# ROLE OF OXIDATIVE STRESS IN BEHAVIORAL, COGNITIVE AND BIOCHEMICAL IMPAIRMENT IN A RAT MODEL OF SOCIAL STRESS

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A Dissertation Presented to

the Faculty of the Pharmacological and Pharmaceutical Sciences Department

University of Houston, College of Pharmacy

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In Partial Fulfillment

of the Requirements for the Degree

Doctor of philosophy

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by

Naimesh Natawarlal Solanki

August 2016

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# ABSTRACT

Psychological stress is known to contribute to anxiety and cognitive impairment in humans. Currently, the drugs of choice for treatment of anxiety disorders are traditional-antidepressants, with modest efficacy and major side effects. Therefore, alternative therapies with higher efficacy and fewer side effects are needed. Recent evidence from our lab has suggested a causal role of oxidative stress (OS) in psychological stress (social defeat)-induced behavioral and cognitive impairments in rats. Basically, psychological stress induces behavioral and cognitive deficits in rats while increasing oxidative stress systemically as well as in the brain. Increase in oxidative stress was associated with reduced systemic and cerebral antioxidant status. Imbalance in oxidant-antioxidant status seemed to have contributed to stress-induced deficits in the social defeat (SD) model. If the rise in oxidative stress causes behavioral and cognitive deficits then interventions mitigating oxidative stress by increasing antioxidant could be useful.

In this study, we employed the rat model of social defeat (SD) which closely resembles societal stress in humans to determine whether increasing antioxidant level using grape powder (GP), with its rich antioxidant content, is able to protect and/or reverse SD-induced behavioral and cognitive deficits in rats. Grape powder is a mixture of a variety of antioxidants. Therefore, it is important to know which antioxidant constituent contributes to potentially protective effects of GP. This was determined in a neuronal cell culture model of

HT22 cells, a hippocampal derived cell line. Finally, underlying mechanism(s) of action of GP also were determined.

Sprague Dawley rats after undergoing 7 days of repeated social defeat developed significant behavioral and cognitive impairments. And, 3 weeks GP treatment (15 g/L in drinking water) protected and reversed SD-induced behavioral and cognitive deficits. Biochemical analysis revealed that GP treatment significantly decreased SD-induced increase in levels of plasma corticosterone (systemic marker of stress), and plasma 8-isoprostane (marker of OS). Furthermore, GP treatment significantly increased SD-induced decrease in cellular pool of key antioxidant enzymes such as glyoxalase-1, glutathione reducatse-1 and superoxide dismutases in specific regions of the brain including the hippocampus and amygdala.

Next, utilizing an in-vitro model of oxidative stress, we examined contribution of Quercetin (Q), Resveratrol (R) and Kaempferol (K), key antioxidants present in grapes, in mediating protective effect. HT22 cells were treated with 1mM BSO (L-Buthionine-sulfoximine, pro-oxidant) for 14 hrs to induce oxidative stress. The cells were treated for 4 hrs with Q, R or K prior to BSO treatment. Q and R but not K were the most effective in protecting BSO-induced decreased total antioxidant capacity, suggesting major contribution of Q and R in protective action of grape powder. Further data suggested that GP protected oxidative stress-induced cell death by preventing oxidative stress-

induced increased calcium influx, mitochondrial dysfunction and release of cytochrome c.

Collectively, animal and cell culture data suggest that GP protected and reversed SD-induced behavioral and cognitive impairments in rats and, that quercetin and resveratrol appear as the most likely major contributors towards beneficial effects of GP. Finally, it seems that GP mitigates oxidative stress by increasing antioxidant pool and preventing cell damage and death.

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# ABBREVIATIONS

OS- Oxidative Stress

SD- Social Defeat

GP- Grape powder

Q- Quercetin

R- Resveratrol

K- Kaempferol

BSO- L-Buthionine-sulfoximine

GABA- gamma-amino butyric acid

SSRI- selective serotonin reuptake inhibitor

BZD- Benzodiazepine

ROS- Reactive oxygen species

RNS- Reactive nitrogen species

ATP- adenosine triphosphate

SOD- superoxide dismutase

GLO- glyoxalase

GSH- glutathione

GSR- glutathione reductase

GSSG- glutathione oxidase

GPX- glutathione peroxidase

DNA- deoxy ribonucleic acid

CNS- central nervous system

ACTH- adreno-corticotrophic hormone

HPA- hypothalamus-pituitary-adrenal

CRH- corticotropin-releasing hormone

GR- glucocorticoid receptor

MR- mineralocorticoid receptor

PFC- pre-frontal cortex

mPFC- medial pre-frontal cortex

BLA- basolateral amygdala

HFS- high frequency stimulation

ER- endoplasmic reticulum

BDNF- brain-derived neurotrophic factor

PTSD- post-traumatic stress disorder

I$\kappa$B- inhibitor of kappa B

Fe- iron

LMWA- low-molecular weight antioxidants

Cu-Zn SOD- Copper-Zinc Superoxide Dismutase

Mn SOD- Manganese Superoxide Dismutase

$H_2O_2$- Hydrogen Peroxide

NADPH- Nicotinamide Adenine Dinucleotide Phosphate

MG- methylglyoxal

AGEs- Adavanced Glycation End Products

AD- Alzheimer's disease

PD- Parkinson’s disease

HD- Huntington’s disease

alphaSyn- alpha Synuclein

mHtt- mutant Huntington protein

$Ca^{2+}$- Calcium

LTP- long-term potentiation

LTD- long-term depression

PVN- para ventricular nucleus

BNST- bed nucleus of stria terminalis

DSM- Diagnostic and Statistical Manual of Mental Disorders

PDE-2- phosphodiesterase-2 inhibitor

X+XO- xanthine-xanthine oxidase

CREB- cyclic response element binding protein

COX-2- cyclooxygenase-2

AP-1- activator protein-1

Nrf2- erythroid 2p45 (NF-E2)-related factor 2 transcription factor

NF-κB – nuclear factor-kappa B

ARE- antioxidant response element

MAPK- mitogen activated protein kinase

JNK- c-Jun-N-terminal kinase

IL- interleukin

SPS- single prolonged stress

HDAC-5- histone deacetylase-5

CTGC- California Table Grape Commission

FDGP- Freeze-dried grape powder

HO-1- heme oxygenase-1

BBB- blood-brain barrier

GP- grape powder

LE- Long-Evans

EPM- elevated plus maze

LD- light dark

OF- open field

MB- marble burrow

RAWM- radial arm water maze

STM- short-term memory

LTM- long-term memory

FST- forced swim test

EDTA- ethylene-diamine-tetra-acetic acid

SDS-PAGE- Sodium dodecyl sulfate-poly acrylamide gel electrophoresis

PVDF- polyvinylidene difluoride

FC- Folin Ciocalteu

PBS- phosphate buffer saline

DMEM- Dulbecco’s Modified Eagle Medium

ANOVA- analysis of variance

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# 1. INTRODUCTION

Stress in life is unavoidable, affecting each one of on a daily basis. Stress can be temporary or it can last over a long period of time. Mammals mainly encounter physiological or psychological stressors. Physiological stressors are defined as any internal or external condition that challenges the homeostasis of a cell or an organism. Examples of psychological stressors include, but are not limited to, exposure to war, trauma, natural disasters, or related to life experiences such as death of a loved one, unemployment or divorce (Sun and Alkon 2014). Psychological stressors-induced neuropsychiatric disorders are marked as one of the most debilitating ailments in the world today. Several clinical studies have reported that psychological stress in humans leads to development of comorbid psychopathologies including anxiety, depression and cognitive impairment (Cohen et al. 2007; Somers et al. 2006). Globally, 1 in 13 and 1 in 20 suffer from anxiety and depression respectively (Ford and Erlinger 2004; Somers et al. 2006). Anxiety disorders are common and disabling multifactorial conditions that affect approximately 40 million people in the US (Greenberg et al. 1999). And, approximately 33%-50% of anxious and depressed individuals are reported to have cognitive impairment (Potter and Steffens 2007). The estimated economic burden of anxiety and depression is 72 billion dollars in the US (Greenberg et al. 1999; WHO). Stress serves as a significant factor that is

known to contribute to anxiety, depression and cognitive impairment (Cohen et al. 2007; Somers et al. 2006).

Although everyone encounters stress almost on a daily basis, the term “stress” is considered ambiguous. Hans Selye, the father of the stress research, borrowed the term “stress” from the field of physics. In physics, stress is defined as any strain on the physical body induced by force. Hans Selye began using the term “stress” in the 1920’s (Viner 1999). Although Selye is regarded as a trailblazer in the field of stress research and provided cogent arguments about the effect of stress on health, not everyone agreed to his opinion about stress as a non-specific phenomenon that led to an interesting debate (Viner 1999).

The next question that came up was: What is psychological stress? The physician John Mason, by his experiment on monkeys, provided the first clue to this fascinating question. In his experiments, he used two groups of food-deprived monkeys. Group 1 monkeys did not watch others receive food, while monkeys in group 2 watched others receive food. Although both groups of monkeys experienced physical stress of hunger, the one who saw others receive food had higher stress hormone levels. This experimental evidence suggested that psychological stress and physical stress were equally powerful. It was still debated that if stress were a non-specific phenomenon than everyone would respond in a similar fashion to the same stressor. On the other hand, the presence of some prevailing factors result in increasing in the stress hormone levels was convincing (Mason 1968).

In humans, any life threatening, physical or emotional event resulting in bodily harm is referred to as stress. Stress arising from physiological stressors is often linked to cardiovascular and psychiatric conditions. On the other hand, anxiety, depression and cognitive impairments result following an exposure to severe emotional events. Therefore, physiological and emotional stress in humans is considered as a causal factor for comorbid psychopathologies (Kinderman et al. 2015).

Various theories have been proposed about association between stress-induced anxiety and depression. Two classical theories that have been studied in great detail are: 1) Involvement of gamma-amino butyric acid (GABA) receptors, 2) Abnormalities of serotonin receptors. The current pharmacotherapy to treat anxiety and depression is developed based on these two theories. The current treatment options include use of traditional anxiolytics, such as benzodiazepines and antidepressants such as selective serotonin reuptake inhibitors (SSRIs). Though they are regarded as “gold standard” for the treatment of anxiety and depression, they are associated with severe side effects including, but not limited to, tolerance, withdrawal effects, memory dysfunction, drug interaction, and significant weight gain. (Ashton 1994; Barker et al. 2004; Ferguson 2001; Gudex 1991; Trindade et al. 1998). The alternate problem with current pharmacotherapy is: there is no one size fits all treatment available as everyone does not respond to a stressor in the same way (Fairburn and Patel 2014). Though anti-anxiety drugs are available, patients are afraid to take them, which limit their adherence

to the prescribed regimen. In addition, a vast majority of anxiety patients remain unresponsive to classical anti-anxiety drugs (Gorman et al. 2002). Therefore, improvement in therapeutic interventions is needed. Alternative therapy with higher efficacy and lesser side effects must be examined. Developing better therapeutic interventions requires comprehensive understanding of the underlying etiology and signaling pathways of anxiety disorders. Among various known traditional mechanisms of anxiety, such as involvement of GABA and serotonin receptors, non-traditional theory of involvement of oxidative stress (OS) in anxiety disorders is achieving consensus (Masood et al. 2008; Oliveira-Dos-Santos et al. 2000; Salim et al. 2011a). Present study focuses on the concept that OS may regulate psychological stress-induced physiological and behavioral health.

## Oxidative Stress:

A chemical process called oxidation is ubiquitous in nature and is carried out by an oxidant. In general, oxidant is defined as any molecule with the capacity to lose one or more electrons to oxidize the other molecule. Oxidants are usually introduced through either endogenous process in mitochondria or via external chemical toxins. These oxidants are known to activate molecular signaling pathways that in turn trigger the generation of pernicious reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Kohen and Nyska 2002). Mitochondrial oxidative phosphorylation serves as a major source of adenosine triphosphate (ATP) production in aerobic organisms. Highly reactive

free radicals, such as, ROS and RNS are generated as a by-product of oxidative phosphorylation (Perez-Pinzon et al. 2012). A molecular defense system consisted of antioxidant enzymes and non-enzymatic antioxidants are known to prevent cell damage by preserving redox homeostasis. The enzymatic antioxidant defense system is comprised of key antioxidant enzymes such as superoxide dismutase (SOD), glyoxalase (GLO) and glutathione (GSH). The examples of non-enzymatic defense system include vitamins A, C, tocopherol, carotenes and selenium (Li et al. 2013b). The balance between the pro-oxidants and antioxidants is critical. Oxidative stress results when the levels of ROS/RNS exceed the counteracting antioxidant defense system. At low concentrations, free radicals are beneficial and known to participate in normal physiological processes such as secondary messenger in the regulation of vascular cell functioning, protect the cell from injury or invading pathogens and mediator of intracellular regulation of calcium concentration (Griendling et al. 2000; Halliwell 2006; Valko et al. 2007). However, when the oxidative insult occurs, increased ROS/RNS levels may have detrimental effects on cellular structures like deoxyribonucleic acid (DNA), protein, lipid and membranes leading to cell damage or cell death (Castellani et al. 2008; Cooke and Robson 2006; Hu et al. 2005).

Of all the organs, the brain is highly susceptible to oxidative damage owing to its high lipid content and its high glucose and oxygen utilization (Li et al. 2013b). Considering deleterious consequences of OS in the central nervous

system (CNS), it is implicated in several neurodegenerative and neuropsychiatric conditions, including anxiety. The neuronal response to this pervasive stress in the brain is not uniform. Although many neurons can tolerate rise in OS to some extent, presence of highly susceptible neurons render vulnerability of specific brain regions to oxidative damage. For example, hippocampus, amygdala and cerebellar granule cells are the most susceptible to OS compared to other neurons (Wang and Michaelis 2010). Owing to such differential neuronal vulnerability to OS, these neurons are usually the first to undergo functional decline leading to cell death in neurodegenerative and neuropsychiatric disorders. Many studies have suggested association between OS and anxiety disorders (Bouayed et al. 2009; Hovatta et al. 2005; Masood et al. 2008; Salim et al. 2010a; Salim et al. 2011a; Vollert et al. 2011). Though several studies including our own have suggested implication of OS in mental disorders, the causal role of OS in these neuropsychiatric conditions is still unclear. Animal studies have shed light on the role of OS in behavioral deficits in rodents. Several investigators have reported the causal role of OS in behavioral deficits using different rodent models (de Oliveira et al. 2007; Hovatta et al. 2005; Masood et al. 2008). However, the question still remains open, whether pharmacologically-induced OS mediated behavioral outcome can be simulated in psychological stress-induced OS.

To answer this intriguing question, we employed the rat model of social defeat to induce psychological stress in rats. The strength of social defeat model

is that it resembles societal stress in humans and represents an ethologically valid stressor as it induces long-lasting physiologic and behavioral changes (Hollis and Kabbaj 2014). Social defeat is also referred to as resident-intruder paradigm in which a social conflict between an aggressive, dominant resident and an intruder results in intruder attaining submissive, supine posture. Social defeat is not solely a model of physical stressor but also of psychological stressor, as the resident and intruder are separated at the end of the defeat session by a transparent plexiglass partition to prevent physical contact. This transparent partition facilitates olfactory, auditory and visual interactions for the remainder of the session to induce psychological stress to the intruder (Patki et al. 2013b). Exposure to a single social defeat session is considered as acute whereas multiple sessions are referred as chronic model of psychological stress. Social defeat is known to induce long-term physiologic and behavioral changes (Hollis and Kabbaj 2014). Several studies have reported that intruder demonstrate signs of stress, including elevated adrenocorticotropic hormone (ACTH) and corticosterone levels, increased glucocorticoid activity, tachycardia, elevated blood pressure and hyperthermia after single social defeat exposure (Tornatzky and Miczek 1994). Rodents exhibit increased anxiety-like and depression-like behavior, impaired memory, social avoidance and decreased locomotor and exploratory activity following four consecutive social defeat exposures (Koolhaas et al. 1997; Meerlo et al. 1997; Tidey and Miczek 1997). Social defeat-induced behavioral impairments are known to last at least 4 weeks

after the last exposure (Berton et al. 2006; Hollis et al. 2010; Tsankova et al. 2006).

Various studies including our own have shown a causal role of OS in behavioral deficits in rodents (Bouayed et al. 2009; Hovatta et al. 2005; Masood et al. 2008; Oliveira-Dos-Santos et al. 2000; Salim et al. 2010a; Salim et al. 2010b; Souza et al. 2007). In separate studies, we reported the protective effect of antioxidant treatment and moderate treadmill exercise against the pro-oxidant-induced oxidative stress mediated anxiety-like behavior in rats (Allam et al. 2013; Salim et al. 2010b). Recently we have shown that social defeat-induced psychological stress leads to behavioral and cognitive impairments in rats (Patki et al. 2013b). However, the mechanism by which social defeat causes these impairments is not clearly understood. We postulate that social defeat-induced OS is a key player and triggers behavioral and cognitive impairments in rats. Therefore, it seems reasonable to test causal role of OS in behavioral and cognitive deficits in social defeat model of rats. Furthermore, if OS causes anxiety, depression and poor cognition, then antioxidant treatment should protect or reverse these behaviors. In the past, we have shown that rats exhibited behavioral and cognitive deficits when subjected to direct induction of OS via pro-oxidant L-buthionine-(S,R)-sulfoximine (BSO) treatment (Allam et al. 2013). In separate studies, treatment with grape powder, natural antioxidant and tempol, synthetic antioxidant reduced OS, attenuated anxiety-like behavior and improved memory of rats, suggesting a causal role of OS in behavioral and cognitive

deficits in rats (Allam et al. 2013; Salim et al. 2011a). Though our findings with tempol are quite interesting, stability issues and unknown side effects pose a major limitation in clinical usefulness of tempol. Therefore, in this study, we focused our attention on natural products with potent antioxidant properties such as grape powder.

Although beneficial effects of grapes in anxiety, depression and memory impairment have been studied in great detail in several studies, none have studied their effects in psychological stress-induced elevated OS conditions. Using rat model of social defeat, we explored the beneficial effects of a standardized freeze-dried grape powder, rich in polyphenols, on psychological stress-induced behavioral and cognitive impairment in rats.

Beneficial effects of grapes in various diseases such as cancer, inflammation, neurodegenerative, neuropsychiatric and diabetes are known for long time. Of all the grape polyphenols, three compounds namely resveratrol, quercetin and kaempferol have been studied in great detail and known for their potent anti-oxidant, anti-proliferative, anti-inflammatory, cardio-protective and neuro-protective properties (Joseph et al. 2009; Shi et al. 2003; Yilmaz and Toledo 2004). Several investigators have reported beneficial effects of grape powder, grape extract or individual grape components such as resveratrol, quercetin and kaempferol in various in-vivo and in-vitro studies. However, which of these grape components could be responsible for the beneficial effects is not clearly understood. In order to investigate the potential bioactive grape

component, we used hippocampus-derived immortalized cell line (HT22), and simulated oxidative stress using BSO. Furthermore, the mechanism by which grape powder modulates oxidative stress pathway and regulates biochemical changes within the hippocampus is unclear. For example, OS-induced hippocampal neuronal death has been reported in the literature (Behl et al. 1997; Liu et al. 2010), but the mechanism is uncertain. Therefore, using the in-vitro model of OS (HT22 cells), we focused our attention on the OS pathway and investigated the mechanism by which grape powder modulates the OS pathway and protects the hippocampal neurons from cell death.

# STATEMENT OF PROBLEM

In humans, stressful life events contribute to development of comorbid psychopathologies including anxiety, depression and poor cognition. The mechanism underlying psychological stress-induced anxiety, depression and cognitive impairment is still not clear. Furthermore, currently available options to treat anxiety and depression are associated with severe side effects (Trindade et al. 1998). We have recently published involvement of oxidative stress in psychological stress (social defeat)-induced behavioral and cognitive impairments in rats (Patki et al. 2013b). Therefore, it seems reasonable to test causal role of oxidative stress in behavioral and cognitive deficits in social defeat model of rats. The aim of the present research is to investigate the role of oxidative stress in psychological stress-induced behavioral and cognitive impairment in rats. The hypothesis to be tested is that grape powder, natural

antioxidant with potent antioxidant properties, reverses and protects against social defeat-induced behavioral and cognitive impairment in rats by engaging oxidative stress pathway involving specific antioxidant enzymes. As part of the study, beneficial effects of grape powder in social defeat-induced behavioral, cognitive and biochemical impairment was tested. A second hypothesis evaluated was to examine which component of the grape powder imparts beneficial effects of grape powder. A validated in-vitro model of oxidative stress of hippocampal derived cell line HT22 was used and then the potential bioactive compound from grape powder was investigated. A third hypothesis evaluated was to reveal potential underlying mechanism of grape powder responsible for exerting beneficial effects.

The significance of this study is that **it sheds light on the causal role of oxidative stress, in the pathophysiology of stress-induced behavioral and cognitive deficits**. In the long term, this research will facilitate identification of target genes/molecules of oxidative stress pathway that can be attractive targets for future pharmacotherapy to treat stress-associated illnesses effectively.

# 2. LITERATURE REVIEW

## 2.1 Stress

Stress in life is inevitable. The word ‘stress’ was obtained from the Latin word *stringere*. The word *stringere* has been extensively used in physics, meaning ‘to draw tight’. It refers to any force-induced ‘strain’ on a material body. In medical terms, stress is always used in a context as any physical or mental strain posing threat to the organism (Sun and Alkon 2014). Strain, in general, is defined as any physiological, emotional and cellular processes. Stress is a predisposing factor for about 75% of all illnesses (AMA 2011). However, not everyone responds to stress in a similar fashion. Although the types of stress experienced by humans are limitless, they mainly involve sensory, social and related life experiences. Examples of each type of stress are listed in table 1.

| Type of stress | Examples |
|---|---|
| Sensory | Pain, noise, bright light, temperature etc. |
| Social | Relationship conflict, break up, divorce or death |
| Life-threatening events | Exposure to war, poverty, natural disaster etc. |

**Table 1. Examples of type of stress experienced by mammals** Adapted from (Sun and Alkon 2014).

Stress, in general, may manifest as one of the following: good stress, which is beneficial and motivating and bad stress that may cause health problems. Organism’s response to stress is called fight-or-flight response, which

is fundamental to their existence. Fight-or-flight response ensures organisms' survival and enables organisms to respond quickly to life-threatening situations. Stressors are referred as any physical or psychological event that induces stress (Koolhaas et al. 2011). Although mammals experience various types of stressors, they mainly encounter physiological or psychological stressors (Sun and Alkon 2014). Physiological stressors are defined as any internal or external situation that offsets the homeostasis of a cell or an organism (Kagias et al. 2012). Physiological changes that occur during the stress response include increased heart rate and respiration, increased sweating and salivation etc. Responses to psychological stressors are known to recruit higher-order neural processing. Of all the organs, brain serves as an initiator of stress response (Lucassen et al. 2014; Sun and Alkon 2014).

### 2.2. Stress and the brain

The stress response initiates in the brain. When someone confronts a stressful event, the auditory and the olfactory system sends the stress signal to amygdala, a brain region known to regulate emotional processing. Up on perceiving a stressful event as a threat, it transmits a distress signal to the hypothalamus. This brain region serves as a command center. The autonomic nervous system facilitates the communication between the hypothalamus and the rest of the body (Kenney and Ganta 2014). Autonomic nervous system regulates involuntary functions such as breathing, blood pressure, dilatation or constriction of blood vessels and certain metabolic functions. Two subdivisions of the

autonomic nervous system are: the sympathetic nervous system and the parasympathetic nervous system (Lucassen et al. 2014). The fight-or-flight response is regulated by the sympathetic nervous system that provides the body with enough energy in order to respond to a threat. The parasympathetic nervous system acts as counterbalance and triggers the “rest and digest” response that help regain the body’s composure once the threat has vanished (Kim and Yosipovitch 2013).

The activation of the sympathetic nervous system following an exposure to stressful events occurs in seconds (Sun and Alkon 2014). The hypothalamus, brainstem and hippocampus are known to regulate the sympathetic nervous response (Resstel et al. 2008; Scopinho et al. 2013). The other system that is involved in stress response is endocrine. The stress-induced-endocrine response is slower and is mediated by hypothalamic-pituitary-adrenal (HPA) axis. Stress-induced activation of HPA axis is known to increase the release of glucocorticoids (Dickerson and Kemeny 2004). The endocrine response is partly regulated by two brain regions: the amygdala and the ventral hippocampus (Bertoglio et al. 2006; Felix-Ortiz and Tye 2014). The cascade of stress-response begins in the hypothalamus when it receives as a stress signal from sensory inputs. The hypothalamus secretes corticotropin-releasing hormone (CRH) that is transported to the pituitary gland. In turn, the pituitary gland releases adrenocorticotropic hormone (ACTH) in to the blood stream activating the adrenal gland to release corticosteroids (Lucassen et al. 2014). Such stress-

induced elevated levels of corticosteroids in the blood are referred as biomarkers of stress (Patki et al. 2013b; Pruett et al. 2008; Solanki et al. 2015). Corticosteroids can readily cross the blood-brain-barrier. Once released, corticosterone (in rodents) and cortisol (in humans) bind to glucocorticoid receptors (GRs) and mineralocorticoid receptors (MRs) in the brain. Their binding affinity for MRs is 10-fold higher than for the GRs. These receptors are highly expressed in the amygdala and the hippocampus (Han et al. 2014; Medina et al. 2013).

Multiple feedback loops play a crucial role in HPA axis regulation. The secretion of CRF and excitatory projections to CRF neurons are inhibited by glucocorticoids (Di et al. 2003). In addition, the limbic brain regions, such as amygdala, hippocampus and pre-frontal cortex (PFC), play a key role in regulating the HPA axis. In response to stressors, the HPA axis is stimulated by amygdala. In contrast, the hippocampus and PFC suppress the axis (Jankord and Herman 2008). The feedback system consists of amygdalar inhibitory projections and excitatory projections from hippocampus to inhibitory neurons of the hypothalamus, specifically the paraventricular nucleus. Under the stress, the net activity of the HPA axis is controlled by increased and decreased inputs from the amygdala and the hippocampus, respectively (Sun and Alkon 2014).

The stress-induced activation of nervous and endocrine systems is known to initiate a multisystem response to cope with the threat. Organism’s normal response to stressors is fundamental to their existence (Koolhaas et al. 2011).

The prolonged activation of the stress response may lead to variety of behavioral and cognitive deficits (Hollis and Kabbaj 2014; Sun and Alkon 2014). The stress responses in mammals and potential targets for pharmacological intervention are depicted in Figure 1.

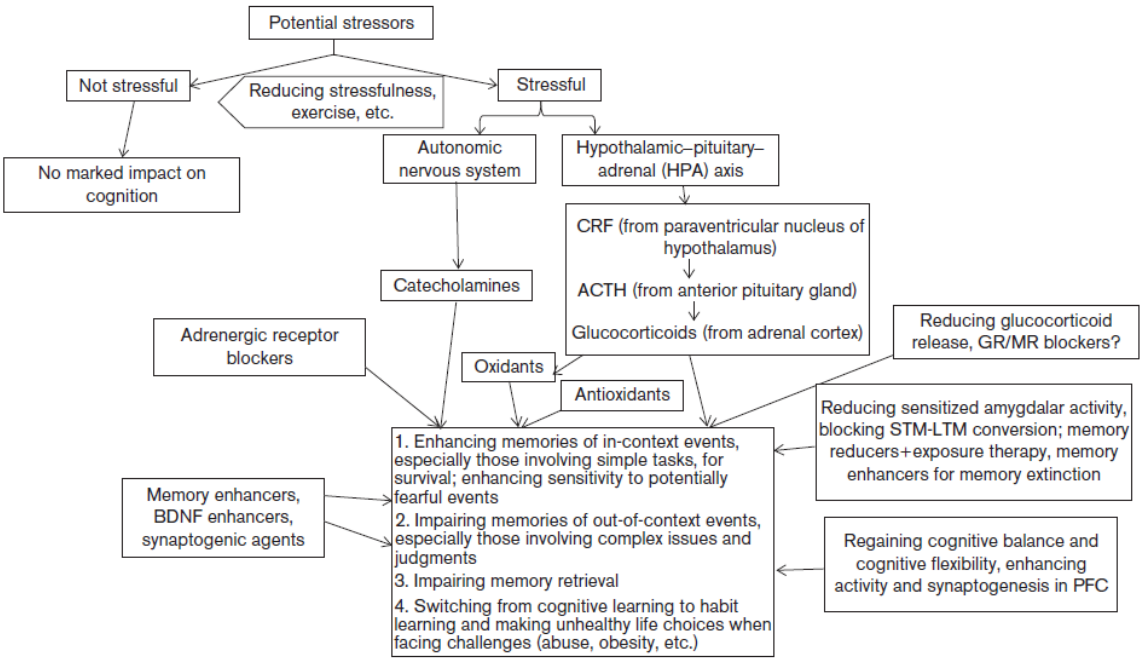



**Figure 1. Stress responses in mammals and potential therapeutic targets.** ACTH, adrenocorticotrophic hormone; BDNF, brain-derived neurotrophic factor; CRF, corticotrophin-releasing factor; GR, glucocorticoid receptor; LTM, long-term memory; STM, short-term memory; MR, mineralocorticoid receptor; PFC, pre-frontal cortex. Taken from (Sun and Alkon 2014).

## 2.3. Acute and chronic effects of stress on the brain

Stress management can be complex and perplexing because there are two major types of stress – acute stress and chronic stress – each with varying symptoms, characteristics, duration and treatment options. In general, acute stress is referred as short-term stress and most commonly it arises from the demands of the recent past or anticipated fear of the future. Generally, acute stress is beneficial, but it is exhaustive when it becomes excessive. Acute stress is easily manageable and highly treatable. The acute stress symptoms are as follows: emotional distress including feeling of irritability, anxiety and depression, musculoskeletal symptoms such as headache, painful back and jaws and tensed muscles (Classen et al. 1998). In addition, increased blood pressure, palpitations, sweaty palms, heartburn, acid stomach, constipation and flatulence are commonly associated with acute stress (Birmes et al. 2003).

While acute stress can be rousing and stimulating, chronic stress is not. It is also called grinding stress, as it destroys bodies, minds and lives. It is devastating mainly because of its extreme nature and long-term duration. It is the stress associated with major life events that could result in psychological distress such as poverty, unhappy marriage, divorce, death of the loved one, unemployment or exposure to war (Sun and Alkon 2014). Chronic stress arises when a person never finds a way out of a doleful situation. It is the stress of never-ending demands and pressures and seems to be present for endless periods of time leaving the individual hopeless (McEwen 2008). Chronic stress

takes toll on the body causing wear-and-tear on the body. The worst characteristic of chronic stress is that people get accustomed to it and because it is persistent people ignore it (McEwen 2008). Chronic stress associated mortality results from suicidal thoughts, heart attack and perhaps, cancer. The physical and mental ability to tolerate the chronic stress diminishes over time, treatment options are limited including extended medical and behavioral therapy (McEwen 2008; Sun and Alkon 2014).

Several studies have reported that exposure to chronic stress often result in unexpected outcomes including altered physiology and biochemistry, poor cognition and behavioral deficits (Hollis and Kabbaj 2014; Zoladz et al. 2012). Several studies including our own have reported that the anxiety- and depression-like behavior and poor cognition could be attributed to three key brain regions – hippocampus, amygdala and pre-frontal cortex (PFC) - most susceptible to the deleterious effects of stress exposure (Arnsten 2009; Hains et al. 2009; Patki et al. 2013b; Shin et al. 2006). Though these brain regions are implicated in anxiety and depression, they regulate different cognitive and behavioral functions in response to stress. The hippocampus mainly regulates spatiotemprol aspects of behavioral (LeDoux 2007). The amygdala mediates the affective and emotional aspects. The PFC is known to regulate cognitive and executive functions (Arnsten 2009; Cerqueira et al. 2008; Czeh et al. 2008; LeDoux 2007). It is known that in response to stress, the amygdala is the first brain region to acquire safety signals (Genud-Gabai et al. 2013). During stress,

the amygdala mediated consolidation of emotional information strengthens mainly due to the increased input from amygdala to the brainstem and hypothalamus resulting in increased release of glucocorticoids. In contrast, the PFC and the hippocampal functioning weakens (Sun and Alkon 2014). Such differential impacts of stress on amygdalar and hippocampal/PFC spines are considered fundamental to stress associated behavioral and cognitive deficits. Interestingly, stress is known to increase amygdalar activity potentially via increasing glutamatergic signaling in the basolateral amygdala (BLA) (Padival et al. 2013a; Padival et al. 2013b). Such stress-induced increased amygdalar activity is associated with increased BDNF expression, increased dendritic branching and spine density in the amygdala (Hill et al. 2013; Mitra et al. 2005). In contrast, stress mediated increased hippocampal glutamate signaling is associated with decreased activity of the pyramidal cells and decreased BDNF expression potentially leading to volume loss and dendritic atrophy in the hippocampus (Boyle 2013; Lakshminarasimhan and Chattarji 2012; Vyas et al. 2002). Therefore, the net outcome of chronic stress leads to strengthening of the structures that provide positive feedback such as amygdala and weakening of the structures that negatively regulate the stress response such as the hippocampus and the PFC (Izquierdo et al. 2006; McEwen 2004). Altogether, it leads to dominance of amygdalar activity on the hippocampus resulting in the increased activation of paraventricular nucleus (PVN) of the hypothalamus via bed nucleus of stria terminalis (BNST). The activation of PVN results in the

increased release of stress hormones such as glucocorticoids (Lucassen et al. 2014). The stress and stress hormones primarily target the higher brain center such as hippocampus (Herman et al. 2005). The stress and stress hormones modulate function of these regions by altering the structure of neurons in these regions. Within the hippocampus, a specific type of circuitry exists. While other brain regions are known to regulate the long-lasting storage of memory, the dentate gyrus-CA3 system is vital for the memory of sequential events (Lisman and Otmakhova 2001). Owing to its function and susceptibility to the damage, the dentate gyrus-CA3 system exhibits a unique characteristic of adaptive structural plasticity. Under the structural plasticity, the dentate gyrus is considered to have regenerative capacity, in which the dentate gyrus undergoes neurogenesis during adulthood. Furthermore, CA3 pyramidal dendrites demonstrate reversible remodeling capacity in chronic stress conditions (McEwen 1999; Popov and Bocharova 1992). Such adaptive plasticity/changes within the hippocampus is thought to be protective in chronic stress-induced permanent damage. The neurogenesis within dentate gyrus is tightly regulated by various hormones, neurochemicals and behavioral tests such as estradiol, brain-derived neurotrophic factor (BDNF) and hippocampal-dependent learning (Aberg et al. 2000; Czeh et al. 2001; Trejo et al. 2001). In terms of stress, acute and chronic stresses are known to inhibit neurogenesis within the dentate gyrus (Gould et al. 1997). The hippocampus undergoes another type of adaptive plasticity - remodeling of dendrites. Various studies have reported that hippocampal CA3

dendrites exhibit retraction and simplification in response to chronic restraint stress (McEwen 1999; Sousa et al. 2000). The CA1 region spine synapse formation increases in response to acute stress, but chronic stress inhibits it (Pawlak et al. 2005; Shors et al. 2001). Such dendritic architectural changes are observed in dominant as well as submissive rats experiencing visible burrow system-induced psychosocial stress (McKittrick et al. 2000). In addition to hippocampus, the amygdala and PFC undergo dendritic alterations during repeated or chronic stress. Dendritic shrinking in medial PFC and dendritic growth in amygdalar neurons following repeated stress exposure has been reported in various studies (Brown et al. 2005; Kreibich and Blendy 2004; Radley et al. 2006; Vyas et al. 2002; Wellman 2001). Using rat model of social conflict, Wood et al. reported that chronic stress-induced increased aggression between cage mates is an indicator of hyperactive amygdala (Wood et al. 2003). The PFC through its extensive projections to other brain regions plays a central role in regulating our thoughts and emotions. In humans and animals, it has been reported that exposure to acute uncontrollable stress can lead to impaired cognition and chronic stress is known to alter prefrontal dendrites architecture (Liston et al. 2009; Luethi et al. 2008). Such stress-induced altered neuronal connections and architecture leads to development of behavioral and cognitive deficits.

