ANTIOXIDANT STATUS IN KIDNEY AND LIVER OF RATS DURING IMMOBILIZATION STRESS AND TREATED WITH CENTELLA ASIATICA (Linn.,)

Arumugam Sarumathi, Nadanam Saravanan*
Department of Biochemistry and Biotechnology, Faculty of Science, Annamalai University, Annamalainagar - 608 002, Tamilnadu, India.

*Division of Biochemistry, Rani Meyyammai College of Nursing, Faculty of Medicine, Annamalai University, Annamalainagar - 608 002, Tamilnadu, India.
E-mail: saravanan_74@rediffmail.com, sarusaran_sss@yahoo.com

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Abstract
Exposure to stress stimulates free radical production thereby reduces the antioxidant system. There exists an imbalance between free radical production and antioxidant defense mechanism, which may lead to cell death during stress. Our study was designed to determine whether extract of Centella asiatica (C.A), an antioxidant, when administered orally (200 mg/kg body weight/day) for 21 days would prevent stress induced changes in antioxidant defense system.

Keywords: Immobilization stress; Centella asiatica; antioxidants.

1. Introduction
Exposure to stressful situations is among the most common human experiences. It is reported that exposure to stress can stimulate many pathways leading to increased production of the oxygen free radicals [1, 2, 3]. These are formed in human body both in physiological and pathological conditions in cytosol, mitochondria, lysosomes, peroxisomes and plasma membrane [4]. Psychological stress is associated with increased reactive oxygen species (ROS) production and oxidative damage and long term exposure to psychological stressors may enhance the risk of many diseases like atherosclerosis, diabetes, rheumatoid arthritis and liver diseases [2, 5, 6]. Stress plays a potential role in aggravating liver diseases viz., hepatic fibrosis and cirrhosis [7]. Other types of stressors are also known to alter antioxidant status and cause tissue damage in experimental animals. For instance, in rats, immobilization for 6 h induced severe bleeding in the stomach and a significant increase in plasma levels of thiobarbituric acid [8]; immobilization for 8 h [9] or 6 h [10] caused a significant increase in lipid peroxidation (LPO), oxidative damage of nuclear DNA, decrease in number of neutrophils and activity of catalase (CAT) and superoxide dismutase (SOD); restraint for 6 h resulted in reduction in the level of reduced glutathione (GSH), oxygen radical absorbance and activity of SOD, glutathione-S-transferase activity (GST) [11]; 48 h cold stress (4°C) caused a significant increase in CAT, glutathione reductase (GR) and GSH and decreased SOD activity [12]. In these studies alterations in the antioxidant status or oxidative damage have been studied after exposure to a single stressor. It is possible that human beings or animals are exposed to several stressors within a day. Whether exposure to a stressor after an initial stressful experience further alters antioxidant status and thereby enhances oxidative damage or the system gets habituated to stress exposure remains to be understood. To protect cells against oxidative damage by oxidants, produced during the oxygen metabolism, an antioxidant system has presumably evolved in aerobic organisms [13]. Major antioxidants like SOD, CAT, GSH, GPx, ascorbic acid, α tocopherol are important for cellular protection due to their ability to detoxify free radicals, such as reactive oxygen species (ROS) [14]. Various synthetic antioxidants have been used, which restricted the use of natural antioxidants as in food, and were proven to be carcinogen [15]. Potential antioxidant therapy should, therefore, include either natural antioxidant or agents, which are capable of augmenting the function of these enzymes [16]. Thus, a need for identifying alternative natural and safer sources of food antioxidant is created. Therefore, search for natural antioxidants, especially of plant origin, has notably increased in recent years [17]. And, none has attempted evaluating therapeutic intervention with the natural antioxidant like Centella asiatica.
asiatica (C.A) in immobilization stress conditions. Phytochemicals have long been recognized to possess many properties including antioxidant, anti allergic, anti inflammatory, antiviral, antiproliferative and anticarcinogenic effects [18]. C.A (L) urban, synonym Hydrocotyle asiatica, belongs to the family Apiaceae and is found almost all over the world. In Ayurveda, an Indian system of medicine, this is used in the management of central nervous system, skin and gastrointestinal disorder. The major principles in the plant are the polyphenols [19] and triterpenes [20]. C.A has been shown to improve memory, general mental ability of mentally retarded children [21]. It was also shown to have wound healing property [22], anticancer property [23], antioxidant property [19] and is also shown to have antitumoric property [24]. Therefore, our study was concentrated on the role of C.A in augmenting the functions of antioxidants in stress condition.

