Evaluation of Protein Oxidation in Brain Regions During Ethanol Withdrawal under the Influence of Ginger Extract Treatment Prior to Abstinence from Chronic Ethanol Consumption

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Abstract: Ethanol withdrawal is linked to elevated oxidative damage to neurons. Protein peroxidation indices serve as indicative markers for the oxidative stress within the cell. Elevated level of protein carbonyls is generally a sign of oxidative stress and moreover a sign of disease derived protein dysfunction. Here we report our findings on the contribution of ginger extract to counterbalance sudden ethanol withdrawal-initiated oxidative events in different rat brain regions. We showed that ethanol withdrawal after 72h after 6 weeks of chronic exposure provoked greater levels of oxidative damage than the preceding ethanol exposure. Ginger extract treatment either during ethanol exposure or ethanol withdrawal, however, reversed oxidative damage, as demonstrated by the restored protein levels and significantly reduced protein carbonylation, compared to ginger extract untreated groups. In addition, the efficacy of the extract was also supported by in vitro studies of the antioxidant properties of the extract. The findings implies that free-radical scavenging may not be the only mechanism to be responsible for the observed neuroprotection and warrants further studies to establish the efficiency and role of the each of the individual components of ginger against EW-related neuronal oxidative stress.

Key words: Free Radicals • Proteins • Peroxidation • Ethanol Withdrawal • Oxidative Stress • Ginger

INTRODUCTION

Unmanaged sudden withdrawal from the excessive consumption of alcohol (ethanol) adversely alters neuronal integrity in vulnerable brain regions such as the cerebellum, hippocampus or cortex. In addition to well known hyperexcitatory neurotransmissions, ethanol withdrawal (EW) provokes the intense generation of reactive oxygen species (ROS) and the activation of stress-responding protein kinases. Ethanol withdrawal is linked to elevated oxidative damage to neurons. EW also inflicts mitochondrial membranes/membrane potential, perturbs redox balance and suppresses mitochondrial enzymes, all of which impair a fundamental function of brain. Moreover, EW acts as a prooxidant-provoking stressor which is implicated in serious cognitive disabilities and neuronal cell damage.

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The protein carbonyl content was increased in the blood of ethanol-dependent patients [10] and in the liver or pancreas of ethanol exposed rats and mice [11-13]. Similarly, an increased content of protein carbonyls was observed in the cerebellum, cortex and hippocampus of ethanol consuming rats [14]. Importantly, the level of protein carbonyls was further increased during EW [14], suggesting that abrupt EW produces more severe protein oxidation than ethanol per se.

In spite of the fact that synthetic drugs such as Benzodiazepines are the most important therapeutic agents known to medicine in treating EW, researchers have been making efforts to find substances from plant sources for the treatment of alcohol withdrawal. The herbal drugs have been prescribed widely because of their effectiveness, fewer side effects and relatively low cost. Several studies revealed the beneficial effects of medicinal plants like ginger. Recent scientific investigation and clinical studies had confirmed the efficacy of some medicinal plants and herbal preparations in the improvement of addiction related disorders.

Ginger (Zingiber officinale, ZO) is one of the most widely consumed spices for the flavoring of food worldwide [15]. It has been that, ginger had reported several beneficial pharmacological effects such as hypoglycemic, insulinotropic and hypolipidemic activities in humans [16, 17] and in experimental animals [18, 19]. The major chemical constituents of ginger rhizome are essential volatile oil and non-volatile pungent compounds, such as gingerols, shogaols, paradols and zingerone [20]. Ginger has enormous health-promoting potential effects as an evident when using it for treating a number of ailments including degenerative disorders (arthritis and rheumatism), digestive health (indigestion and constipation), cardiovascular disorders (atherosclerosis and hypertension), diabetes mellitus and cancer. Also it has anti-inflammatory properties and these properties are beneficial in controlling the process of aging, as well as it is recommended for sore throat and vomiting. Moreover, it has antimicrobial potential, which can help in treating infectious diseases and helmintiasis [21-25]. The health-promoting perspective of ginger is often attributed to its rich phytochemistry [21].

In this study a comprehensive evaluation of the role of ZO in curtailing of alcohol withdrawal stress mediated oxidative product formation has been elucidated through detailed studies with respect to it’s in vitro antioxidant property. The cerebellum, cortex and hippocampus were specifically examined because of the known susceptibility of these brain regions to ethanol and EW insult and to oxidative stress [26-28].

