Sub-acute oral toxicity of Roundup® and ammonium nitrate with special reference to oxidative stress indices in wistar rats

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ABSTRACT
The aim of present study was to unravel the single and interactive toxic potential of Roundup® and ammonium nitrate in rats after their oral administration for a period of 28 days. The animals were randomly divided into four groups with six rats in each group. Group I served as control and were orally administered with water. Group II animals were orally exposed to Roundup @ 500mg/Kg/day. Animals in group III were orally treated with ammonium nitrate @ 220mg/Kg/day while as group IV received both Roundup and ammonium nitrate @ 500mg/Kg/day and 220mg/Kg/day respectively. After 28th day of treatment, blood samples were taken and analysed for various oxidative stress parameters. Significant increase in LPO was observed in group II, III and IV as compared to control. GSH decreased significantly in all treated groups in comparison to control animals. The activity of anti-oxidant enzymes SOD, CAT, GSH-Px decreased significantly in group II, III and IV as compared to group I. GST increased non significantly in group II but decreased significantly in group III and IV . Concomitant exposure to both of these chemicals in group IV showed more significant alterations in oxidative stress indices even in comparison to group II and III.

Key words: Ammonium nitrate, Oxidative stress, Rats, Roundup®

INTRODUCTION
Roundup® (Glyphosate), largest selling agrochemical product worldwide accounts for share of about 25 per cent of the global herbicide market (Business Report, 2011). It is a wide spectrum non selective and post emergent herbicide used for food/ non food crops and relatively high use of this herbicide (0.5 to 2.0 Kg/ha/application) is often used in growing season (Duke et al., 2012). Glyphosate as herbicide is described by manufacturers as pesticide of low toxicity and environmently friendly (Franz et al., 1997). It has been rated as least dangerous in comparison to other herbicides and pesticides such as those from organochlorine family and has been assigned as class III on toxic scale for oral and inhalation exposure (US EPA, 1993). However, recent study has shown that glyphosate can cause serious health problems like birth defects, blood cancer as well as disturbances in reproductive hormone secretions (Sood, 2011). There is a broad scope to study this chemical which is considered safe but is controversial emerging chemical of great concern. There is also dearth of studies describing its interactive toxic potential with fertilizers like ammonium nitrate that has been used extensively for agricultural purposes and has increasingly contaminated the environment with nitrate or nitrite ions (Bensoltane et al., 2006). This assessment of deleterious or toxic effects produced by concurrent exposure to commonly encountered chemicals is of great significance in order to find out toxicological consequences arising out of their interactions. Such understanding will help in comprehensive management of untoward effects produced by these chemicals. Therefore, present study was an attempt to study interactive toxic potential of Roundup® and ammonium nitrate in wistar rats after their repeated oral administration on oxidative stress indices for a period of 28 days.

MATERIALS AND METHODS
Experimental animals and designs: The study was conducted on healthy wistar rats of either sex weighing about 200-250 gram. The animals were provided with standard ration and clean drinking water ad libitum. The experiment was conducted strictly in accordance to the Institutional Animal Ethics committee. The animals used were randomly divided into four groups with six rats in each group. Group I served as control and were provided with water only. Group II animals were treated orally with Roundup® @ 500mg/Kg b.wt (10 percent LD50) (Stout and Rucker, 1993). The animals of Group III were orally administered with ammonium nitrate @ 220mg/Kg b.wt (10 percent LD50) (Bensoltane et al., 2006). Group IV received both Roundup® @ 500mg/Kg b.wt and

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ammonium nitrate @ 220mg/Kg b.wt. Dosing of chemicals was done early in the morning continuously for 28 days and body weight recorded at 7 days interval to adjust the dosage of application according to body weight.

**Chemicals:** Roundup® (Glyphosate 41 % EC) was commercially obtained from the local market and ammonium nitrate obtained from SD Fine Chemicals Mumbai were used in the present study.