2. Materials and method

2.1 Experimental animals

Eight-week-old adult male albino rats of Wistar strain, weighing approximately 160 to 180 g, were acclimatized for 7 days at room temperature (25 ± 3°C) and relative humidity (55%) in a 12-hour light/dark cycle in a room under hygienic condition. The animals reared in Central Animal House, Department of Experimental Medicine, Rajah Muthiah Medical College, Annamalai University were used for the experiment. Male animals were used throughout the investigation to avoid complications due to the estrous cycle. The animals were allowed free access to water and standard pellet diet (Amrut Laboratory Animal Feed, Pranav Agro Industries Ltd., Bangalore, India). Animal handling and experimental procedures were approved by the Institutional Animal Ethics Committee, Annamalai University (Registration Number: 166/1999/CPCSEA, Proposal No. 543) and animals were cared in accordance with the guidelines by the “Committee for the purpose of control and supervision on experimental animals” (CPCSEA, 2004).

2.2 Plant Collection and Identification

The fresh leaves of CA leaves were collected in Thanjavur, Tamilnadu, INDIA. The accuracy of plant selection was proved and authenticated by Department of Botany, Annamalai University, Tamilnadu, India.

2.3 Extraction

The fresh leaves of the plant were air-dried at 40°C and ground to powder, which was then subjected to exhaustive extraction using water in a Soxhlet apparatus. The dark green liquid extract was concentrated under vacuum and the resulting dried extract was lyophilized and preserved in a refrigerator at 4°C until use in the experiments.

2.4 Induction of Stress.

Stress was induced in rats by placing the animals in 20 cm × 7 cm plastic tubes for 2 h/day for 21 days. There are several 3mm holes at the far end of the tubes for breathing that allows ample air but animals will be unable to move [25].

2.5 Experimental design

The rats were randomly divided into four groups with six rats each. The test extract (C.A) was completely dissolved in Water to the volume of 10 ml.

- **Group I**: Control rats
- **Group II**: Control+ C.A (200 mg/kg bw, for 21 days)
- **Group III**: Stress
- **Group IV**: Stress + C.A (200 mg/kg bw, for 21 days)

The total duration of the study was 21 days. On 21th day, the rats were sacrificed by cervical dislocation. Blood samples were collected in a dry test tube and allowed to coagulate at ambient temperature for 40 min. Serum was separated by centrifugation at 2000 rpm for 10 min. The tissues was excised immediately, washed in chilled isotonic saline and used for the various parameters analysis.

2.6 Assay of superoxide dismutase (SOD, EC. 1.15.1.1)

The activity of SOD was assayed by the method of Kakkar et al. [26]. The assay is based on the inhibition of the formation of NADH-phenazine methosulphate-nitroblue tetrazolium formazone. The reaction was initiated by the addition of NADH. After incubation for 90 s adding glacial acetic acid stopped the reaction. The colour developed at the end of the reaction was extracted into n-butanol layer and measured. The enzyme concentration required to inhibit the chromogen produced by 50% in one min under standard conditions was taken as one unit. (Units/min/mg protein for tissue).

