CONCOMITANT SUPPLEMENTATION OF GOSSYPIN: A BIOFLAVONOID PROTECTS LEAD AND ETHANOL INDUCED OXIDATIVE STRESS IN RATS

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Abstract: The objective of the present study was to evaluate the protective efficacy of gossypin co-administration during lead-ethanol co-exposure. In one of our earlier study we reported the protective efficacy of gossypin against lead induced oxidative stress and it was of interest to observe the efficacy in the situation of combined lead-ethanol exposure. Animals were exposed to lead as lead nitrate (2 mg/kg, i.p. once daily), ethanol (5%) was given in drinking water or the combination, while gossypin was administered orally dissolved in polyethyleneglycol (PEG). Oxidative stress variables were determined in blood, liver, kidney and brain. Gossypin administration was able to restore blood δ-aminolevulinic acid dehydratase (ALAD) activity and reduced glutathione (GSH) levels. Significant reduction in reactive oxygen species (ROS) in blood was also noted when gossypin was co-administered during lead-ethanol co-exposure. The levels of reduced and oxidized glutathione, thiobarbituric acid reactive substances (TBARS) and ROS were also carried out in brain, liver and kidney and showed significant depletion on lead, ethanol or lead-ethanol co-exposure. Co-administration of gossypin significantly protected these changes suggesting protection against lead-ethanol and lead-ethanol induced oxidative stress. Thus, the present study suggests that gossypin protects lead induced oxidative stress irrespective of individual or combined lead-ethanol co-exposure.

Key words: Lead toxicity, Oxidative stress, Gossypin, Rats

INTRODUCTION

Lead and alcohol are two toxins in nature out of which, the population is occupationally exposed to the former while the latter is deliberately taken. Both of them are neurotoxic and exert its effect on the developing brain. Ethanol has the ability to diffuse across biological membranes and can affect the absorption of heavy metals such as lead. As the production and consumption of ethanol as well as environmental lead pollution are increasing, there is a likelihood of exposure of both these contaminants in general population, especially the industrial workers. There are very few studies that relate the toxic effects of lead and alcohol in combination. Flora and Tandon [1] were among the first to study the interactive effects between lead and ethanol on the variables that are primarily related to lead toxicity as well as on those more characteristic of ethanol toxicity. Evidences support that lead induces oxidative stress and is involved in the pathophysiology of lead toxicity [2,3]. Several epidemiological studies among workers with high occupational exposure to lead have reported associations between lead exposure and oxidative stress markers [4,5]. Oxidative stress occurs when generation of free radicals (i.e. substances with one or more unpaired electrons) exceed the capacity of antioxidant defense mechanisms (i.e. pathways that provide protection against harmful effect of free radicals). The depletion of glutathione and protein bound sulphhydryl groups and the changes in the activity of various antioxidant enzymes indicative of
lipid peroxidation have been implicated in lead induced oxidative tissue damage [2]. It has also been observed that the ingestion of ethanol cause depletion of endogenous glutathione (GSH), calcium and magnesium in animals exposed to lead. [6,7]. Ethanol administration has been reported to alter hepatic GSH metabolism and cause GSH reduction attributable to its lowered synthesis [8,9]. The decrease in the GSH concentration and increase in the absorption of lead under the influence of ethanol seem to enhance the toxic effects of lead [10].

Flavonoids are well known to activate key enzymes in mitochondrial respiration by acting as antioxidants, thus breaking the vicious cycle of oxidative stress and tissue damage. Antioxidants can play an extremely important role in abating toxic effects of lead as well as alcohol. Gossypin (3, 3’, 4’, 5, 7, 8- hexahydroxy flavone 8-glucoside) is a flavonoid present in Hibiscus species. It is usually found in the flowers of Gossypium indicum, Hibiscus vitifolius and Hibiscus esculentus. Hibiscus vitifolius is the richest source of gossypin. Gossypin has anti-inflammatory [11], analgesic [12] properties. Co-administration of gossypin with lead has some protective efficacy against brain oxidative stress and heme synthesis pathway but has only limited effect on the depletion of lead from the target organs [13]. Along with alcohol drinking it is of interest to determine the interactive relation between lead and ethanol co-exposure. In the present study we intended to see whether gossypin is able to protect against lead-ethanol induced oxidative stress, alone as well as in combination.