### 2.3.1 Neural circuits underlying anxiety behavior

While the involvement of the amygdala, the BNST, the hippocampus and the PFC in anxiety and depression is known, the contributions of regional microcircuits in regulating these behaviors are not properly understood. In order to identify a situation as a potential threat and to exhibit an anxiety-like response, the threatening stimuli must first be detected through sensory systems (Mathew and Charney 2008). Such interpretation of threating/stressful stimuli is facilitated by coordinated activity in the amygdala, the BNST, the hippocampus and the PFC. These structures are highly interconnected and possess multiple reciprocal projections known to regulate vigilance and defensive behaviors via involvement of brainstem (Calhoon and Tye 2015). The information about the threatening stimuli is transmitted via both forward (amygdala → BNST → hippocampus → mPFC) and backward (mPFC → hippocampus → the amygdala and BNST) mechanisms. While the forward direction facilitates the detection and interpretation of stressful stimuli causing enhanced vigilance, the reverse direction evaluates the initial interpretation (Calhoon and Tye 2015). The basolateral amygdala (BLA), a main input nucleus in the amygdala, receives sensory information of the potential threat from the thalamus and sensory cortices (Calhoon and Tye 2015; Lucassen et al. 2014). Then, projections from the BLA targeting the BNST are activated in response to fear or anxiety-inducing conditions. The sensory neurons do not exclusively regulate the activity in the BLA, however; the monosynaptic neuronal inputs from the PFC and the

hippocampus to interneurons in the BLA and the reciprocal projections from the BLA to both of these regions are well described (Calhoon and Tye 2015). The anxiety response is tightly regulated by the increased activity in these pathways. The recruitment of BNST is critical to sustain the anxiety response which is partly due to the direct projections from the BLA and through the glutamatergic projections from the hippocampus (Cullinan et al. 1993; Davis et al. 2010; Dong et al. 2001) and the mPFC (Stamatakis et al. 2014). Furthermore, another pathway known to regulate anxiety-like behavior is the BLA-ventral hippocampus circuit. For example, the glutamatergic projections from the BLA to the pyramidal neurons in the CA1 region of the ventral hippocampus reciprocally control anxiety-like behaviors in the elevated plus maze test and the open field test (Felix-Ortiz et al. 2013). The reciprocal projections between the amygdala and the PFC have been studied in great details in both humans and rodents (Kim et al. 2011; Ochsner et al. 2002). The acquisition of aversive memory is attributed to the cumulative reciprocal activity between the dorsal anterior cingulate cortex of the PFC and BLA (Calhoon and Tye 2015). Altogether, based on literature, it is safe to note that anxiety responses are tightly regulated by three main brain regions i.e. the hippocampus, amygdala and the PFC.

### 2.4. Animal models of stress

Chronic stress is difficult to study in humans, as it is a multifactorial condition leading to development of comorbid psychopathologies. Understanding the relationship between the exposure to extreme stressor and psychological

consequences was made possible by development of animal models. Various animal models of stress have provided insights into the pathogenesis of stress-induced psychiatric ailments such as anxiety, depression, mood disorders and post-traumatic stress disorder (PTSD). Furthermore, most of the symptoms of stress-induced psychiatric conditions are associated with activated HPA axis and elevated glucocorticoid biomarkers. Several studies including our own have reported that rodents exhibit anxiety and depression-like behavior and poor cognition after single or multiple exposures to a stressor (Koolhaas et al. 1997; Meerlo et al. 1997; Patki et al. 2013b; Solanki et al. 2015). The majority of the animal models of stress include exposure to predators, physical shock and movement restriction (Campos et al. 2013b). These animal models mainly differ from each other in two aspects: duration of exposure (acute versus chronic) and nature of stressor (Campos et al. 2013b). Several well-established stress protocols are listed in table 2.

| **Examples of protocols used in stress research** | | |
|---|---|---|
| Physical stress models | Main Features | Few studies |
| a. Restraint stress | Animal is placed in a cylindrical tube for 2-3 hours. | (Campos et al. 2010; Padovan and Guimaraes 2000) |
| b. Immobilization | The movement of | (Hill et al. 2009; |

| | | |
|---|---|---|
| stress | animal's limbs and head is restricted by gentle wrapping. | Shansky et al. 2009) |
| c. Temperature variation stress | Subject the animal to the cold water or freezing temperature | (Jaggi et al. 2011; Kvetnansky et al. 1977) |
| d. Electric foot shock stress | Subject the animal to a unpredicted, mild intensity foot shock | (Golub et al. 2009; Herrmann et al. 2012) |
| Psychosocial stress models | | |
| a. Neonatal isolation | For 8 consecutive days, litter is placed in a separate cage for 1 hour (protocol usually starts on 2$^{nd}$ day of the birth) | (Babygirija et al. 2012; Francis and Meaney 1999; Kosten and Kehoe 2005; Lai et al. 2008; Maniam and Morris 2010) |
| b. Noise stress | Subject the animals to noise intensity of 100 dB of higher | (File and Fernandes 1994; Manikandan and Devi 2005; Naqvi et al. 2012; Ravindran et al. 2005) |

| | | |
|---|---|---|
| c. Circadian rhythm change | Subject the animal to unexpected alteration in day-night light cycle | (Castro et al. 2005; Fonken et al. 2009; Nicholson et al. 1985; Tapia-Osorio et al. 2013) |
| d. Predator stress | Subject animal is placed in proximity to the predator or its odor for 5 – 30 minutes. (Widely employed to study PTSD-like symptoms) | (Adamec et al. 2004; Adamec and Shallow 1993; Baisley et al. 2011; Campos et al. 2013a; Matar et al. 2006) |
| e. Social defeat | Resident-intruder paradigm. Animal is subjected to aggressive encounter. | (Hollis and Kabbaj 2014; Miczek 1979; Patki et al. 2013b; Wood et al. 2010) |

**Table 2. Examples of protocols used in stress research.**

## 2.5. Social defeat

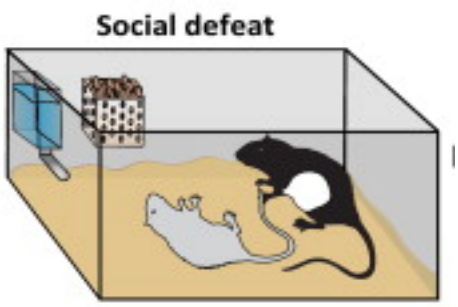
Although humans experience variety of stressors, the majority of them are psychological and emotional in nature (Almeida and Nardi 2002; Bjorkqvist 2001; Kessler 1997). Exposure to such stressors render the human vulnerable to neuropsychiatric and neurodegenerative conditions. Psychological stressor-induced social stress is known to play a central role in the pathogenesis of neuropsychiatric disorders including anxiety and depression (Huhman 2006; Kessler 1997; Patten 1999). Social stress in humans is assessed with two aspects: the number and magnitude of life threatening events and social status. Social status serves as an important index representing the likelihood of experiencing higher number of stressful events (Blanchard et al. 2001). In humans, low social status is thought to have profound impact on almost all aspects of the individual's life including access to resources. In addition to material consequences of low status, when an individual ranks his or her status with reference to others also induce stress (de Ridder 2000). Since social stress effects are chronic and powerful, it is employed extensively to study stress mechanisms (Blanchard 2002).

Most of the animal models of social stress involve single, intermittent, or chronic encounter between the conspecific members (Berton et al. 2006; Blanchard et al. 1995; Tsankova et al. 2006). Of all the rodent models of social stress, the social defeat model has gained popularity in last few decades (Koolhaas et al. 1997). Social defeat, a resident-intruder paradigm, was originally

developed by Miczek (Miczek 1979). Social defeat employs social conflict between a subject and a conspecific animal to induce psychological stress (Hollis and Kabbaj 2014). Because of its ability to generate persistent emotional and psychogenic response, social defeat is considered as an ethologically valid stressor and the most robust model of stress-induced neuropsychopathies including PTSD, depression and anxiety (Berton et al. 2006; Krishnan et al. 2007; Patki et al. 2013b; Tidey and Miczek 1997; Wood et al. 2010). The strength of this paradigm is that it induces persistent psychological and emotional stress without physical harm and habituation (Hollis and Kabbaj 2014; Tidey and Miczek 1997). In contrast to the social defeat, animals exposed to physical stressor such as restraint stress quickly adapt by the multiple presentations (Girotti et al. 2006; Harris et al. 2004). In this model, a male rodent (intruder) is placed in to the home cage of the aggressive dominant male (resident). In other models, the cohabitating female is replaced by the intruder in the aggressive resident cage. In all models, the agonistic encounter occurs between the resident and intruder resulting in intruder attaining submissive posture for the remainder of the procedure. Following the exposure to intimidations and aggressive encounters by the resident, intruder emits frequent signs of distress and exhibit freezing behavior along with assuming subordinate, submissive, supine position (Figure 2A) (Blanchard and Blanchard 1977; Patki et al. 2013b; Wood et al. 2010). The social defeat, however, is not only a model of physical stress. To allow the psychogenic exposure to the intruder, the resident and the intruder is often

separated by a transparent plexiglass partition with holes following the defeat exposure. The transparent partition prevents the physical contact but allows the intense auditory, olfactory and visual interactions between the resident and the intruder for the remainder of the session (Figure 2B) (Patki et al. 2013b; Wood et al. 2010). In some studies, following the social defeat attack, the intruders were placed in a protective cage to induce psychological stress.

**A**




**B**

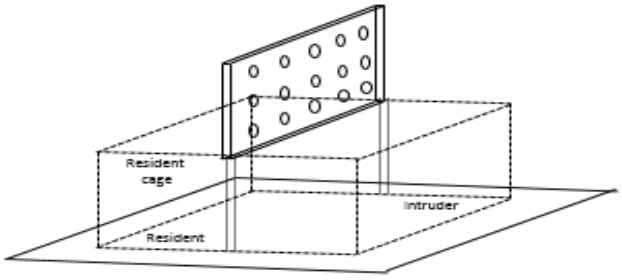



**Figure 2. Schematic representation of the social defeat event (A) and apparatus (B).** Taken from (Opendak and Gould 2015; Patki et al. 2014).

Social defeat-induced psychological stress leads to development of long-lasting physical changes and behavioral deficits (Hollis and Kabbaj 2014; Patki et al. 2013b). Several studies have reported that socially defeated rats exhibit symptoms of activated sympathetic nervous system including increased heart rate and blood pressure and elevated corticosterone levels following activation of HPA axis (Patki et al. 2013b; Tornatzky and Miczek 1993; Wood et al. 2012; Wood et al. 2010). The socially defeated rats also illustrate signs of stress, such as tachycardia and hyperthermia (Tornatzky and Miczek 1993). Even a single agonistic encounter can induce profound physiological and behavioral alterations including retarded growth and increased sensitivity to other stressors (Meerlo et al. 1996a) and anxiety-like behavior (Meerlo et al. 1996b; Ruis et al. 1999). Therefore, it is not unusual that chronic social defeat exposure results in persistent physiological and behavioral changes. Various lines of evidence suggest that following four to seven consecutive social defeat exposures, the rats demonstrate decreased locomotor and exploratory activity (Koolhaas et al. 1997; Meerlo et al. 1996a; Patki et al. 2013b), increased anxiety-like behavior (Jin et al. 2015; Kinsey et al. 2007; Patki et al. 2013b; Ruis et al. 1999) as well as reduced aggression and mating behavior (Hollis and Kabbaj 2014; Meerlo et al. 1996b). Rats subjected to chronic social defeat illustrated depression-like behavior observed by decreased mobility in a force swim test and decreased preference for sweetened solution (anhedonia) (Patki et al. 2013b; Riga et al. 2015; Rygula et al. 2005). In some studies, mice subjected to 10 days of social defeat exhibited

social avoidance (Berton et al. 2006; Hollis et al. 2010). Such chronic social defeat-induced behavioral and emotional alterations are long lasting, persisting at least four weeks after the last defeat session (Berton et al. 2006; Tsankova et al. 2006). Social defeat-induced behaviors such as social avoidance and anhedonia are reversible with chronic antidepressant administration (Berton et al. 2006; Tsankova et al. 2006).

At the molecular level, social defeat can also induce profound morphological and neuronal changes. Several studies including our own have reported that hippocampus, amygdala and prefrontal cortex are most vulnerable to chronic social defeat stress. Furthermore, studies have reported that socially defeated rats had hippocampal and medial prefrontal cortex atrophy and decreased cell proliferation in the hippocampus, which are reversible with antidepressant treatment (Becker et al. 2008; Van Bokhoven et al. 2011). Chronic social defeat also induces significant alterations in the morphology of the dendrites including increased number of mushroom or stubby spines along with decreased postsynaptic density (Christoffel et al. 2011). Such alteration in dendritic morphology was found to be associated with activation of an inhibitor of kappa B (IkB) kinase signaling pathway, known to regulate neuronal morphology (Christoffel et al. 2011).

HPA axis dysregulation is commonly observed in patients with neuropsychiatric conditions, including anxiety, depression and PTSD. Similarly, animal studies have reported that psychological stress-induced by social defeat

also results in dysregulation of the HPA axis. Several studies including our own have reported significantly increased levels of corticosterone after three to seven consecutive exposures to social defeat (Patki et al. 2013b; Razzoli et al. 2007; Wood et al. 2010). In the case of four-five consecutive exposures, thymus, adrenal, heart and bladder weights were significantly increased (Becker et al. 2008; Calvo et al. 2011; Kinsey et al. 2007; Wohleb et al. 2014; Yu et al. 2016). Chronic social defeat-induced HPA axis dysregulation and elevated corticosterone levels lasted at least two weeks following the last defeat session (Becker et al. 2008).

Such persistent activation of HPA axis and significantly increased levels of glucocorticoids have been reported as the cause for the hippocampal dysfunction and decreased hippocampal neurogenesis (Cameron and Gould 1994; Gould and Tanapat 1999; Sapolsky 2000). In order to explore the functional outcomes of HPA axis, Lehmann's group subjected adrenalectomized mice to the social defeat exposure for two weeks. The authors reported that adrenalectomized mice exhibited increased resilience to the behavioral deficits as compared to the sham mice exposed to social defeat (Lehmann et al. 2013). In the same study, the authors observed significantly decreased hippocampal neurogenesis in the sham mice as compared to both control and defeated adrenalectomized mice, suggesting the critical role of hyperactive HPA axis on hippocampal neurogenesis (Lehmann et al. 2013). In one study by Becker et al, 10% and 25% decrease in hippocampal volume and neurogenesis was observed respectively,

after four weeks of social defeat (Becker et al. 2008). Chronic imipramine treatment during social defeat was found to be protective against deleterious effects of defeat (Becker et al. 2008). In a separate study, five weeks of social defeat significantly decreased neurogenesis in the mPFC that was again protected by chronic antidepressant administration (Czeh et al. 2007). Though social defeat-induced behavioral deficits have been reported to last at least four weeks after the last exposure, morphological changes such as decreased neurogenesis have been reported to be transient. A study by Legace et al. reported that 10 consecutive days of defeat exposure resulted in marked decrease in hippocampal neurogenesis that restored to normal levels in 24 hours (Lagace et al. 2010). Altogether, these finding suggest that social defeat-induced increased glucocorticoids, activated HPA axis, and morphological changes in the brain areas such as within the hippocampus and mPFC, could be responsible for the behavioral and cognitive deficits.

While physiological and behavioral alterations are reported to be induced by social defeat, it is also known to induce profound immunological responses (Kinsey et al. 2008). Pertinent to this, exposure to repeated social defeat stress in rats is known to elicit strong inflammatory responses including dysfunctional cytokine cascades and NF-κB activation (Hodes et al. 2014; Niciu et al. 2014). The activation of NF-κB is tightly regulated by primary inflammatory mediators such as Interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α). Such activation of NF-κB results in the production of cytokines including interleukin-6

(IL-6), interleukin-8 (IL-8), interferon (IFN) and C-reactive protein (CRP). The downstream effects of NF-κB activation result in the increase in oxidative stress and neuro-inflammation (Gordon and Martinez 2010). In a recent study, repeated social defeat in mice led to elevated inflammatory cytokines and increased microglia activation in the hippocampus with marked decrease in the number of young and mature neurons. Altogether, the authors suggested that social defeat-induced hippocampal neuro-inflammation and impaired neuroplasticity could be responsible for behavioral and cognitive deficits (Niraula 2015). Nevertheless, these findings suggest that rodent model of social defeat may serve as an excellent tool to study neuropsychiatric conditions and may provide mechanistic insights of the psychological stress-induced behavioral and cognitive deficits.

### 2.6. Oxidative stress

Several studies have reported that exposure to chronic stress generates excessive reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Devaki et al. 2013; Gerecke et al. 2013; Wang et al. 2012). Highly reactive ROS are generated as a by-product in mitochondria during oxidative phosphorylation. All forms of ROS are generated following the activation of molecular oxygen. Using various catalytic pathways, metallo-enzymes facilitate ROS generation upon interaction between redox metals such as Iron (Fe) and $O_2$ (Uttara et al. 2009). The example of generation of free radicals and ROS as a consequence of reaction between metal ion and $O_2$ is depicted below:

Step 1: $Fe^{3+} + \bullet O_2^{-} \rightarrow Fe^{2+} + O_2$

Step 2: $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \bullet OH$ [known as Fenton reaction (Lloyd et al. 1997)]

Generation of ROS after combining both steps

$\bullet O_2^- + H_2O_2 \rightarrow \bullet OH + HO^- + O_2$

ROS formation is inevitable under physiological conditions and is vital for normal physiological functions, such as protection from invading pathogens, regulation of intracellular calcium concentration, protein phosphorylation-dephosphorylation (Halliwell 2006; Valko et al. 2007). ROS are vital for regulation of cell signaling pathways, including cell survival, cell differentiation and proliferation (Catarzi et al. 2011; Gamou and Shimizu 1995; McCubrey et al. 2006; Rao and Clayton 2002). ROS is also thought to play a central role as a regulator of cardiac and vascular cell functioning (Griendling et al. 2000; Valko et al. 2007). However, when in excess, ROS/RNS may prove to be deleterious. Such reactive species are known to oxidize all major cellular macromolecules including DNA, RNA, proteins and lipids (Kohen and Nyska 2002). ROS attack the base and the sugar moieties in the DNA to induce single-and double-stranded breaks, and render the DNA mutated or cross-linked to other molecules blocking the DNA replication (Konat 2003). Single-stranded RNA is more susceptible to oxidative insult than double-stranded DNA and has higher permeability to the mitochondria (Hu et al. 2005). Lipids also serve as a major target in oxidative stress. Free radicals initiate lipid peroxidation and alter membrane properties by targeting polyunsaturated fatty acids in the cell

membrane (Cabiscol et al. 2000). ROS damage the proteins by modification of amino acids and peptides, oxidation of sulfhydryl groups and peptide fragmentation (Lockwood 2000; Stadtman and Levine 2003). Thus, free radicals alter cellular functions by modifying cellular biomolecules including lipids, DNA and proteins, resulting in apoptosis or necrosis (Aksenova et al. 2005; Berk et al. 2008; Filomeni and Ciriolo 2006).

In order to protect the cell from the detrimental effects of excessive free radicals; the cells have a counteracting defense system, antioxidant system in place (Li et al. 2013b). An effective antioxidant system is important to check the over production of ROS/RNS. Oxidative stress results when homeostasis between the ROS/RNS production and counteracting antioxidant defense system is disturbed (Figure 3) (Gandhi and Abramov 2012). Such oxidative insult is marked potentially due to uncontrolled production of reactive species or malfunctioning of antioxidant defense system. Antioxidant defense mechanism consists of antioxidant enzymes and non-enzymes. The primary antioxidant defense is enzymatic including superoxidse dismutase (SOD), glutathione reductase (GSR) and peroxidase (GPX), Glyoxalase (GLO) and catalase (Valko et al. 2007). The non-enzymatic antioxidants include but not limited to Vitamin A, Vitamin C, Vitamin E, selenium, taurine and zinc (Massaad and Klann 2011). These antioxidant enzymes and non-enzymatic antioxidants work synergistically to remove the free radicals (Perumal et al. 2005).

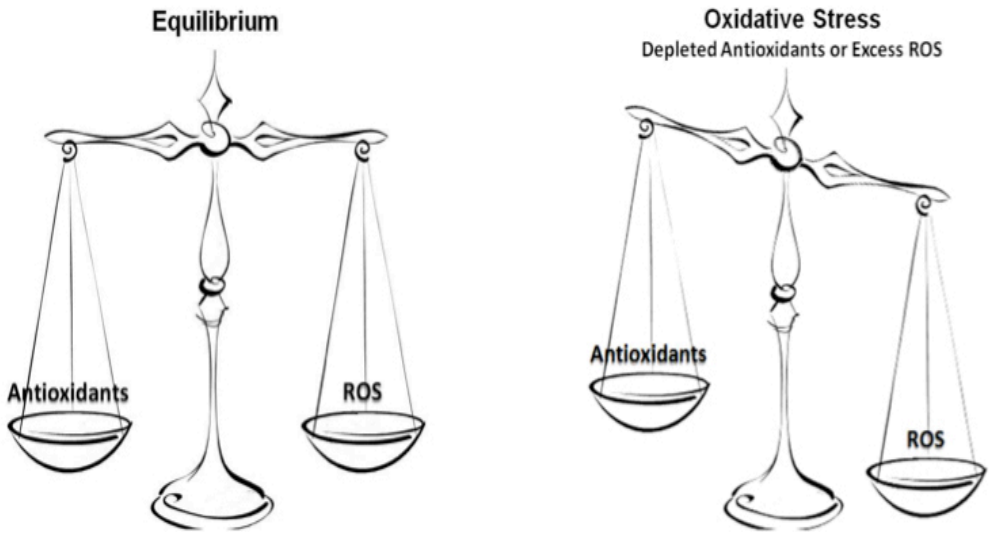



**Figure 3. Schematic representation of equilibrium and oxidative stress conditions.** Taken from (www.horsehealthproducts.com).

The cell, against oxidative stress, may employ the two approaches of defense mechanisms, direct and indirect. While the direct approach is the most efficient repair system consisting of antioxidant enzymes and low-molecular-weight antioxidants (LMWA), the indirect approach regulates the endogenous production of ROS (Kohen et al. 1999; Kohen et al. 2000). Owing to its ability to direct removal of free radicals and ROS, the antioxidant enzymes and small molecules serves as a first line of defense and provides maximum protection to cellular biomacromolecules (Kohen and Nyska 2002). Superoxide dismutase (SOD) enzyme acts primarily on highly reactive superoxide radical and convert it to $H_2O_2$ (Fridovich 1995). Two forms of this enzyme, Cu-Zn SOD and Mn-SOD, are widely distributed in the prokaryotic and eukaryotic cytoplasm and

mitochondria, respectively. This enzyme facilitates the spontaneous dismutation of superoxide radical to generate $H_2O_2$. The end product $H_2O_2$ is further removed by the activity of catalase and glutathione peroxidase (Forman 2016). While catalase can remove higher concentrations of $H_2O_2$, glutathione peroxidase can remove low concentrations efficiently (Chance et al. 1979; Halliwell 2006). The direct and indirect reactive capacity with ROS makes the low-molecular-weight antioxidants (LMWA) unique and highly effective against oxidative insult (Kohen and Nyska 2002). The indirect acting molecules facilitates the chelation of transition metals thereby no free metal is available to participate in the metal-induced Haber-Weiss reaction (Saso and Firuzi 2014). On the other hand, via direct mechanism, they act as scavengers and protect the biological target from free radical attack. Scavengers have several advantages over enzymatic antioxidants: firstly, their small molecular weight facilitates entry through the cellular membranes and allows them to localize near the cellular macromolecules. Secondly, cells have the capability to generate as well as regulate their optimum concentration. Thirdly, their wide spectrum activities enable them to fight against variety of ROS (Bagchi et al. 2014). While endogenous processes in the cell such as, biosynthetic pathway produces scavengers; it is exogenously obtained from diet. Some examples of the LMWA scavengers are glutathione, uric acid, ascorbic acid and melatonin (Kohen and Nyska 2002). The low-molecular weight tripeptide (glutamic acid-cysteine-glycine) – glutathione is found as reduce form (GSH) and oxidized form (GSSG).

In humans, higher levels of GSH serve as a cofactor for other enzymes such as glyoxylase and peroxidase (Lu 2013; Morris et al. 2014). In addition, GSH by acting through indirect mechanism donates electrons to scavenge $H_2O_2$. Glutathione is known to regulate various cellular functions such as metabolism and inter- and intra-cellular communications (Currais and Maher 2013; Lu 2013; Morris et al. 2014). Furthermore, GSH serves as a scavenger as it possess the most reactivity with wide varieties of ROS, including •OH, $ROO^{\bullet}$, and $RO^{\bullet}$ radicals (Haenen and Bast 2014). Following the reaction with a radical, GSH is converted to thiol radical and then dimerization of two newly-formed thiol radicals generates oxidized glutathione (GSSG). The endogenous ratio of GSH:GSSG is an important indicator of cellular redox homeostasis (Berk et al. 2008; Haenen and Bast 2014; Lu 2013; Morris et al. 2014). The glutathione reductase (GR) facilitates the reduction of GSSG to GSH and maintains the optimum levels of reduced GSH via nicotinamide adenosine dinucleotide phosphate (NADPH) (Frasier et al. 2013; Gul et al. 2000; Halliwell 2006). Additionally, cells also have various cytosolic proteins to protect it from oxidative damage. For example, Glyoxalase I (GLO-1) play a central role in detoxification of methylglyoxal (MG) (Distler and Palmer 2012). MG is thought to be involved in generation of advanced glycation end-products (AGEs). AGEs are group of compounds with high oxidative capacity implicated in variety of chronic ailments including cardiovascular disorders and inflammation. AGEs are primarily generated via a non-enzymatic reaction called Maillard or browning reaction. Typically, AGEs are

produced following a reaction between reducing sugars and free amine groups of cellular macromolecules such as lipid, protein and DNA. AGEs are generated during normal metabolism; however, when in excess they can be detrimental to the cell. Owing to its pro-oxidant nature, AGEs are known to induce oxidative stress and inflammation. Higher oxidative stress further damages the lipids and proteins and initiates glycoxidation. This results in the vicious cycle of generation of pro-oxidant AGEs and damaged cellular structures (Rabbani et al. 2016; Uribarri et al. 2010). Altogether, free radicals including ROS and RNS, and AGEs primarily target protein, lipid and nucleic acid within the cell and alter their structure or function leading to cell death.

### 2.7. Oxidative stress and the brain

Generation of free radical (ROS/RNS) is inevitable during oxidative phosphorylation in living cells. While low or moderate concentrations of free radicals are vital for physiological functions, higher levels of ROS/RNS under oxidative stress conditions could be detrimental. Oxidative stress is implicated in a variety of diseases including cardiovascular, inflammatory, cancer, diabetes, respiratory, neurodegenerative and neuropsychiatric (Favaro et al. 2010; Johnson et al. 2013; Kaneto et al. 2010; Montano et al. 2010; Narendra et al. 2010; Subramanian et al. 2008). Owing to its high oxygen consumption capacity, the central nervous system (CNS) is highly vulnerable to oxidative insult (Halliwell 2006). The brain is particularly vulnerable to oxidative damage for the following reasons: First, presence of higher content of unsaturated lipids and fatty

acids that can undergo peroxidation and oxidative modification. Second, the brain has poor antioxidant defense system compared to other tissues. Third, certain brain regions are rich in metal ions such as iron that can initiate generation of free radicals under higher oxidative stress conditions. Lastly, its high oxygen consumption and specific features of the neurons such as terminal-differentiation, makes the brain highly susceptible to such pernicious stress (Butterfield et al. 2002; Li et al. 2013b; Wang and Michaelis 2010). Therefore, it is not unusual that generation of free radicals and ROS under oxidative stress conditions is considered real culprits for neurodegenerative and neuropsychiatric conditions. Most common types of neurodegenerative disorders – late-onset diseases are Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's disease (HD). AD is a chronic neurodegenerative disorder characterized by brain atrophy, neurofirbrillary tangles and neuronal amyloid β plaques (Gandhi and Abramov 2012). In PD, degeneration of dopaminergic neurons of substantia nigra results in tremor, rigidity and progressive loss of balance and muscle coordination (Obeso et al. 2008). The pathological hallmark of HD is involuntary movements called chorea affecting muscle coordination (Kipps et al. 2005). All of these neurodegenerative disorders are associated with dementia or cognitive deficits and/or behavioral impairments leading to psychiatric problems. Neurodegenerative diseases such as AD, PD and HD share a common feature, aggregation of the disease-specific hallmark proteins in the CNS. For example, aggregation of misfolded Tau and amyloid β for AD,

alpha-synuclein (alphaSyn) for PD and mutant Huntington protein (mHtt) for HD are considered histopathological hallmark proteins. The causal relationship between the oxidative stress and formation of misfolded proteins is well established (Narendra et al. 2010; Shelat et al. 2008). ROS play a crucial role in regulating neurotoxicity via oxidative modification of the hallmark proteins (Figure 4).

Several studies have reported that accumulation of amyloid β leads to ROS production and specifically generates superoxide radical via activating pro-oxidant enzyme NADPH-dependent oxidase (Abramov and Duchen 2005; Hernandes and Britto 2012; Shelat et al. 2008). Amyloid β-mediated ROS production is known to induce lipid peroxidation resulting in impaired membrane permeability and increased calcium ($Ca^{2+}$) influx (Butterfield et al. 2013; Demuro et al. 2010; Fonseca et al. 2015; Gunn et al. 2016). Excessive intracellular $Ca^{2+}$ is reported to cause excitoxicity. Some reports have demonstrated the ROS as the regulator of amyloid β-induced impaired long-term potentiation (LTP) (Kamat et al. 2016; Ma et al. 2011; Ma and Klann 2012; Parajuli et al. 2013). Long-lasting increase in neuronal transmission following synchronous stimulation is referred to as LTP. LTP is widely accepted as a cellular mechanism underlying learning and memory (Dumont et al. 2009).

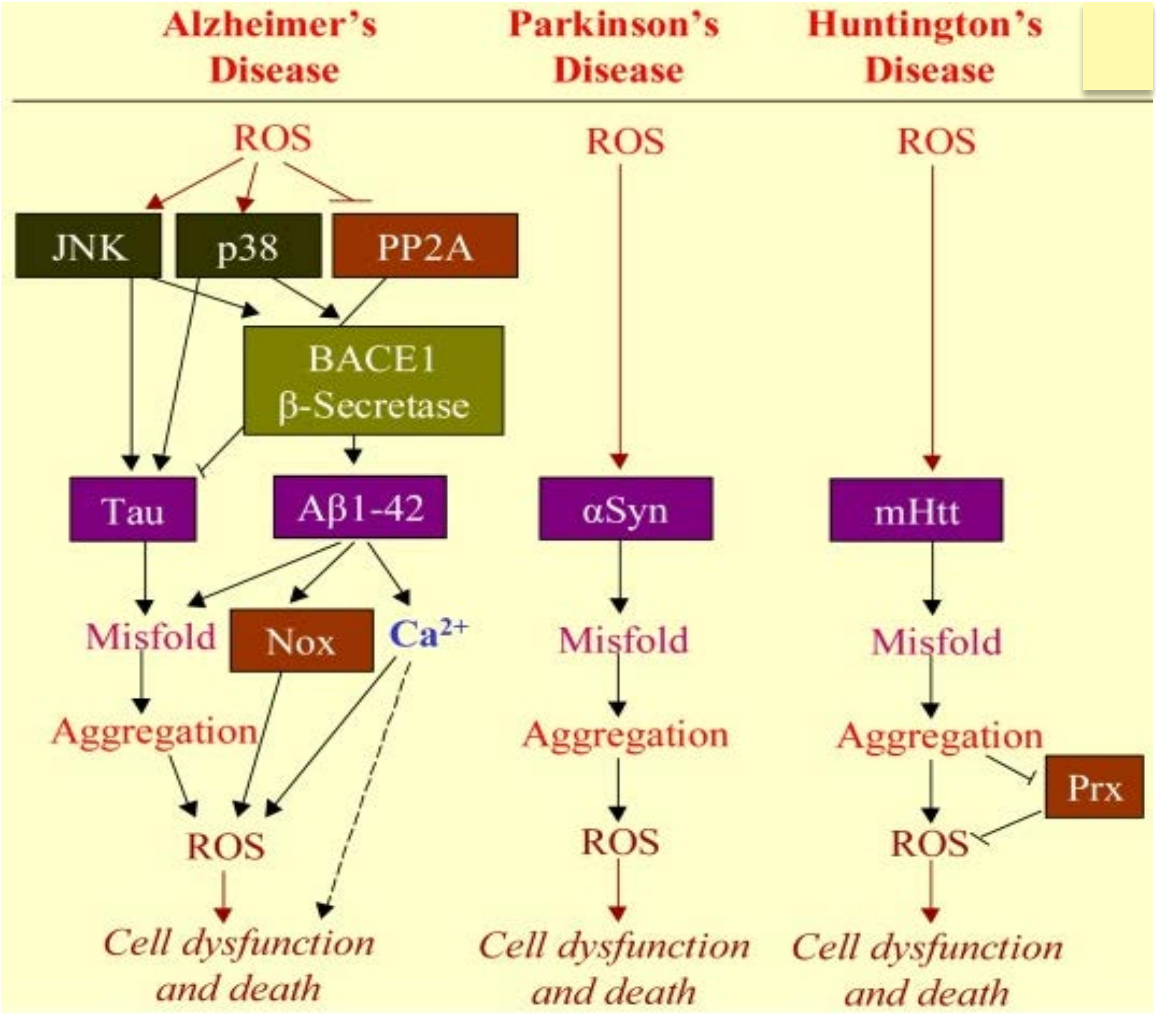



**Figure 4. The causal relationship between ROS and misfolded proteins leading to neurodegenerative disorders.** ROS-reactive oxygen species, JNK-c-jun *N*-terminal kinase, PP2A-protein phosphatase 2A, BACE1-Amyloid-β precursor protein cleaving enzyme1, Nox-NADPH oxidase, $Ca^{2+}$- calcium, α-Syn- alpha-synuclein, mHtt-mutant Huntingtin protein, Prx- peroxiredoxin, TDP-43- TAR DNA binding protein. Taken from (Li et al. 2013b).