MATERIAL AND METHODS

Procurement of Chemicals and Animals

Drugs and Chemicals: Standards like Gallic acid, Alpha Lipoic acid, Ascorbic acid, Citric acid and BSA were purchased from Sigma Chemicals (St. Louis, MO, USA). All other chemicals used are of analytical grade unless otherwise mentioned.

Collection of Plant Material: Aqueous ginger extract is prepared from locally available ginger roots. Ginger rhizomes were purchased fresh from the local markets and authenticated by the staff, Department of Botany, Sri Venkateswara University, Tirupati, India.

Extract Preparation: Whole rhizome of ginger is thoroughly washed, sliced, grated and grind to fine paste. A weighed quantity (30g) of the paste is subjected to continuous hot extraction in soxhlet apparatus using double distilled water. The extract is evaporated under reduced pressure using rotary evaporator and then lyophilized to give an extract sample and stored at 4°C for further studies.

Animals: The study involved young (3–4 months old; 200 - 220g) male albino rats of wistar strain purchased from Sri Venkateswara Traders Pvt. Limited, Bangalore, maintained in the animal house of the department in polypropylene cages. Standard conditions of humidity (50±9% relative humidity), room temperature (25-28°C) and 12 h light/ dark cycle (6:00 AM to 6:00 PM) are maintained. A standard rodent diet (M/s Hindustan Lever Ltd., Mumbai) and water were provided ad libitum. All experimental procedures were approved by the CPCSEA on Animal Care, Govt. of India, bearing the CPCSEA No. 438 / 01/a / CPCSEA / IAEC / SVU / KSR-1 (dt: 11.09.2008).

Treatment Protocol: After a two week adaptation period, animals were divided six per group and grouping involved 3 batches, first batch includes control group given normal saline. Second batch involves two groups of rats given 20% Alcohol(p.o.) at a dose of 2gm/kg body weight and another group is set to receive alcohol at same dose along with ginger extract (200 mg/kg body weight) while third batch is also treated the same way as the second batch except that they are allowed for withdrawal. All the treatments are carried out for 6 weeks with the exception that withdrawal groups were allowed for 3 days of withdrawal from the drug after the last dose.
Tissue Collection: After the experimental period, the animals were sacrificed by cervical dislocation. The whole brain is removed, washed with ice-cold saline, blotted, dried in liquid nitrogen and immediately transferred to the ice chamber at -80°C. Cerebrum (CC), Hippocampus (HC), Pons Medulla (PM) and Cerebellum (CB) were separated as described by Nayak and Chatterjee [29].

Biochemical Assays
Preparation of Synaptosomes: Synaptosomal fraction is prepared from the brain homogenate following the method of Hajos [30]. In brief the preparation is as follows, after sacrifice, the brain regions were dissected out on ice. The tissue is minced and homogenized gently in 10 volumes w/v of ice-cold 0.32M sucrose with a Teflon homogenizer. After centrifugation of the homogenate at 1000g for 10 min at 4°C, the supernatant is again centrifuged at 12,500g for 20 min. The pellet is finally resuspended in 10 volumes of 0.32M sucrose and used as the crude synaptosomal fraction.

In vitro Antioxidant Studies
Peroxynitrite Scavenging Assay: Peroxynitrite (ONOO⁻) is synthesized by the method described by Beckman et al., [31] and Evans Blue bleaching assay is used to measure peroxynitrite scavenging activity. The assay is performed by a standard method of Bailly et al., [32]. Gallic acid is used as the reference compound.

Singlet Oxygen Scavenging Assay: The production of singlet oxygen (¹O) is determined by monitoring N, N-dimethyl-4-nitrosoaniline (RNO) bleaching, using a previously reported spectrophotometric method [33] and compared with Lipoic acid, used as a reference compound.

Hypochlorous Acid Scavenging Assay: Hypochlorous acid (HOCl) assay is carried out as described by Aruoma and Halliwell[34] against Ascorbic acid, a potent HOCl scavenger, that is used as a reference [35].

Protein metabolism
Estimation of Protein profiles: The total protein content is estimated by the method of Lowry et al., [36].

Estimation of Protein peroxidation Indices: Protein Oxidation is evaluated by measuring Protein Carbonyls (PC), a marker of free radical activity according to Lenz et al., [37] and Levine et al., [38]. Protein Thiol content (P-SH) is measured spectrophotometrically using 5,5-dithiobis (2-nitrosobenzoic acid) (DTNB) as described by Ellman [39].