**MATERIALS AND METHODS**

Gossypin was purchased from M/s Research Organics (India) with more than 95% purity. å-aminolevulenic acid was purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other analytical laboratory chemicals and reagents were purchased from E. Merck (Germany), Sigma (USA), Fluka (Germany), or BDH Chemicals (India).

**Animals and Treatment:** All the experiments were performed on male Wistar rats weighing 100±10 g. Animals were obtained from animal house facility of Defense Research and Development Establishment (DRDE), Gwalior. Animal ethical committee of DRDE, Gwalior, India approved the protocols for experiments. All animals received human care in compliance with the guidelines of the committee for the purpose of control and supervision of experiments on animals (CPCSEA). Prior to dosing, they were acclimatized for 7 days to light from 0600 to 1800 h alternating with 12 h darkness. The animals were housed in stainless steel cages in an air-conditioned room with temperature maintained at 25 ± 2 °C. Rats were allowed a standard rat chow diet (Amrut Feeds, Pranav Agro, New Delhi India; metal contents of diet, in ppm dry weight, were Cu 10.0, Zn 45.0, Mn 55.0, Co 5.0, and Fe 75.0) throughout the experiment. Forty animals were randomly put into eight groups of six rats each and were treated as below for a period of three weeks.

- **Group I** - No treatment
- **Group II** - Lead nitrate 2mg/kg i.p. once daily for three weeks
- **Group III** - Ethanol (5% in drinking water)
- **Group IV** - Lead + Ethanol
- **Group V** - Lead + Gossypin dissolved in PEG (200 mg/kg oral)
- **Group VI** - Ethanol + Gossypin dissolved in PEG (200 mg/kg oral)
- **Group VII** - Lead + Ethanol + Gossypin
- **Group VII** - PEG (Polyethylene Glycol)

Lead nitrate was administered 5hrs prior to the oral dose of gossypin (200mg/kg). Six animals were sacrificed from each group under light ether anesthesia, 48 hrs after last dosing. Blood was collected in heparinized vials and liver, kidney and brain were rinsed in cold saline and blotted.

**Biochemical assays:** Activity of blood ALAD was assayed according to the procedure of Berlin and Schaller [14] while the analysis of blood GSH concentration was performed with method described by Ellman [15] and as modified by Jollow et al. [16]. Amount of reactive oxygen species (ROS) in blood was measured using 2’,7’-dichlorofluorescein diacetate (DCF-DA) which gets converted into highly fluorescent DCF by cellular peroxides (including hydrogen peroxide).

White blood cells (WBC), red blood cells (RBC), hematocrit (Hct), hemoglobin (Hb), mean cell volume (MCV), mean cell hemoglobin (MCH) and mean cell hemoglobin concentration (MCHC) was measured on Sysmex Hematology Analyzer (model K4500).

Amount of ROS in liver, kidney and brain were measured using 2’, 7’-dichlorofluorescein diacetate (DCF-DA) that gets converted into highly fluorescent...
Fig. 1: Effect of gossypin administration on TBARS levels in soft tissues of lead-ethanol co-exposed rats.

Abbreviations and units-
TBARS - Thiobarbituric acid reactive substance as µg/g tissue. Values are mean ± SE (n=6). *p<0.05 vs. normal animals, †p <0.05 vs. respective control (Lead+ gossypin vs. lead; ethanol + gossypin vs. ethanol; lead+ ethanol+ gossypin vs. lead+ ethanol).

Fig. 2: Effect of gossypin administration on ROS levels in soft tissues of lead-ethanol co-exposed rats.

Abbreviations and units-
ROS reactive oxygen species as µmol min⁻¹(mg protein)⁻¹ Values are mean ± SE (n=6). *p<0.05 vs. normal animals, †p <0.05 vs. respective control (Lead+ gossypin vs. lead; ethanol + gossypin vs. ethanol; lead+ ethanol+ gossypin vs. lead+ ethanol).

Fig. 3: Effect of gossypin administration on GSH in soft tissues of lead-ethanol co-exposed rats.

Units - Reduced Glutathione as mg/g tissue. Values are mean ± SE (n=6). *p<0.05 vs. normal animals, †p <0.05 vs. respective control (Lead+ gossypin vs. lead; ethanol + gossypin vs. ethanol; lead+ ethanol+ gossypin vs. lead+ ethanol).
DCF by cellular peroxides (including hydrogen peroxide). The assay was performed as described by Socci et al. [17]. Urinary δ-aminolevulinic acid was estimated as described by the method of Davis et al. [18]. Tissue lipid peroxidation was measured by method of Ohkawa et al [19]. Reduced glutathione levels were measured fluorometrically using the method of Hissin and Hilf [20].