**Blood collection and analysis:** The rats were anaesthetized with diethyl ether and blood samples were collected from retro-orbital fossa using capillary tubes in aliquots containing heparin @ 10 IU/ml of blood. Prior to centrifugation, 200µl whole blood was used for estimation of blood glutathione (GSH) (Beutler, 1975). Then 1 % haemolysate was used for estimation of superoxide dismutase (SOD) (Marklund and Marklund, 1974), catalase (CAT) (Aebi, 1983), glutathione-S-transferase (GST) (Habig et al., 1974) and glutathione peroxidise (GSH-Px) (Hafeman et al., 1974). Lipid peroxidation in terms of malondialdehyde (MDA) level was estimated in 33% haemolysate (Shafiq-Ur-Rehman, 1984).

**Statistical analysis:** The data was subjected to analysis of variance applying completely randomized design (CRD) at 5% level (P<0.05) of significance (Duncan, 1955)

### RESULTS AND DISCUSSION

The results on the effect of repeated oral administration of Roundup® and ammonium nitrate for a period of 28 days on various oxidative stress indices are presented in Table 1. Statistically significant (P<0.05) increase in mean value of MDA level was observed in group II (Roundup®) and group III (ammonium nitrate) as compared to control animals. The animals of group IV receiving both Roundup® and ammonium nitrate showed more significantly (P<0.05) increased value of MDA as compared to group I (control), group II (Roundup®) and group III (ammonium nitrate). Blood glutathione level decreased significantly (P<0.05) in group II (Roundup®), group III (ammonium nitrate) and in combination group IV (Roundup® and ammonium nitrate) as compared to control. The concentration of superoxide dismutase (SOD) decreased significantly (P<0.05) in group II (Roundup®) and group III (ammonium nitrate) as compared to control group I with more significant (P<0.05) decreased trend was observed in group IV (Roundup® and ammonium nitrate) as compared to group I (control) and group II (Roundup®). The activity of catalase (CAT) decreased significantly (P<0.05) in all the treated animals group II, III, IV with latter group showing more pronounced change even compared to groups II and III. Statistically significant (P<0.05) decreased value was observed enzymatic activity of glutathione peroxidise (GSH-Px) in group II (Roundup®) and group III (ammonium nitrate) as compared to control and co-administered group IV (Roundup® and ammonium nitrate) as compare to group I, II and III. As compared to control, glutathione-S-transferase (GST) increased non significantly in group II (Roundup®) and decreased significantly (P<0.05) in group III (ammonium nitrate) and group IV (Roundup® and ammonium nitrate).

Oxidative stress is associated with generation of toxic reactive oxygen species and mammalian cells are endowed with extensive antioxidant defence mechanisms which counteract the damaging effects of these toxic reactive oxygen species (Halliwell and Gutteridge, 1989). It is well known that MDA is a terminal product of lipid peroxidation, so the content of MDA can be used to estimate extent of lipid peroxidation. This can indirectly reflect the degree to which the lipid membranes of cells are attacked by free radicals (Raina et al., 2009). Enhanced MDA levels in the present study, therefore, indicated that rats receiving glyphosate and ammonium nitrate were in oxidative stress. Glutathione (GSH) is the most abundant non protein thiol in organism and it plays role in intracellular protection against toxic compounds such as reactive oxygen intermediates and other free radicals (Anderson and Luo, 1998). It plays a major role in antagonizing the oxidative action of the herbicides or insecticides and has been reported that when GSH concentration is decreased to 20% of its original level, it results in enhanced lipid peroxidation (Younes and Siegers 1981; Parke and Piotrowski, 1996). Decreased blood glutathione level as seen in the present study, therefore, might be responsible for increased lipid peroxidation of membranes.

**Table 1:** Effect of repeated oral administration of Roundup® and ammonium nitrate alone and in combination on oxidative stress parameters in rats