In PD, oxidative stress has a causal role in generation of alpha-synuclein aggregates. Aggregated alpha-synuclein in dopaminergic neurons is neurotoxic and further triggers the generation of intracellular ROS, leading to cell death that

was protected by antioxidant treatment (Xiang et al. 2013). Furthermore, in HD, free radicals are implicated in misfolding and accumulation of mHtt-induced neurotoxicity in mHtt PC12 cells. While accumulation of mHtt led to decrease in antioxidant protein Prx1, the overexpressed wildtype Prx1 significantly reduced mHtt-induced toxicity (Pitts et al. 2012). Such great deal of scientific literature describes the implication of oxidative stress in neurodegenerative disorders such as AD, PD and HD.

### 2.8. Oxidative stress in synaptic plasticity and memory

Several lines of evidence suggest that reactive oxygen species (ROS) regulate mammalian learning and memory. Previously, oxidative stress-induced free radicals had been known for their deleterious effects on neuronal function and were implicated in aging-induced cognitive deficits (Brewer 2002; Hu et al. 2006) and neurodegenerative disorders (Abramov et al. 2004; Mattson 2002). However, recent work has suggested that ROS play a central role in normal cognitive function. Specifically, oxidative stress-induced free radicals are vital for long-term potentiation (LTP), a form of synaptic plasticity, learning and memory and for various biochemical signaling that are thought to underlie memory formation (Kishida and Klann 2007). Synaptic plasticity is thought be the cellular mechanism underlying learning and memory. Among all the types of synaptic plasticity, LTP is widely studied. Both LTP and learning and memory share various molecular processes (Lynch 2004). Thus, LTP is widely used as a tool to study molecular mechanisms underlying learning and memory behavior (Kishida

and Klann 2007). Several pharmacological approaches have been used to study the involvement of ROS in synaptic plasticity and memory formation. A study by Klann et al reported that high-frequency stimulation (HFS)-induced hippocampal LTP was blocked upon scavenging superoxide radicals, suggesting that superoxide is critical for HFS-induced LTP. In contrast, inhibition of LTP has been reported with overproduction of $H_2O_2$, a byproduct of superoxide dismutase (SOD) activity (Gahtan et al. 1998). Furthermore, Kamsler et al reported that $H_2O_2$, in a concentration-dependent manner, could either stimulate or inhibit HFS-induced LTP (Kamsler and Segal 2003). The use of transgenic animals has shed light on the involvement of ROS in learning and memory. Several studies have reported that SOD-1 overexpressing mice exhibited increased anxiety-like behavior in open field test and decreased escape latencies in hippocampal dependent-Morris water maze test suggesting scavenging of superoxide led to impaired learning and memory (Gahtan et al. 1998; Levin et al. 1998; Thiels et al. 2000). Altogether these studies suggest that free radicals play a central role in learning and memory.

While optimum levels of free radicals are beneficial for synaptic plasticity and learning and memory, the effect of higher concentrations of free radicals on learning-memory function are not. Neurons are particularly vulnerable to oxidative stress as axons, dendrites and synaptic terminals are rich in the apoptotic molecular machinery such as death receptors, calcium, cytochrome-c and caspase-3 (Mattson et al. 1998a; Mattson et al. 1998b). Pertinent to this,

several studies have reported that oxidative stress in synaptic terminals and dendrites are known to impair mitochondrial function and lead to activation of caspases causing apoptotic neuronal death (Mattson 1998a; 1998b). Furthermore, oxidative stress-induced depleted neurotrophic factors such as BDNF in the synaptic terminals may also trigger apoptotic events (Guo and Mattson 2000). Altogether, these events may alter regenerative/remodeling capacity and may cause degeneration of axons and dendrites leading to impaired synaptic plasticity. Furthermore, increase in oxidative stress is known to impair NMDA receptor function with concomitant increase in $Ca^{2+}$ influx and decrease in BDNF expression (Lu et al. 2001; Roceri et al. 2002). In addition, rise in oxidative stress is known to induce $Ca^{2+}$ influx from voltage-dependent calcium channel (VDCC) and internal stores such as endoplasmic reticulum (ER) (Kumar and Foster 2004). The increased intracellular $Ca^{2+}$ concentrations may directly promote the induction of long-term depression (LTD) or it may suppress the activation of NMDA receptors leading to longer inhibition of LTP. Both of these events are known to cause cognitive dysfunction (Foster 2007). Furthermore, Oxidative stress-induced elevated intracellular calcium leads to induction of various cellular pathways such as activation of phospholipases (calcineurin) and proteases such as calpains and caspases. The oxidative stress mediated overproduction of any of these molecules may alter the synaptic plasticity by altering the cytoskeletal components leading to dendritic spine loss (Arundine and Tymianski 2004). Altogether, physiological concentrations of ROS play a

central role in regulating synaptic plasticity and at higher concentrations they may affect cognitive function by altering synaptic plasticity.

## 2.9. Oxidative stress in neuropsychiatric conditions

Anxiety, depression and cognitive impairments are highly comorbid and the most prevalent neuropsychiatric conditions affecting patient's physiological, psychological and emotional health (Hovatta et al. 2010; Lereya et al. 2015). In humans, such co-occurrence of these conditions results following exposure to a stressful event. While low or moderate level of anxiety is considered protective or beneficial, higher level of anxiety is pathological leading to anxiety disorders (Wilson et al. 2007). As per *Diagnostic and Statistical Manual of Mental Disorders* (DSM) anxiety disorders are divided into four categories: generalized anxiety disorder, panic disorder, post-traumatic stress disorder and obsessive-compulsive disorder (APA 2000). There are several factors including genetic, environmental and developmental that may lead to anxiety disorders. Various studies suggest the association of depression and anxiety disorders with cognitive impairment (Burt et al. 1995; Karam and Itani 2014). As far as the molecular mechanism for the pathogenesis of anxiety and depression are concerned, involvement of GABA and serotonin is widely accepted and has been studied in great detail (Frodl et al. 2015; Petit et al. 2014; Varani and Balerio 2012; Varani et al. 2014). The current pharmacotherapy to treat anxiety and depression includes benzodiazepines (BZDs) and selective serotonin reuptake inhibitors (SSRIs). Though BZDs and SSRIs are the drug of choice to treat

anxiety and depression, they have modest efficacy and are associated with severe side effects such as tolerance, withdrawal effects and cognitive impairments that limit their use as prescribed regimen (Husain and Mehta 2011; Uzun et al. 2010). Pharmacotherapy for improving cognition is not adequate either. Several cognitive enhancers such as psychostimulants are associated with toxicity and physical or psychological dependence (Bisagno et al. 2016). Therefore, improvement in therapeutic interventions is needed. Alternative therapies with higher efficacy and lesser side effects must be examined.

Among various known mechanisms of anxiety, such as involvement of GABA and serotonin receptors, a non-traditional concept of implication of oxidative stress in anxiety disorders is gaining consensus (Masood et al. 2008; Oliveira-Dos-Santos et al. 2000; Salim et al. 2011a). Various studies including our own have suggested an association between oxidative stress and behavioral deficits in rodents (Bouayed et al. 2009; Hovatta et al. 2005; Masood et al. 2008; Oliveira-Dos-Santos et al. 2000; Salim et al. 2010a; Salim et al. 2010b; Souza et al. 2007). Few studies involving genetic manipulation and pharmacological intervention have reported oxidative stress as a potential regulator of anxiety-like behavior in rodents (de Oliveira et al. 2007; Hovatta et al. 2005; Masood et al. 2008; Salim et al. 2010a). Interestingly, genetic manipulation study by Hovatta et al. (Hovatta et al. 2005) reported increased and decreased anxiety-like behavior in mice following local overexpression and inhibition of the two antioxidant genes glyoxalase-I (GLO1) and glutathione reductase I, respectively. On the other

hand, using pharmacological induction of oxidative stress via pro-oxidant BSO, Masood et al. demonstrated increased anxiety-like behavior in mice that was reversed by phosphodieserase-2 (PDE) inhibitor suggesting it as a potentially novel therapeutic target for treating anxiety disorders (Masood et al. 2008).

Various studies from our lab using pharmacological and psychological induction of oxidative stress have also resulted in behavioral and cognitive deficits in rats (Allam et al. 2013; Patki et al. 2013a; Patki et al. 2013b; Salim et al. 2010b; Solanki et al. 2015; Vollert et al. 2011). These behavioral and cognitive impairments were protected using antioxidant intervention or moderate treadmill exercise indicating a causal role of oxidative stress. Earlier, we have reported increased anxiety-like behavior and decreased antioxidant enzymes in hippocampus and amygdala of rats, using two separate pharmacological inductions of oxidative stress via pro-oxidants BSO and xanthine plus xanthine oxidase (X + XO) (Salim et al. 2010a; Salim et al. 2010b). Interestingly, antioxidant tempol treatment mitigated oxidative stress and improved anxiety-like behavior of rats. In separate studies, our laboratory reported the protective effect of antioxidant treatment and moderate treadmill exercise against the pro-oxidant-induced oxidative stress-mediated anxiety-like behavior in rats (Allam et al. 2013; Salim et al. 2010b). These studies have demonstrated the causality of pharmacologically-induced oxidative stress in anxiety-like behavior in rats. On the other hand, when rats were subjected to non-pharmacological induction of oxidative stress such as sleep-deprivation also resulted in elevated oxidative

stress and behavioral deficits in rats (Vollert et al. 2011). Furthermore, a recent study from our lab using a rat model of social defeat we have shown that increase in oxidative stress was associated with behavioral and cognitive impairments in rats (Patki et al. 2013b). Interestingly, in these studies we reported significant decrease in the expression of antioxidant enzymes such as glyoxalase-1 (GLO-1) and glutathione reductase-1 (GSR-1) in the hippocampus, amygdala and the pre-frontal cortex. These studies in rodents suggest oxidative stress as a potential regulator of anxiety-like behavior. In another study using an acute model of PTSD, we reported similar behavioral and cognitive deficits associated with increased oxidative stress that was prevented by antioxidant treatment (Solanki et al. 2015). Altogether these studies suggest that exposure to psychological stress results in malfunctioning of antioxidant defense system leading to elevated oxidative stress.

### 2.10. Antioxidants

Oxidative stress has been implicated in many mental disorders. It is an area of active research, and perhaps an avenue for drug intervention. Several studies have reported implication of oxidative stress in neuroinflammation, mitochondrial dysfunction, impaired synaptic plasticity and poor cognition suggesting an interrelationship between oxidative stress and neuropsychiatric conditions including, anxiety and depression. The prevalence of neuropsychiatric disorders has increased in recent years (Rivera et al. 2015). Along with it the

need for safe and effective treatment options has been noted. Various life style related factors such as diet and exercise influence physiological and mental health. Emerging research has reported that healthy diet and moderate exercise help maintain neuronal integrity and promote brain health (Conlon and Bird 2015; Laitman and John 2015). This association between brain health and life style factors has led the scientists to study the benefits of nutrition as potential therapeutic alternatives to improve neuronal function and cognition. Nutrients, in general, are known to be neuroprotective (de Wilde et al. 2011; Keunen et al. 2015). Owing to their broad-spectrum physiological, behavioral, molecular as well as cellular activity against neurological disorders, plant derived polyphenols have been studied in great detail. Plants produce natural organic compounds–polyphenols to protect against pathogens and ultraviolet radiation. More than 8000 plant-derived polyphenols have been identified and studied for their various activities, including brain-protecting activity (Hadi et al. 2000; Mandel et al. 2007; Proestos et al. 2005). Plant-derived polyphenols have garnered the attention of many pharmaceuticals and nutraceuticals as they offer relatively safe and side effect free intervention that is inexpensive and non-invasive.

Chemically, polyphenols consist of various phenolic groups. Depending on the number of phenolic rings they possess, polyphenols are divided into various groups such as flavonoids, flavanols, flavanones, flavones, anthocyanins and isoflavanoids. Among these groups, flavonoids are most abundantly found polyphenols with a specific 2-phenyl-1, 4-benzopyrone structure (Graf et al.

2005). In nature, flavonoids are present in glycosylated forms resulting in poor absorption in the body. The flavonoids must undergo conversion to aglycones, in order to pass through the gut wall effectively. After the intake of dietary flavonoids, few components are absorbed through small intestine. Usually, flavonoids undergo hydrolysis in small intestine to release aglycone. Before entering the circulatory system, aglycones are subjected to extensive metabolism resulting in sulfate, methylated and glucuronide metabolites (Crozier 2009). In the liver, these metabolites undergo phase II metabolism leading to further conversions. The unabsorbed flavonoids and their metabolites can be absorbed in the large intestine following the conversion of aglycones to phenolic acids in the colonic microflora (Georgiev et al. 2014). Depending on the different susceptibilities to gut enzymes, the bioavailability of polyphenols (Wollen 2010).

Antioxidants are known to reduce or slow down the oxidation of other molecules. In other words, they are able to combat oxidative stress. There are various ways by which antioxidants may overcome oxidative stress: (1) by inhibition of free radical generation, (2) by interrupting autoxidation chain reactons, (3) by up-regulating and protecting cellular antioxidant defense mechanism, (4) by neutralizing the action of metal pro-oxidant ions, (5) by inhibiting the action of pro-oxidant enzymes, and (6) by increasing the activity of other antioxidants (Georgiev et al. 2014). Flavonoids, in general, are low molecular weight compounds with potent antioxidant properties. Different in vitro studies have reported different mechanism of action of flavonoids. In one study,

flavonoids decreased oxidative stress as preventive and chain breaking antioxidant (Pietta 2000). In another in vitro study, they were found to act as metal chelator and enzyme inhibitors to combat oxidative stress (Ferlazzo et al. 2016). In contrast, the in vivo studies reported flavonoids as indirect anti-oxidants as they were found to up-regulate anti-oxidant defense system (Carocho and Ferreira 2013; Khlebnikov et al. 2007; Pietta 2000).

Polyphenols are known to act as antioxidants through their ability to convert their hydroxyl radical to phenoxyl radical via donating a hydrogen atom. The phenoxyl radical is unstable and can generate a stable compound either by losing hydrogen or initiating a reaction with other radical (Rossi et al. 2008). In plants, polyphenols are known to confer color and can stabilize the free radicals by scavenging unpaired electrons (Ng et al. 2006; Quideau et al. 2011). Though they possess antioxidant properties, polyphenols also work as neuro-protective agents via modulating molecular crosstalk associated with cognitive function such as synaptic plasticity. Polyphenols are known to induce cAMP-response element-binding protein (CREB), a transcription factor and activate brain-derived neurotrophic factor (BDNF) thereby modulating signaling pathways associated with neuroprotection and learning and memory function (Williams et al. 2008). Furthermore, polyphenols are known to regulate nuclear factor-kappa B (NF-$\kappa$B) signaling pathway. Redox-sensitive transcription factor such as NF-$\kappa$B is a known regulator of various cellular and physiological functions such as expression of cytokines, cyclo-oxygenase 2 (COX-2) and growth factors. The

impairment in the NF-$\kappa$B signaling results in inflammatory and neurodegenerative diseases, autoimmune disorder and cancer. Several natural polyphenols such as curcumin, resveratrol and ellagic acid are potent inhibitors of NF-B (Karunaweera et al. 2015; Ren et al. 2013; Yang et al. 2016). The activator protein-1 (AP-1), a transcription factor highly sensitive to oxidative imbalance, is critical for stress response and normal growth. AP-1 activation is associated with growth regulation, apoptosis and inflammation. Phenolic compounds such as quercetin, resveratrol and curcumin are known to inhibit the AP-1 activation (Kubota et al. 2010; Mishra et al. 2015; Yan et al. 2013). Furthermore, anthocyanins are known to induce phase-II antioxidant enzymes and reported to possess tumor suppressor activity. The binding between erythroid 2p45 (NF-E2)-related factor 2 (Nrf2) transcription factor and the antioxidant response element (ARE) in the promoter region of several antioxidant genes serves as the prerequisite for the induction of phase-II antioxidant enzymes (Talalay 2005; Yang and Xiao 2013). Curcumin and resveratrol are reported to have chemoprotective activity via induction of antioxidant enzymes through Nrf2 signaling (Jaramillo and Zhang 2013; Whitlock and Baek 2012). Moreover, polyphenols have significant effect on signaling proteins such as mitogen-activated protein kinase (MAPK). Such MAPK proteins are classified in to three major groups: (1) the extracellular signal-regulated protein kinase (Erk), (2) c-Jun N-terminal kinase (JNK) and (3) $p38^{MAPK}$ (El-Mowafy and White 1999; Vanamala et al. 2011). Typically, environmental stressors and pro-inflammatory

cytokines promote inflammation, pain and apoptosis through activating JNK and $p38^{MAPK}$. MAPKs are also involved in activation of phase II antioxidant enzymes. Catechin and quercetin are reported to have cardioprotective activity through stimulation of Erk, JNK and p38 signaling pathways (Arumugam et al. 2012; Chen et al. 2013; Dreger et al. 2008). Additionally, polyphenols exert their pharmacological effects through acting on various cellular and molecular targets including DNA, enzymes and hormone transporters. Polyphenols are known to catalyze electron transport reaction through its effect on metal ions such as $Fe^{2+}$, $Cu^{2+}$ and $Zn^{2+}$ (Martinez-Florez et al. 2002).

Diet including plants, herbs, vegetables, fruits and beverages are the major source of natural polyphenols. Several observational studies have reported lower incidence of manifesting degenerative diseases, such as cancer and cardiovascular diseases, with increased consumption of fruit and vegetables (Ness and Powles 1997; Riboli and Norat 2003). While vegetables are the key source of polyphenols, several fruits including grape, olive, blueberry and citrus fruits are known to have high content of polyphenols (Deng et al. 2012; Fu et al. 2011; Fu et al. 2010; Li et al. 2013a). Historically, grapes, among all the other fruits, have garnered special attention due to its medicinal benefits (Vislocky and Fernandez 2010). Several clinical studies have reported the protective or preventive effects of grapes and grape products in certain diseases (Castilla et al. 2008; Lekakis et al. 2005; Mursu et al. 2008; Vigna et al. 2003).

Grapes, a member of berry family, are the most commonly consumed and widely used in the beverage industry (Yadav et al. 2009). Hundreds of varieties of grapes are available, each differ in the chemical content as per the geographic origin (Monagas et al. 2003). Several grape products such as grape juice, grape extract, wine and grape pomace are widely used as dietary supplements. As per American Dietetic Association and American Diabetes Association, a serving of fresh grapes provides approximately 45 calories, 12g of carbohydrates and negligible amount of fiber, protein and fats. Glucose and fructose are the sugars predominantly found in the grapes (USDA-ARS 2009; Wheeler and Pi-Sunyer 2008).

Grape polyphenols are reported to exert antioxidant effects via neutralizing or inhibiting the generation of free radicals (Vislocky and Fernandez 2010). Flavonoids, a subclass of polyphenols, are highly abundant in grape seeds, skin and pulp (Georgiev et al. 2014). Of all the phenolic content of grapes, anthocyanins, proanthocyanidins, resveratrol, quercetin, kaempferol and phenolic acids are the most common (Leifert and Abeywardena 2008a; 2008b). The content of these polyphenols varies based on the geographic origin and grapes species. Several lines of evidence suggest the antioxidant activity of grape flavonoids could be the central player in mediating beneficial effects (Cui et al. 2002; Graf et al. 2005; Hooper et al. 2008; Mursu et al. 2008). Furthermore, other bioactivities of grape polyphenols have been reported, including antiproliferative,

antimicrobial, anti-aging, anti-inflammatory, cardio and neuroprotective (Georgiev et al. 2014).

ROS serves as a “double-edged sword” in cellular and physiological functions. In other words, the optimum concentration is beneficial whereas the higher concentrations are known to disturb the homeostasis leading to induction of oxidative stress. Oxidative stress is found to be the major culprit for several diseases such as cancer, diabetes, cardiovascular, autoimmune diseases and atherosclerosis. Higher oxidative stress in the brain is known to alter neuronal activity and transmission. Furthermore, oxidative stress-induced impaired membrane integrity results in neuronal dysfunction (Bouayed et al. 2009; Halliwell 2006; Valko et al. 2007). As a result, oxidative stress may serve as a critical factor in etiology of variety of neuropsychiatric disorders including anxiety, depression and cognitive impairments (Bouayed et al. 2009; Halliwell 2006; Ng et al. 2008; Valko et al. 2007). Though several studies including research from our laboratory have reported an association between increased oxidative stress and behavioral and cognitive deficits in rodents, the causal role was unclear until recently (Hovatta et al. 2005; Masood et al. 2008; Salim et al. 2010a; Salim et al. 2011a; Vollert et al. 2011). Previous work from our laboratory has shown that pharmacological induction of oxidative stress via BSO resulted in increased anxiety-like behavior in rats that was attenuated by antioxidant tempol treatment. In the same study decreased oxidative stress in the brain regions was implicated in regulating anxiety (amygdala, hippocampus and locus coeruleus) following

tempol treatment indicating the causality of oxidative stress in behavioral deficits in rats (Salim et al. 2010a). On the other hand, in another study from our laboratory, sleep deprived rats exhibited behavioral and cognitive impairments and increased oxidative stress (Vollert et al. 2011). Though results with tempol are quite interesting, synthetic antioxidants such as tempol are associated with unknown side effects thereby limiting its clinical use. Therefore, we focused our attention to natural compound with antioxidant properties such as grape powder. The use of the grape powder is important as it offers safe and side effect free intervention. In the past, using two different rat models we have reported the beneficial effects of grape powder. In one study, pro-oxidant BSO-induced oxidative stress resulted in behavioral and cognitive impairments as well as increased oxidative stress in rats that was attenuated by 3 weeks of grape powder (15 g/L in tap water) supplement (Allam et al. 2013). In another study using overiectomized rats – a rodent model of estrogen depletion, behavioral and cognitive deficits in the female rats were observed. And, overiectomy-induced behavioral and cognitive deficits were prevented by grape powder (15 g/L in tap water for 3 weeks) treatment (Patki et al. 2013a). Overall, these studies suggest the beneficial effects of grape powder in direct and indirect induction of oxidative-stress. Furthermore, using an acute model of PTSD, single-prolonged stress (SPS), we have reported the preventive effects of grape powder in PTSD-induced behavioral and cognitive deficits (Solanki et al. 2015). Furthermore, grape powder normalized SPS-induced increased plasma corticosterone and 8-

isoprostane levels suggesting its beneficial effects on HPA axis dysfunction and antioxidant activity, respectively. In the same study, we reported that grape powder protected SPS-induced decreased BDNF protein expression in the amygdala of the SPS rats. Furthermore, grape powder treatment increased hippocampal and amygdalar expression of acetylated histone 3 as well as histone deacetylase 5 (HDAC5) in the SPS rats (Solanki et al. 2015). Overall, this study suggested that grape powder though epigenetic regulation of BDNF mediates beneficial effects in SPS-induced behavioral and cognitive deficits. While, the beneficial effects of the grape powder in pro-oxidant or acute PTSD model are now known, no reports are available about its effects on psychological stress (social defeat)-induced behavioral and cognitive deficits.

The grape powder, used in these studies, was provided by California Table Grape Commission (CTGC) and is a mixture of freeze-dried and grounded black, red and green grapes. Similar to fresh grapes, freeze-dried grape powder (FDGP) contains various phenolics such as resveratrol, quercetin, kaempferol, catechin and anthocyanins. The complete list of known FDGP phytoconstituents reported by CTGC is listed below in table 2.

Though grapes are rich in hundreds of polyphenolic compounds, three phenolic compounds, namely resveratrol, quercetin and kaempferol have received considerable attention as potential therapeutic intervention for a large number of diseases (Care et al. 2016; Das and Maulik 2006; Das et al. 2016; Gong et al. 2014; Malavolta et al. 2016; Park et al. 2015; Yang et al. 2015).

These compounds have been studied in great detail and reported to have antioxidant, estrogenic, antiproliferative and anti-inflammatory properties (Bhat et al. 2001; P. Iacopinia 2008; Schneider et al. 2000). While the exact molecular mechanism of action of these compounds is not known, resveratrol, quercetin and kaempferol are known for their potent free radical scavenging and anti-peroxidative properties (Khanduja and Bhardwaj 2003). In an in-vitro study, quercetin was found to possess the strongest anti-radical activity followed by resveratrol and kaempferol (Quercetin > Resveratrol > Kaempferol). In the same study, resveratrol was reported to possess stronger anti-peroxidative activity than kaempferol (Khanduja and Bhardwaj 2003). Furthermore, these three compounds are reported to serve as hydroxyl and superoxide radical scavenger (Rossi et al. 2013). It is likely that grape polyphenols specifically resveratrol, quercetin and kaempferol possess wide spectrum activities in preventing or protecting against oxidative insult.

**Phytochemicals Analyzed in Freeze-Dried Preparation**

*Note: The following analysis does not represent the complete phytochemical profile of grapes*

| Compounds | Total | Individual |
|---|---|---|
| | | |
| **Catechins** | 57.2 mg/kg | |
| Catechin | | 36.4 mg/kg |
| Epicatechin | | 20.8 mg/kg |
| **Anthocyanins** | 566 mg /kg | |
| Peonidin | | 38.3 mg/kg |
| Cyanidin | | 266 mg/kg |
| Malvidin | | 261.7 mg/kg |
| **Flavonols** | | |
| Quercetin | | 16 mg/kg |
| Kaempferol | | 3.4 mg/kg |
| Isorhamnetin | | 3.5 mg/kg |
| **Stilbenes** | | |
| Resveratrol | | 1.8 mg/kg |
| **Total Polyphenols** | | 448 mg/100g |

**Table 3. Phyto-constituents reported to be present in freeze-dried grape powder.** Taken from California Table Grape Commission report.

Several lines of evidence from in-vitro studies suggest that resveratrol exert neuroprotective effect mainly through its antioxidant activity (Fukui et al. 2010; Han et al. 2004; Kim et al. 2007; Singh et al. 2013; Zhang et al. 2014). In one study using primary neuronal cells - hippocampal cells from rat, oxidative stress was induced by sodium nitroprusside, nitric oxide free radial donor, leading to increased oxidative stress and intracellular ROS accumulation. This was significantly attenuated by resveratrol and other polyphenols resulting in cell protection (Bastianetto et al. 2000). In other hippocampal-derived neuronal cells, HT22 cells, resveratrol protected from glutamate-induced cytoxicity via increased heme oxygenase-1 (HO-1) expression (Kim et al. 2012). Similarly, HT22 cells were protected from oxidative stress-induced cell death by resveratrol through

significant upregulation of mitochondrial SOD (Fukui et al. 2010). Several in vivo studies have reported neuroprotective effects of resveratrol could be potentially due to its ability to cross blood-brain barrier (BBB) (Mokni et al. 2007; Wang et al. 2002). In one study, a positive relationship between resveratrol intake and improved cognitive deficits was observed in streptozotocin-induced damage in rats. This was associated with increased brain glutathione levels (Sharma and Gupta 2002). Similar results were obtained with another neurotoxic agent, colchicine-induced cognitive impairments and increased oxidative stress (Kumar et al. 2007). Furthermore, resveratrol-induced improved cognition was observed in Tg2576 mice, transgenic mouse model of AD (Wang et al. 2008). Overall, data from animal and cell culture studies suggests that resveratrol may serve as an important therapeutic intervention owing to it neuroprotective and antioxidant properties.

Neuroprotective effects of quercetin and kaempferol have also been investigated using both *in-vivo* and *in-vitro* approach. In separate studies, kaempferol and quercetin were found to be protective against glutamate-induced increased oxidative stress mediated HT22 neuronal cell death (Yang et al. 2014; Yang et al. 2013). Furthermore, anti-inflammatory properties of quercetin and kaempferol have also been reported (Bureau et al. 2008). On the other hand, studies have also reported anxiolytic effects of kaempferol in mice (Grundmann et al. 2009). Interestingly, quercetin was found to ameliorate cognitive and emotion function in a transgenic mouse model of Alzheimer's disease (Sabogal-

Guaqueta et al. 2015). Overall, these studies suggest that grapes and grape polyphenols possess potent antioxidant and neuroprotective activity making them potential candidates for therapeutic intervention in several neurodegenerative and neuropsychiatric conditions.

# 3.0 METHODS AND MATERIALS

## 3.1. Freeze-dried grape powder

Freeze-dried grape powder used in this study was prepared and supplied in small, sealed sachets, by California Table Grape Commission. The GP is a composite of fresh seeded and seedless red, green and black grapes that were freeze-dried, grounded and processed to retain the integrity of the bioactive compounds. The GP contains resveratrol, quercetin, catechins, and various simple phenolics as depicted in Table 2. The GP was stored at -80$^{o}$C following its receipt. For the experimental purpose, the grape powder, at a concentration of 15 g/L, was dissolved in tap water. The GP solution was prepared fresh daily and fed orally *ad libitum* to the rats during the course of the treatment. This dose was attentively chosen based on the pilot dose-response studies. In our recent studies, the chosen dose has been reported to produce most pronounced effects on rat behavior (Allam et al. 2013; Patki et al. 2013a; Solanki et al. 2015). Additional information about rat's average daily consumption of grape powder and/or its constituents is summarized in table 5 (see appendix).

## 3.2. Animals and housing conditions

Adult male Sprague-Dawley rats and male Long-Evans (LE) retired breeders rats were bought from Charles River Laboratories, Wilmington, MA. Sprague-Dawley rats weighing 275-300 g served as control or intruders and retired breeders LE rats weighing 400-500 g were used as resident for social defeat model. Rats were singly housed in a climate-controlled room on a

12hr/12hr light/dark cycle and provided regular chow diet and water/grape powder/placebo *ad libitum*. All animal experiments followed NIH guidelines and approved protocols from University of Houston Animal Care Committee.

**3.3. Social defeat (SD):**

Miczek originally developed the resident-intruder paradigm of the social defeat model in 1979 (Miczek 1979). In this study, we used the modified version of the resident-intruder paradigm. Basically, intruder Sprague-Dawley rats were subjected to 7 social defeat encounters over 7 consecutive days with a pre-screened aggressor LE rat. To prevent the habituation between the resident and the intruder, each intruder was subjected to defeat exposure with six different aggressors (Bhatnagar et al. 2006; Golden et al. 2011; Patki et al. 2013b). An ideal social defeat was marked when the intruder attains submissive supine posture for approximately 3 seconds. At the end of the physical encounter session, a transparent plexiglass partition with holes was placed in the resident cage for 30 minutes to stop the fight. While avoiding the direct contact between the resident and the intruder, this partition facilitated the intense auditory, olfactory and visual interactions. If social defeat is not observed within 10 minutes after placing the intruder in the resident's cage, rats were separated with plexiglass partition for the remainder of the 30-min session. Naïve control rats were placed behind the plexiglass barrier in a fresh cage for 30 min daily. At the end of each defeat or control exposure, all Sprague-Dawley rats were returned to their home cage. All Sprague-Dawley rats were used for behavioral tests and

sacrificed thereafter for collection of brains. Experimental scheme is depicted in the Figure 5.

### 3.3.1. Screening of aggressor Long-Evans (LE) rats

The 3-day screening process for selection of appropriate LE rats was dependent on the LE rats that exhibited consistent levels of aggression. Such steady dominant behaviors of LE rats are vital to successfully model chronic social defeat stress in rats. The aggressive behavior among all male retired breeder LE rats differed in the intensity, extent and quality. Only those aggressors that met the following criteria were included in the social defeat paradigm: aggressive resident performing a defeat as demonstrated by the intruder attaining supine position for approximately 3 seconds. If the residents were to be used for multiple defeats, all aggressors were subjected to single screening session before the initiation of consecutive social defeat exposures (Golden et al. 2011).

