined using the glucose oxidase method. The method is based on the ability of glucose oxidase to catalyse the oxidation of β -D-glucose to D-glucono- σ -lactone with the concurrent release of hydrogenperoxide (H_2O_2). And quantitative formation of a quinoneimine dye complex which is measured at 510 nm. [22]

Determination of SGOT and SGPT

The measurement of transaminase levels in serum by Ambica diagnostic kit studied. The kit utilize the colorimetric procedure of Reitman and Frankel¹ in which the oxaloacetate and/or pyruvate formed in either the GOT or GPT reaction is combined with 2, 4-nitrophenylhydrazine to yield a brown-coloured hydrazone which is measured at 505 nm.[23]

Determination of Adrenal gland weight

Animals were weighed just before termination, sacrificed by decapitation, Adrenals were quickly removed, Cleaned and weighed. [24]

Statistical Analysis

Values are expressed as mean \pm SD (n=6). Statistical significance was determined by one way analysis of variance (ANOVA) followed by Dunnet's post-hoc test. $P < 0.01$ and $P < 0.05$ were considered statistically significant when compared with Negative control.

RESULTS

Table No. 1. Shows the level of anxiety in the socially isolated rat for 21 and 84 days by elevated plus maze. There was significant decrease in the ($p < 0.01$) % open arm entry, Time spent at the center and increase in the % close arm entry of group II when compare to group I which signifies that there was increase in the level of anxiety in socially isolated rat. Even there was significant decrease in the ($p < 0.01$) % open arm entry, Time spent at the center and increase in the % No. of close arm entry at 84 days social isolation when compare to 21 days social isolation. There was significant ($p < 0.001$) decrease in the % No. of open arm entry and time spent at the center and increase in the % no. of close arm entry of group III and IV compare to Group II at 21 and 84 days social isolation.

The level of anxiety in the socially isolated rat for 21 and 84 days by Open field test shown in Table No. 2. There was significant decrease in the ($p < 0.01$) No. of occurrence at the Center, Time spent at the center in group II when compare to group I. Even there was significant decrease in the ($p < 0.01$) No. of occurrence at the Center, Time spent at the center at 84 days of social isolation when compare to 21 days of social isolation. There was

significant ($p < 0.001$) decrease in the No. of occurrence at the Center, Time spent at the center in group III and IV compare to Group II at 21 and 84 days social isolation.

Table 1: Behavioral measurement after social isolation of 21 and 84 days in rats exposed to the elevated plus-maze by percentage number of open and close arm entries.

Sr. No.	Group	21 days			84 Days		
		% open arm entries	% close arm entries	Time spent at the open arm	% open arm entries	% close arm entries	Time spent at the open arm
1	Group I	22.66 ± 1.21	77.33 ± 0.81	16.5 ± 1.04	24.43 ± 1.58	76.82 ± 0.05	16.81 ± 0.24
2	Group II	11.66 ± 1.03 [@]	88.33 ± 0.81 [@]	6.91 ± 0.64 [@]	4.41 ± 0.17 ^{@£}	95.60 ± 0.35 ^{@£}	2.63 ± 0.33 ^{@£}
3	Group III	17.66 ± 1.21**	82.5 ± 1.04**	15.33 ± 1.86**	10.23 ± 0.53**	89.70 ± 0.04**	15.35 ± 0.76**
4	Group IV	20.33 ± 0.81**	79.66 ± 1.21**	19.16 ± 1.16**	18.26 ± 0.85**	81.76 ± 0.15**	15.65 ± 0.76**

Data are expressed as the Mean ±SD (n=06), where
[@] p<0.01, compared to control group, ** P < 0.01, compared to negative control group
[£] p<0.01, Compared to 21 days reading

Table 2: Behavioral measurement after social isolation of 21 and 84 days in rats exposed to the Open field test by Time spent at centre and No. of Occurrence at the center.

Sr. No.	Groups	21 days		84 Days	
		Time spent at centre (sec)	No. of occurrences at center	Time spent at centre (sec)	No. of occurrences at center
1	Group I	13.85 ± 0.78	4.50 ± 1.37	14.00 ± 0.48	5.66 ± 0.81
2	Group II	8.66 ± 0.20 ^{@£}	1.83 ± 0.75 ^{@£}	6.05 ± 0.48 ^{@£}	1.33 ± 0.51 ^{@£}
3	Group III	13.15 ± 0.49**	3.66 ± 1.36**	13.15 ± 0.66**	4.66 ± 0.81**
4	Group IV	13.65 ± 0.19**	5.00 ± 0.89**	14.13 ± 0.33**	5.50 ± 0.83**

Data are expressed as the Mean ±SD (n=06), where
[@] p<0.01, compared to control group, ** P < 0.01, compared to negative control group
[£] p<0.01, Compared to 21 days reading

As shown in Table No. 3. there was increase in the levels of Oxidative stress markers in the socially isolated rats. There were significant increase ($p < 0.01$) in the level of LPO and Decrease in the level of GSH, CAT and SOD of isolated rat (isolated for 21 and 84 days) compare to normal rat. There were significant alteration ($p < 0.01$) in the levels of oxidative stress markers in socially isolated rat on 84th day compare to 21 day of social isolation. In group III and group IV rats there were decrease in the levels of LPO and increase in the level of GSH, CAT and SOD compare to Group II rat (isolated for 21 and 84 days).