<table>
<thead>
<tr>
<th>Parameters/Units</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid Peroxidation (nmol MDA formed/ml erythrocytes)</td>
<td>5.7±0.58a</td>
<td>8.19±0.64a</td>
<td>7.89±0.71b</td>
<td>10.10±0.61c</td>
</tr>
<tr>
<td>GSH (nmol/ml)</td>
<td>62.78±2.20a</td>
<td>44.39±4.35b</td>
<td>49.58±3.37b</td>
<td>40.69±3.79b</td>
</tr>
<tr>
<td>SOD (Units/mg protein)</td>
<td>48.63±4.39a</td>
<td>37.20±2.98b</td>
<td>38.72±3.59b</td>
<td>29.22±1.99b</td>
</tr>
<tr>
<td>CAT (µmol of H₂O₂ decomposition/min/mg protein)</td>
<td>61.95±4.44a</td>
<td>36.75±2.58b</td>
<td>38.10±4.27b</td>
<td>26.30±1.76c</td>
</tr>
<tr>
<td>GSH-Px (Units/mg protein)</td>
<td>13.31±1.01a</td>
<td>11.53±1.08b</td>
<td>10.58±0.55b</td>
<td>8.58±0.62b</td>
</tr>
<tr>
<td>GST(µmol of conjugate GSH-CDNB/min/mg protein)</td>
<td>0.041±0.005a</td>
<td>0.054±0.003a</td>
<td>0.021±0.007b</td>
<td>0.022±0.004b</td>
</tr>
</tbody>
</table>

Values given are mean ± SE of the results obtained from 6 animals unless otherwise stated. Means with at least one common superscript do no differ significantly at 5% (P<0.05 ) level of significance.
of erythrocytes (Schafer and Buettner, 2001). Similar findings of enhanced lipid peroxidation and decreased glutathione by glyphosate and ammonium nitrate toxicity are also reported elsewhere (Contraradio-Jara et al., 2009; Lushchak et al., 2009; Guilherme et al., 2010). Compared to control group, the activity of SOD, CAT, GSH-Px and GST decreased significantly in glyphosate and ammonium nitrate treated rats. Superoxide dismutase is considered the first and major line of defence against the action of \( \text{O}_2^\cdot \) and other ROS (Dubey et al., 2012). It converts the superoxide radicals into hydrogen peroxide which is decomposed by catalase to water and oxygen (McCord and Fridovich, 1969; Chelikani et al., 2004). Superoxide dismutase and catalase are considered as main antioxidant enzymes in oxidative stress produced by xenobiotics (Abdollahi et al., 2004). The direct inhibition of these enzymes by glyphosate or ammonium nitrate alone and in combination or increased utilization due to excess formation of free radicals could be possible reasons for the resultant depletion of these antioxidant enzymes (Eraslan et al., 2007). The results, therefore, suggested that excess free radical generation might cause decrease in superoxide dismutase which in turn was essential for activity of catalase.

Glutathione Peroxidase is a seleno-enzyme that protects biomembranes and other cellular components against oxidative damage (Mills, 1957; Lillte and Brien, 1968). The enzyme catalyses the reduction of a variety organic hydroperoxides and lipid hydroperoxides using glutathione as the reducing equivalent (Liu and Luo, 2003). Likewise, GST catalyses the nucleophilic adding of reduced glutathione to a variety of electrophiles. Additionally, GST bind with compounds like polycyclic aromatic hydrocarbons, pesticides, herbicides (Singh et al., 2009; Lushchak et al., 2009; Ortiz-Ordonez et al., 2011). This catalytic activity of combined glutathione with electrophiles helps in excretion of toxicant from the cells and protects the tissues from oxidative stress (Hayes and Paiford, 1995). Reduced blood glutathione level as observed in the present study, therefore, suggests decreased activity of GSH-Px and GST.

In the present study, the co-administered group receiving both Roundup\textsuperscript{*} and nitrate showed more significant alterations in various oxidative stress parameters as compared to other groups. Even though no direct in vivo relation between Roundup\textsuperscript{*} and nitrate could be established. However, the concomitant exposure Roundup\textsuperscript{*} in the presence of nitrates might have generated more free radicals as nitrates are involved in conversion of haemoglobin into methaemoglobin which in turn can aggravate the oxygen deficiency in cells. (Azhipa et al., 1990; Bauret et al., 2004; Vivian and Claudia, 2007; Aly et al., 2010; Cavalla et al., 2013).

**CONCLUSION**

In conclusion, Roundup\textsuperscript{*} & ammonium nitrate after repeated oral administration alone or in combination produced marked alterations in oxidative stress parameters (LPO, GSH, SOD, CAT, GSH-Px and GST). Such changes were more prominent and consistent with co-administration of these two toxicants.

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