### 3.3.2. Experimental design

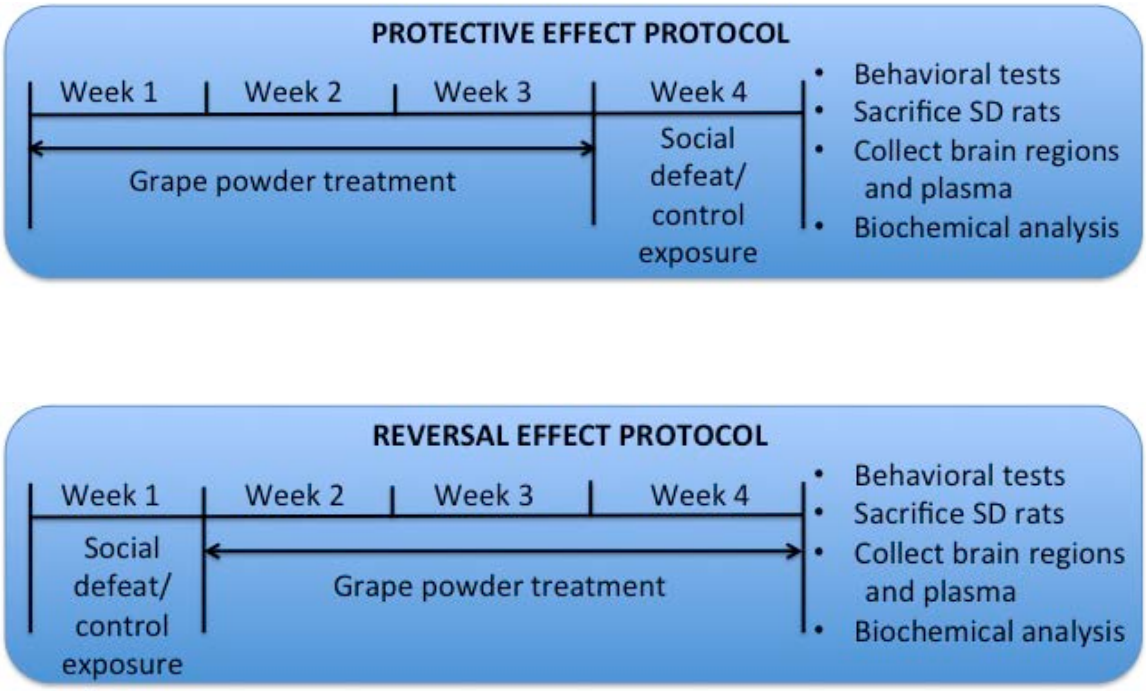



**Figure 5. Schematic representation of the experimental regimen.**

Two experimental designs were followed. In one, reversal effect of GP was examined and in another protective effect of GP was investigated. Rats were randomly assigned to the following groups in both experimental designs:

| **Protective Effect** |
| --- |
| **NC** – Naïve control rats<br>**GPNC** – Naïve control rats pretreated with grape powder for 3 weeks<br>**CE** – Control exposure where intruders subjected to the residents cage when the residents were not present<br>**SD** – Socially defeated rats<br>**GPSD** – Socially defeated rats pretreated with grape powder for 3 weeks |
| **Reversal Effect** |
| **NC** – Naïve control rats<br>**NCGP** – Grape powder treated naïve control rats (3 weeks of GP treatment)<br>**CE** – Control exposure where intruders subjected to the residents cage when the residents were not present.<br>**SD** – Socially defeated rats<br>**SDGP** – Socially defeated rats treated with grape powder for 3 weeks<br>**SDPL** – Socially defeated rats treated with placebo for 3 weeks |

### 3.4. Behavioral assessments

#### 3.4.1. Measurement of Anxiety-like behavior

A battery of non-cognitive behavior tests was performed to study the reversal and protective effects of grape powder in social-defeat induced behavioral deficits. These tests were done in the following order:

3.4.1.1 Elevated plus maze (EPM) test

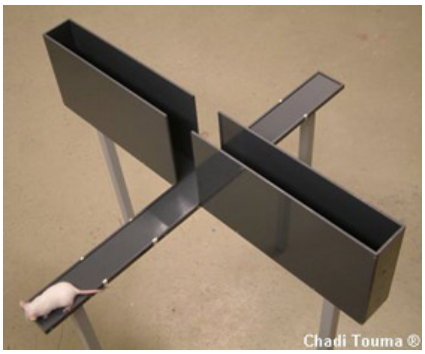



**Figure 6. The elevated plus maze apparatus**

The standard rat EPM apparatus consists of four arms (10 cm x 50 cm length) (two open and two closed) intersecting in a way that create a plus shape (Figure 6) was obtained from Med Associates Inc., (St. Albans, VT). The arms of the EPM were elevated about 60 cm off the ground. The EPM procedure was performed as described (Salim et al. 2010b; Vollert et al. 2011). Briefly, the rat was placed in the intersection area facing the open arms of the maze and allowed to explore the maze for 5 minutes. The movement of the rat was tracked visually to measure the amount of time the rat spent in the open arm. Reduced time spent by a rat in the open arm is an indication of anxiety-like behavior.

3.4.1.2. Light-dark (LD) test

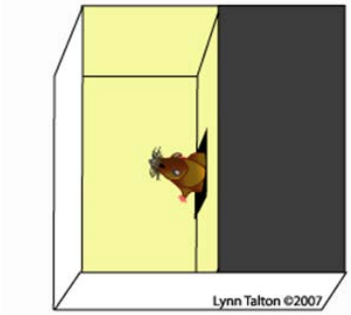



**Figure 7. The light-dark box**

The light-dark test is marked as a valid and sensitive test to measure anxiety-like behavior in rodents (Arrant et al. 2013). The LD box consisted of two compartments: a lit compartment (27 x 27 x 27 cm) and a dark compartment made up of blackened wall and floor (27 x 18 x 27 cm) (Figure 7). These two compartments were separated by a single partition with an opening (7 x 7 cm) to facilitate the movement of rat between the two compartments (Salim et al. 2010b). Each rat was given 5 minutes to explore both compartments and the total time spent in the lit area was recorded manually as previously published (Salim et al. 2010b; Vollert et al. 2011).

3.4.1.3 Open field (OF) test

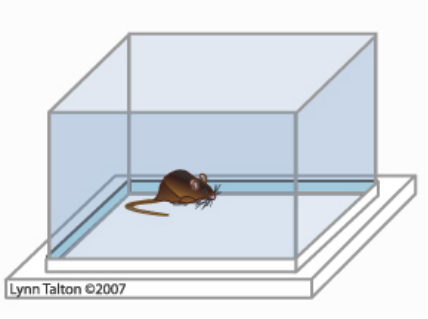



**Figure 8. The open field arena**

The OF test is widely used to study anxiety level, exploratory as well as locomotor activity in rodents (Gould 2009). As depicted in Figure 8, the OF apparatus consisted of open arena (60 x 40 cm) surrounded by transparent plexiglass walls. Rats were placed in the center of the arena and allowed to move freely in the arena for 15 minutes. The infrared light sensors detected the movement, which was quantified using Opto-Varimex Micro Activity Meter v2.00 software (Optomax, Columbus Instruments; OH). The light intensity was kept at 300 lux. The time spent in the center of the arena was analyzed and reported as percentage. The less time a rat spent in the center of the arena versus periphery of the arena is an indicator of anxiety-like behavior. The apparatus was wiped down with alcohol in between each test animal.

3.4.1.4 Marble burying test

**Figure 9. The marble burying test**

Marble burying test is also marked as a test to measure anxiety-like behavior in rodents. In this test, a standard cage with bedding was used. The opaque glass marbles were evenly spaced on the bedding (Figure 9). Subsequently, each rat was placed in a cage with marbles on the bedding for 30 min. The rats with higher anxiety levels have tendency to engage in a digging behavior resulting in covering a greater percentage of marbles. Thus, the greater the number of marbles buried, the higher the anxiety-like behavior (Njung'e and Handley 1991).

### 3.4.2 Measurement of Cognitive function

3.4.2.1 Radial Arm Water Maze test (RAWM)

The RAWM test was developed as a hybrid test between Morris water maze and Radial arm maze. The RAWM is widely used to study cognitive function in rodents such as learning and memory. In this test, a circular black water pool with six swimming arms and an open central area (Figure 10A) was used. The test was performed in a dimly lit room with visual cues on the surrounding walls. Each rat was randomly assigned a goal arm with a hidden platform submerged 1 cm under water at the end of the arm. Each rat was released from one of the arms different from the goal arm and allowed to swim to find the hidden platform. In each learning trial and memory test, the rats were allowed a maximum time of 1 minute. Quantification of the cognitive function was done in terms of the number of errors rats made in finding the hidden platform. An error was marked when the rat entered the halfway in to any arm other than the goal arm or in the goal arm but failed to reach the platform. The number of errors ranges from 1 to 7, as the rat can only swim into 7 arms within 1 min. When the rat failed to find the platform in 1 minute, the rat was given a score of 7 errors and manually guided to the platform. Once on the platform, the rat was given 15 seconds rest prior to initiating the next learning trial. The timeline for all learning trials, short-term memory (STM) and long-term memory (LTM) tests is depicted in the schematic representation in Figure 10B.

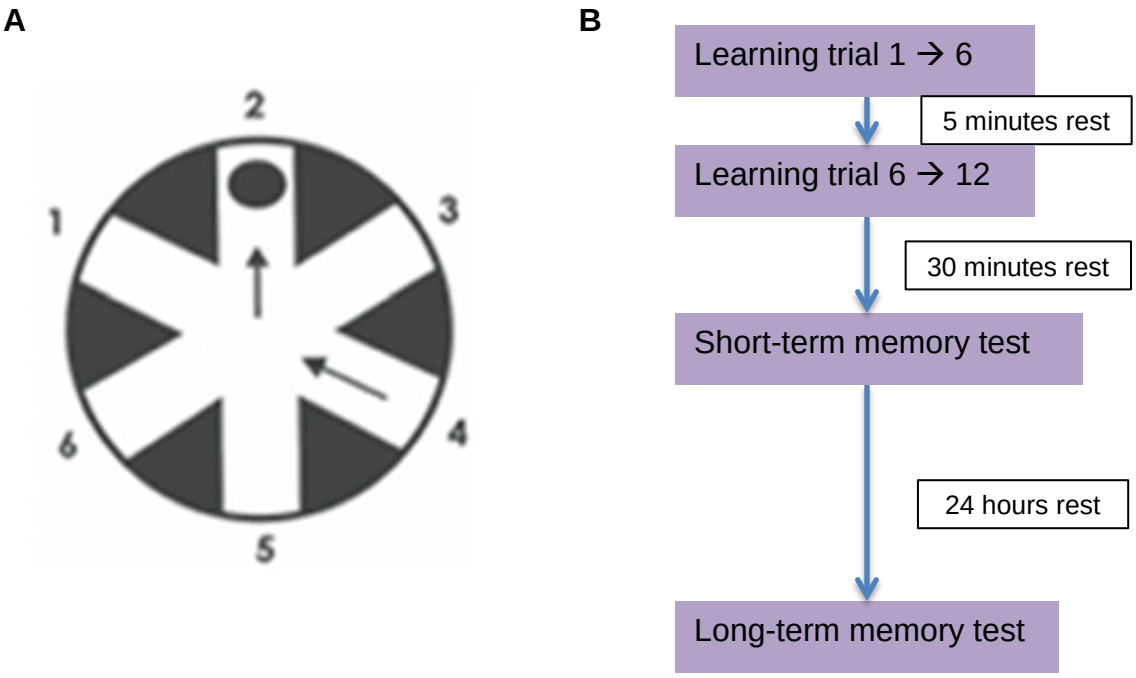



**Figure 10. A) The radial arm water maze pool. B) Schematic of learning trials and short-term and long-term memory test in the RAWM.** The animals received 5 minutes as rest period between two sets of learning trials. 30 minutes and 24 hours after the 12$^{th}$ learning trial, the short-term and long-term memory was tested, respectively. The same test animals were used for all the learning trials, STM and LTM tests.

### 3.4.3 Measurement of depression-like behavior

3.4.3.1 Forced Swim Test (FST)

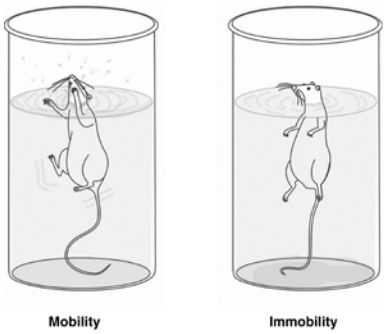



**Figure 11. The forced swim test apparatus**

Depression-like behavior in rat was assessed using FST. In this test, each rat was put in a water (25$^{o}$C) filled tank (24 cm in diameter and 30 cm high) for 5 minutes (Figure 11). Soon after being placed in the water tank, rats exhibit immobile posture and assume motionless floating (Slattery and Cryan 2012). The total time spent being immobile was recorded as previously published by us (Solanki et al. 2015). The more time a rat spent being immobile in the FST, higher the depression-like behavior.

### 3.5. Brain dissections and collection of plasma

Rats were anesthetized (Isoflurane, #57319-479-06, Phoenix Pharmaceuticals) 24 hours after the conclusion of all the behavioral tests. The brains were quickly removed and rapidly frozen and stored at -80$^{o}$C until analysis. Blood collection and plasma separation was performed immediately and stored at -80$^{o}$C. Three brain regions namely; hippocampus, amygdala and

pre-frontal cortex (PFC) were identified according to Paxinos and Watson (Paxinos, 1986) and grossly dissected out.

### 3.6. Corticosterone levels and indices of oxidative stress

Stress-induced release of corticosterone levels was measured in plasma using an EIA based kit (cat # 500655, Cayman Chemical, MI) per manufacturer's instructions. Plasma corticosterone levels are considered as systemic marker of stress. The plasma level of 8-isoprostane was also measured using EIA kit (cat # 516351, Cayman Chemical, MI). Oxidative stress-induced random oxidation of tissue phospholipids generates isoprostanes. 8-isoprostane is a known marker of oxidative stress.

### 3.7. Western blotting

3.7.1. Tissue homogenization and protein estimation

The brain tissue homogenates were prepared in the lysis buffer containing 20mM Tris-HCL, 4mM EDTA, protease inhibitors, 100ug/ml PMSF, 1 ug/ml leupeptin, 1ug/ml aprotinin and 1ug/ml pepstatin (Salim and Dessauer 2004). The estimation of protein concentration of the lysates was performed using microBCA assay kit from Pierce (Pierce, Rockford, IL).

3.7.2. Immunoblotting and detection

The homogenates were diluted in 2x Laemmli buffer (50 mM Tris-HCL, pH 6.8, 10% glycerol, 2% SDS and 0.1 mg/ml bromophenol blue). The samples (approximately 30 μg of total protein per sample) were resolved on standard 10-well 8-16% SDS-PAGE gels. The proteins were transferred to PVDF membrane

(Amersham Pharmacia Biotech, Buckinghamshire, England). The detection of the proteins as immunoreactive bands was performed using specific primary antibodies and horseradish peroxidase-conjugated secondary antibody. Beta-actin was used as a loading control. Antibodies dilutions were as detailed in table 4. The protein blots were developed using commercial chemiluminescence reagents (Bio-rad). Alpha Innotech imaging system (Alpha Innotech, San Leandro, CA, USA) was used to detect the chemiluminescence and densitometric analysis was performed using Fluorochem FC8800 software.

| Antibody | Primary antibody | Secondary antibody |
|---|---|---|
| GLO-1 | Ab81461<br>Abcam<br>1:1000 | Cell signaling<br>1:2000<br>Goat Anti-Rat |
| GSR-1 | 1:200<br>Obtained from Dr. Iris Hovatta (Finland)<br>(Hovatta et al. 2005) | Cell signaling<br>1:2000<br>Goat Anti-Rabbit |
| Mn SOD | 06-984<br>Millipore<br>1:1000 | Cell signaling<br>1:2000<br>Goat Anti-Rabbit |
| Cu/Zn-SOD | 07-403<br>Millipore<br>1:1000 | Cell signaling<br>1:2000<br>Goat Anti-Rabbit |
| β-actin | sc-47778<br>Santa Cruz Biotech<br>1:1000 | Cell signaling<br>1:2000<br>Goat Anti-Mouse |

**Table 4: Antibodies, dilutions, and sources. Details of primary and secondary antibodies used in detecting the levels of specific proteins/molecules.**

### 3.8. Enzyme Activity assay

GLO-1 activity (Cat# MAK114, Sigma-aldrich, MO), total GSH and SOD activity (Cat# 703002, 706002 Cayman, MI) in plasma was measured using EIA kit per manufacturer's instructions.

- The glyoxalase (GLO-1) assay uses the glyoxalase enzyme capacity to induce production of S-lactoylglutathione which is measured at 240 nm.
- The total glutathione (GSH) assay uses glutathione reductase enzyme for the quantification of GSH. Up on interaction with the DNTB (5,5'-dithio-bis-2-(nitrobenzoic acid), Ellman's reagent), the sulfhydryl group of GSH produces 5-thio-2-nitrobenzoic acid (TNB). The spectrophotometric absorbance of TNB at 405 nm provides an estimation of total GSH in the sample.
- The superoxide dismutase (SOD) assay uses a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine.

### 3.9. Total antioxidant capacity measurement

Total antioxidant capacity in plasma and in the hippocampus tissue was measured using EIA kit (Cat# 709001, Cayman, MI) per manufacturer's instructions. This kit estimates the capacity of the total antioxidants in the sample to prevent ABTS (2,2'-Azino-di-[3-ethylbenzthiazoline sulphonate]) oxidation that is compared with that of Trolox, a tocopherol analogue, and is quantified as millimolar Trolox equivalents.

### 3.10. Total phenolic content measurement

In order to estimate the presence of grape polyphenols in the hippocampus the total phenolic content was measured spectrophotometrically using Folin Ciocalteu's (FC) method (Iva Juranović Cindrić et al. 2011; Jaromír Lachman 2010). Briefly, tissue homogenate was pipetted into a volumetric flask containing 0.5mL of Folin Ciocalteu's reagent (Sigma-aldrich, Cat # F9252), 5mL of distilled water and 1.5mL of 20% $Na_2CO_3$ solution. During the oxidation of phenolic compounds, the F-C reagent undergoes reduction and produce blue-colored oxides. Similarly, the standard gallic acid solutions were prepared as (50, 100, 150, 250, 500 mg/L). After 2 hours, the spectrophotometric measurement at 765 nm wavelength was performed. The measurements were compared to a standard curve obtained from gallic acid solutions and expressed as mg/L of gallic acid equivalents.

### 3.11. Cell culture

The immortalized mouse hippocampal cell line (HT22) was received as a generous gift from Dr. Dave Schubert from the Salk Institute, La Jolla. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) containing 4.5 g of glucose/liter. In addition, penicillin/streptomycin (50 units/mL), glutamate (2 mM) and 10% fetal bovine serum (Atlanta Biologicals, GA) were used as DMEM supplements. Cells were grown under humidified atmosphere at $37^oC$ with 10% $CO_2$.

### 3.12. Experimental Scheme

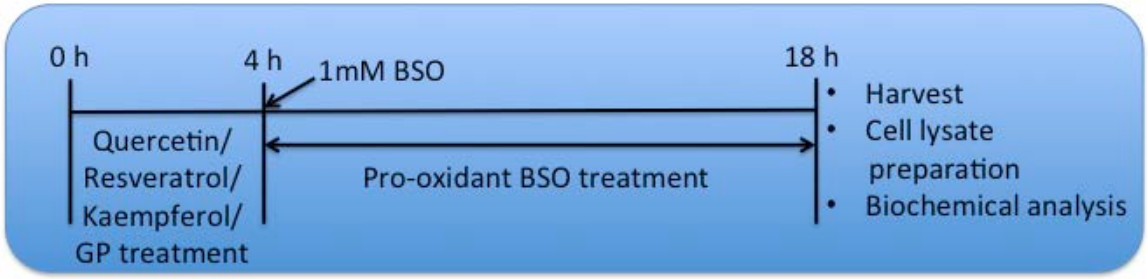



**Figure 12. A schematic representation of the experimental design**

HT22 cells were seeded into six well cell culture plates and divided into five groups: control (PBS), BSO alone (1mM in PBS), Ethanol alone, Resveratrol (R)/Quercetin (Q)/Kaempferol (K)(1-20μM) with BSO and grape powder (GP)(2400-10000 μg/mL) with BSO. BSO was purchased from Sigma-Aldrich (St. Louis, MO). 1mM dose of BSO was chosen based on previous studies from our group (Salim et al. 2011b; Salvi et al. 2016). The treatment of BSO or vehicle (DMEM media) was initiated when cells were 60%-70% confluent. Cells were pretreated with either R/Q/K or GP for 4 hours followed by BSO treatment that lasted 14 hours (Figure 12). For mechanistic studies, cells were seeded into six well cell culture plates and divided into four groups: control (PBS), BSO alone, placebo pretreatment for 4 hours followed by 14 hours of BSO treatment (PL + BSO) and 14 hours of BSO treatment following 4 hours of GP treatment (GP + BSO). All experiments were conducted at least 3-4 times.

### 3.13. HT22 cell lysate

Harvesting of HT22 cells were done by trypsinization and cell pellet (approximately 7.5 x $10^5$ cells) was obtained by centrifugation. The cell pellet was homogenized either using PBS or specific assay buffer. The homogenate was subjected to centrifugation at 10,000xg for 15 minutes at 4$^o$C and supernatant was collected. This supernatant was used for determining total antioxidant capacity.

### 3.14. Total antioxidant capacity measurement

Measurement of total antioxidant capacity in HT22 cell lysates was performed using EIA based kit following manufacturer's protocol (Cayman, MI, Cat # 709001).

### 3.15. Calcium assay

Calcium assay (Abcam, Cat # ab102505) was performed using HT22 cell lysates following manufacturer's protocol. Both procedures used colorimetric spectrophotometry at λ 450 nm and λ 575 nm respectively.

### 3.16. Mitochondrial membrane potential measurement by JC-1

HT22 cells were cultured at 1.5 x $10^4$ in a 96 well plate in 200 μL of culture medium. Following the overnight incubation of cells in a humidified environment at 37$^o$C with 10% $CO_2$, mitochondrial membrane potential was analyzed using a kit based assay per the manufacturing protocol (Cayman, MI, cat # 600880). Upon entering the mitochondria, the JC-1 dye forms either J-aggregates or monomers. While J-aggregates were measured with excitation and emission at

560 and 590 nm, respectively, monomers were analyzed with excitation and emission at 485 nm and 535 nm, respectively. The ratio of J-aggregate (595 nm) to monomer (535 nm) intensity indicates the mitochondrial health and depolarization of mitochondrial membrane potential (Chowdhury et al. 2013).

### 3.17. Caspase-3 assay

Caspase-3 assay (Abcam, Cat # ab39401) was performed using HT22 cell lysates per following manufacturer's protocol. This procedure used colorimetric spectrophotometry at λ 400nm or λ 405nm.

### 3.18. Western blotting

HT22 cell homogenates were prepared in RIPA buffer (Sigma-Aldrich, MO). The estimation of protein concentration of the cell lysates was performed using microBCA assay kit from Pierce (Pierce, Rockford, IL). The homogenates were then diluted with 2x Laemelli buffer and then subjected to SDS-PAGE and western blotting as described above in 3.7.2. The primary and secondary antibodies used in western blotting are listed in Table 4.

### 3.19. Statistical analysis

All values are reported as mean $\pm$ SEM. Comparison between groups were made either by student's t-test or one-way ANOVA with subsequent Tukey's post-hoc test where appropriate (GraphPad Software, Inc., San Diego, CA). $p < 0.05$ was used to denote statistically significant groups.

# 4. RESULTS

## 4.1 Behavioral assessments

Psychological stress, in humans, is known to contribute to comorbid psychopathologies including anxiety, depression and cognitive impairment. It is well recognized that the hippocampus, amygdala and pre-frontal cortex are the primary brain regions implicated in psychological stress, which are considered as key areas responsible for causing behavioral and cognitive changes. These phenotypes of psychological stress were successfully reproduced in several rodent models; one such model is the rat model of social defeat. In the past, we have reported that rats subjected to social defeat for one week exhibited anxiety- and depression-like behavior and cognitive impairments. Interestingly, behavioral and cognitive deficits were accompanied with an increase in oxidative stress in these animals (Patki et al. 2013b). In agreement with our previously reported work, in this study, we observed that one-week of social defeat in rats led to behavioral and cognitive deficits. Importantly, three weeks of grape powder treatment prevented and reversed social defeat-induced cognitive and behavioral impairments while decreasing oxidative stress. Further, using a simulated in-vitro model of oxidative stress, we investigated which antioxidant constituents of grape powder impart protective activity to grape powder. Quercetin, resveratrol and kaempferol are three major antioxidants present in grape powder. Our data suggested that grape powder exerted its protective effects by engaging quercetin and resveratrol but not kaempferol. It seems that quercetin and resveratrol

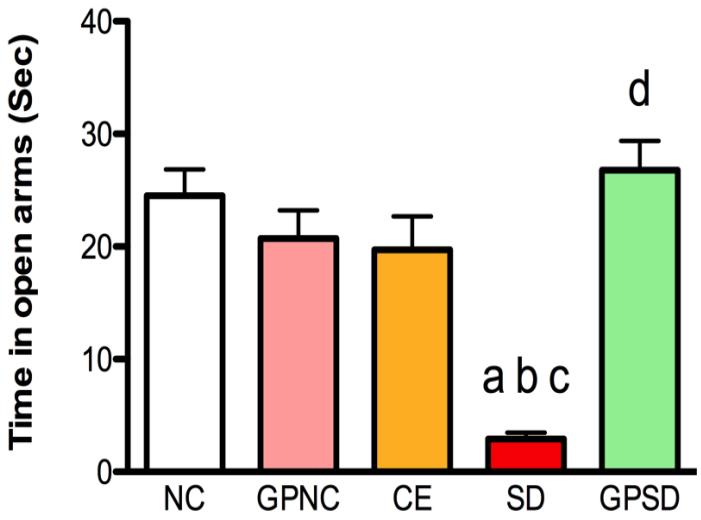
modulate calcium levels, limit mitochondrial dysfunction and cause deactivation of caspases, collectively controlling stress-induced oxidative stress and exerting an overall protective effect.

### *4.1.1 Social defeat-induced anxiety-like behavior is reversed and protected by grape powder treatment*

#### 4.1.1.1 Examination of anxiety-like behavior in the elevated plus maze (EPM)

Rodents, in general, have aversion for elevated and open areas. Using the advantage of this aversion the EPM assesses the anxiety-like behavior in rodents (Xiang et al. 2011). Duration of time spent in the open arm is the measure of anxiety. Anxious rats spend less time in the open arm. In this study, socially defeated rats experienced increased anxiety as compared to control groups as these rats spent significantly more time in the closed arm (NC: 24.50 $\pm$ 2.36 seconds, GPNC: 20.70 $\pm$ 2.52 seconds, CE: 19.70 $\pm$ 2.99 seconds, NCGP: 23.5 $\pm$ 4.11, SD: 2.9 $\pm$ 0.58 seconds). Grape powder treatment protected (Figure 13A) (GPSD: 26.80 $\pm$ 2.58 seconds) and reversed (SDGP: 24.0 $\pm$ 3.18 seconds) (Figure 13B) social defeat-induced anxiety-like behavior suggesting its anxiolytic effect.

A


Time in open arms (Sec)
40
30
20
10
0
a b c
d
NC
GPNC
CE
SD
GPSD

B

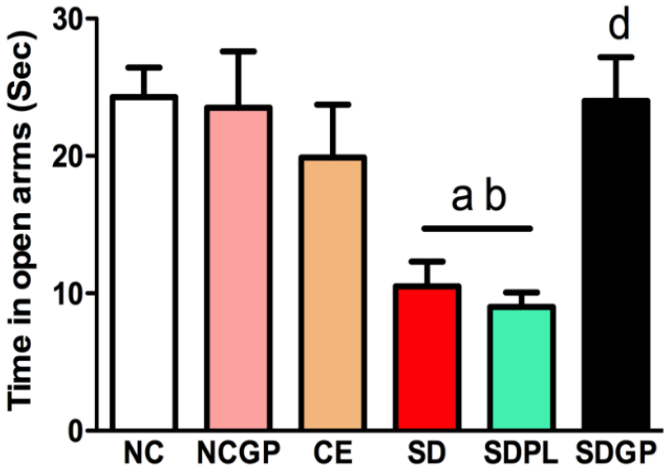
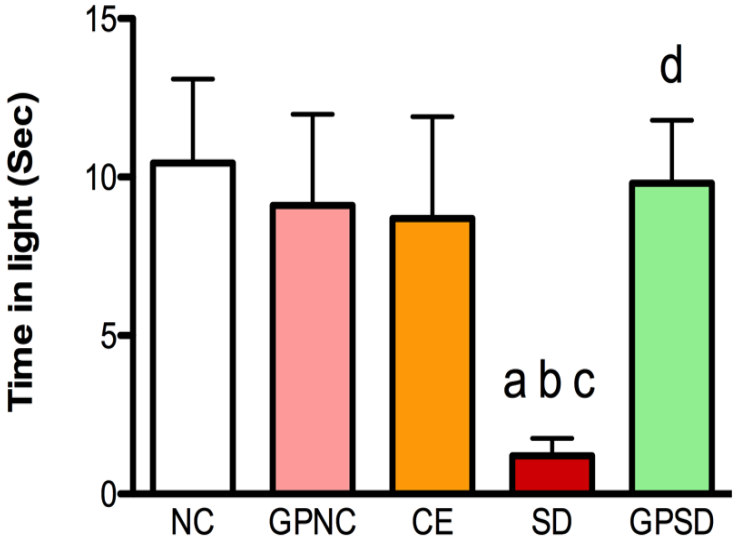

Time in open arms (Sec)
30
20
10
0
a b
d
NC
NCGP
CE
SD
SDPL
SDGP

**Figure 13. Examination of anxiety-like behavior in the elevated plus maze (EPM) test in the control or socially defeated rats with/without grape powder treatment.** Rats subjected to social defeat spent significantly less time in the open arm of the EPM that was protected (A) and reversed (B) with grape powder treatment. NC-naïve control, GPNC-naïve control rats pre-treated with grape powder, CE- control exposure, SD-social defeat, GPSD-socially defeated rats pre-treated with grape powder, NCGP-grape powder treated naïve control rats, SDPL-socially defeated rats treated with placebo, SDGP-socially defeated rats treated with grape powder. a=significantly different from NC, b=significantly different from GPNC/NCGP, c=significantly different from CE, d=significantly different from SD/SDPL. $p < 0.05$. Values are mean $\pm$ SEM., n = 10-12 rats/group.

#### 4.1.1.2 Examination of anxiety-like behavior in the light-dark (LD) test

In the LD test, the more amount of time rats spend in the dark area is an indication of anxiety-like behavior (Salim et al. 2010b; Vollert et al. 2011). We observed that NC, GPNC, CE and NCGP groups spent a similar length of time in the lit compartment while SD rats spent significantly less time in the lit area (NC: 10.44 $\pm$ 2.65 seconds, GPNC: 9.10 $\pm$ 2.88 seconds, CE: 8.7 $\pm$ 3.2 seconds, SD: 1.20 $\pm$ 0.55 seconds). Interestingly, control exposure rats exhibited anxiety-like behavior as indicated by decreased amount of time spent in the lit area as compared to naïve control group (CE: 14.23 $\pm$ 1.02 seconds, NC: 19.01 $\pm$ 1.92 seconds). Similar to EPM test, in the LD test the grape powder treatment before (Figure 14A) (GPSD: 9.8 $\pm$ 1.99 seconds) and after (Figure 14B) (SDGP: 23.25 $\pm$ 2.0 seconds) social defeat significantly increased the time spent in the lit area suggest its protective and reversal effects in mitigating social defeat-induced anxiety-like behavior.

A


Time in light (Sec)
15
10
5
0
a b c
d
NC
GPNC
CE
SD
GPSD

B

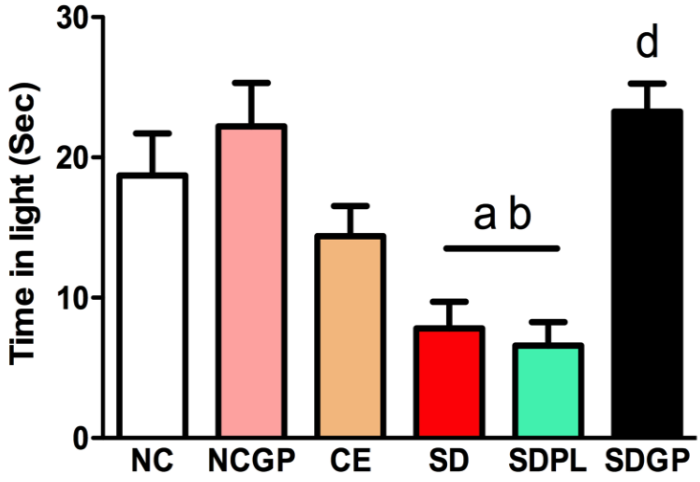
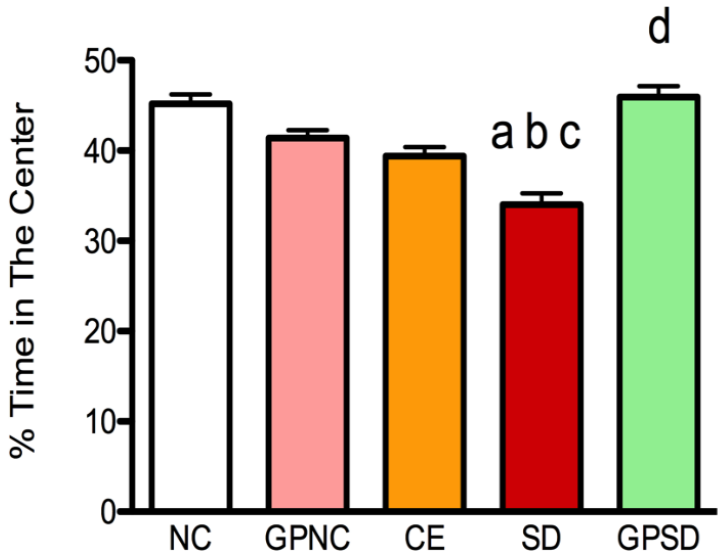

Time in light (Sec)
30
20
10
0
a b
d
NC
NCGP
CE
SD
SDPL
SDGP

**Figure 14. Examination of anxiety-like behavior in the light dark test (LD) test in the control or socially defeated rats with/without grape powder treatment.** Rats subjected to social defeat spent significantly less time in the lit area of the LD box that was protected (A) and reversed (B) with grape powder treatment. NC-naïve control, GPNC-naïve control rats pre-treated with grape powder, CE- control exposure, SD-social defeat, GPSD-socially defeated rats pre-treated with grape powder, NCGP-grape powder treated naïve control rats, SDPL-socially defeated rats treated with placebo, SDGP-socially defeated rats treated with grape powder. a=significantly different from NC, b=significantly different from GPNC/NCGP, c=significantly different from CE, d=significantly different from SD/SDPL. $p < 0.05$. Values are mean $\pm$ SEM., n = 10-12 rats/group.

#### 4.1.1.3 Examination of anxiety-like behavior in the open field (OF) test

The OF test is widely employed to study anxiety levels in rodents. Reduced time spent in the center of the arena is correlated with high anxiety levels (Gould 2009; Salim et al. 2010b). In this study SD rats spent an average of 34.04 $\pm$ 1.21 percent time in the center of the arena, which is significantly shorter than the control groups (NC: 45.17 $\pm$ 1.05 %, GPNC: 41.38 $\pm$ 0.88 %, CE: 39.42 $\pm$ 0.99 %, NCGP: 46 $\pm$ 2.85 %). Socially defeated rats treated with grape powder spent significantly greater percentage of time in the center of the arena suggesting its anxiolytic effects (Figure 15A, GPSD: 45.94 $\pm$ 1.21 %, Figure 15B, SDGP: 49.3 $\pm$ 1.9 %).