**Statistical analysis:** Data are expressed as means ± SEM. Data comparisons were carried out using student ‘T’ test to compare means between the different treatment groups. Difference between unexposed (with or without chelation) with a p value < 0.05 was considered significant.

**RESULTS**

**Effect on hematological variables:** Table-1 shows the changes in hematological variables during concomitant administration of gossypin to lead, ethanol or lead-ethanol exposed animals. We could observe a significant depletion in the ALAD activity in the lead or ethanol exposed animals but a pronounced depletion was seen in lead-ethanol co-exposed rats. Concomitant administration of gossypin showed significant increase in ALAD activity in lead or ethanol exposed animals. Moreover, co-administration of gossypin also provided limited recovery in ALAD activity. A significant decrease was noted in reduced glutathione (GSH) in lead or ethanol exposed group while gossypin co-administration showed a significant increase in GSH content.

Gossypin was also able to restore GSH contents in lead-ethanol co-exposed rats. A significant increase in reactive oxygen species (ROS) was seen in the lead, ethanol and lead-ethanol exposed groups. We could observe a significant decrease in the ROS during concomitant administration of gossypin in lead and ethanol exposed animals. However, significant changes were also seen when gossypin was

<table>
<thead>
<tr>
<th></th>
<th>ALAD</th>
<th>GSH</th>
<th>ROS</th>
<th>U-ALA</th>
</tr>
</thead>
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<tr>
<td>Normal animals</td>
<td>11.75±1.2</td>
<td>1.08±0.03</td>
<td>0.006±0.002</td>
<td>0.03±0.001</td>
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<tr>
<td>Lead</td>
<td>0.76±0.27*</td>
<td>0.48±0.03*</td>
<td>0.028±0.002*</td>
<td>0.52±0.01*</td>
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<tr>
<td>Ethanol</td>
<td>7.21±0.42*</td>
<td>0.56±0.03*</td>
<td>0.027±0.003*</td>
<td>0.06±0.001*</td>
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<tr>
<td>Lead+ Ethanol</td>
<td>0.45±0.35*</td>
<td>0.28±0.02*</td>
<td>0.042±0.001*</td>
<td>0.74±0.001*</td>
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<tr>
<td>Lead+ Gossypin</td>
<td>1.46±0.17*</td>
<td>0.76±0.01*</td>
<td>0.018±0.006*</td>
<td>0.14±0.005*</td>
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<tr>
<td>Ethanol+ Gossypin</td>
<td>9.42±0.63</td>
<td>0.76±0.01*</td>
<td>0.016±0.007</td>
<td>0.03±0.005*</td>
</tr>
<tr>
<td>Lead+ Ethanol+ Gossypin</td>
<td>1.22±0.07†</td>
<td>0.49±0.02†</td>
<td>0.024±0.004†</td>
<td>0.37±0.001†</td>
</tr>
<tr>
<td>PEG</td>
<td>10.73±0.97</td>
<td>0.91±0.01</td>
<td>0.006±0.002</td>
<td>0.075±0.001</td>
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</table>

Abbreviations and Units: ROS- Reactive oxygen species as ηmoles/ min/ ml RBC, GSH-Reduced Glutathione as mg/ml, ALAD-Aminolevulinic acid dehydratase as nmol/min/ml erythrocytes, U-ALA – urinary aminolevulinic acid as µg/ml urine. Values are mean ± SE (n=6). *p< 0.05 vs. normal animals, †p <0.05 vs. respective control (Lead+ gossypin vs. lead; ethanol + gossypin vs. ethanol; lead+ ethanol+ gossypin vs. lead+ ethanol)

Table 1. Beneficial effects of gossypin on biochemical variables related to porphyrin metabolism and oxidative stress in lead-ethanol co-exposed rats.

Table 2: Beneficial effects of gossypin on clinical hematological variables in lead-ethanol co-exposed rats.