2.7 Estimation of catalase (CAT, EC 1.11.1.6)

The activity of CAT in the tissues was determined by the

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**Table 1** Level of SOD, CAT and GPx of liver and kidney in control and experimental animals

<table>
<thead>
<tr>
<th>Groups</th>
<th>Liver (U/mg protein)</th>
<th>Kidney (U/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SOD U***</td>
<td>CAT U***</td>
</tr>
<tr>
<td></td>
<td>Stress</td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>3.84 ± 0.18a</td>
<td>5.73 ± 1.20a</td>
</tr>
<tr>
<td>Stress</td>
<td>1.48 ± 0.06b</td>
<td>39.67 ± 0.84b</td>
</tr>
<tr>
<td>Stress + C.A</td>
<td>2.85 ± 0.08a</td>
<td>52.74 ± 1.17a</td>
</tr>
<tr>
<td>Stress + C.A</td>
<td>2.92 ± 0.17a</td>
<td>53.02 ± 1.62a</td>
</tr>
</tbody>
</table>

**Table 2** Level of GSH, Vit C and Vit E of liver and kidney in control and experimental animals

<table>
<thead>
<tr>
<th>Groups</th>
<th>Liver (U/mg protein)</th>
<th>Kidney (U/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SOD U***</td>
<td>Vit C U***</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>35.65 ± 2.13a</td>
<td>1.38 ± 0.14a</td>
</tr>
<tr>
<td>Stress</td>
<td>28.59 ± 0.55b</td>
<td>1.12 ± 0.08b</td>
</tr>
<tr>
<td>Stress + C.A</td>
<td>35.15 ± 0.91a</td>
<td>1.28 ± 0.14a</td>
</tr>
<tr>
<td>Stress + C.A</td>
<td>34.33 ± 1.13a</td>
<td>1.28 ± 0.12a</td>
</tr>
</tbody>
</table>

**Table 3** Level of SOD, CAT and GPx of liver and kidney in control and experimental animals

<table>
<thead>
<tr>
<th>Groups</th>
<th>Liver (U/mg protein)</th>
<th>Kidney (U/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SOD U***</td>
<td>CAT U***</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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<tr>
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</tr>
</tbody>
</table>

U= Units/min/mg protein for tissue. U= μmol of H2O2 consumed/min/mg of protein for tissues. U*** = μmoles of GSH consumed/min/mg protein. CA= Centella asiatica 200 mg/kg/bw.
method of Sinha [27]. Dichromate in acetic acid was converted to perchromic acid and then to chromic acetate, when heated in the presence of H₂O₂. The chromic acetate formed was measured at 620 nm. The catalase preparation was allowed to split H₂O₂ for various periods of time. The reaction was stopped at different time intervals by the addition of dichromate-acetic acid mixture and the remaining H₂O₂ as chromic acetate was determined colourimetrically. The specific activity was expressed as μmol of H₂O₂ consumed/min/mg of protein for tissues.

2.8 Estimation of glutathione peroxidase (GPx, EC. 1.11.1.19)

GPx activity was estimated by the method of Rotruck et al. [28]. A known amount of enzyme preparation was allowed to react with H₂O₂ in the presence of GSH. GPx utilize GSH for the decomposition of H₂O₂. After a specific time period, the remaining GSH content was measured. The specific activity was expressed as μmoles of GSH consumed/min/mg protein for tissue.

2.9 Estimation of reduced glutathione (GSH)

GSH in the tissues was estimated by the method of Ellman [29]. This method was based on the formation of 2-nitro-5-thiobenzoic acid (a yellow colour compound) when 5, 5’-dithio-bis (2-nitrobenzoic acid) (DTNB) was added to compounds containing sulphhydryl groups. The amount of glutathione was expressed as mg/100g of tissue.

2.10 Estimation of ascorbic acid (vitamin C)

Ascorbic acid in the tissues was estimated by the method of Roe and Kuether [30]. The ascorbic acid was converted to dehydroascorbic acid by mixing with norit and then coupled with 2, 2’ dipyridyl. The coupled dinitrophenylhydrazine (DNPH) in the presence of thiourea, a mild reducing agent. The coupled dinitrophenylhydrazine was converted into a red coloured compound when treated with sulphuric acid. The values were expressed as mg/100 g tissue.