A


% Time in The Center
50
40
30
20
10
0
a b c
d
NC
GPNC
CE
SD
GPSD

B

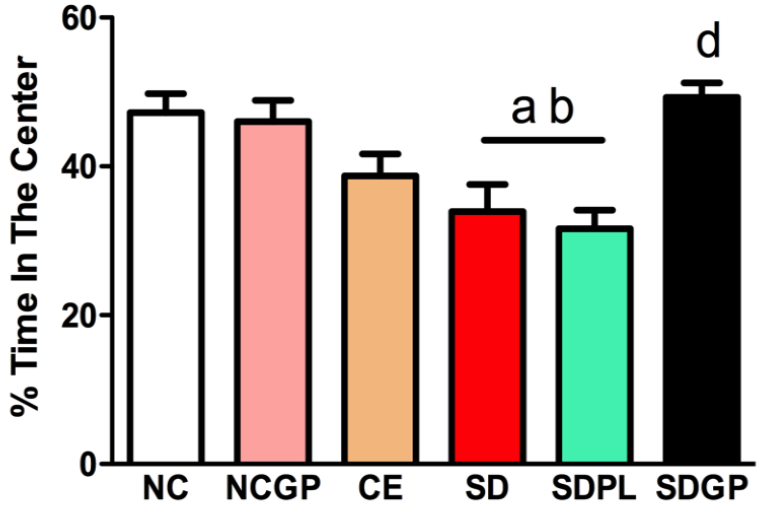
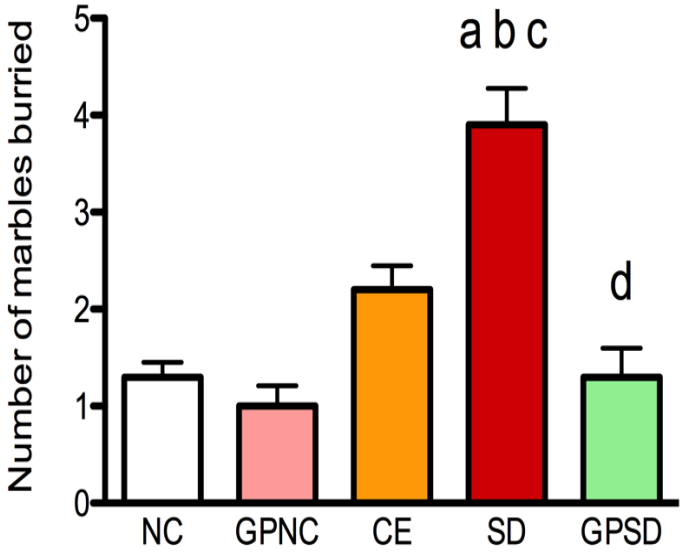

% Time In The Center
60
40
20
0
a b
d
NC
NCGP
CE
SD
SDPL
SDGP

**Figure 15. Examination of anxiety-like behavior in the open field (OF) test in the control or socially defeated rats with/without grape powder treatment.** Rats subjected to social defeat spent significantly less time in the center area of the open arena that was protected (A) and reversed (B) with grape powder treatment. NC-naïve control, GPNC-naïve control rats pre-treated with grape powder, CE- control exposure, SD- social defeat, GPSD-socially defeated rats pre-treated with grape powder, NCGP-grape powder treated naïve control rats, SDPL-socially defeated rats treated with placebo, SDGP-socially defeated rats treated with grape powder. a=significantly different from NC, b=significantly different from GPNC/NCGP, c=significantly different from CE, d=significantly different from SD/SDPL. $p < 0.05$. Values are mean $\pm$ SEM., n = 10-12 rats/group.

#### 4.1.1.4 Examination of anxiety-like behavior in the Marble Burying (MB) test

Rats with higher levels of anxiety demonstrate digging behavior thus end up covering the greater number of marbles under the bedding. Therefore, a higher number of buried marbles is considered indicative of anxiety-like behavior (Njung'e and Handley 1991). In our study, SD rats buried more marbles as compared to the control groups (NC: 1.3 $\pm$ 0.15, GPNC: 1.0 $\pm$ 0.21, CE: 2.2 $\pm$ 0.24, NCGP: 1.4 $\pm$ 0.3, SD: 3.9 $\pm$ 0.37). Interestingly, control exposure rats exhibited anxiety-like behavior as indicated by increased number of marbles buried as compared to naïve control group (CE: 2.2 $\pm$ 0.24, NC: 1.3 $\pm$ 0.15). Additionally, similar to what was observed in the EPM, LD and OF, the MB data showed that grape powder treatment in socially defeated rats protected (Figure 16A, GPSD: 1.3 $\pm$ 0.3) and reversed (Figure 16B, SDGP: 1.22 $\pm$ 0.27) anxiety-like behavior as demonstrated by marked decrease in number of marbles buried. Overall, these data suggest that social defeat-induced anxiety-like behavior was protected and reversed by grape powder treatment.

A




B

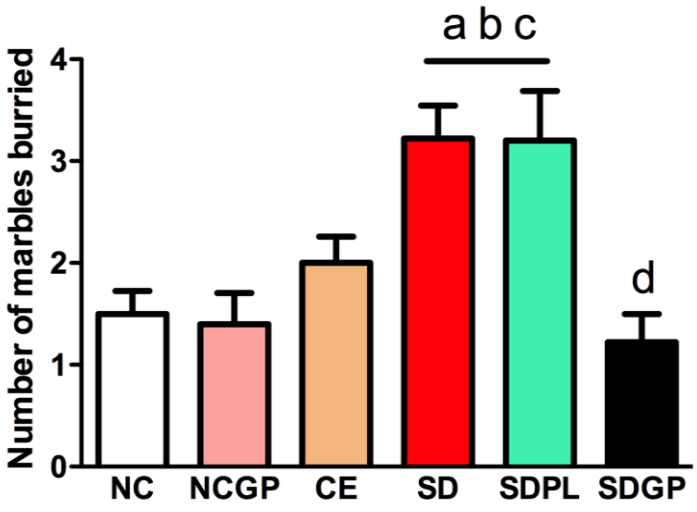



**Figure 16. Examination of anxiety-like behavior in the marble burying (MB) test in the control or socially defeated rats with/without grape powder treatment.** Rats subjected to social defeat buried more number of marbles as compared to control groups that was protected (A) and reversed (B) with grape powder treatment. NC-naïve control, GPNC-naïve control rats pre-treated with grape powder, CE- control exposure,

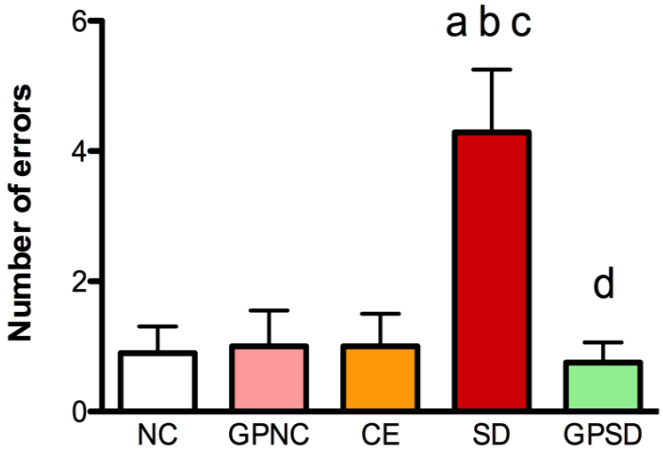
SD- social defeat, GPSD-socially defeated rats pre-treated with grape powder, NCGP-grape powder treated naïve control rats, SDPL-socially defeated rats treated with placebo, SDGP-socially defeated rats treated with grape powder. a=significantly different from NC, b=significantly different from GPNC/NCGP, c=significantly different from CE, d=significantly different from SD/SDPL. $p < 0.05$. Values are mean $\pm$ SEM., n = 10-12 rats/group.

### 4.1.2 Examination of short- and long-term memory function in the Radial Arm Water Maze (RAWM) test

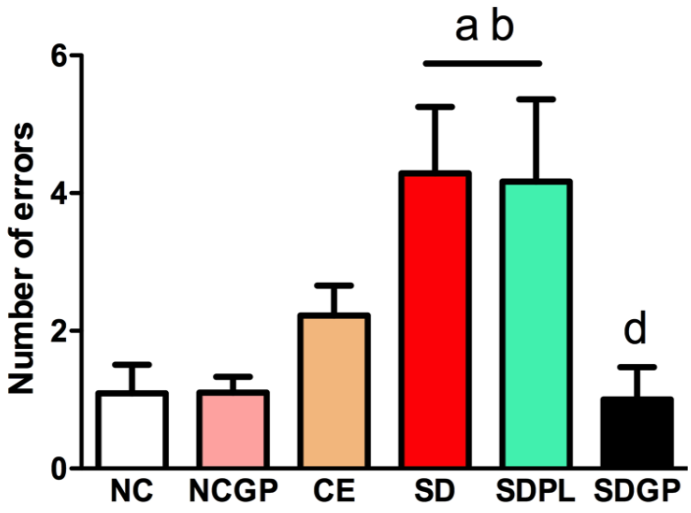
In the STM test, performed 30 minutes after the end of the 12$^{th}$ learning trial, the SD rats made significantly higher number of errors than control groups (NC: 0.9 $\pm$ 0.4, GPNC: 1.0 $\pm$ 0.55, CE: 1.0 $\pm$ 0.50, NCGP: 1.10 $\pm$ 0.23, SD: 4.28 $\pm$ 0.96). Three weeks of grape powder treatment significantly reduced the number of errors in both protection (Figure 17A, GPSD: 0.75 $\pm$ 0.31) and reversal (Figure 17B, SDGP: 1.0 $\pm$ 0.47) protocol. Similar results were obtained in the LTM test, which was performed 24 hours after the 12$^{th}$ learning trial. Socially defeated rats made an average of 5.37 $\pm$ 0.77 errors in the LTM task that was significantly higher compared to all other control groups. The grape powder treatment significantly improved their long-term memory as indicated by decreased number of errors in the LTM task (Figure 17C, GPSD: 1.5 $\pm$ 0.59; Figure 17D, SDGP: 1.80 $\pm$ 0.38). It is noteworthy that grape powder treatment failed to improve performance in control rats as both the control groups with and without grape powder treatment performed similarly on STM and LTM task. However, grape powder treatment significantly enhanced short-term and long-term memory of socially defeated rats.

Short-term memory test

A

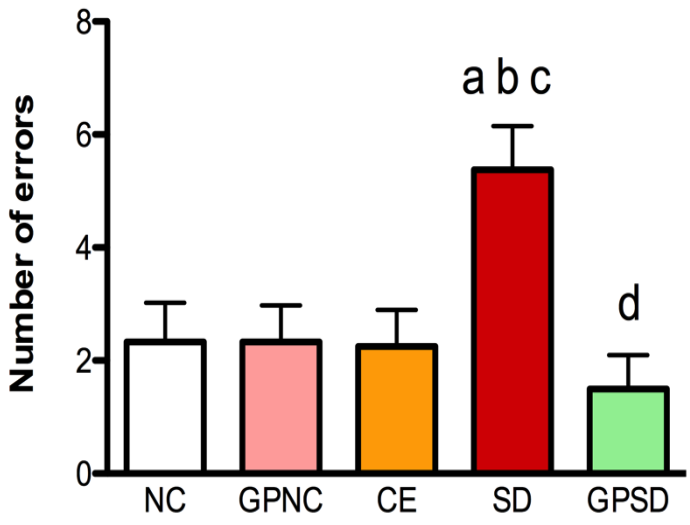



B



Long-term memory test

C




D

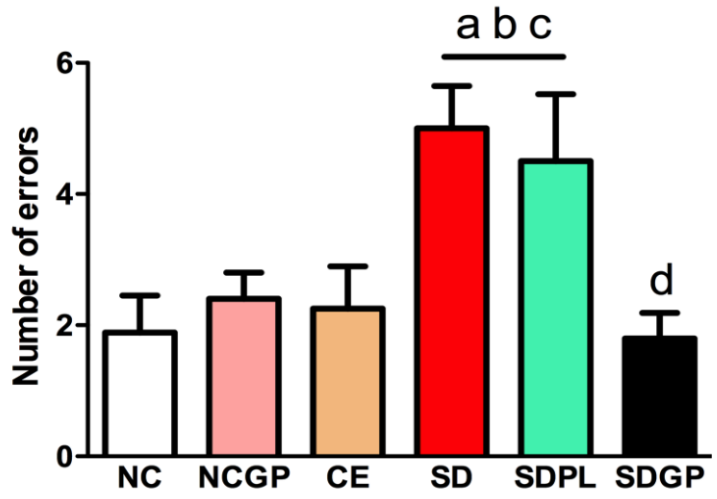


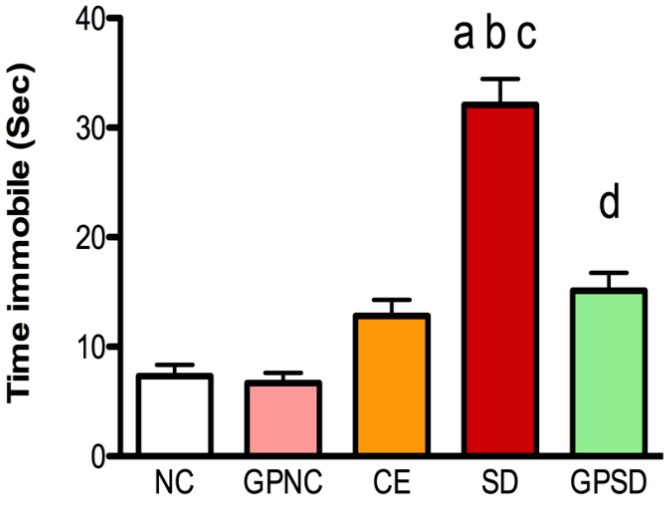
**Figure 17. Radial arm water maze (RAWM) performance in control or socially defeated rats with/without grape powder treatment.** Rats subjected to social defeat buried revealed impaired short and long-term memory as compared to control groups that was protected (A) and reversed (B) with grape powder treatment. NC-naïve control, GPNC-naïve control rats pre-treated with grape powder, CE- control exposure, SD-social defeat, GPSD-socially defeated rats pre-treated with grape powder, NCGP-grape powder treated naïve control rats, SDPL-socially defeated rats treated with placebo, SDGP-socially defeated rats treated with grape powder. a=significantly different from NC, b=significantly different from GPNC/NCGP, c=significantly different from CE, d=significantly different from SD/SDPL. $p < 0.05$. Values are mean $\pm$ SEM., n = 10-12 rats/group.

### 4.1.3 Examination of depression-like behavior in the Forced Swim Test (FST)

The Forced swim test (FST) is widely used to test depression-like behavior in rodents (Slattery and Cryan 2012). In this test, the rat is put in the inescapable water tank for 5 minutes. After a brief period of active behavior such as swimming and climbing, rats assume passive behavior such as immobility – an indicator of despair. The more amount of time rat spends immobile in FST is marked as depression-like behavior. In this study, the socially defeated rats exhibited significantly higher immobility as compared to control groups (NC: 7.3 $\pm$ 1.0 seconds, GPNC: 6.7 $\pm$ 0.9 seconds, CE: 12.8 $\pm$ 1.4 seconds, NCGP: 20.67 $\pm$ 4.21 seconds, SD: 32.10 $\pm$ 2.33 seconds). Interestingly, control exposure rats exhibited depression-like behavior as indicated by higher immobility as compared to naïve control group (CE: 12.8 $\pm$ 1.4 seconds, NC: 7.3 $\pm$ 1.0 seconds). The grape powder treated socially defeated rats showed significantly decreased immobility that was similar to that of control groups (Figure 18A, GPSD: 15.10 $\pm$

1.65 seconds, Figure18B, SDGP: 20.38 ± 2.86 seconds). This data suggest the anti-depressant like effect of grape powder in socially defeated rats.

A




B

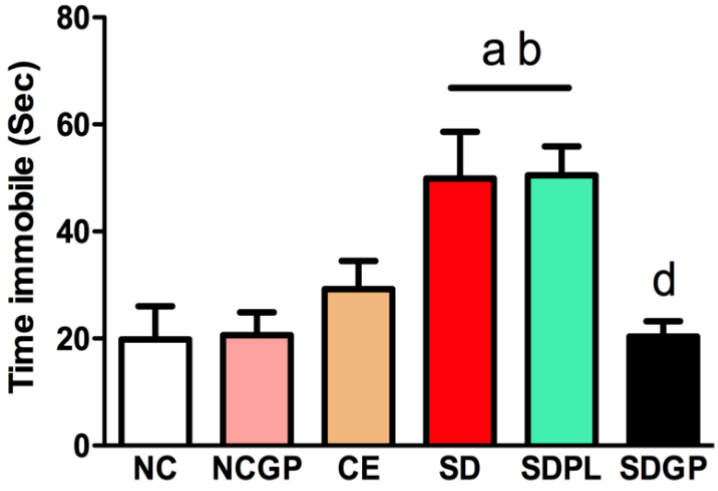


**Figure 18. Examination of depression-like behavior in the forced swim test (FST) in the control or socially defeated rats with/without grape powder treatment.** Rats subjected to social defeat spent significantly increased amount of time immobile as compared to control groups that was protected (A) and reversed (B) with grape powder treatment. NC-naïve control, GPNC-naïve control rats pre-treated with grape powder, CE- control exposure, SD- social defeat, GPSD-socially defeated rats pre-treated with grape powder, NCGP-grape powder treated naïve control rats, SDPL-socially defeated rats treated with placebo, SDGP-socially defeated rats treated with grape powder. a=significantly different from NC, b=significantly different from GPNC/NCGP, c=significantly different from CE, d=significantly different from SD/SDPL. $p < 0.05$. Values are mean $\pm$ SEM., n = 10-12 rats/group.

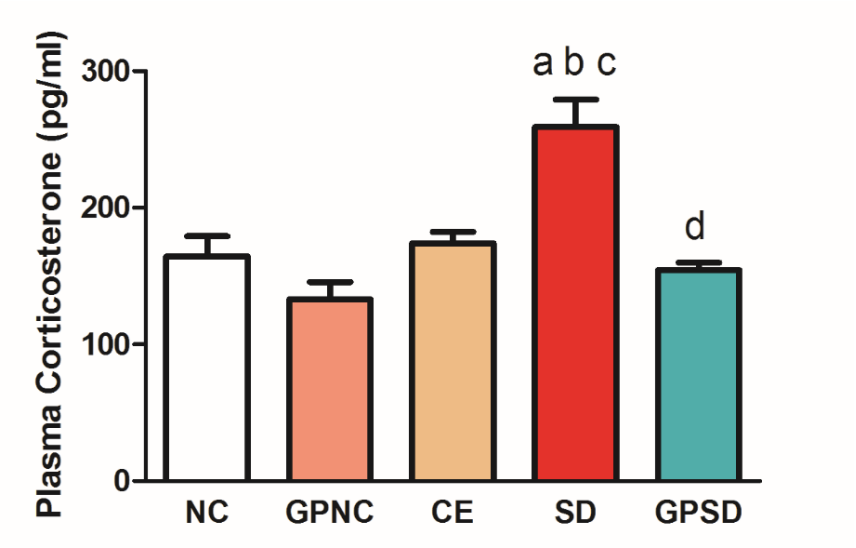
### 4.2 Examination of plasma levels of corticosterone and 8-isoprostanes

The activation of the hypothalamic-pituitary-adrenal axis (HPA axis) following the exposure to a stressful event results in increased glucocorticoids, specifically corticosterone in rodents. Such stress-induced increased levels of corticosterone in the blood stream is regarded as a biomarker of stress (Dickerson and Kemeny 2004). In this study, rats subjected to social defeat stress exhibited increased levels of plasma corticosterone as compared to control groups (NC: 164.4 $\pm$ 14.75 pg/mL, GPNC: 132.9 $\pm$ 12.66 pg/mL, CE: 173.9 $\pm$ 8.44 pg/mL, NCGP: 174.1 $\pm$ 24.9 pg/mL, SD: 259.1 $\pm$ 19.9 pg/mL). Interestingly, control exposure rats also had hyperactive HPA axis as indicated by increased levels of plasma corticosterone as compared to naïve control group (CE: 173.9 $\pm$ 8.44 pg/mL, NC: 164.4 $\pm$ 14.75 pg/mL). Grape powder treatment significantly decreased the social defeat-induced increase in plasma corticosterone levels (Figure 19A, GPSD: 154.3 $\pm$ 5.58 pg/mL, Figure 19B, SDGP: 140.8 $\pm$ 9.66 pg/mL).

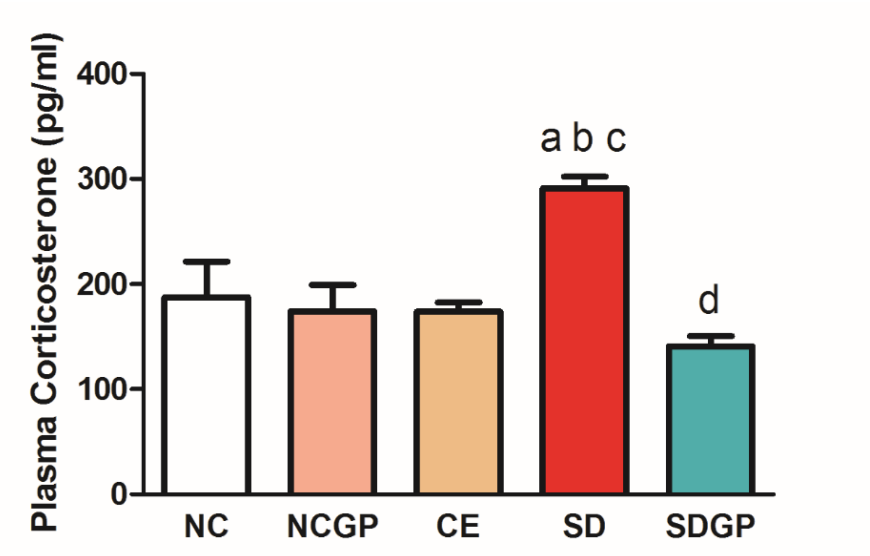
Several lines of evidence suggest that exposure to chronic stress generates high levels of reactive oxygen species (ROS) and reactive nitrogen species (RNS) leading to oxidative stress conditions (Gerecke et al. 2013; Kanarik et al. 2011; Moretti et al. 2012). Cellular biomolecules such as DNA, protein and lipids are primary targets of ROS and RNS. Oxidative stress-induced random oxidation of tissue phospholipids generates isoprostanes. 8-isoprostane is a known marker of oxidative stress (Montuschi et al. 2004; Patki et al. 2013b; Solanki et al. 2015). In this study, plasma levels of 8-isoprostanes were found to be significantly higher in socially defeated rats as compared to control groups (NC: 65.83 $\pm$ 7.06 pg/mL, GPNC: 84.49 $\pm$ 6.36 pg/mL, CE: 99.3 $\pm$ 3.47 pg/mL, NCGP: 76.66 $\pm$ 4.27 pg/mL, SD: 155 $\pm$ 18.95 pg/mL). Interestingly, control exposure rats also had increased oxidative stress as indicated by increased levels of plasma 8-isoprostane as compared to naïve control group (CE: 99.3 $\pm$ 3.47 pg/mL, NC: 65.83 $\pm$ 7.06 pg/mL). Furthermore, three weeks of grape powder treatment significantly diminished plasma 8-isoprostane levels in socially defeated rats (Figure 19C, GPSD: 103.7 $\pm$ 5.7 pg/mL, Figure 19D, SDGP: 74.26 $\pm$ 4.3 pg/mL). This data suggest that social defeat stress results in increased oxidative stress in rats that was decreased with antioxidant treatment.

Plasma corticosterone levels

A

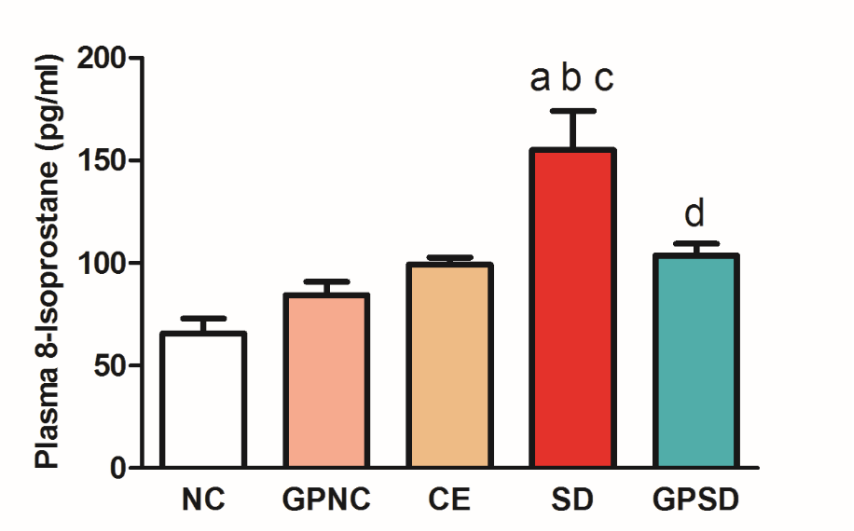



B



Plasma 8-isoprostane levels

C




D

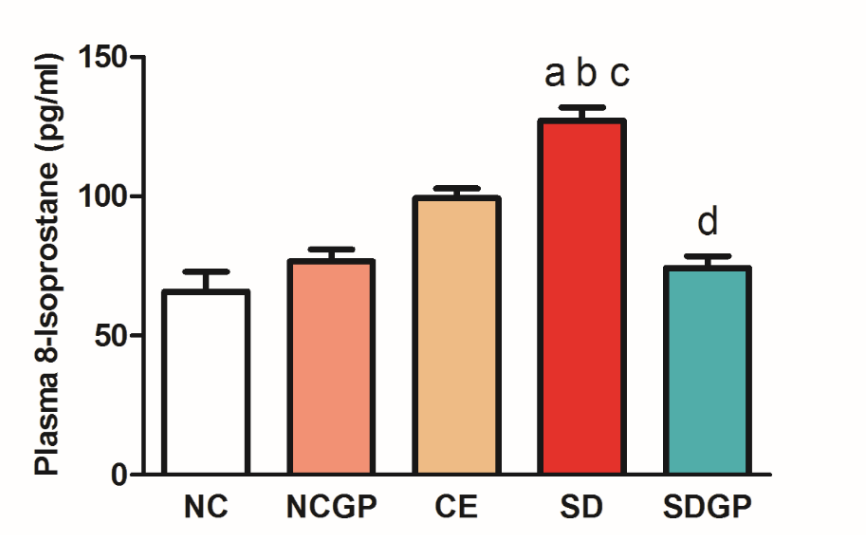



**Figure 19. Analysis of beneficial effects of grape powder on corticosterone and 8-isoprostane levels in socially defeated rats.** Rats subjected to social defeat showed marked increase in plasma levels of corticosterone (A,B) and 8-isoprostane (C,D) as compared to control groups that was protected and reversed with grape powder

treatment. NC-naïve control, GPNC-naïve control rats pre-treated with grape powder, CE- control exposure, SD- social defeat, GPSD-socially defeated rats pre-treated with grape powder, NCGP-grape powder treated naïve control rats, SDGP-socially defeated rats treated with grape powder. a=significantly different from NC, b=significantly different from GPNC/NCGP, c=significantly different from CE, d=significantly different from SD. $p < 0.05$. Values are mean $\pm$ SEM., n = 4-5 rats/group.

## 4.3 Molecular analysis

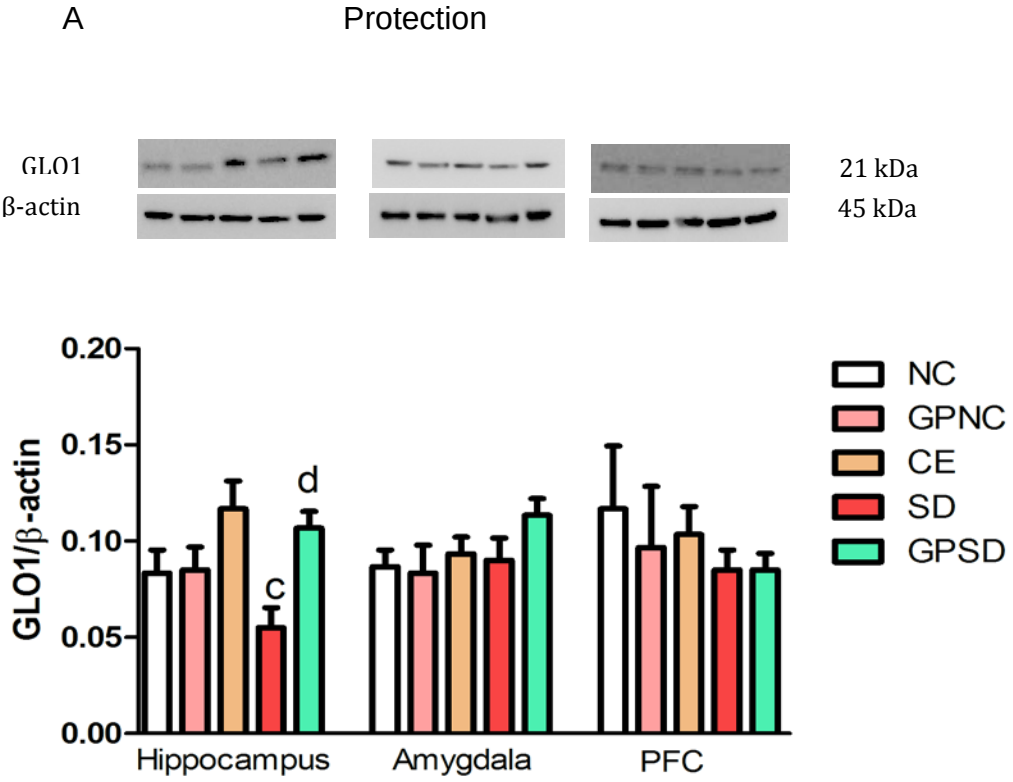
### 4.3.1 Examination of beneficial effects of grape powder treatment on the levels of antioxidant enzymes in the hippocampus, amygdala and pre-frontal cortex of socially defeated rats

The antioxidant enzymes and non-enzymatic antioxidants make up the antioxidant defense system, which is utilized to combat oxidative stress. Among all the antioxidant enzymes, glyoxalase-I (GLO-1), glutathione reductase-I (GSR-1), copper-zinc SOD (Cu-Zn SOD) and manganese SOD (Mn SOD) serve as the first line of defense against oxidative stress. These enzymes work synergistically to inhibit the deleterious effects of free radicals and provide maximum protection to cell or organism (Kohen and Nyska 2002). In this study, protein expression levels of GLO-1, GSR-1, Cu-Zn SOD and Mn SOD were examined in the hippocampus, amygdala and the pre-frontal cortex (PFC) of controls and socially defeated rats treated with/without grape powder. While GSR-1 (NC: 0.152 $\pm$ 0.016, GPNC: 0.097 $\pm$ 0.0085, CE: 0.122 $\pm$ 0.016, NCGP: 0.170 $\pm$ 0.046, SD: 0.086 $\pm$ 0.008), Cu-Zn SOD (NC: 0.78 $\pm$ 0.09, GPNC: 0.760 $\pm$ 0.054, CE: 0.715 $\pm$ 0.045, NCGP: 1.01 $\pm$ 0.07, SD: 0.39 $\pm$ 0.04) and Mn SOD (NC: 1.73 $\pm$ 0.06, GPNC: 1.95 $\pm$ 0.09, CE: 1.29 $\pm$ 0.12, NCGP: 1.66 $\pm$ 0.05, SD: 1.14 $\pm$ 0.10) protein expressions were significantly decreased in the hippocampus of socially

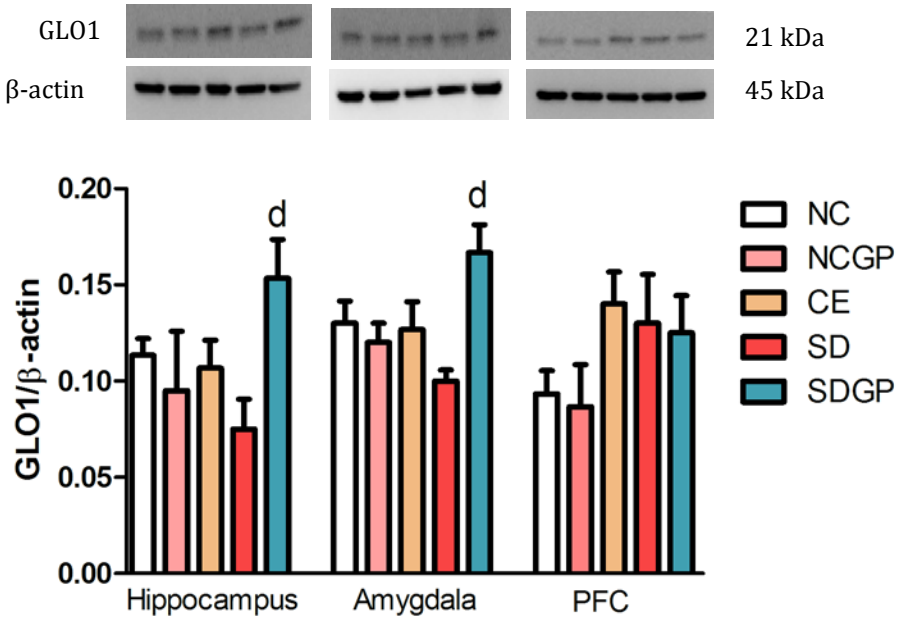
defeated rats, a decreased trend was observed for GLO-1 expression (NC: 0.08 ± 0.01, GPNC: 0.08 ± 0.01, CE: 0.11 ± 0.01, NCGP: 0.12 ± 0.01, SD: 0.05 ± 0.01). Grape powder treatment prevented social defeat-induced decreased GLO-1 (Figure 20A, GPSD: 0.10 ± 0.008), GSR-1 (Figure 20C, GPSD: 0.28 ± 0.02), Cu-Zn SOD (Figure 20E, GPSD: 0.75 ± 0.07) and Mn SOD (Figure 20G, GPSD: 1.93 ± 0.03) in the hippocampus of socially defeated rats. While grape powder treatment protected from social defeat-induced decreased expression of GSR-1 (GPSD: 0.28 ± 0.02), Cu-Zn SOD (GPSD: 0.876 ± 0.06) and Mn SOD (GPSD: 1.937 ± 0.03) in the amygdala of socially defeated rats, a similar trend was observed in amygdalar expression of GLO-1 (GPSD: 0.113 ± 0.008). No significant protective effect of grape powder was observed in the PFC of socially defeated rats.