<table>
<thead>
<tr>
<th></th>
<th>WBC</th>
<th>RBC</th>
<th>Hb</th>
<th>HCT</th>
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</thead>
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<tr>
<td>Normal animals</td>
<td>12.26±1.86</td>
<td>5.73±0.35</td>
<td>10.5±0.62</td>
<td>32.12±1.86</td>
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<td>Lead</td>
<td>15.1±0.97*</td>
<td>6.25±0.24</td>
<td>11.63±0.61</td>
<td>35.2±0.43</td>
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<td>Ethanol</td>
<td>14.3±0.5*</td>
<td>5.91±0.44</td>
<td>10.52±0.73</td>
<td>33.8±1.34</td>
</tr>
<tr>
<td>Lead+ Ethanol</td>
<td>18.26±0.46*</td>
<td>6.30±0.57</td>
<td>11.4±1.28</td>
<td>34.8±3.04</td>
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<tr>
<td>Lead+ Gossypin</td>
<td>12.1±1.48†</td>
<td>6.47±0.39</td>
<td>10.98±0.51</td>
<td>34.58±1.20</td>
</tr>
<tr>
<td>Ethanol+ Gossypin</td>
<td>13.1±1.14</td>
<td>5.39±0.39</td>
<td>11.32±0.48</td>
<td>33.7±1.41</td>
</tr>
<tr>
<td>Lead+ Ethanol+ Gossypin</td>
<td>12.66±0.21</td>
<td>6.46±0.23</td>
<td>11.62±0.46</td>
<td>35.57±0.32</td>
</tr>
<tr>
<td>PEG</td>
<td>22.66±1.24</td>
<td>5.96±0.02</td>
<td>10.9±0.11</td>
<td>33.96±0.57</td>
</tr>
</tbody>
</table>

WBC, white blood cell (10³/µl), RBC, red blood cell (10⁶/µl); Hb, hemoglobin (g/do); Hct, hematocrit (%). Values are mean ± SE (n=6). *p< 0.05 vs. normal animals, †p <0.05 vs. respective control (Lead+ gossypin vs. lead; ethanol + gossypin vs. ethanol; lead+ ethanol + gossypin vs. lead + ethanol)
administered to the lead-ethanol co-exposed animals. A significant increase in the excretion of aminolevulinic acid (ALA) in urine was observed in lead and ethanol exposed animals with a more pronounced increase in the lead-ethanol co-exposed group. However a significant decrease in the ALA excretion was noticed when gossypin was administered in the lead or ethanol exposed group and significant changes were also noted when gossypin was administered to the lead-ethanol co-exposed group.

**Effect on clinical hematological variables:** Table-2 depicts the changes in clinical hematological variables. Only marginal changes were observed which were found to be not significant in any of the groups. Only significant changes in WBC’s were noted in lead, ethanol and lead-ethanol co-exposed animals and recovery was seen when gossypin was administered.

**Effect on TBARS level in soft tissues:** Fig. 1 shows the effect of gossypin co-administration on lead, ethanol and lead-ethanol co-exposed animals in liver kidney and brain. A significant increase in TBARS was observed during lead, ethanol alone and also during their co-exposure in liver, kidney and brain. Significant decrease in TBARS was noted (compared with respective controls) when gossypin was co-administered to lead, ethanol and lead-ethanol co-exposed rats in liver kidney and brain.

**Effect on generation of ROS in soft tissues:** Figure 2 depicts the generation of ROS as a result of lead, ethanol and lead-ethanol co-exposure. We observed a significant increase in ROS in liver kidney and brain, due to lead, ethanol or lead-ethanol co-exposure. Co-administration of gossypin significantly reduced ROS in lead and ethanol exposed rats. It was also noted that gossypin administration significantly reduced ROS in liver, kidney and brain of lead-ethanol co-exposed animals.

**Effect on reduced glutathione in soft tissues:** Changes in reduced glutathione level are shown in Fig. 3. GSH level reduced significantly due to lead, ethanol and lead-ethanol co-exposure in liver, kidney and brain. Co-administration of gossypin showed significant increase in GSH in liver, kidney and brain of lead or ethanol exposed rats. Significant changes in GSH content were also noted in liver, kidney and brain during concomitant administration of gossypin in lead-ethanol co-exposed animals.

**DISCUSSION**

Flavonoids are most ubiquitous in plants. They are polyphenolic compounds that possess antioxidant properties. The antioxidant potency and specific effect of flavonoids in promoting human health depend on flavonoid type (chemical, physical, and structural properties). Beneficial effects of flavonoids on human health are partly explained by their antioxidant property. Due to their antioxidant property, it is suggested that flavonoids may delay or prevent the onset of diseases induced by free radicals. Flavonoids possess a highly reactive hydroxyl group that becomes oxidized by electron donation, thus stabilizing the radical to a less reactive molecule. One way of reaction is the direct scavenging of free radicals, e.g., superoxide anions, singlet oxygen, and lipid peroxy radicals. Flavonoids have been shown to activate key enzymes in mitochondrial respiration and to protect neuronal cells by acting as antioxidants, thus breaking the vicious cycle of oxidative stress and tissue damage. Our group for the first time reported the antioxidant potential of gossypin against lead toxicity [13].