2.11 Estimation of α-tocopherol (vitamin E)

α–Tocopherol in the tissues was estimated by the method of Baker et al [31]. The method involves the reduction of ferric ions to ferrous ions by α–tocopherol and the formation of a red coloured complex with 2, 2’ dipryridyl. Absorbance of the chromophore was measured at 520 nm. The values were expressed as mg/100g tissue.

2.12 Statistical Analysis

Data were analyzed by one way analysis of variance (ANOVA) followed by Duncan’s multiple range test (DMRT) using a commercially available statistics software package (SPSS for Windows, V. 11.0, Chicago, USA). Results were presented as means ± SD. P values <0.05 were regarded as statistically significant.

3. Results

When compared with control there was a significant decrease in liver and kidney enzymic antioxidants such as SOD, CAT, and GPx activities in stress induced animals at the same time enzymic antioxidant status of stress group treated with C.A having normal level of SOD, CAT, GPx which were showed in Table 1. Table 2 showed that level of liver and kidney nonenzymic antioxidant such as GSH, Vit C, Vit E. Stress induced group had significant decrease in GSH, Vit C, Vit E activities, treated group remain unchanged when compared with normal.

4. DISCUSSION

Free radical generation is controlled by a large number of antioxidant systems that act as protection against free radicals. Free radical scavenging enzyme such as SOD, CAT, and GPx are the first line of cellular defense against oxidative injury which are involved in the disposal of superoxide anions and H₂O₂ [32]. Immobilization stress is a well known method for the production of chronic stress [33] and acute stress. The original point of this study is examining the tissue from immobilization stress for the changes in antioxidant enzyme activities (SOD, CAT and GPx), GSH, protein oxidation and lipid peroxidation levels. Stressful conditions are known to interfere with antioxidant system by interfering with production or inactivation of antioxidant enzymes [34, 35]. Hence, changes in the activity of antioxidant enzymes are considered in the present study to assess the effect of repeated acute stress exposure. The SOD converts superoxide radicals into hydrogen peroxide. The ROS scavenging activity of SOD is effective only when its activity is followed by the actions of CAT and GPx, because hydrogen peroxide generated by SOD is further scavenged by CAT and GPx [36]. Therefore, it is hypothesized that an imbalance in the SOD/CAT results in oxidative alterations. The reduction in the activities of SOD and CAT was observed following immobilization stress. The present study indicates how decreased antioxidant status due to stress induced by immobilization. Further, decreased CAT activity indicates reduced hydrogen peroxide scavenging. In addition, there was also a decrease in potency of secondary antioxidant defense system in stressed rats as shown by decreased activities of Vit C, Vit E and GST. It is evident from earlier studies that decreased antioxidant status was observed following on exposure to an acute stressor. At the mean time stress group treated with C.A, it reverts the reactions in antioxidant system. C.A to directly react with reactive oxygen species especially effective in scavenging free radicals. As studies have also reported administration of free radical scavengers to senescent animals reverses protein oxidation in rat [37]. The phenolic compounds (quercetin and catechins) present in the C.A [19] may have different functional property such as scavenging of reactive oxygen species [38], inhibition of the generation of free radicals and chain-breaking activity [39]. They may act as hydrogen-donating radical scavengers by scavenging lipid alkoxy and peroxyl radical [40]. Veerendra kumar and Gupta in 2002[41] also reported that the C.A was found to increase the level of glutathione tripeptide in cognitive deficit rat. Our results are in accord with the study of Arivazhagan et al. [42] indicating the depletion of antioxidants was rectified by administration of C.A.

5. Conclusion

This study provides convincing evidence that C.A treatment can effectively protect against the free radical formation to maintain the antioxidant activities. Apparently it may offer safe and inexpensive means of preventing decline of antioxidants, by means of this it may act as an antistress agent.
6. Reference


[34] Y.Kono,and I.Fridovich, Inhibition and reactivation of Mn-catalase: implications for valence changes at the active site manganese, JBC. 258, (1983)13646-13648


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