In the case of reversal effect protocol, social defeat exposure resulted in decreased GSR-1 (NC: 0.190 ± 0.03, CE: 0.11 ± 0.017, NCGP: 0.170 ± 0.04, SD: 0.07 ± 0.02), Cu-Zn SOD (NC: 1.04 ± 0.03, CE: 0.64 ± 0.04, NCGP: 1.01 ± 0.07, SD: 0.39 ± 0.043) and Mn SOD (NC: 1.73 ± 0.06, CE: 1.71 ± 0.11, NCGP: 1.66 ± 0.05, SD: 1.19 ± 0.09) expression in hippocampus as compared to control groups and a non-significant decreased trend of hippocampal GLO-1 expression. Grape powder treatment was able to reverse social defeat-induced decreased GLO-1 (Figure 20B, SDGP: 0.166 ± 0.014), GSR-1 (Figure 20D, SDGP: 0.17 ± 0.01), Cu-Zn SOD (Figure 20F, SDGP: 0.9 ± 0.05), and Mn SOD (Figure 20H, SDGP: 1.49 ± 0.07) protein expression in the hippocampus and the amygdala of rats subjected to social defeat. While no significant reversal effect of grape

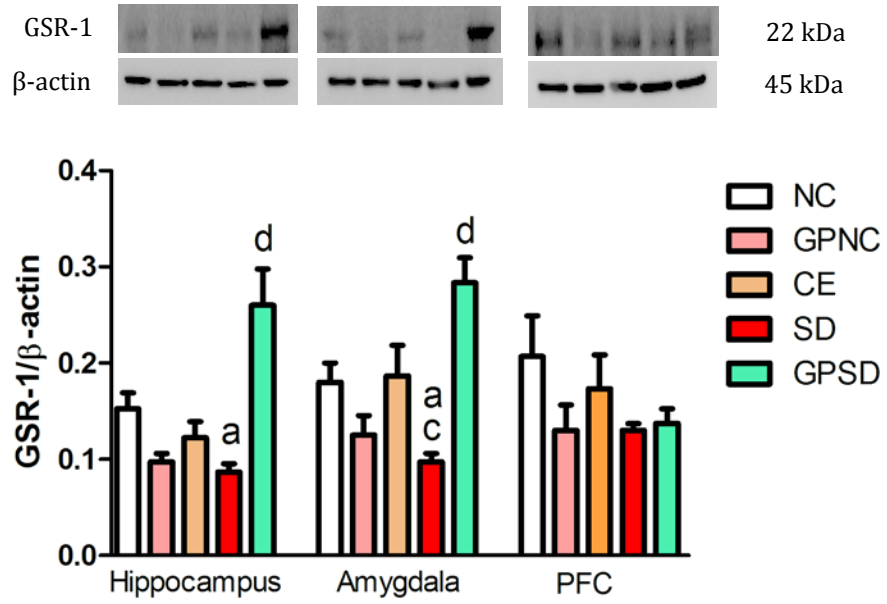
powder in PFC was observed for GLO-1, GSR-1 and Cu-Zn SOD expression, Mn SOD expression in PFC was successfully reversed with grape powder treatment. Overall, this data suggest that grape powder treatment reversed and protected social defeat-induced decrease in protein expression of key antioxidants in the hippocampal and the amygdala.



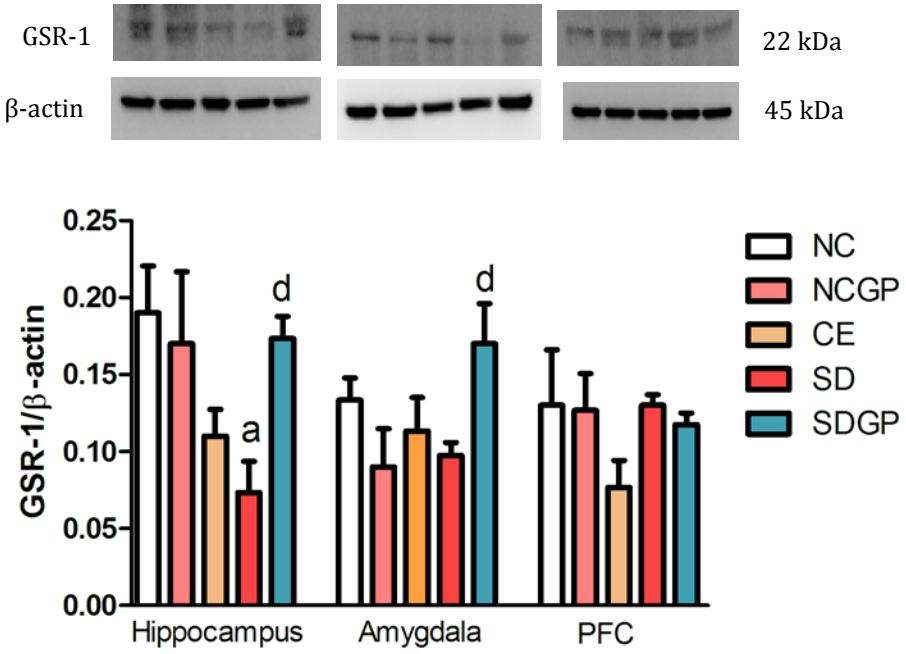
B Reversal



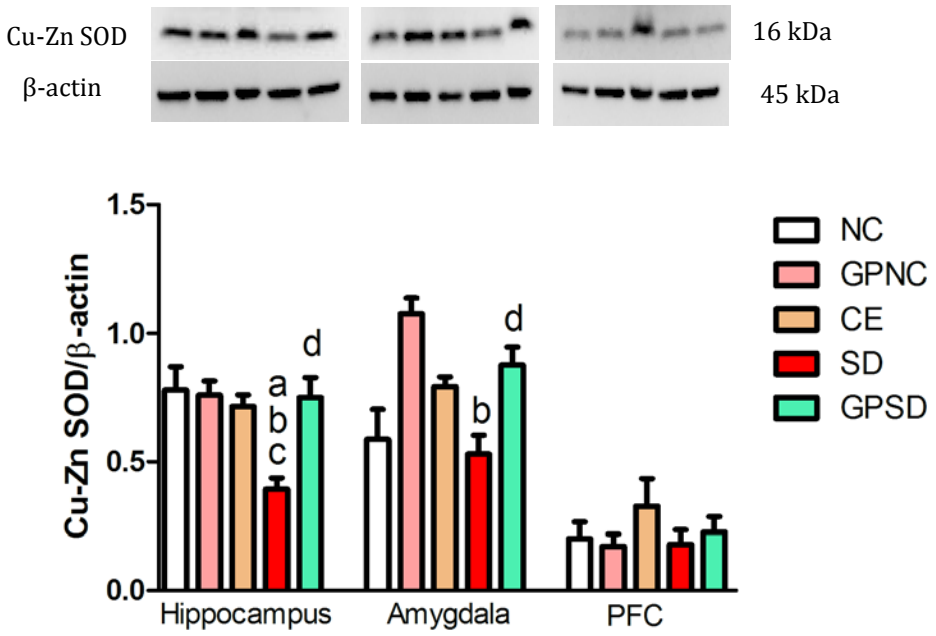

C Protection



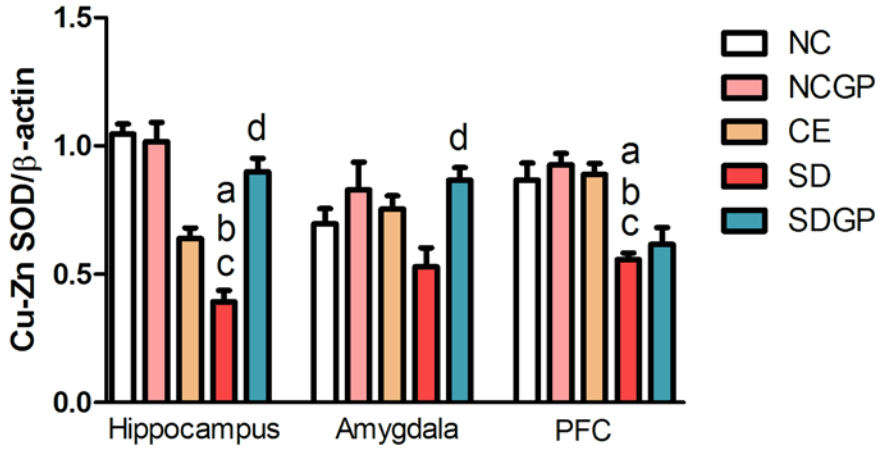
D Reversal



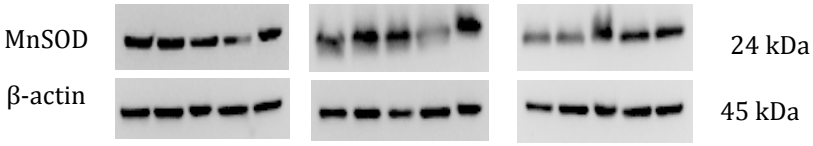

E Protection

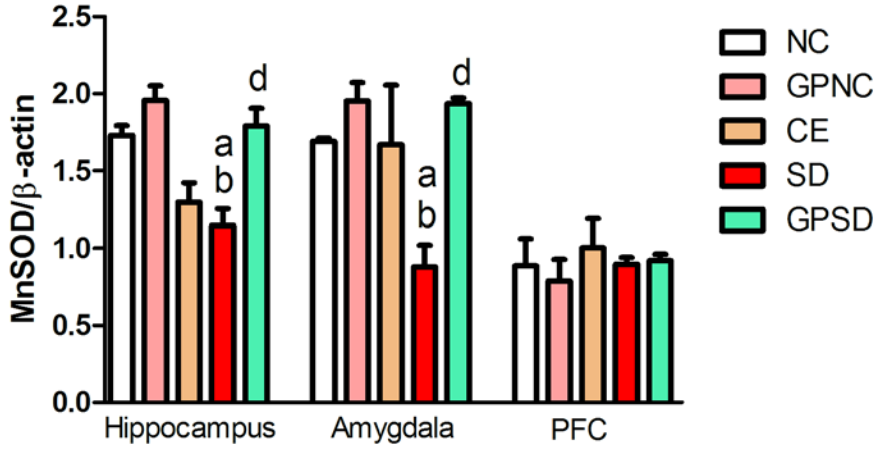


F Reversal


G Protection



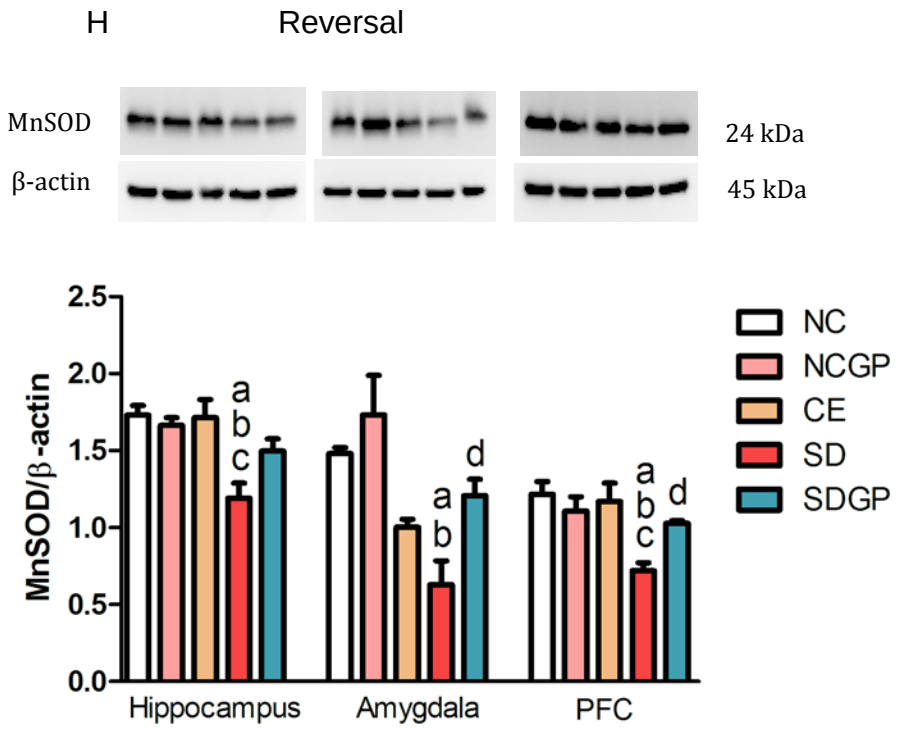
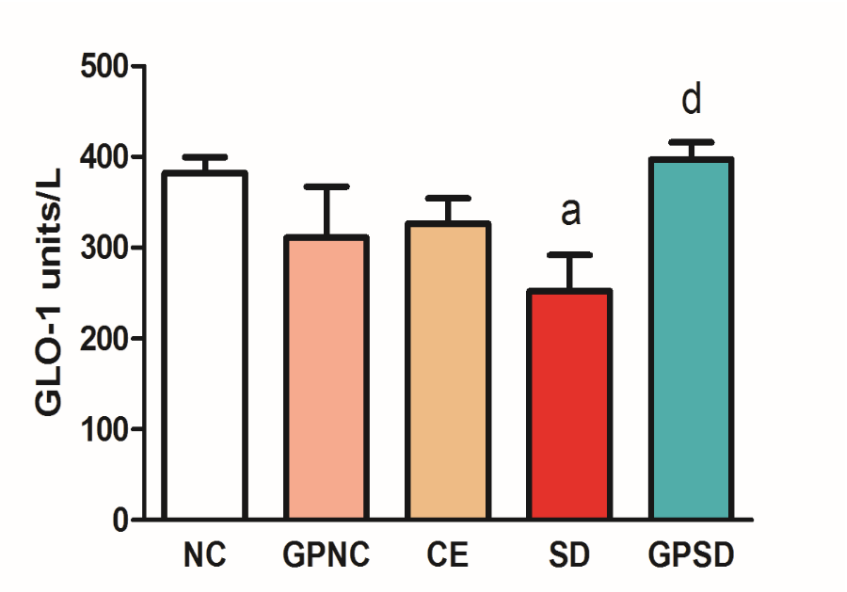



**Figure 20. Examination of GLO-1, GSR-1, Cu-Zn SOD and Mn SOD protein levels in the hippocampus, amygdala and pre-frontal cortex of socially defeated rats treated with grape powder.** Rats subjected to social defeat showed marked decrease in the expression of these enzymes, which was protected (A, C, E, G) and reversed (B, D, F, H) with grape powder treatment. The upper panels are representative blots of GLO-1, GSR-1, Cu-Zn SOD, Mn SOD and lower panels are protein loading control $\beta$ – actin, respectively. NC-naïve control, GPNC-naïve control rats pre-treated with grape powder, CE- control exposure, SD- social defeat, GPSD-socially defeated rats pre-treated with grape powder, NCGP-grape powder treated naïve control rats, SDGP-socially defeated rats treated with grape powder. a=significantly different from NC, b=significantly different from GPNC/NCGP, c=significantly different from CE, d=significantly different from SD. $p < 0.05$. Values are mean $\pm$ SEM., n = 4-5 rats/group.

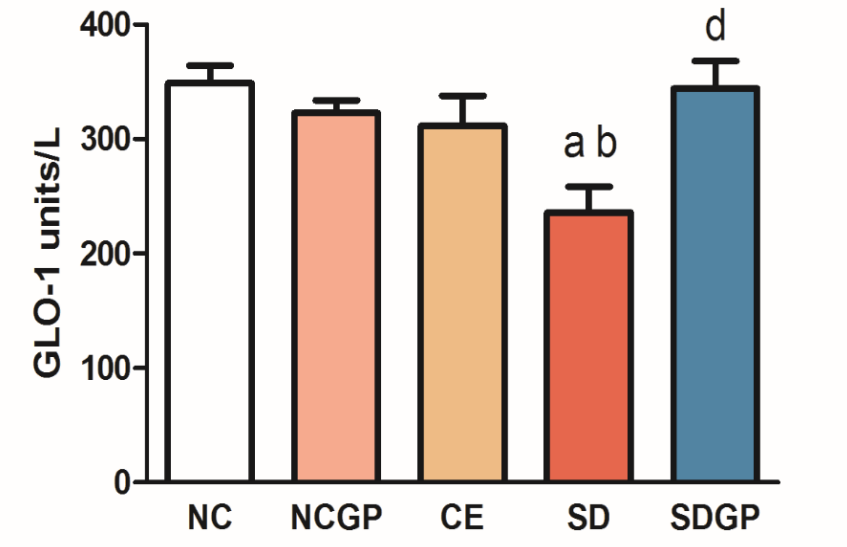
### 4.3.2 Examination of beneficial effects of grape powder treatment on GLO-1, total GSH and SOD activity in plasma

In this study, social defeat exposure in rats resulted in marked decrease in plasma activity of key antioxidant enzymes such as GLO-1 (NC: 382.1 ± 17.85 units/L, NCGP: 311 ± 56.22 units/L, SD: 252.3 ± 39.46 units/L), total glutathione (GSH) (NC: 1.60 ± 0.25 μM, GPNC: 1.27 ± 0.10 μM, SD: 0.409 ± 0.094 μM) and SOD (NC: 2.66 ± 0.08 U/mL, GPNC: 2.45 ± 0.09 U/mL, CE: 2.52 ± 0.08 U/mL, SD: 2.18 ± 0.08 U/mL). Grape powder treatment increased social defeat-induced decreased GLO-1 (Figure 21A, GPSD: 397.4 ± 18.56 units/L, Figure 21B, SDGP: 344.2 ± 23.86 units/L), total GSH (Figure 21C, GPSD: 1.44 ± 0.15 μM, Figure 21D, SDGP: 1.78 ± 0.08 μM) and SOD activity (Figure 21E, GPSD: 2.96 ± 0.18 U/mL, Figure 21F, SDGP: 2.15 ± 0.144 U/mL) in plasma. These data suggest that social defeat-induced decreased activity of key antioxidants in plasma was reversed and protected by grape powder treatment.

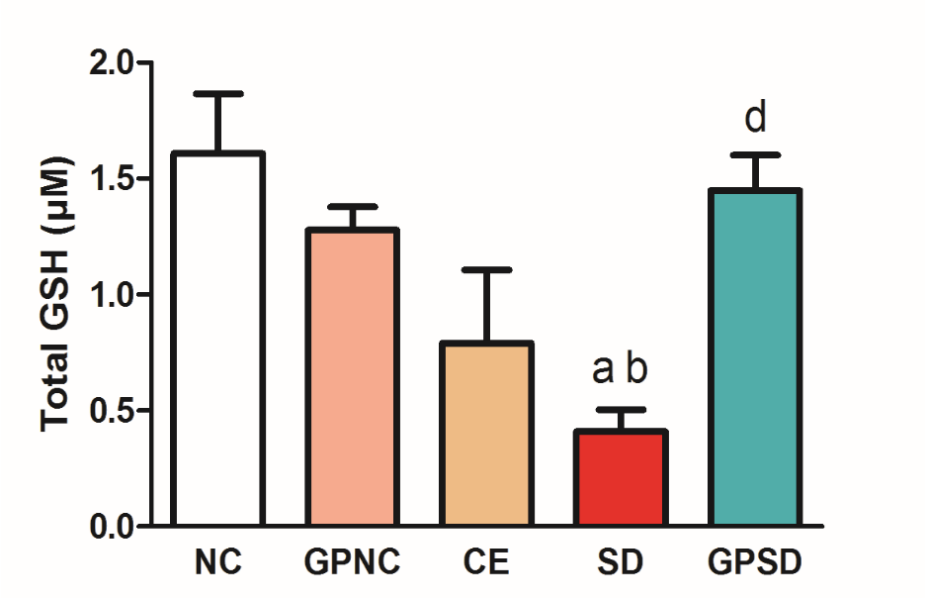
A


500
400
300
200
100
0
GLO-1 units/L
d
a
NC
GPNC
CE
SD
GPSD

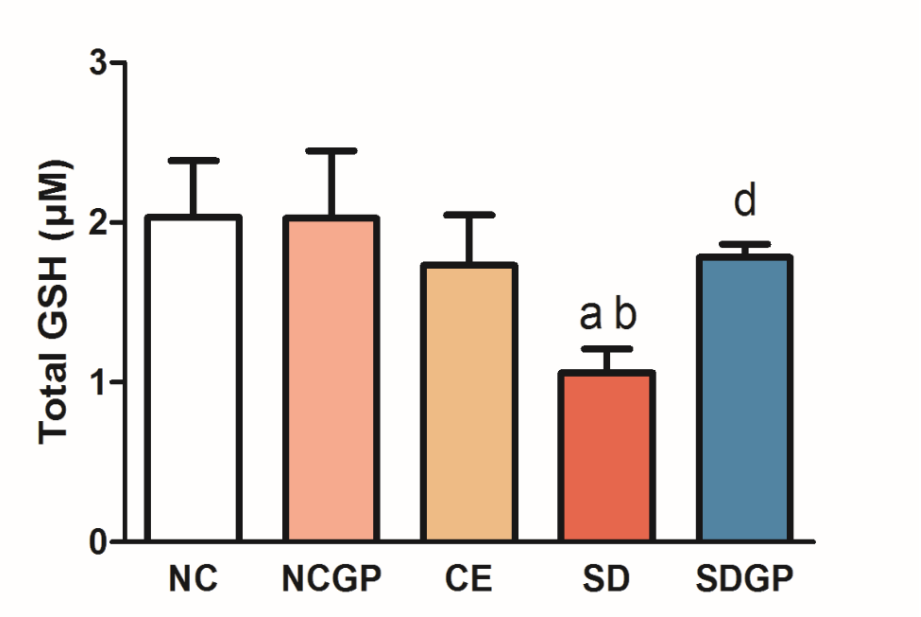
B


400
300
200
100
0
GLO-1 units/L
d
a b
NC
NCGP
CE
SD
SDGP

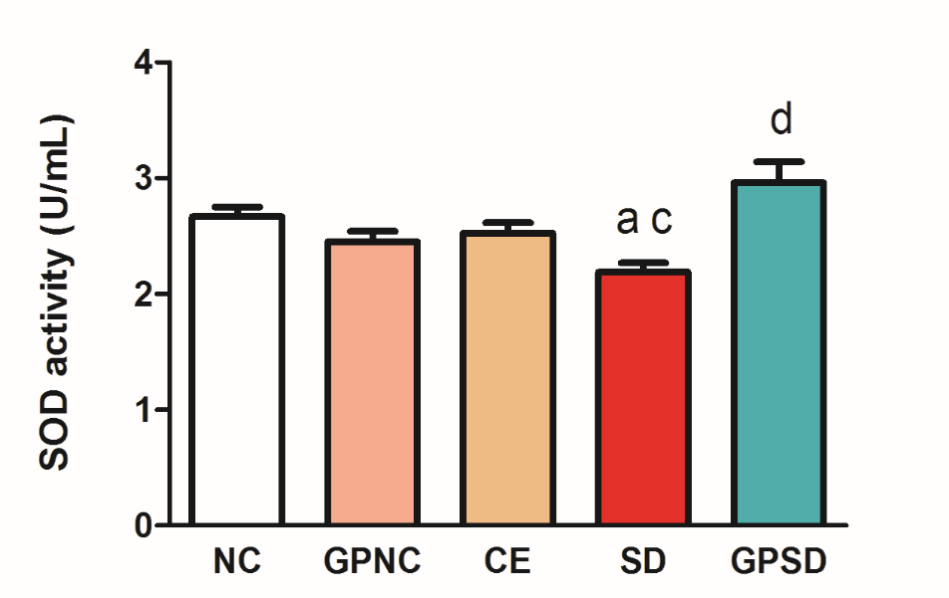
C


Total GSH (µM)
2.0
1.5
1.0
0.5
0.0
a b
d
NC
GPNC
CE
SD
GPSD

D


Total GSH (µM)
3
2
1
0
a b
d
NC
NCGP
CE
SD
SDGP

E


SOD activity (U/mL)
4
3
2
1
0
a c
d
NC
GPNC
CE
SD
GPSD

F

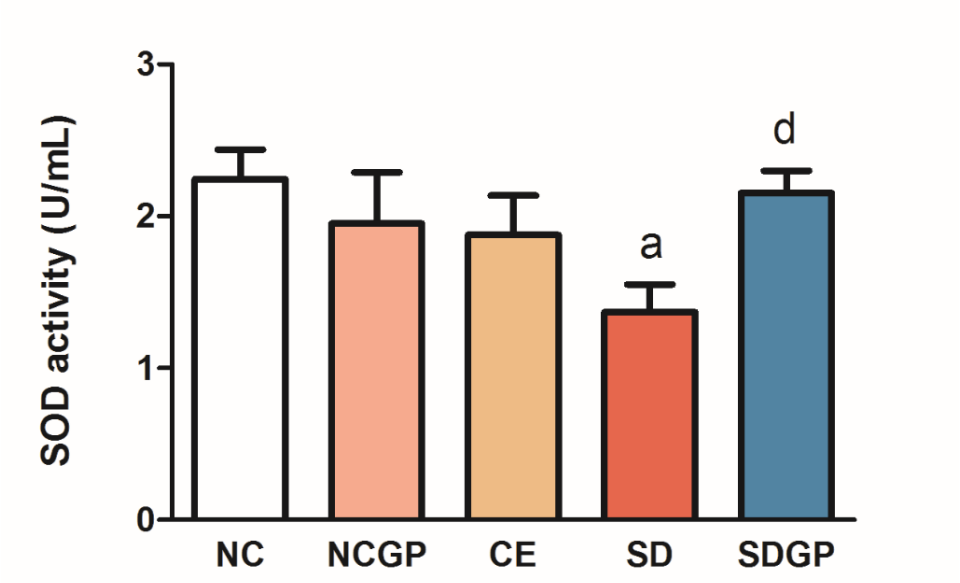
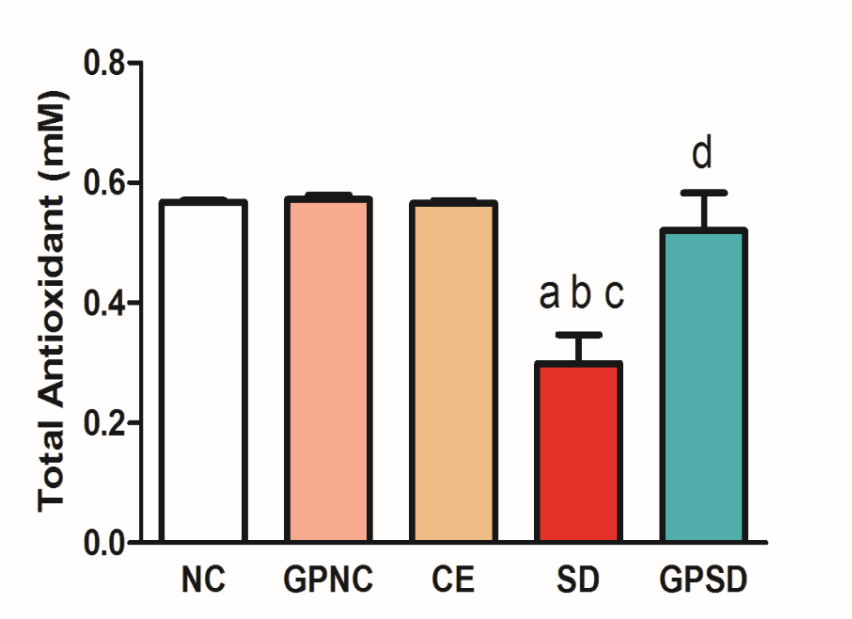

SOD activity (U/mL)
3
2
1
0
a
d
NC
NCGP
CE
SD
SDGP

**Figure 21. Examination of GLO-1, total GSH, and SOD activity in plasma of socially defeated rats treated with/without grape powder.** Rats subjected to social defeat showed marked decrease in the activity of these antioxidant enzymes, which was protected (A, C, E) and reversed (B, D, F) with grape powder treatment. NC-naïve control, GPNC-naïve control rats pre-treated with grape powder, CE- control exposure, SD- social defeat, GPSD-socially defeated rats pre-treated with grape powder, NCGP-grape powder treated naïve control rats, SDGP-socially defeated rats treated with grape powder. a=significantly different from NC, b=significantly different from GPNC/NCGP, c=significantly different from CE, d=significantly different from SD. $p < 0.05$. Values are mean $\pm$ SEM., n = 4-5 rats/group.

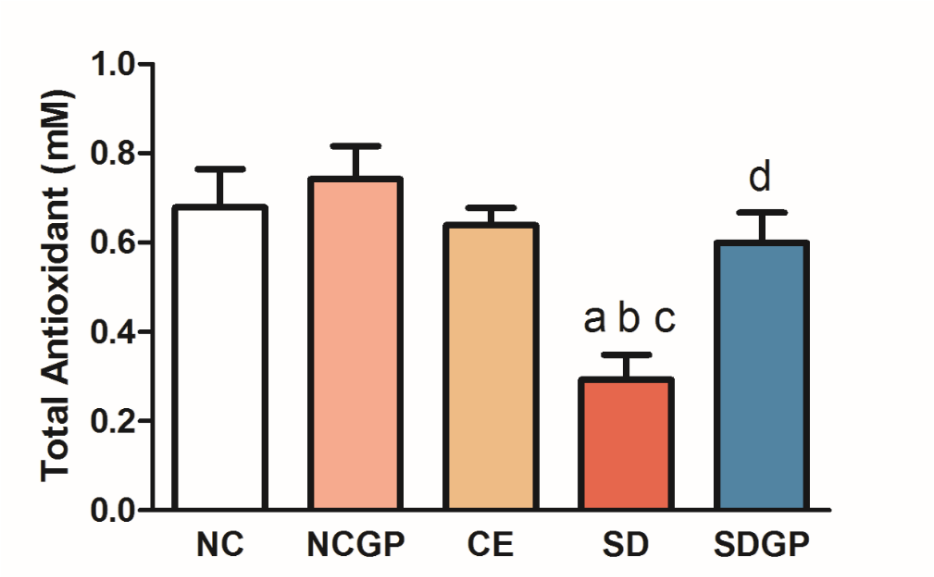
### 4.3.3 Examination of beneficial effects of grape powder treatment on total antioxidant capacity in plasma and hippocampus

The presence of antioxidant defense system in living organisms helps them combat oxidative damage. The antioxidant defense system consists of various antioxidant enzymes and non-enzymatic antioxidants. The synergism between different antioxidants offers greater protection against oxidative stress than any single antioxidant. Thus, total antioxidant capacity (TAC) may serve as an indicator of cumulative effects of antioxidants present in the system (Bartosz 2003). In this study, marked decrease in TAC in plasma (NC: 0.567 $\pm$ 0.003 mM, GPNC: 0.572 $\pm$ 0.007 mM, CE: 0.565 $\pm$ 0.004 mM, NCGP: 0.742 $\pm$ 0.074 mM, SD: 0.298 $\pm$ 0.047 mM) and hippocampus (NC: 0.216 $\pm$ 0.002 mM, GPNC: 0.224 $\pm$ 0.012 mM, CE: 0.198 $\pm$ 0.008 mM, NCGP: 0.143 $\pm$ 0.017 mM, SD: 0.153 $\pm$ 0.009 mM) was observed in rats exposed to social stress. Grape powder treatment replenished the TAC in both plasma (Figure 22A, GPSD: 0.520 $\pm$ 0.06 mM, Figure 22B, SDGP: 0.599 $\pm$ 0.067 mM) and hippocampus (Figure 22C, GPSD: 0.200 $\pm$ 0.003 mM, Figure 22D, SDGP: 0.170 $\pm$ 0.007 mM).

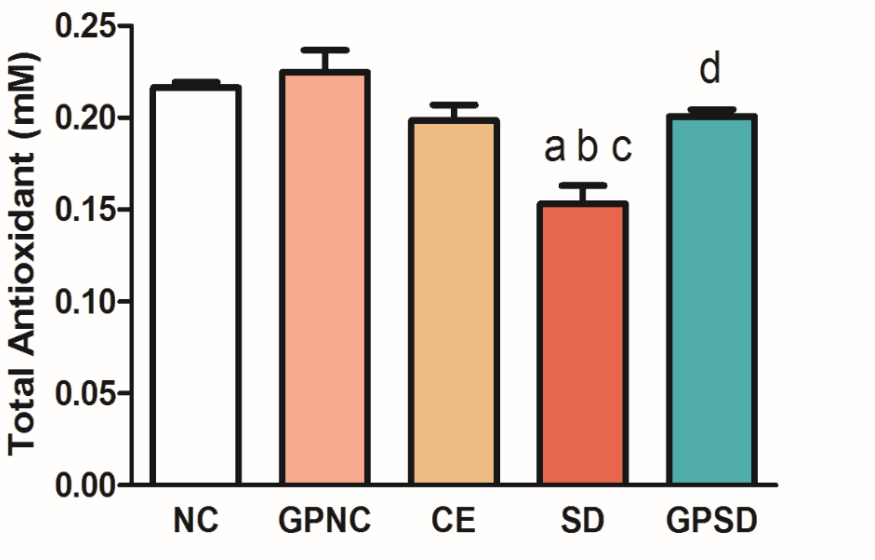
A Plasma




B Plasma



C Hippocampus




D Hippocampus

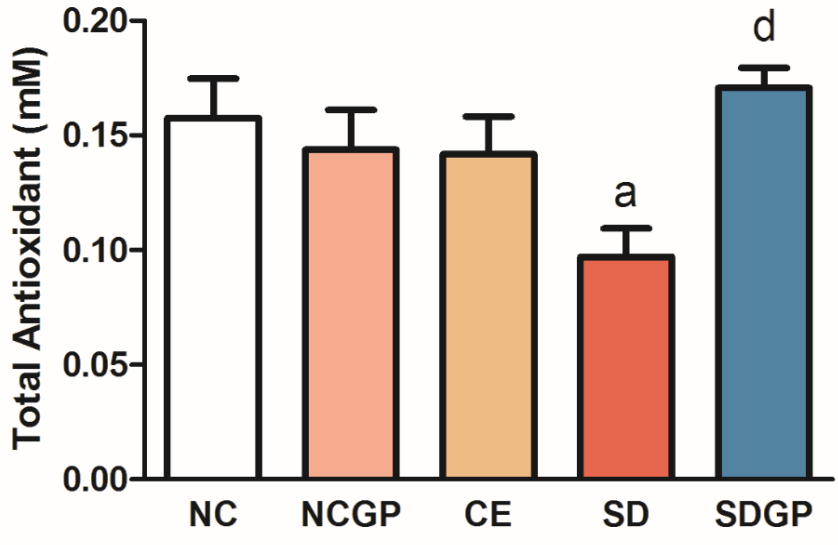
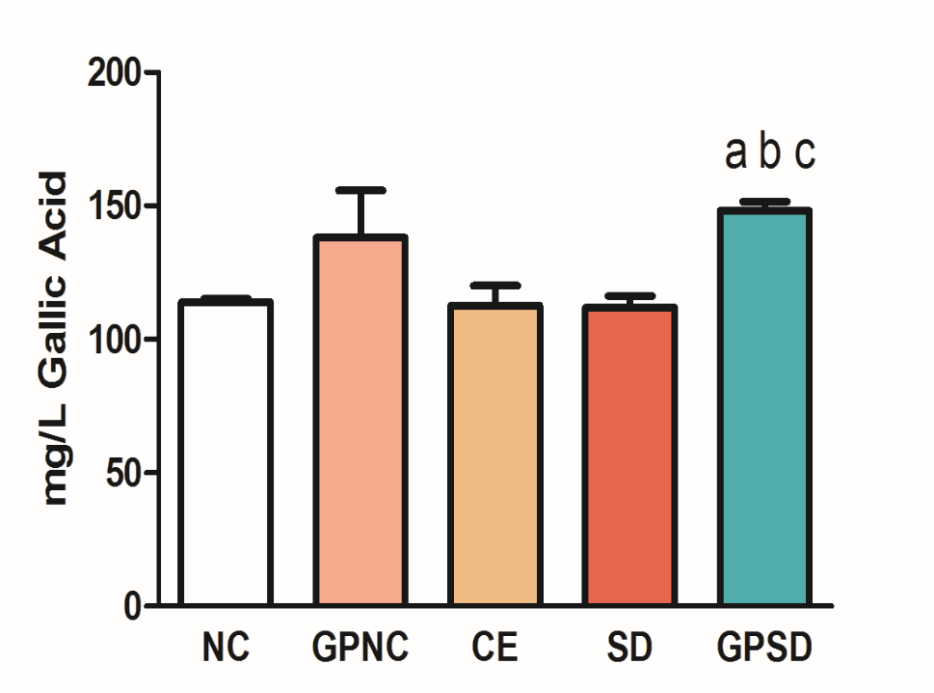


**Figure 22. Examination of total antioxidant capacity (TAC) in plasma and hippocampus of socially defeated rats treated with/without grape powder.** Rats subjected to social defeat showed marked decrease in the TAC, which was protected (A, plasma; C; hippocampus) and reversed (B, plasma; D, hippocampus) with grape powder treatment. NC-naïve control, GPNC-naïve control rats pre-treated with grape powder, CE- control exposure, SD- social defeat, GPSD-socially defeated rats pre-treated with grape powder, NCGP-grape powder treated naïve control rats, SDGP-socially defeated rats treated with grape powder. a=significantly different from NC, b=significantly different from GPNC/NCGP, c=significantly different from CE, d=significantly different from SD. $p < 0.05$. Values are mean $\pm$ SEM., n = 4-5 rats/group.