The antioxidant, antitumour and anticarcinogenic properties of the flavonoid gossypin has been studied in vitro and scavenged the in vitro free radicals, i.e. superoxide, hydroxyl and nitric oxide radicals [21]. ALAD is a crucial enzyme in lead toxicity because the inhibition of ALAD lowers heme production and increases levels of the substrate delta-aminolevulinic acid (ALA). Elevated levels of ALA, found both in the blood and urine of subjects with lead exposure, are known to stimulate ROS production [22]. We could observe a significant recovery in the ALAD activity when gossypin was given to lead exposed animals. This is in accordance with our previous study and gossypin could also increase the ALAD activity in ethanol exposed animals. Significant change in ALAD activity was also seen when gossypin was given to the lead-ethanol co-exposed animals. Gossypin could significantly reduce the U-ALA excretion in lead, ethanol and lead-ethanol co-exposed animals. This suggests that gossypin could however reduce the U-ALA excretion thereby restoring the ALAD activity to some extent. Gossypin could reduce ROS in soft tissues of lead, ethanol and lead-ethanol co-exposed animals. This suggests that although gossypin possesses good antioxidant activity and is able to reduce oxidative stress in lead, ethanol and lead-ethanol co-exposed animals.
GSH is one of the most important compounds, which helps in the detoxification and excretion of heavy metals. It binds with heavy metals. The resulting water soluble chemical is more easily filtered out of the body [23]. GSH is a tripeptide containing free SH group of cysteine and displays protective effect against oxidative stress by acting both as a direct scavenger of ROS and as a cofactor in metabolic detoxification [24] and is generally seen at significantly reduced levels in blood on exposure to lead. Lead binds exclusively to the SH group [25, 26] which decreases the GSH levels [27] and can interfere with the antioxidant activity of GSH. We could observe a significant depletion in GSH content of lead exposed animals but gossypin administration could increase the GSH content thereby implying that gossypin exerts antioxidant effects. Moreover, significant changes were also noted when gossypin was administered to ethanol exposed animals showing that it exerts antioxidant effect in ethanol induced oxidative stress also. Co-administration of gossypin also resulted in increased GSH level in lead-ethanol co-exposed rats implying that it may be a good antioxidant in reducing oxidative stress during lead-ethanol co-exposure.

Significant increase in ROS in lead, ethanol and lead-ethanol exposed animals are indicative of oxidative stress. It is well known that lead-induced cellular damage is mediated by the formation of ROS [28, 29, 30]. Gurer and colleagues [31, 32] using animal models demonstrated that lead increases the pro-oxidant/antioxidant ratio in a concentration-dependent manner. Recent studies have shown that lead inhibits the activity of antioxidant enzymes, including glutathione (GSH) peroxidase, catalase, and superoxide dismutase (SOD) [33]. Furthermore, it generates ROS, stimulates lipid peroxidation, and depletes antioxidant reserves, which have been postulated to be the major contributors to lead exposure-related diseases [34, 35]. Gossypin is a known antioxidant and can cross the cell membrane; therefore, it provides an intracellular protective effect. Gossypin could reduce lipid peroxidation in lead exposed animals but no change was seen when gossypin was administered to lead-ethanol co-exposed animals. Similar results were observed in GSH. However, we could observe that ROS decreased in gossypin administered groups as compared to their respective toxic groups suggestive of the fact that gossypin could provide protection to renal and hepatic variables in lead-ethanol co-exposed animals. Although we observed a significant reduction of ROS GSH and TBARS in gossypin administered groups in liver, kidney and brain variables.

**CONCLUSION**

Gossypin is a known antioxidant and in our study it showed significant protection against lead-ethanol combined toxicity. Gossypin could effectively protect against lead toxicity which is in accordance with our previous study and also in ethanol. The present study suggest that even during the situation of co-exposure to lead and ethanol, gossypin could effectively reduce oxidative stress. Although the mechanism underlying such an effect is unknown but use of a chelator along with gossypin could be beneficial in reducing the oxidative stress and lead decoporation.

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**REFERENCES**