Data Analysis: Statistical analyses are carried out by one-way ANOVA with multiple comparison measurements used to assess the statistical significance of effects using the Statistical Package for Social Sciences (SPSS) software 12.0. Scheffe’ post hoc analyses were used when appropriate.

RESULTS

In vitro antioxidant studies are given from Figures A1-A3.

Peroxynitrite Scavenging Assay: From the peroxynitrite anion scavenging activity of ginger extract and the standard Gallic Acid it is found that the extract has an IC₅₀ = 391.05±32.25 µg/ml. The IC₅₀ value of the standard is 464.05±56.96 µg/ml (Fig A1).

Singlet Oxygen Scavenging Assay: Effects of Gingerroot extract and the standard Lipoic Acid on the scavenging of singlet oxygen has been studied. IC₅₀ of Alpha Lipoic acid is found to be 76.45±5.36 µg/ml. The IC₅₀ value of the ginger extract is 57.69±1.16 µg/ml (Fig A2).

Fig. A1: Peroxynitrite Scavenging Assay of Zingiber officinale extract and the standard Gallic Acid. Each value represents mean±S.D. (n = 6).
Fig. A2: Singlet Oxygen Scavenging Assay of *Zingiber officinale* extract and the standard ALA. The results are mean±S.D. of six parallel measurements.

![Singlet Oxygen Scavenging Assay](image)

Fig. A3: Hypochlorous acid scavenging capacity of *Zingiber officinale* and the standard Citric acid. All data is expressed as mean±S.D. (n = 6).

![Hypochlorous Acid Scavenging Assay](image)

**Hypochlorous Acid Scavenging Assay:** The Hypochlorous acid scavenging activities of Ginger extract and the standard Ascorbic Acid predict an IC₅₀ of 47.99±6.26µg/ml and 148±5.75 µg/ml for the extract and Ascorbic Acid respectively (Fig A3).

**Protein Metabolism:** The changes in protein profiles during withdrawal stress can be summarized as follows.

**Protein Levels:** Total proteins decreased with significant changes in different regions of rat brain during ethanol-induced withdrawal. An increase in the levels of different protein fractions are recorded during pre-treatment with *ZO* extract compared with withdrawal group alone.

During ethanol-induced withdrawal, the decrease in the structural protein content is as follows.

Protein: HC (-12.98) > CC (-9.95) > PM (-7.63) > CB (-6.07).

The total protein content is increased in all the brain regions during treatment with aqueous *ZO* extract, whereas, pre-treatment with *ZO* extract and antioxidant combination resulted in a significant non depletion in the structural protein content in all the brain regions (Fig B1).

**Protein Carbonyls:** The changes in the protein Carbonyl content in different regions of rat brain during EtOH-induced withdrawal and pre-treatment with aqueous extract of Ginger is represented in Fig C1.

The percent change of exaggeration in the protein Carbonyl content during EtOH-induced withdrawal when compared with ethanol (Positive control) can be represented as:

PC: HC (55.078) > PM (49.636) > CC (48.797) > CB (46.576)

Protein Carbonyl levels are significantly decreased in all the brain regions during treatment with ginger extract.

**Protein Thiols:** The changes in the protein thiol content in different regions of rat brain during EtOH-induced withdrawal and pre-treatment with aqueous extracts of Ginger is given in Fig D1.
Fig. B1: The changes in the structural protein content in different regions of rat brain during EW and pre-treatment with extract of *Zingiber officinale*
All the values are mean ± SD of six individual observations
*Significant at *P* < 0.001, ** significant at *P* < 0.01, ***significant at *P* < 0.05 in comparison with the control.

Fig. C1: The changes in the protein Carbonyl content in different regions of rat brain during EtOH induced withdrawal and pre-treatment with aqueous extract of *Zingiber officinale*
All the values are mean ± SD of six individual observations
*Significant at *P* < 0.001, ** significant at *P* < 0.01, ***significant at *P* < 0.05 in comparison with the control.

Fig. D1: The changes in the protein thiol content in different regions of rat brain during EtOH induced withdrawal and pre-treatment with aqueous extract of *Zingiber officinale*
All the values are mean ± SD of six individual observations
*Significant at *P* < 0.001, ** significant at *P* < 0.01, ***significant at *P* < 0.05 in comparison with the control.