### 4.3.4 Examination of beneficial effects of grape powder treatment on total phenolic content in hippocampus

In order to confirm the presence of grape phenols and polyphenols in the brain, total phenolic content assay was performed. Total phenolic content in the hippocampus was measured spectrophotometrically using Folin Ciocalteu's (FC) method using gallic acid as standards (Iva Juranović Cindrić et al. 2011). In this study, groups treated with vehicle had similar basal levels of phenolic content in the brain (Figure 23A, NC: 113.9 $\pm$ 1.42 mg/L gallic acid, CE: 112.7 $\pm$ 7.43 mg/L gallic acid, SD: 112.7 $\pm$ 7.43 mg/L gallic acid) (Figure 23B, NC: 110 $\pm$ 10.28 mg/L gallic acid, CE: 114.3 $\pm$ 5.98 mg/L gallic acid, SD: 103.7 $\pm$ 3.80 mg/L gallic acid). However, grape powder treatment led to a significant increase in the total phenolics in the hippocampus (GPSD: 148.3 $\pm$ 3.32 mg/L gallic acid, NCGP: 152.7 $\pm$ 5.11 mg/L gallic acid, SDGP: 152.7 $\pm$ 8.41 mg/L gallic acid). These data suggest that grape polyphenols reach brain by crossing blood-brain barrier and exert their antioxidant effects to provide protection.

A


200
150
100
50
0
mg/L Gallic Acid
a b c
NC
GPNC
CE
SD
GPSD

B

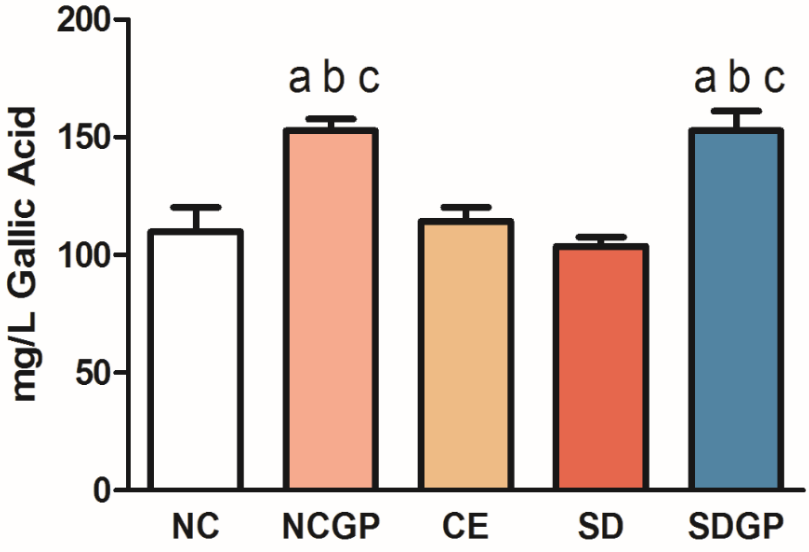

200
150
100
50
0
mg/L Gallic Acid
a b c
a b c
NC
NCGP
CE
SD
SDGP

**Figure 23. Examination of total phenolic content (TPC) in hippocampus of socially defeated rats treated with/without grape powder.** Rats subjected to grape powder treatment showed marked increase in the total phenolic content of hippocampus in both protection (A) and reversal (B) protocol. NC-naïve control, GPNC-naïve control rats pre-treated with grape powder, CE- control exposure, SD- social defeat, GPSD-socially defeated rats pre-treated with grape powder, NCGP-grape powder treated naïve control rats, SDGP-socially defeated rats treated with grape powder. a=significantly different from NC, b=significantly different from GPNC/NCGP, c=significantly different from CE, d=significantly different from SD. $p < 0.05$. Values are mean $\pm$ SEM., n = 4-5 rats/group.

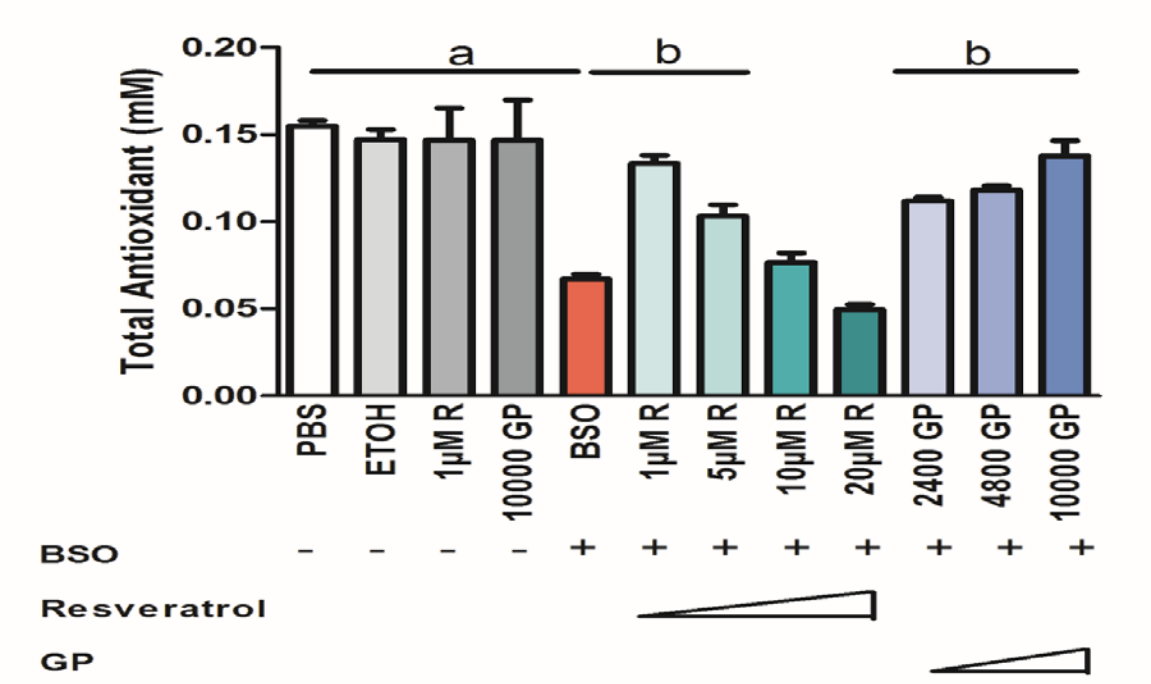
## 4.4 In-vitro studies

### 4.4.1 Assessment of beneficial effects of resveratrol, quercetin and kaempferol in protecting BSO-induced decreased total antioxidant capacity in HT22 cells

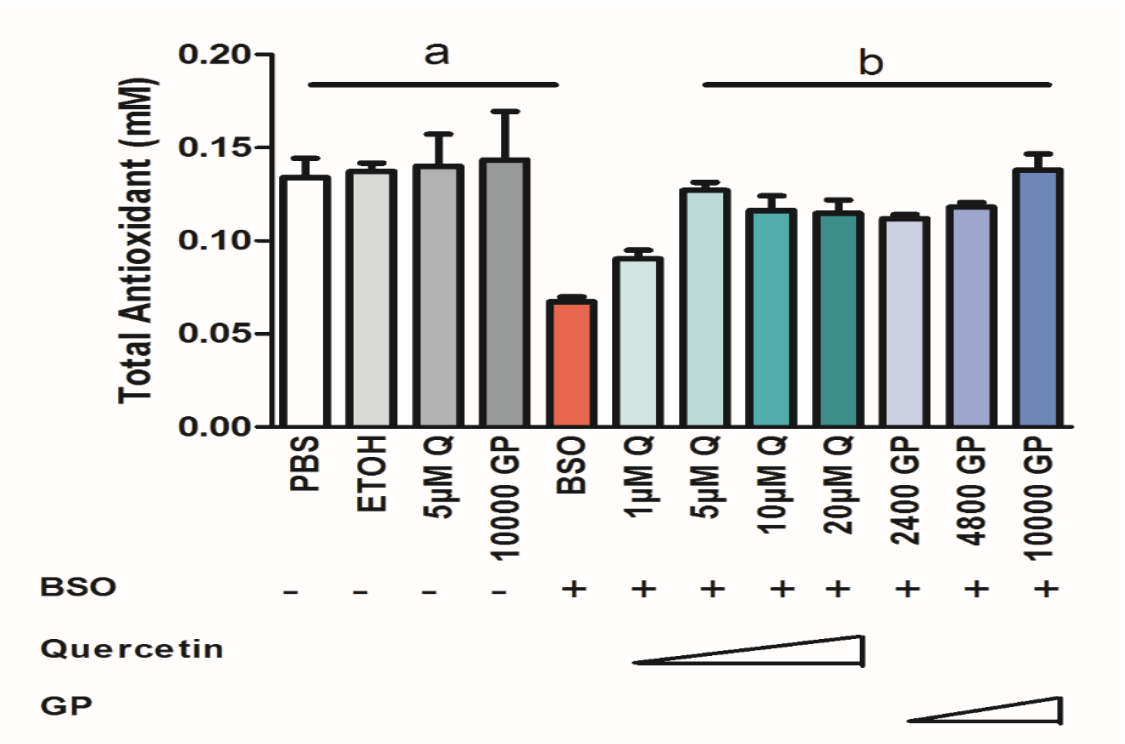
In this study, pro-oxidant BSO treatment significantly decreased TAC in HT22 cells (PBS: 0.154 $\pm$ 0.003 mM, BSO: 0.067 $\pm$ 0.002 mM). Decrease in TAC was prevented with grape powder treatment (2400 GP: 0.111 $\pm$ 0.002 mM, 4800 GP: 0.118 $\pm$ 0.002 mM, 10000 GP: 0.137 $\pm$ 0.008 mM). Similarly, resveratrol at 1μM and 5μM concentrations also prevented BSO-induced decreased TAC (1μMR: 0.133 $\pm$ 0.004 mM, 5μMR: 0.103 $\pm$ 0.006 mM) (Figure 24). This data suggests that resveratrol was effective at the lowest concentration in maintaining TAC levels. In addition, the ability of quercetin and kaempferol in protecting BSO-induced decreased TAC was investigated. While, quercetin was found to be effective at 5μM, 10μM and 20μM concentrations (PBS: 0.133 $\pm$ 0.01 mM, BSO: 0.067 $\pm$ 0.002 mM, 5μMQ: 0.127 $\pm$ 0.004 mM, 10μMQ: 0.116 $\pm$ 0.007 mM, 20μMQ: 0.114 $\pm$ 0.007 mM), kaempferol failed to protect BSO-induced decreased TAC (1μM, 5μM, 10μM and 20μM) concentrations (1μMK: 0.07 $\pm$

0.003 mM, 5μMK: 0.07 ± 0.006 mM, 10μMK: 0.065 ± 0.009 mM, 20μMK: 0.082 ± 0.001 mM). The protective effect of quercetin was comparable to that of grape powder treatment (2400GP: 0.111 ± 0.002 mM, 4800GP: 0.118 ± 0.002 mM, 10000GP: 0.137 ± 0.008 mM). Overall, these data suggest that resveratrol and quercetin were effective in preventing TAC levels from declining upon induction of oxidative insult. TAC levels were normalized with resveratrol and quercetin treatment.

A



B


Total Antioxidant (mM)
0.20
0.15
0.10
0.05
0.00
a
b
PBS
ETOH
5μM Q
10000 GP
BSO
1μM Q
5μM Q
10μM Q
20μM Q
2400 GP
4800 GP
10000 GP
BSO
- - - - + + + + + + + +
Quercetin
GP

C

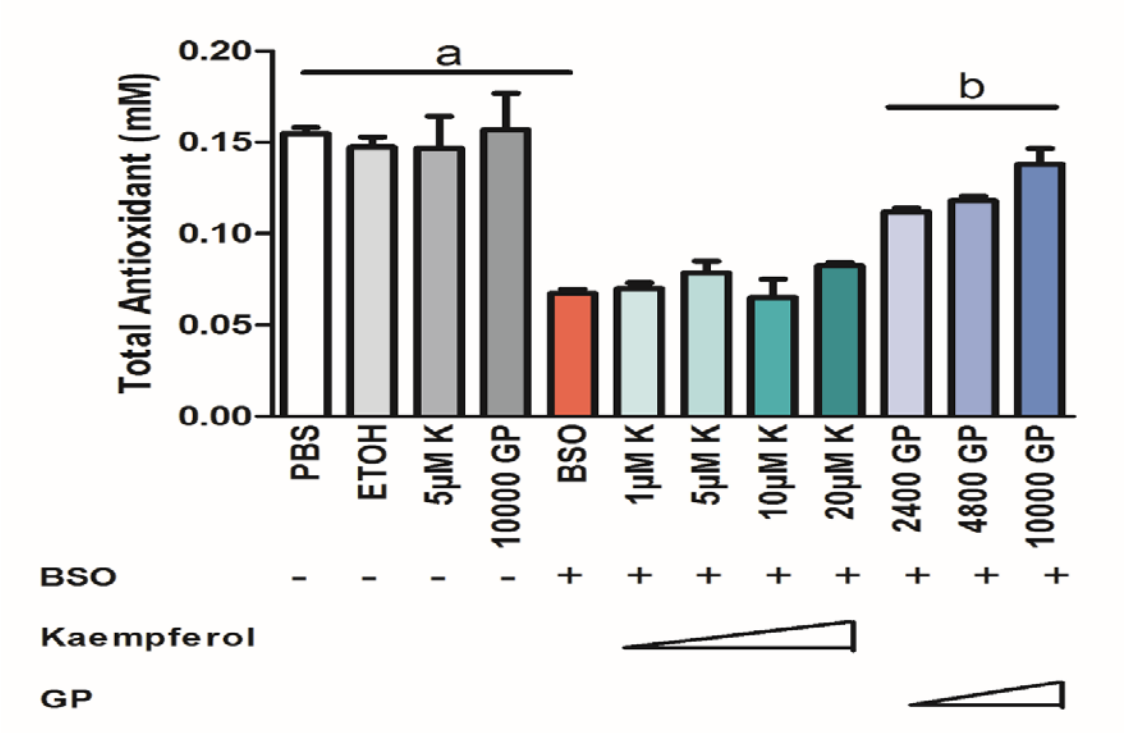

Total Antioxidant (mM)
0.20
0.15
0.10
0.05
0.00
a
b
PBS
ETOH
5μM K
10000 GP
BSO
1μM K
5μM K
10μM K
20μM K
2400 GP
4800 GP
10000 GP
BSO
- - - - + + + + + + + +
Kaempferol
GP

**Figure 24. The protective effects of resveratrol (R), Quercetin (Q) and Kaempferol (K) on total antioxidant capacity (TAC) in BSO-induced oxidative stress.** Representative graphs show BSO-induced decreased TAC was effectively protected by (A) resveratrol at 1μM and 5μM concentrations, (B) quercetin at 5μM, 10μM and 20μM concentrations, (C) kaempferol – failed to protect TAC at 1μM-20μM concentrations. PBS-control, ETOH-ethanol treated HT22 cells, 1μM-20μM R/K/Q- 1μM-20μM resveratrol/quercetin/kaempferol treated HT22 cells with/without BSO treatment, 2400GP-10000 GP- 2400-10000 μg/mL grape powder treated HT22 cells with/without BSO treatment. a=significantly different from all control groups; b=significantly different from BSO group. $p < 0.05$. Values are mean $\pm$ SEM., n = 3-4.

### 4.4.2 Assessment of protective effect of grape powder in BSO-induced increased calcium concentrations in HT22 cells

Calcium is essential for all living organisms as it serves as a messenger for a large number of cellular processes. Oxidative stress-induced altered calcium homeostasis is a known initiator of cellular apoptotic processes (Salido et al. 2009). We observed that BSO treatment led to marked increase in calcium levels in HT22 cells (PBS: 0.143 $\pm$ 0.002 μg/ μL, BSO: 0.249 $\pm$ 0.037 μg/ μL, PL + BSO: 0.238 $\pm$ 0.013 μg/ μL), while it was significantly decreased and normalized with grape powder treatment (Figure 25, GP + BSO: 0.136 $\pm$ 0.005 μg/ μL).

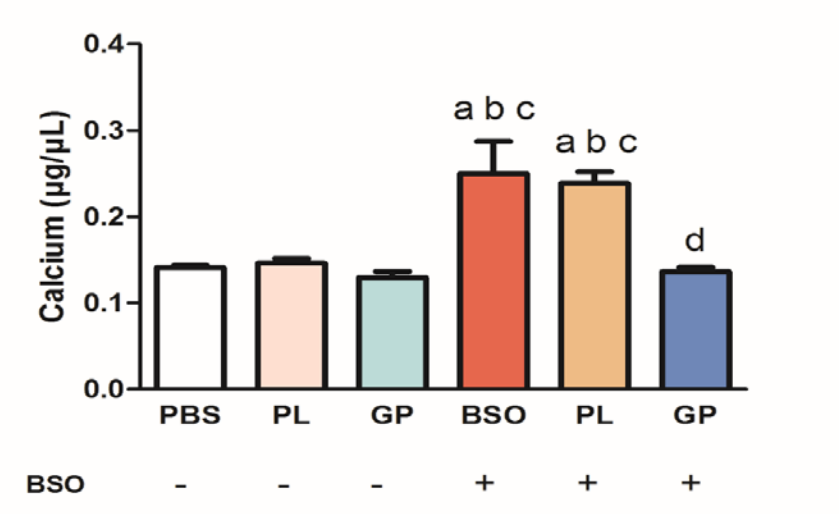



**Figure 25. Analysis of protective effects of grape powder on BSO-induced calcium levels in HT22 cells.** BSO treatment showed marked increase in calcium levels, while grape powder pretreatment prevented HT22 cells from such increase. PBS-control, PL-placebo treated HT22 cells with/without BSO treatment, GP-grape powder treated HT22 cells with/without BSO treatment, BSO- 1mM BSO treated HT22 cells. a=significantly different from PBS, b=significantly different from PL alone, c=significantly different from GP alone, d=significantly different from BSO. $p < 0.05$. Values are mean $\pm$ SEM., n = 3-4.

### 4.4.3 Assessment of protective effect of grape powder in BSO-induced impaired mitochondrial membrane potential in HT22 cells

Under oxidative stress conditions, free radicals target a large number of macromolecules and organelles including mitochondria. Mitochondria play a central role in regulation of apoptosis. Mitochondrial membrane potential is an indicator of cell health or injury. Cells undergoing oxidative stress or apoptosis have higher mitochondrial membrane potential (Gogvadze et al. 2006). JC-1 dye, an indicator of mitochondrial membrane potential, is widely used to study mitochondrial health in apoptosis. JC-1 dye is mitochondrial membrane specific and highly sensitive to its depolarization. Potential dependent accumulation of

JC-1 in mitochondria is indicated by fluorescence ratio. In our study, BSO treatment lowered mitochondrial membrane potential (PBS: 100%, BSO: 51 ± 4.04%, PL + BSO: 44.33 ± 6.17%). Grape powder pretreatment reestablished mitochondrial membrane potential (Figure 26, GP + BSO: 91 ± 5.68%).

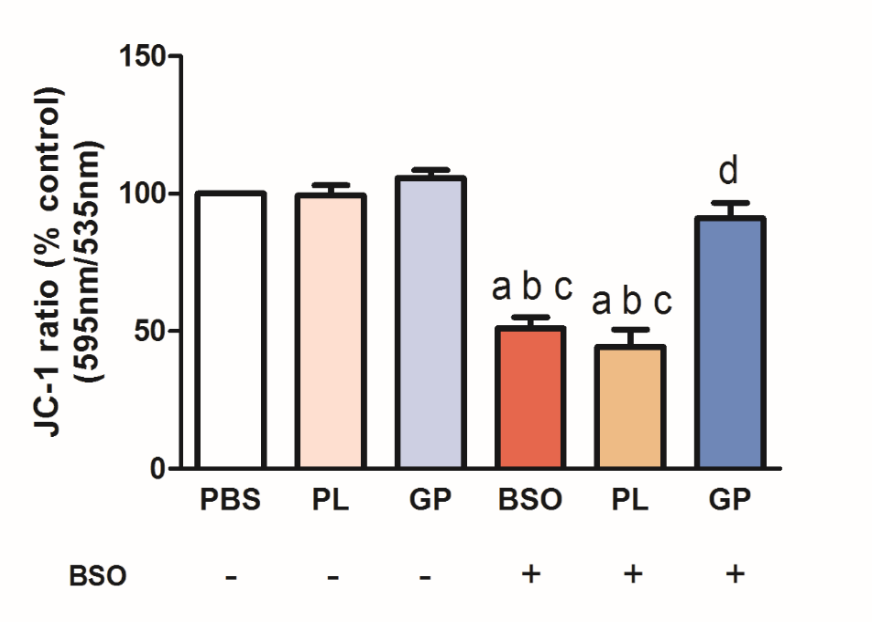



**Figure 26. Analysis of protective effects of grape powder on BSO-induced impaired mitochondrial membrane potential in HT22 cells.** Mitochondrial membrane potential was depicted as a ratio of J-aggregates to monomer intensity (595nm/535nm). The JC-1 ratio was presented as % control (n= 3-4). PBS-control, PL-placebo treated HT22 cells with/without BSO treatment, GP-grape powder treated HT22 cells with/without BSO treatment, BSO- 1mM BSO treated HT22 cells. a=significantly different from PBS, b=significantly different from PL alone, c=significantly different from GP alone, d=significantly different from BSO. $p < 0.05$. Values are mean ± SEM., n = 3-4.

### 4.4.4 Assessment of protective effect of grape powder in BSO-induced increased cytochrome-c release in HT22 cells

Oxidative stress-induced mitochondrial impairment results in a cascade of events including release of cytochrome-c to cytosol. Release of cytochrome-c in the cytosol is considered as the initiator of the apoptotic process leading to cell death (Ott et al. 2002). In our study, 14 hours of BSO treatment led to increase in the levels of cytosolic cytochrome-c (PBS: 0.730 $\pm$ 0.06, BSO: 1.51 $\pm$ 0.05, PL + BSO: 1.45 $\pm$ 0.02). 4 hours of grape powder treatment prior to BSO treatment prevented release of cytochrome-c (Figure 27, GP + BSO: 0.753 $\pm$ 0.08).

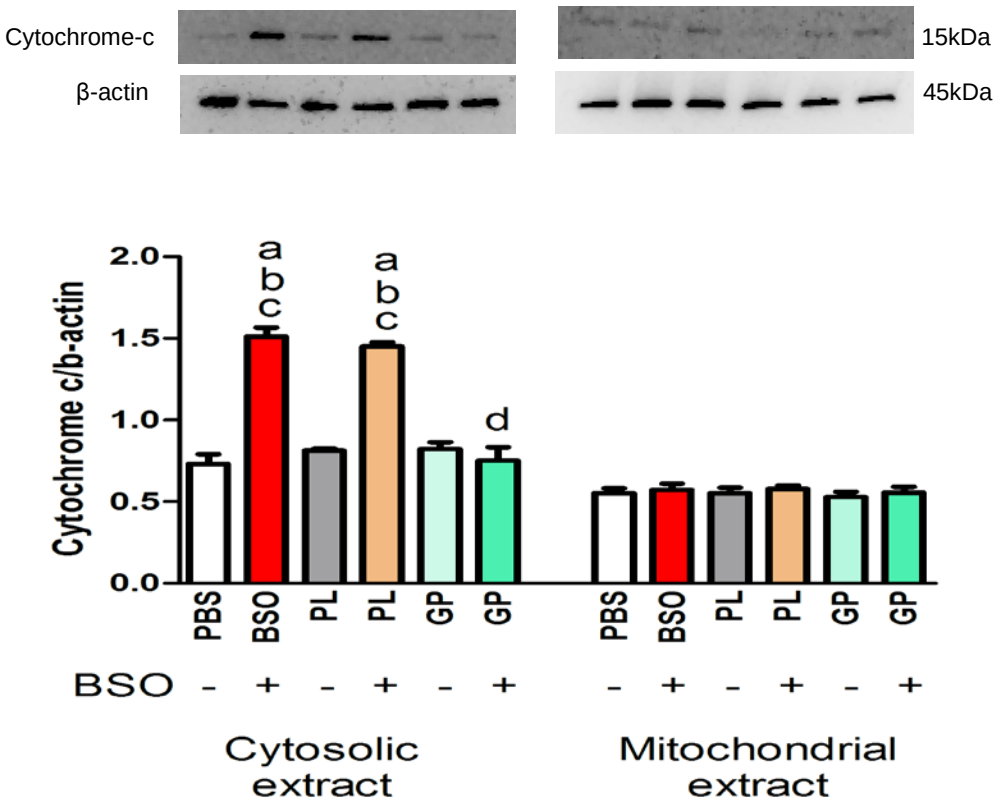


**Figure 27. Analysis of protective effect of grape powder on BSO-induced increased cytochrome-c release.** 4 hours of grape powder treatment prevented BSO-induced increase in release of cytochrome-c. PBS-control, PL-placebo treated HT22 cells with/without BSO treatment, GP-grape powder treated HT22 cells with/without BSO treatment, BSO- 1mM BSO treated HT22 cells. a=significantly different from PBS, b=significantly different from PL alone, c=significantly different from GP alone, d=significantly different from BSO. $p < 0.05$. Values are mean $\pm$ SEM., n = 3-4.

### 4.4.5 Assessment of protective effect of grape powder in BSO-induced increased caspase-3 activity in HT22 cells

Oxidative stress-induced cytochrome-c release from mitochondria activates caspases. Caspase activation is the initiator of the death cascade. Activation of caspases, specifically caspase-3, is a known marker of cell death (McIlwain et al. 2013). In our study, 14 hours of BSO treatment increased caspase-3 activity in BSO treated cells (PBS: 0.078 $\pm$ 0.002, BSO: 0.144 $\pm$ 0.012. PL + BSO: 0.138 $\pm$ 0.025). BSO-induced increased activity of caspase-3 was absent in the group treated for 4 hours with grape powder prior to BSO treatment (Figure 28, GP + BSO: 0.080 $\pm$ 0.009).

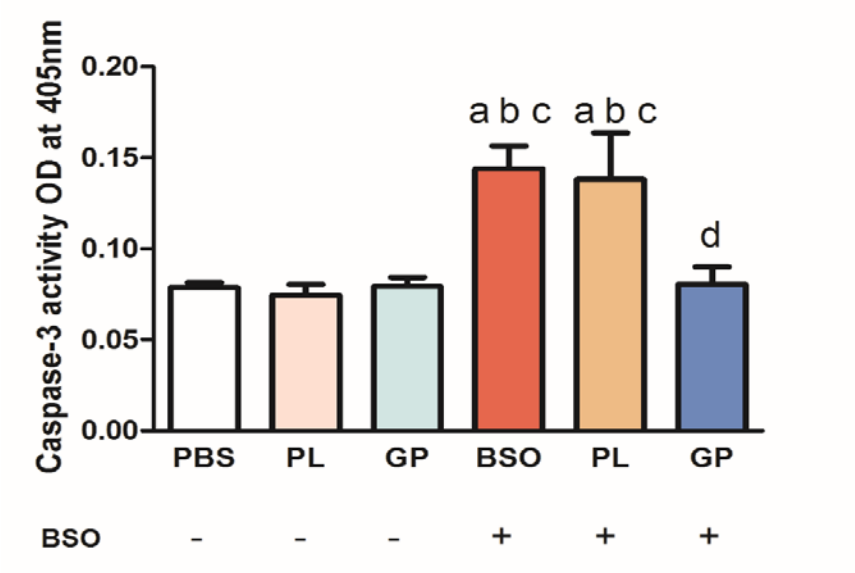


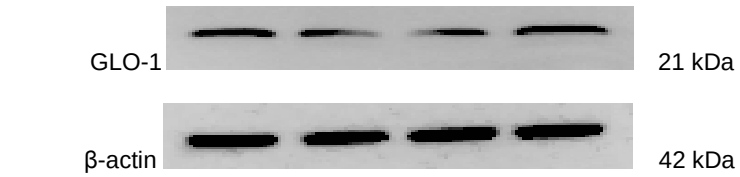

**Figure 28. Analysis of protective effect of grape powder on BSO-induced increased caspase-3 activity.** 4 hours of grape powder treatment prevented BSO-induced increase in caspase-3 activity. PBS-control, PL-placebo treated HT22 cells with/without BSO treatment, GP-grape powder treated HT22 cells with/without BSO treatment, BSO- 1mM BSO treated HT22 cells. a=significantly different from PBS, b=significantly different from PL alone, c=significantly different from GP alone, d=significantly different from BSO. $p < 0.05$. Values are mean $\pm$ SEM., n = 3-4.

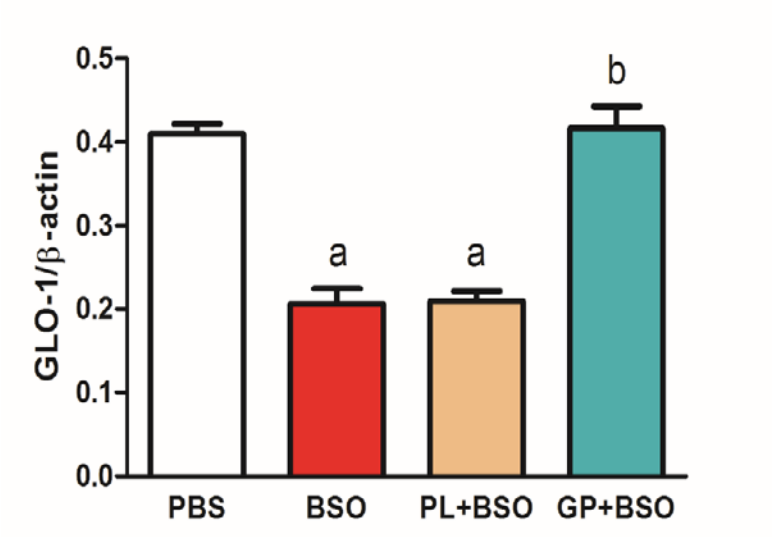
### 4.4.6 Examination of beneficial effects of grape powder treatment on BSO-induced decreased levels of antioxidant enzymes in HT22 cells

Oxidative stress results when there is excessive production of free radicals or depletion of antioxidants. It has been reported that higher oxidative stress conditions lead to malfunctioning of the antioxidant defense system primarily the antioxidant enzymes. Recently, we have reported that BSO treatment induces oxidative stress in HT22 cells via decreasing protein levels of antioxidant enzymes (Salvi et al. 2016). In our study, western blot analysis of HT22 cell lysate indicated that the grape powder treatment prevented the BSO-induced decreased protein expression levels of (i) GLO-1 (Figure 29A, PBS:

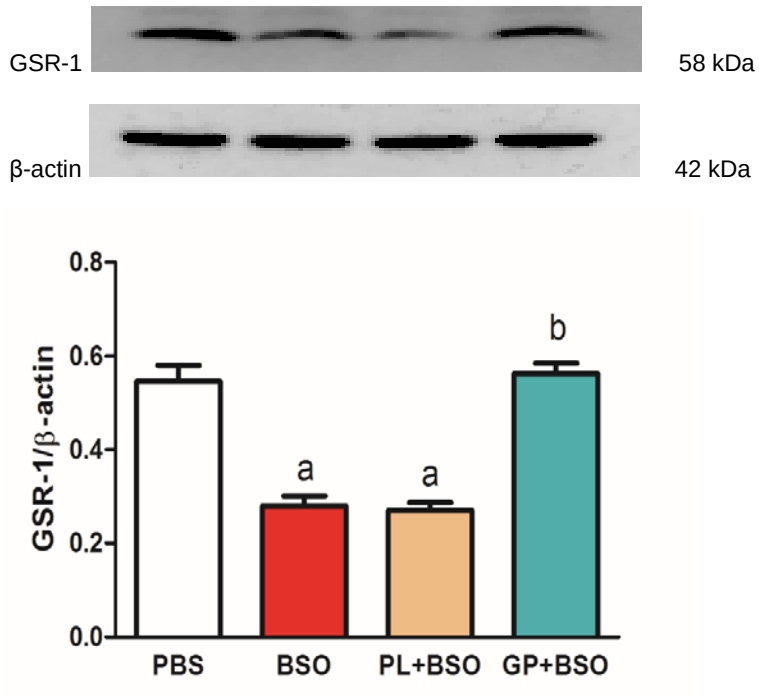
0.410 ± 0.011, BSO: 0.206 ± 0.017, PL + BSO: 0.210 ± 0.011, GP + BSO: 0.416 ± 0.026) (ii) GSR-1 (Figure 29B, PBS: 0.546 ± 0.033, BSO: 0.280 ± 0.020, PL + BSO: 0.270 ± 0.017, GP + BSO: 0.563 ± 0.021), (iii) Mn SOD (Figure 29C, PBS: 0.456 ± 0.031, BSO: 0.310 ± 0.017, PL + BSO: 0.293 ± 0.014, GP + BSO: 0.453 ± 0.023), and Cu-Zn SOD (Figure 29D, PBS: 0.470 ± 0.020, BSO: 0.280 ± 0.011, PL + BSO: 0.296 ± 0.018, GP + BSO: 0.466 ± 0.023).