Table 3: Study of oxidative stress markers after social isolation of 21 and 84 days in rats

Sr. No.	Groups	21 days				84 Days			
		LPO (nMMDA/g hb)	GSH (µM/mg protein)	CAT (U/mg protein)	SOD (Unit/mg protein)	LPO (nMMDA/g hb)	GSH (µM/mg protein)	CAT (U/mg protein)	SOD (Unit/mg protein)
1	Group I	3.15 ± 0.43	8.58 ± 0.49	251.16 ± 0.75	49.23 ± 0.53	3.74 ± 0.08	8.82 ± 0.10	251.56 ± 0.87	50.05 ± 0.48
2	Group II	5.81 ± 0.07 [@]	5.66 ± 0.40 [@]	220.16 ± 0.75 [@]	30.36 ± 1.02 [@]	7.87 ± 0.37 ^{@£}	4.67 ± 0.16 ^{@£}	206.30 ± 2.40 ^{@£}	21.75 ± 0.66 ^{@£}
3	Group III	5.46 ± 0.08*	6.31 ± 0.21**	229.83 ± 0.75**	33.5 ± 1.04**	7.05 ± 0.12**	5.73 ± 0.16**	221.90 ± 1.89**	34.31 ± 2.64**
4	Group IV	4.03 ± 0.08**	7.23 ± 0.38**	239.83 ± 0.75**	40.00 ± 1.09**	5.53 ± 0.49**	6.88 ± 0.29**	238.98 ± 0.90**	45.31 ± 2.86**

Data are expressed as the Mean ±SD (n=06), where
[@] p<0.01, compared to control group, ** P < 0.01, compared to negative control group
[£] p<0.01, Compared to 21 days reading

The biochemical parameters like SGOPT, SGPT, Blood glucose level in socially isolated rats were studied on 21 and 84 days (Table No. 4). On 21 days of social isolation there was no significant changes in the level of SGOPT, SGPT and Blood Glucose compare to Group I but on 84th day of social isolation there was significant ($p < 0.05$, 0.01) increase in the level of SGOT, SGPT and blood glucose level of group II compare to Group I also Group II and Group IV shows significant ($p < 0.001$) decrease in the levels of SGOT, SGPT and Blood glucose level. There were significant increase ($p < 0.01$) in the level of LPO and Decrease in the level of GSH, CAT and SOD in liver of socially isolated rats for 84 days compare to normal rats. In group III and group IV rats there were decrease in the levels of LPO and increase in the level of GSH, CAT and SOD compare to Group II rat (Table No. 5).

Table 4: Study of Biochemical Parameters after social isolation of 21 and 84 days in rats

Sr. No.	Groups	21 days			84 Days		
		SGOT (IU/L)	SGPT (IU/L)	Glucose (mg/dl)	SGOT (IU/L)	SGPT (IU/L)	Glucose (mg/dl)
1	Group I	16.83 ± 0.75	27.33 ± 0.81	75.98 ± 0.36	16.16 ± 0.75	27.33 ± 0.51	77.46 ± 0.92
2	Group II	17.33 ± 0.51 ^{ns}	26.83 ± 0.75 ^{ns}	75.2 ± 0.50 ns	18.83 ± 0.40 [@]	32.00 ± 1.78 [@]	135.83 ± 6.37 [@]
3	Group III	16.66 ± 0.51 ^{ns}	26.16 ± 0.98 ^{ns}	74.85 ± 1.04 ns	17.33 ± 0.51 ^{**}	30.00 ± 0.63 ^{**}	103.31 ± 3.13 ^{**}
4	Group IV	16.50 ± 0.83 ^{ns}	26.16 ± 0.75 ^{ns}	74.68 ± 0.63 ns	16.83 ± 0.75 ^{**}	29.50 ± 0.54 ^{**}	85.85 ± 0.92 ^{**}

Data are expressed as the Mean ±SD (n=06), where
 $\# p < 0.05$, $@ p < 0.01$, compared to control group
 $* p < 0.05$, $** p < 0.01$, ns $p > 0.05$, compared to negative control group,