The percent change of depletion in the protein thiol content during EtOH-induced withdrawal when compared with ethanol (Positive control) can be represented as:

Thiols: CB (-54.23) > HC (-42.63) > CC (-38.81) > PM (-29.42)

**DISCUSSION**

EW stimulates the pathways of unstable oxidative molecules and stress-responding signalling proteins *viz.* PKC and P38. While ethanol itself directly generates ROS during its metabolism, EW produces oxidative stress
indirectly through the excitatory neurotransmitter system or excitatory molecules such as Ca²⁺ [40]. The mechanisms by which EW provokes oxidative stress/damage induced cellular injury is often associated with oxidative insults [41, 42] including EW-induced cellular oxidation [43]. The oxidative stress was more severe during EW than ethanol exposure per se and was accompanied with cell death [44]. Taken together, these findings suggest that EW induces destructive oxidative stress both in vivo and in vitro conditions.

The oxidative damage to proteins is manifested by increase in levels of protein carbonyls (PCO) [45] and decrease in levels of protein thiols (PSH) [46]. MDA, which is an end product of lipid peroxidation, is also known to cross-bind or induce secondary oxidative damage in the plasma proteins[47]. Thiol compounds, such as glutathione (GSH), cysteine (CSH) and homocysteine (HCSH) form a natural reservoir of the reductive capacity of the cell. Thiols in vivo function as components of the intracellular and extracellular redox buffer. A characteristic hallmark of many pathophysiologic conditions is a decrease in the GSH: GSSG ratio. A diminished cellular GSH level accompanies multifarious pathological states such as diabetes, alcoholism, immunological diseases, gastric erosions, cataract, neurological diseases, malnutrition and has also been observed during aging [48-54].

So this comparison explained that the ethanol treated and withdrawal animal groups displayed a significant dramatic, drastic and random alterations in their protein levels and protein carbonylation pointing to the fact that ethanol and ethanol withdrawal treatments severely damage the biological system and result in induced stress that disintegrates the membrane properties and also imbalances the antioxidant systems/levels vital for the neural conduction and no significant changes are observed in groups that are efficient scavenger of HOCl. The oxidative stress was more severe during EW than ethanol exposure per se and was accompanied with cell death [44]. Taken together, these findings suggest that EW induces destructive oxidative stress both in vivo and in vitro conditions.

Studies indicate that Z. officinale hizome extract contains significant amounts of flavonoids and phenolic compounds. Both these classes of compounds have good antioxidant potential and their effects on human nutrition and health are quite considerable. The mechanism of action of flavonoids is through scavenging or chelation [65]. Phenolic compounds are also very important plant constituents because their hydroxyl groups impart scavenging ability [66]. Polyphenolic compounds are known to have antioxidant activity and it is very much likely that the neuroprotective antioxidant activity of the extract is due to these compounds [67-72]. This activity is assumed to be mainly due to their redox properties, which plays a significant role in adsorbing and neutralizing free radical formation. The oxidative stress was more severe during EW than ethanol exposure per se and was accompanied with cell death [44]. Taken together, these findings suggest that EW induces destructive oxidative stress both in vivo and in vitro conditions.
radicals, quenching singlet and triplet oxygen, or decomposing peroxides [73, 74]. Two of ginger's most important antioxidants, curcumin and gingerol, have been shown to inhibit and even reverse the deposition of the amyloid plaques in the brain that are associated with Alzheimer's disease [75]. Moreover, research has established that zingerone, another of ginger's antioxidants, neutralizes the powerful oxidant, peroxynitrite, which has also been implicated as an aggravating factor in Alzheimer's and other neurodegenerative diseases [76]. Ginger's prominent role in reducing lipid oxidation by enhancing the activities of crucial internally produced antioxidants, such as superoxide dismutase has been validated by contemporary research. In particular, melatonin found in ginger [77, 78] is not only a highly effective free-radical scavenger itself, but also stimulates production of the main antioxidant enzyme of the brain, glutathione peroxidase [79].

CONCLUSION

It is concluded that oral administration of aqueous ginger extract along during chronic ethanol ingestion significantly ameliorates ethanol-induced protein peroxidation in both the cases of ethanol induced and ethanol withdrawal induced oxidative stress, possibly by the antioxidant protective properties of polyphenol and flavonoid components of the extract thereby increasing the antioxidative defence mechanism of the cells.

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REFERENCES