A



B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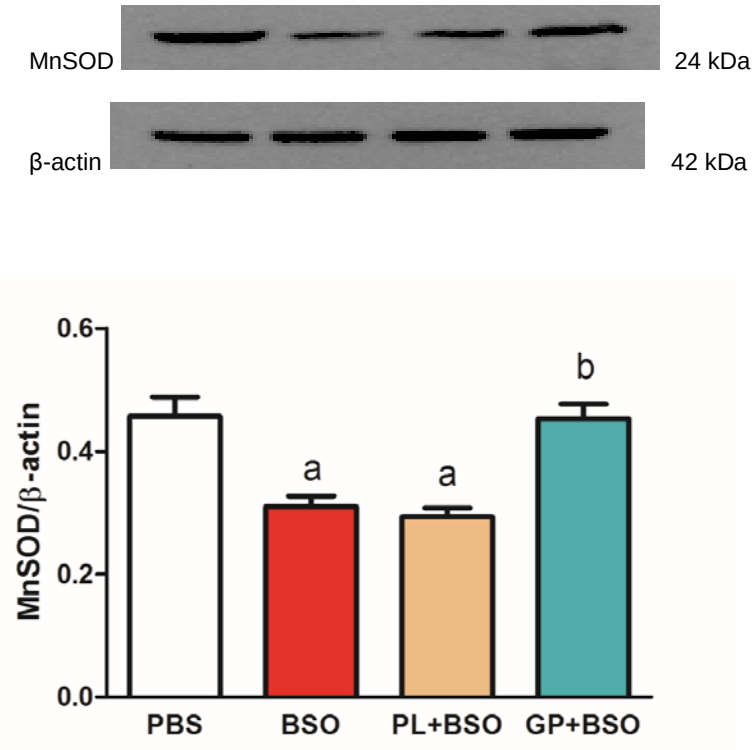
β-actin
42 kDa
GSR-1/β-actin
0.8
0.6
0.4
0.2
0.0
a
a
b
PBS
BSO
PL+BSO
GP+BSO

C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24 kDa
β-actin
42 kDa
MnSOD/β-actin
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0.4
0.2
0.0
a
a
b
PBS
BSO
PL+BSO
GP+BSO

D

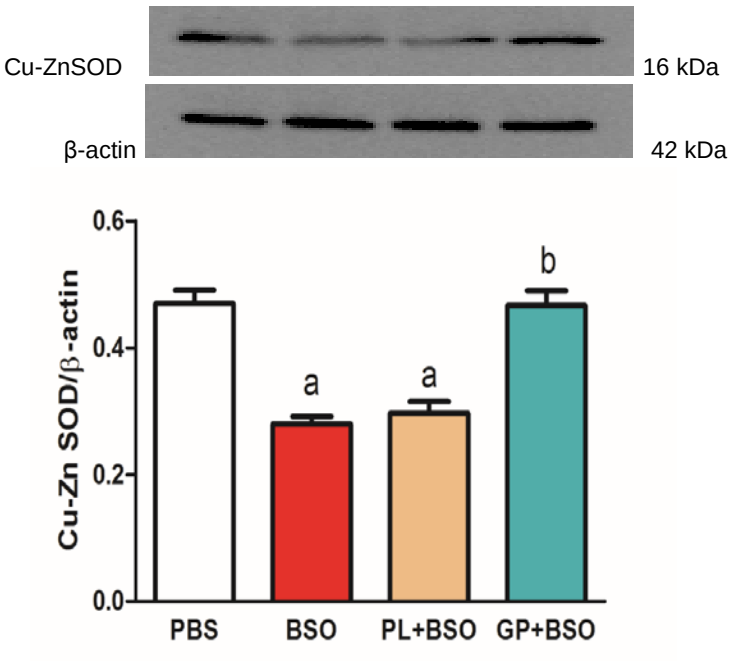


**Figure 29. Analysis of protective effects of grape powder on antioxidant protein levels by western blotting.** Grape powder treatment effectively protected BSO-induced decreased protein levels of (A) GLO-1, (B) GSR-1, (C) MnSOD, and (D) Cu-Zn SOD. PBS-control, BSO- 1mM BSO treated HT22 cells, PL+BSO-placebo treated HT22 cells prior to BSO treatment, GP+BSO-grape powder treated HT22 cells prior to BSO treatment. a=significantly different from PBS; b significantly different from BSO. $p < 0.05$. Values are mean $\pm$ SEM., n = 4-5.

# 5. DISCUSSION

## 5.1 Protective and reversal effects of grape powder treatment in social defeat-induced behavioral and cognitive deficits

Evidence from our lab suggests that direct induction of oxidative stress via pro-oxidant BSO results in behavioral and cognitive deficits in rats (Allam et al. 2013; Salim et al. 2010a; Salim et al. 2011a). On the other hand, induction of physical or psychological stress in rats also led to behavioral and cognitive deficits as well as increase in oxidative stress (Patki et al. 2013b; Patki et al. 2014; Solanki et al. 2015; Solanki et al. 2016). In separate studies from our laboratory protective effects of antioxidant tempol treatment and moderate treadmill exercise against BSO-induced oxidative stress-mediated anxiety-like behavior also was reported in rats (Allam et al. 2013; Salim et al. 2010b). These studies suggest a causal role of oxidative stress in direct/indirect induction of oxidative stress-mediated behavioral and cognitive deficits in rats. Furthermore, previous data from our lab reported that social defeat stress (7 encounters for 7 consecutive days) in rats produced behavioral and cognitive impairments (Patki et al. 2013b). In the same study we also reported increase in oxidative stress markers and depleted key antioxidants protein expression in the hippocampus and the amygdala of socially defeated rats. Therefore, it seems reasonable to test causal role of oxidative stress in behavioral and cognitive deficits in social defeat model of rats. Furthermore, if oxidative stress causes behavioral and cognitive deficits, then antioxidant treatment should prevent or reverse these behaviors. While observations with tempol, a synthetic antioxidant, are

significant, clinical use of tempol is limited due to stability issues and unknown side effects. Therefore, we focused on natural products such as grape powder with known antioxidant properties. In the past, we have reported that grape polyphenols mimic the anxiolytic effects of tempol in either direct or indirect induction of oxidative stress (Allam et al. 2013; Patki et al. 2013a; Solanki et al. 2015). However, whether grape polyphenols can reduce or eliminate social defeat-induced increase in oxidative stress and thereby provide protection from behavioral and cognitive deficits is unknown. Therefore, in the present study, protective and reversal effects of grape powder were vigorously tested in socially defeated rats.

In the present study, we employed the social defeat model to induce psychological stress in the male rats. The social defeat model is widely used to study social stress effects in male subjects, as females exhibit relatively little or no aggression within-sex fighting (Hollis and Kabbaj 2014). This limits the use of social defeat model in female subjects. Therefore, we chose male rat model of social defeat in the present study. In agreement with previous report from our laboratory, socially defeated rats exhibited increased anxiety levels as indicated by decreased time spent in the open arms of EPM, lit area of LD box and in the center of the OF arena. Interestingly, 3 weeks of grape powder treatment in socially defeated rats significantly improved anxiety-like behavior suggesting its anxiolytic effects. Moreover, socially defeated rats exhibited depression-like behavior as marked by increased time spent immobile in FST, which was successfully reversed and prevented with grape powder treatment indicating its

antidepressant-like effects. Interestingly, in the present study, control exposure (CE) rats, exposed to the resident's cage without the resident's presence, exhibited anxiety-like behavior on the LD and MB test and depression-like behavior on FST. These data suggest that exposure of rats to the psychogenic components such as fur, bedding and litter of the aggressive LE rats was equally stressful as social defeat in inducing behavioral deficits. Similar to this, others have also reported psychogenic stressors-induced anxiety-like behaviors in rodents (Adamec et al. 2006; Zoladz et al. 2008). Consistent with this, exposure of rats to natural or synthetic predator odors is also known to elicit behavioral deficits (Dielenberg and McGregor 2001). Such predator odor-induced behavioral deficits were associated with increased Fos expression in the olfactory and the hypothalamic system specifically in the amygdala, a regulator of fear and emotions and hypothalamus, respectively (Dielenberg et al. 2001). Perhaps, the psychogenic stress-induced hyper-activation of limbic and hypothalamic circuitry results in the activation of sympathetic tone leading to behavioral deficits in control exposure rats. Furthermore, in congruence with our previous report, socially defeated rats exhibited impaired short- and long-term memory in RAWM that was significantly improved with grape powder treatment suggesting its beneficial effects on learning and memory functions. In agreement with earlier reports, beneficial effects of grapes and grape components such as resveratrol have been reported in behavioral and cognitive deficits (Ge et al. 2015; Gocmez et al. 2016; Singleton et al. 2010; Sonmez et al. 2007). In addition, drugs with potent antioxidant properties have been reported to prevent anxiety-like behavior

(de Oliveira et al. 2007; Masood et al. 2008; Salim et al. 2011a; Solanki et al. 2016). The aforementioned studies used pharmacological induction of oxidative stress and antioxidant treatment via reducing or eliminating oxidative stress protected from behavioral deficits and cognitive impairments. Earlier, relevant to these observations, we have reported the protective role of grape powder in three different models of direct or indirect induction of oxidative stress (Allam et al. 2013; Patki et al. 2013a; Solanki et al. 2015). The hippocampus and amygdala seem to be the most susceptible brain regions to both direct and indirect induction of oxidative stress (Allam et al. 2013; Patki et al. 2013a; Solanki et al. 2015). Relevant to this, hippocampus and amygdala are implicated in anxiety disorders (Charney et al. 2002; Shin et al. 2006) and cognition (Femenia et al. 2012). Furthermore, the neural circuitry underlying the anxiety-like behavior includes coordinated activity within the amygdala, hippocampus, BNST and the PFC. The anxiety response is known to be regulated by reciprocal monosynaptic neuronal projections from the BLA to the hippocampus and the PFC with potential involvement of BNST to sustain the anxiety response (Calhoon and Tye 2015). In the present study, we suggest that social defeat-induced behavioral deficits could be attributed to increased activation of this monosynaptic circuitry following the exposure to social defeat stress.

Interestingly, higher vulnerability of the hippocampus and amygdala to social defeat-induced oxidative stress and breakdown of antioxidant defense system is evident from our results obtained from the present study. Therefore, it seems highly plausible that social defeat-induced oxidative stress in the brain

compromises the biochemical integrity of selected brain areas i.e. the hippocampus and amygdala. It is well known that the hippocampal dentate gyrus-CA3 system regulates structural plasticity, regenerative/remodeling capacity as well as neurogenesis factors such as BDNF (Popov and Bocharova 1992). It has also been suggested that the pyramidal cells of CA1 and CA3 and granule cells of DG are highly susceptible to oxidative damage (Sarnowska 2002; Vornov et al. 1998; Wilde et al. 1997). Thus, social defeat-induced oxidative damage of DG-CA function may diminish cell proliferation, impair remodeling capacity, alter structural plasticity and disrupt neurogenesis, collectively disturbing normal synaptic neurotransmission. And, oxidative stress-initiated neuroendocrine alterations within the amygdala including amygdalar hyperactivity and dendritic shrinking (Brown et al. 2005; Kreibich and Blendy 2004; Radley et al. 2006; Vyas et al. 2002; Wellman 2001; Wood et al. 2010) can further potentiate synaptic disturbances by disrupting the hippocampus-amygdala projections. Furthermore, free radicals are known to oxidize the extracellular sites of glutamatergic N-methyl-D-aspartate (NMDA) receptors resulting in the inhibition leading to attenuation of LTP and synaptic neurotransmission (Haxaire et al. 2012; Lee et al. 2012; Rai et al. 2013). Collectively, these events offer an attractive explanation for social defeat-induced behavioral and cognitive impairment. And, grape powder potentially by mitigating oxidative stress protected and reversed social defeat-induced behavioral and cognitive deficits.

## 5.2 Impact of grape powder treatment on protection and reversal against social defeat-induced biochemical impairments

Stress-induced activation of HPA axis is a pathological hallmark of anxiety and depression. HPA axis activation results in increased glucocorticoids levels specifically corticosterone. Therefore, increased plasma level of corticosterone is considered as a biomarker of stress (Pruett et al. 2008). Socially defeated rats had significantly increased plasma level of corticosterone. Furthermore, stress-induced increase in amygdalar dominance over the hippocampus results in increased activation of HPA axis (Izquierdo et al. 2006). Perhaps, in the present study, socially defeated rats had increased amygdalar activity with reduced inhibitory inputs from the hippocampus resulted in the hyperactive HPA axis. Interestingly, control exposure (CE) rats also had hyperactive HPA axis as indicated by elevated plasma corticosterone levels. Exposure to cues associated with predator is known to trigger psychogenic stress-induced endocrine response (Blanchard et al. 1998; Masini et al. 2005; Munoz-Abellan et al. 2011). It is likely that predator threat-induced hyperactive amygdala increases the paraventricular nucleus activity in the hypothalamus resulting in the activation of HPA axis in the CE rats. Oxidative stress is implicated in several neuropsychiatric conditions including anxiety and depression. Oxidative stress results when the balance between the free radical generation and defensive antioxidant mechanism is altered (Halliwell 2006). The excessive free radical generation or depleted antioxidant enzymes are thought to be the underlying cause for oxidative stress-induced anxiety and depression. Similar to our previous studies, in this study we

observed increased level of 8-isoprostane, a marker of oxidative stress, in plasma of socially defeated rats and reversal and protection from elevated oxidative stress with grape powder treatment suggesting its antioxidant effects (Allam et al. 2013; Patki et al. 2013b). Similar to what we observed with plasma corticosterone levels, plasma 8-isoprostane levels were found to be elevated in control exposure rats. Pertinent to this, elevated glucocorticoids-induced glutamate release is associated with mitochondrial dysfunction and increase in oxidative stress (Mitsui et al. 2002). Perhaps, psychogenic stress-induced increased glucocorticoids led to marked increase in oxidative stress potentially via promoting glutamate release. Moreover, we assessed the antioxidant protein levels in three key brain regions, namely hippocampus, amygdala and PFC. These brain regions are highly susceptible to oxidative stress and are implicated in anxiety, depression and cognitive impairments (Mathew and Charney 2008; Patki et al. 2013b; Solanki et al. 2015). In this study, we observed that the expression of 2 key antioxidant enzymes, GLO-1 and GSR-1 were reduced in the hippocampus and amygdala of socially defeated rats, which was reversed but not protected in amygdala with grape powder treatment. Furthermore, Cu-Zn SOD and Mn SOD expression were significantly decreased in socially defeated rats, which were restored in hippocampus and amygdala of rats, subjected to social stress. This data is in agreement with our previous studies wherein induction of oxidative stress via either pro-oxidant, estrogen deficiency or acute single prolonged stress (SPS) led to depletion of antioxidant enzymes in the hippocampus and the amygdala of rats which was reported to be restored with

grape powder treatment. Interestingly the expression of these proteins did not change in the PFC of socially defeated rats except for Mn SOD. Therefore, it seems that hippocampus and amygdala are the most susceptible regions to stress-induced oxidative damage followed by PFC. The rationale for existence of such regional differences is not clearly understood. Based on the literature it is clear that the hippocampus, amygdala and the PFC are implicated in anxiety disorders and cognitive deficits (Mathew and Charney 2008; Patki et al. 2013b; Solanki et al. 2015). However, under oxidative stress conditions which brain region is the first to undergo adaptive and architectural changes leading to behavioral impairment and consequently responsive to the nutritional intervention is difficult to comment on. Perhaps, the presence of specific circuitry within the hippocampus, the dentate gyrus-CA3 system, may attribute to the adaptive structural plasticity. Under the structural plasticity, the dentate gyrus is known for its regenerative capacity (Popov and Bocharova, 1992). Perhaps, social defeat-induced decreased cell proliferation within the dentate gyrus could impair the remodeling capacity. Such hippocampal neurogenesis is tightly regulated by various neurochemicals including BDNF (Czeh et al., 2001). Interestingly, in the past, we have reported decreased hippocampal BDNF expression in socially defeated rats (Patki et al., 2013). Overall, these studies suggest that social defeat-induced decreased neurogenesis within dentate gyrus-CA3 system could lead to hippocampal dysfunction resulting in cognitive deficits. Furthermore, chronic stress-induced inhibition of synapse formation within the hippocampal CA1 neurons is known (Pawlak et al., 2005; Shors et al., 2001). In addition to

hippocampus, the amygdala and PFC also exhibit dendritic alterations such as dendritic shrinking in response to chronic stress (Brown et al., 2005; Radley et al., 2006). Additionally, social defeat-induced hyperactive HPA and increased glucocorticoids result in reduced hippocampal function (Keeney, A, 2006). Interestingly, grape powder treatment significantly decreased the social defeat-induced increased corticosterone levels suggesting its protective effects on neuroendocrine system. In the past, we have reported increase in oxidative stress and concomitant decrease in antioxidant protein expression in the hippocampus and amygdala of socially defeated rats (Patki et al., 2013). Interestingly, in this study grape powder normalized social defeat-induced increased oxidative stress as evident by increased antioxidant protein expression in the hippocampus and amygdala of socially defeated rats suggesting its neuroprotective effects. It seems that grape power exerts its neuroprotective effects potentially via strengthening antioxidant defense mechanisms. Furthermore, co-occurrence of decreased antioxidant protein expression and reduced antioxidant enzyme activity under oxidative stress condition has been reported (Lih-Brody et al. 1996). Similar to this, here, GLO-1, total GSH and SOD antioxidant enzyme activity in plasma were found to be decreased in socially defeated rat which was reinstated with grape powder treatment. This data suggest that rats subjected to social defeat had failing antioxidant defense mechanism, which led to behavioral and cognitive deficits and grape powder by strengthening antioxidant defense system improved behavioral and cognitive alterations. Grape powder treatment not only decreased the oxidative stress

marker 8-isoprostane, increased antioxidant enzyme activity and restored the antioxidant protein expression in specific brain regions, but it also led to significant increase in the total antioxidant capacity. Grape powder potentially exerts its beneficial effects either via directly strengthening antioxidant defense system or by modulating oxidative stress pathway. While in this study, the beneficial effects of grape powder are potentially attributed to its antioxidant properties, we cannot rule out the possible involvement of anti-inflammatory activity of grape powder. Several lines of evidence suggest that social defeat in rats leads to concomitant increase in oxidative stress and pro-inflammatory cytokines (Patki et al. 2013b; Song and Wang 2011). Perhaps In the present study social defeat-induced behavioral deficits in rats could be attributed equally to increased oxidative stress and neuro-inflammation. Furthermore, natural polyphenols including resveratrol are known for their anti-inflammatory activity (Nichols and Katiyar 2010; Zhang et al. 2010). Resveratrol serves as NF-κB inhibitor and attenuates inflammatory responses (Csiszar et al. 2006; Ren et al. 2013). Perhaps in the present study, grape polyphenols served as anti-inflammatory agents and reduced neuro-inflammation specifically in the hippocampus and amygdala, the most susceptible brain regions under social defeat-induced oxidative stress conditions, potentially leading to correction of behavioral and cognitive deficits in socially defeated rats. Furthermore, studies on bioavailability of the grape polyphenols suggest that grape polyphenols cross blood-brain-barrier and reach to the site of action (Janle et al. 2010; Krikorian et al. 2010). In this study, we established that grape polyphenols reach the brain as

indicated by marked increase in total phenolic content in the hippocampus of the grape powder treated rats. Overall, these data suggest that upon reaching the brain, grape polyphenols decrease oxidative stress, strengthen antioxidant defense mechanisms and increase total antioxidant capacity thereby providing maximum protection against oxidative damage leading to improved behavioral and cognitive function in rats.

### 5.3 Investigating potential grape powder constituents responsible for beneficial effects

In this study, hippocampus was found to be highly vulnerable to social defeat-induced oxidative stress as indicated by decreased expression of key antioxidant enzymes and total antioxidant capacity in the hippocampus. While the reversal and protective effect of grape powder under elevated oxidative stress conditions such as social defeat is evident, the two questions still remained unanswered. *First,* which components of grape powder could be responsible for beneficial effects? *Second,* by what mechanism grape powder exerts the beneficial effects? To answer these questions, a series of experiments were performed using HT22 cell line by simulating elevated oxidative stress as observed in defeated rats. Grapes are rich in polyphenols such as anthocyanins, proanthocyanidins, resveratrol, quercetin and kaempferol. Among all the grape polyphenols, resveratrol, quercetin and kaempferol have garnered the attention of researchers for their potent antioxidant activity (Bouayed and Bohn 2010; Graf et al. 2005; P. Iacopinia 2008). Numerous in-vitro and in-vivo studies have reported the neuroprotective effects of resveratrol, quercetin and kaempferol

(Albani et al. 2009; Curin et al. 2006; Farooqui and Farooqui 2009; Ndiaye et al. 2005; Yang et al. 2013). Therefore, in this study using HT22 cells, we tested the effects of these three pure synthetic compounds on pro-oxidant (BSO)-induced oxidative stress and compared with grape powder. Similar to our previously reported work (Salvi et al. 2016), pro-oxidant BSO treatment for 14 hours at 1mM concentration significantly increased oxidative stress. Using this as a cell culture model of oxidative stress, HT22 cells were treated for 4 hours with either quercetin/resveratrol/kaempferol (1μM – 20μM concentrations) or grape powder (2400 μg/mL – 10000 μg/mL) prior to 1mM BSO treatment for 14 hours. The dose of polyphenols or grape powder was chosen based on the pilot studies performed in our lab and also by the others (Fukui et al. 2010; Yang et al. 2014). In this study, BSO-induced decreased total antioxidant capacity was normalized with 1μM resveratrol and 5μM quercetin that was similar to grape powder treatment suggesting its antioxidant effects. This is in agreement with other studies where resveratrol and quercetin were found to be protective at these concentrations against glutamate-induced oxidative stress (Fukui et al. 2010; Yang et al. 2013). Interestingly, in the present study, resveratrol was found to be most effective at the lowest concentration but not at higher concentrations in protecting BSO-induced decreased total antioxidant capacity. A dose-dependent decrease in the ability of resveratrol to protect against BSO-induced oxidative stress could be attributed to its potential toxicity as evident by increased number of detached cells/non-viable cells observed under the microscope. Pertinent to this, concentration-dependent inhibition of cell viability by resveratrol has been

reported in HT22 cells as well as in breast and prostate cancer cell lines (Hsieh 2009; Zhou et al. 2009). Although resveratrol is a weak apoptotic agent, it is known to induce apoptosis at higher concentrations potentially via inhibiting mitogen-activated protein kinase kinase (MEK) (Zhou et al. 2009). It is likely that, resveratrol-induced apoptosis at 20μM concentration causes decreased total antioxidant capacity against oxidative insult. Grape polyphenols are known to induce phase-II antioxidant enzymes via facilitating the binding between Nrf2 and ARE in the promoter region of several antioxidant genes (Yang and Xiao 2013). Moreover, polyphenols regulate various signaling pathways via acting on signaling proteins such as MAPK (Vanamala et al., 2011). MAPKs are also known to induce phase-II antioxidant enzymes. Perhaps, resveratrol and quercetin increased total antioxidant capacity after oxidative insult partly due to its ability to induce phase-II antioxidant enzymes potentially through its effects on MAPKs. While others have reported neuroprotective effects of kaempferol against oxidative stress, in our study, kaempferol failed to protect and maintain total antioxidant capacity of HT22 cells from oxidative insult at these concentrations. This observation could be partly due to the different mode of induction of oxidative stress. While others have used glutamate (Yang et al. 2014), we have used pro-oxidant BSO to induce oxidative stress. At higher concentrations, glutamate acts as neurotoxicant and induces oxidative stress via increase in intracellular ROS generation with concomitant decrease in total antioxidant capacity and regulation of expression of apoptosis-inducing factor and MAPK (Vyas et al. 2013; Yang et al. 2014). Kaempferol was found to protect

HT22 cells from glutamate-induced apoptosis. In contrast, in our model, BSO exerts its effects via disrupting glutathione synthesis (Marengo et al. 2008). Impaired glutathione synthesis results in poor detoxification of $H_2O_2$ leading to its accumulation in the cell (Dunning et al. 2013). Toxic concentrations of $H_2O_2$ is known to inhibit superoxide dismutase (SOD), an enzyme known to dismutase the highly reactive superoxide radical, leading to increased concentration of deleterious superoxide radical (Gottfredsen et al. 2013). Furthermore, cytosolic protein GLO-1 is known to be GSH-dependent antioxidant (Thornalley 2003). Therefore, functional glutathione system is likely to play a critical role in maintaining the redox homeostasis. In our oxidative stress model, BSO-induced impaired glutathione system could have rendered SOD and GLO-1 enzymes dysfunctional causing cumulative damage to the antioxidant defense system. Perhaps kaempferol failed to reinstate BSO-induced glutathione deficit in our model and therefore did not exert any beneficial effects. Furthermore, others have reported neuroprotective effects of kaempferol at the dose of 25μM-50μM that was higher than what we have used (1μM-20μM) in our study. Overall, these data suggest that beneficial effects of grape powder could be attributed to resveratrol and quercetin.

**5.4 Possible mechanism of grape powder on neuroprotection**

Based on our recent studies, it is safe to postulate that beneficial effects of grape powder are largely attributed to its antioxidant activity and ability to strengthen the antioxidant defense system. Pertinent to this, in this in-vitro study, we have observed significant decrease in the expression of key antioxidant

proteins after induction of oxidative stress via pro-oxidant BSO. Interestingly, in agreement with our data from social defeat model, in this study grape powder normalized the oxidative stress-induced decreased expression of antioxidant proteins suggesting its antioxidant effects. Others have also reported the beneficial role of grape powder either via regulating free radical generation (Campos-Esparza et al. 2009) and $Ca^{2+}$ concentrations (Ishige et al. 2001), mitochondrial dysfunction (Long et al. 2009) or regulation of sirtuins (Sirtuin-1) (Bruckbauer and Zemel 2014; Pandey and Rizvi 2014). However, the exact molecular mechanism of grape powder still remains unclear. Therefore, in this study we traced the oxidative stress pathway of cell death and investigated the potential molecular mechanism of protective effect of grape powder. Under oxidative stress conditions increased cellular $Ca^{2+}$ concentration triggers cell death. In our study, grape powder treatment normalized BSO-induced increase in cellular $Ca^{2+}$ concentration. Similar to this, others have reported protective effects of grape components such as resveratrol and kaempferol in glutamate-induced increased $Ca^{2+}$ concentrations (Fukui et al. 2010; Yang et al. 2014). Cellular Ca2+ signals are vital for various physiological processes, cell injury and apoptosis (Smaili et al. 2000). Though mitochondria serve as a regulator of intercellular Ca2+ signals, increased $Ca^{2+}$ concentrations result in mitochondrial dysfunction leading to impaired mitochondrial membrane potential and opening of mitochondrial permeability transition pore (Carraro and Bernardi 2016). In congruence with the existing knowledge, in this study 15 hours of pro-oxidant BSO treatment induced mitochondrial dysfunction as demonstrated by impaired

mitochondrial membrane potential in HT22 cells. And, mitochondrial impairment was prevented with 4 hours of grape powder treatment. Furthermore, mitochondrial intermembrane space is occupied by cytochrome c. Several stress signals including oxidative stress is known to induce release of cytochrome c. Once in the plasma, cytochrome c regulates the activation of apoptosis-inducing factor-1 (AIF-1), which serves as a precursor for activation of caspases. Activation of caspases is marked as a signal for cell death (Garrido et al. 2006). Pertinent to this, 15 hours of BSO treatment significantly impaired mitochondrial function and activated caspase-3 in our study. Interestingly, grape powder found to be neuroprotective by preventing BSO-induced elevation in $Ca^{2+}$ levels, mediated mitochondrial impairment and subsequently prevented cell death.

# 6. SUMMARY AND CONCLUSIONS

1. In the present study, we examined the protective and reversal effects of grape powder in a rat model of social defeat. We employed the chronic model of psychological stress (social defeat) where Sprague-Dawley rats were exposed to aggressive encounters by LE rats for 7 consecutive days. Socially defeated rats exhibited anxiety- and depression-like behaviors as well as cognitive impairment.
2. In two separate studies, socially defeated rats were treated with grape powder for 3 weeks either before (protection) or after (reversal) the defeat exposure. Grape powder treatment successfully prevented and reversed social defeat-induced anxiety- and depression-like behavior and cognitive impairment in rats.
3. At the biochemical level, social defeat stress in rats resulted in elevated levels of plasma corticosterone suggesting stress-induced activation of hypothalamic-pituitary-adrenal (HPA) axis. Interestingly, grape powder treatment restored corticosterone to normal levels in socially defeated rats. This data suggests protective and reversal effects of grape powder on neuroendocrine system. Furthermore, we found elevated plasma levels of oxidative stress marker 8-isoprostane in socially defeated rats, which returned to normal levels with grape powder treatment. Overall, this data suggest that grape powder used in this study exhibits strong in-vivo antioxidant activity.

4. At the molecular level, expression of key antioxidant enzymes GLO-1, GSR-1, Mn SOD and Cu-Zn SOD were found to be significantly decreased in the hippocampus and amygdala of socially defeated rats. And, decreased antioxidant levels were restored to normal levels with grape powder treatment. These data also indicated that the hippocampus and the amygdala of socially defeated rats exhibited impairment of antioxidant defense system and hence were deficient in combating social defeat-induced oxidative stress. Furthermore, decreased activity of these antioxidants in plasma was normalized with grape powder treatment. Social defeat-induced increased oxidative stress in the brain and poor antioxidant defense system specifically within the hippocampus and the amygdala could be responsible for causing behavioral and cognitive deficits in rats.
5. Total antioxidant capacity was significantly decreased in the plasma and the hippocampus of socially defeated rats suggesting that elevated levels of oxidative stress supersede/overwhelm total antioxidant capacity. Grape powder treatment normalized the total antioxidant capacity in the plasma and hippocampus suggesting its antioxidant effect in the brain as well as in the periphery. Grape powder most likely delivers its antioxidant contents and replenishes stress-induced depleted antioxidant pool.
6. Estimation of total phenolic content in the hippocampus of rats treated with or without grape powder revealed that rats treated with grape powder

exhibited elevated levels of polyphenols suggesting that grape polyphenols reach their site of action in the brain.

7. We successfully mimicked social defeat-induced increased oxidative stress condition in HT22 cells, a hippocampal derived cell line, using pro-oxidant BSO. Using this simulated model of oxidative stress, we established that protective effects of grape powder were most likely attributed to resveratrol and quercetin, as evident from their ability to protect total antioxidant capacity of HT22 cells from BSO-induced oxidative stress.
8. We also investigated the potential underlying mechanism for neuroprotective effects of grape powder using the HT22 model of oxidative stress. Data revealed that grape powder normalized oxidative stress-induced increased $Ca^{2+}$ concentrations, prevented mitochondrial damage and inhibited the cascade of apoptotic events leading to cell death.
9. Altogether, behavioral, biochemical and in-vitro findings indicate that grape powder treatment reversed and protected social defeat-induced behavioral and cognitive deficits potentially via inhibiting oxidative stress pathway of cell death as depicted in Figure 30. And beneficial effects of grape powder could be attributed to two grape components, resveratrol and quercetin. Thus, daily moderate grape powder consumption may serve as a useful adjuvant therapy for chronic conditions such as psychological stress-induced anxiety and depression.

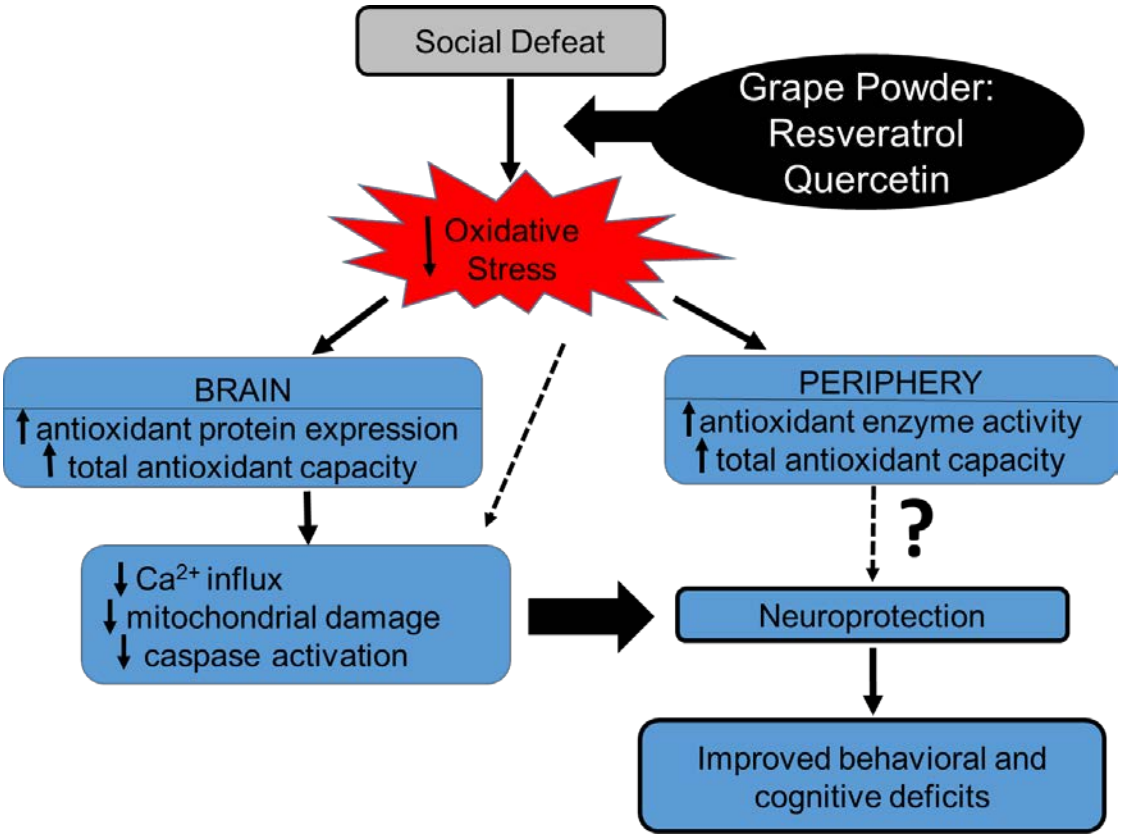


**Figure 30. Schematic representation of the events potentially responsible for the beneficial effects of grape powder in social defeat-induced behavioral and cognitive impairments.**

# 7. APPENDIX

| **Estimated average daily consumption of grape powder: ~ 400 mg/day** | |
|---|---|
| **Grape constituents** | **Average consumption/day** |
| Catechin | 0.0145 mg |
| Epicatechin | 0.0083 mg |
| Peonidin | 0.0153 mg |
| Cyanidin | 0.1064 mg |
| Malvidin | 0.1046 mg |
| Quercetin | 0.0064 mg |
| Kaempferol | 0.00136 mg |
| Isorhamnetin | 0.0014 mg |
| Resveratrol | 0.00072 mg |
| Total Polyphenols | 1.792 mg |

**Table 5. Summary of important information regarding estimated average daily consumption of grape powder and its constituents.** This table extends from page 59.

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