Table 5: Study of markers of oxidative stress in rat's liver after social isolation of 84 days

Sr. No.	Groups	LPO (nMMDA/mg tissue)	SOD (Unit/mg protein)	CAT (U/mg protein)	GSH (nmol/mg protein)
1	Group I	23.10 ± 0.79	97.95 ± 0.41	330.91 ± 1.32	24.15 ± 0.46
2	Group II	31.56 ± 0.49 [@]	61.25 ± 0.75 [@]	245.28 ± 4.59 [@]	15.03 ± 0.63 [@]
3	Group III	27.55 ± 0.51 ^{**}	76.00 ± 0.89 ^{**}	264.51 ± 3.97 ^{**}	18.63 ± 0.37 ^{**}
4	Group IV	24.90 ± 0.52 ^{**}	87.83 ± 0.98 ^{**}	299.71 ± 5.58 ^{**}	21.18 ± 0.64 ^{**}

Data are expressed as the Mean ±SD (n=06), where
 $@ P < 0.01$, compared to control group
 $** P < 0.01$, compared to negative control group

Table 6: Measurement of weight of adrenal gland after social isolation in rats

Sr. No.	Groups	weight of adrenal gland in mg	weight of adrenal gland - mg/100 g body weight
1	Group -I	47.26 ± 0.55	13.65 ± 0.17
2	Group -II	50.33 ± 0.24 [@]	15.58 ± 0.13 [@]
3	Group -III	49.61 ± 0.31 ^{**}	14.96 ± 0.12 ^{**}
4	Group -IV	47.95 ± 0.15 ^{**}	14.50 ± 0.18 ^{**}

Group -II is compared with remaining groups. Data are expressed as the Mean ±SD (n=06), where
 $@$ = The P value is < 0.01 , considered extremely significant as compared to control group
 $**$ = The P value is < 0.01 , considered extremely significant as compared to negative control group

Weight of Adrenal gland and Adrenal gland Index were checked in 84 Days socially isolated rat. There was significant increase in the weight of Adrenal gland (50.33 ± 0.24) and Adrenal gland index (15.58 ± 0.13) in Group II as compare to Group I. Whereas in the Group III and IV there was significant decrease ($p < 0.001$) in the weight of Adrenal gland and Adrenal Index compare to Group II (Table No. 6).

DISCUSSION

Diazepam belongs to a class of medicines called benzodiazepines. Diazepam is commonly used to treat anxiety and panic attacks. Benzodiazepines also have a relatively low toxicity in overdose. In this study, we examined the influence of diazepam on the duration of immobility in forced swim test and tail suspension method after short term and long term isolation. Anxiety reduces immobility time in forced swim test and tail suspension test, as well as cellular and oxidative damage. Our data suggest the antioxidant effect of anxiety along with antianxiety effect. Anxiety may contribute to the production of and exposure to reactive oxygen species, and thus leads pathogenesis of multiple diseases. Anxiety is associated with the activation of innate immune response and subsequent production of phagocytes. Activated Phagocytes are significant sources of reactive oxygen species and increased oxidative damage may represent a common mechanism for pathogenesis of multiple diseases. [25]

We consider the possibility that the observed increased lipid peroxidation and decreased CAT, SOD and GSH were a part of a broader oxidative process occurring among the anxious rats. We studied persistent oxidative stress may damage liver tissue confirmed by measuring SGOT and SGPT (biomarkers of liver function test) at long term anxiety. The raised levels of these enzymes in anxiety might be as the result of cortisol induced gluconeogenesis in the liver. During stressful condition there might be altered membrane permeability which contributes to release of these transaminases. The observed increase in the blood glucose level may be due to the release of glucocorticoids during persistent anxiety in two ways either by promoting gluconeogenesis in liver from amino acids or by inhibiting glucose utilization by peripheral cells. Increased release of glucocorticoids may be a consequence of increased weight of adrenal gland. The findings in the present investigation derives its importance from the evidence that oxidative stress has an important role in development and progression of chronic liver disease and altered glucose level. By modulating oxidative stress diazepam might thus affect the progression and development of several hepatic pathologies.

MDA was decreased and GSH, SOD and CAT were increased in blood and liver by diazepam. This might suggest that diazepam can be administered for its antioxidant effect and treatment of liver disease in patients with anxiety.

CONCLUSION

Social isolation leads to anxiety, long term anxiety induced increase in glucose, SGOT, SGPT are mediated through the increased generation of free radicals. This alteration of oxidant and antioxidant balance is due to altered behavior. Pretreatment with diazepam an antianxiety drug, controls such behavior and thereby prevents generation of free radical.

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