Lipid peroxidation is an oxidative deterioration of polyunsaturated lipids (1). Amphipathic polyunsaturated lipids are the major constituents of biological membranes that are prone to lipid peroxidation in presence of metal ions and other prooxidants (1). Lipid peroxidation results in the generation of stereospecific endoperoxides and hydroperoxides by enzymatic and non-enzymatic involvement of ‘reactive oxygen species’ like super oxide radical (O$_2^-$), hydroxyl radical (·OH), thiyl radical (RS·), nitric oxide (NO·), singlet oxygen (1O$_2$), ozone (O$_3$) etc. that can decompose to yield a wide range of cytotoxic products most of which are aldehydes, as exemplified by malondialdehyde (MDA), 4-hydroxy-2-nonenal (4-HNE) etc. (2, 3). In oxidative stress the endogenous enzymatic (e.g., superoxide dismutase, catalase, peroxidase etc.) and non-enzymatic (e.g., ascorbic acid, α-tocopherol, glutathione etc.) antioxidant level is not sufficient to cope with the generated oxygen species (1, 3).

In oxidative stress exogenous administration of antioxidants has proven helpful to overcome oxidative damage in certain disease conditions, e.g., probucol in atherosclerosis, ebselen in inflammation, iron ion chelators like deferoxamine in thalassemia, leukemia (3). It has been also reported that vitamin E and C may be of benefit to prevent onset or progression of cardiovascular disease (4), disabling eye disease (5), cancer (6), maternal age associated infertility in human (7) etc.

The use of non-steroidal anti-inflammatory drugs (NSAIDs) is common in alleviating pain, pyrexia and inflammation, and in patients with rheumatoid arthritis and osteoarthritis. However, the drugs are associated with high incidence of gastrointestinal ulceration (8), bleeding (9) and kidney damage (10, 11).

MDA is a highly toxic byproduct generated \textit{in vivo} on different biochemical pathways like prostaglandin synthesis, or lipid peroxidation. Due to lipid peroxidation, where MDA intensity depends on oxygen-derived free radical production (12), MDA exerts extremely deleterious effects on cells and tissues by inducing damages to DNA or cellular proteins (13). Proteins may be dramatically modified by MDA through glycoxidation (12, 14) that are potentially as deleterious as free MDA and could be involving in aging (15), as well as in degenerative complications of diseases with increased oxidative stress such as atherosclerosis (16) and diabetes mel-
litus (12, 16). In those cases, MDA production, serum concentration and tissue content of MDA are increased concomitantly with the intensity of glycoxidation and atheroma lesions (17). Biological half-life of free MDA is short in vivo, since MDA binds to proteins very quickly after its generation (12). Long-lived proteins such as type I collagen demonstrated major targets of MDA binding and accumulation, leading to the formation of crosslinks and to alterations of functions (18). Free MDA induces apoptosis only in tumor cells, i.e., colorectal and lung carcinoma cells (19).

A rise in MDA content may be taken as an index of lipid peroxidation process. It can be assayed by measuring the quantity of 2-thiobarbituric acid (TBA) chromogenic complex of MDA formed during the reaction. Thus, it was of interest to specifically study the measurement of MDA which may be generated through NSAID (diclofenac sodium, ibuprofen, flurbiprofen, paracetamol, indomethacin, nimesulide, celecoxib)-induced lipid peroxidation and attempt has been made to study the potential of ascorbic acid as possible suppressor of NSAID-induced lipid peroxidation (if any) to provide a scope of further investigation on possible consideration of this as prospective candidates for co-therapy with drugs to reduce NSAID-induced toxicities that are possibly mediated by free radical mechanism.

EXPERIMENTAL

Chemicals
Trichloroacetic acid, thiobarbituric acid were purchased from Ranbaxy, S.A.S. Nagar. Diclofenac sodium and paracetamol were gifted by Nicholas Piramal India Ltd., Mumbai, India. Ibuprofen and nimesulide were gifted by Albert David Limited, Kolkata and flurbiprofen, indomethacin and celecoxib were gifted by Abbott India Limited; Goa, Wintac Limited; Bangalore and Cipla Ltd.; Mumbai, India, respectively. All other reagents were of analytical grade.

Liver was collected from goat (Capra capra) and used as the lipid source. Goat liver was selected because of its easy availability and close similarity of its lipid constituents to that of human liver (20).

Work was carried out according to the guidelines of Institutional animal ethical committee.

Preparation of tissue homogenate
Liver was perfused with normal saline through hepatic portal vein. Liver was harvested and its lobes were briefly dried between filter papers (to remove excess of blood) and were thin-cut with a sharp blade. These small pieces were then transferred to the glass-teflon homogenizing tube to prepare homogenate (1g/mL) in phosphate buffer saline (pH= 7.4) under cold conditions. It was centrifuged at 2000 rpm for 10 min. The supernatant was collected and finally suspended in PBS to contain approximately 0.8-1.5 mg of protein in 0.1 mL of suspension to perform the in vivo experiments.

Incubation of tissue homogenate with drug and/or ascorbic acid
For each drug, the tissue homogenate was divided into four different parts of 50 mL each in a glass stoppered 250 mL conical flask. The first portion was kept as the control (C) while the second portion was treated with drug (D). The third portion was treated with both drug and ascorbic acid (DA). The fourth portion was treated with only ascorbic acid (A). After treatment with drug and/or antioxidant, liver homogenates were stirred for 1 h below 20OC on a mechanical shaker and then incubated at 15°C for 4 h along with the control sample.

Effective concentration of drugs and ascorbic acid
Considering therapeutic dose of drugs per average weight of human liver (i.e. 1500 g) diclofenac sodium (0.03 mg/g of liver homogenate), ibuprofen (0.267 mg/g of liver homogenate), flurbiprofen (0.03 mg/g of liver homogenate), paracetamol (0.33 mg/g of liver homogenate), nimesulide (0.067 mg/g of liver homogenate), celecoxib (0.067 mg/g of liver homogenate), indomethacin (0.016 mg/g of liver homogenate) and ascorbic acid (0.17 mg/g of liver homogenate) were taken to carry out the experiments.

Estimation of lipid peroxidation breakdown products measured as malondialdehyde from tissue homogenate
Different sets of experiment were performed at 15°C for each drug-ascorbic acid pair and it was repeated in five animal sets. In each set liver homogenates were incubated for 4 h and MDA content was determined at 2 and 4 h of incubation and a mean of five observations was considered for the determination of MDA content of each sample at a particular hour. In each case five samples of 2.5 ml of an incubation mixture was transferred into a tube containing 2.5 mL of 10% w/v trichloroacetic acid. It was stirred by glass rod for good mixing. Then the tubes were centrifuged at 3000 rpm for 30 min to precipitate the protein part. The TCA soluble frac-
tion was fully separated and then a color reaction was developed (21) with slight modification. The filtrate was then treated with 5 mL of 0.002 M TBA solution and the volume was made up to 10 mL with distilled water and the resultant mixture was boiled for half an hour. After that it was cooled to room temperature and its absorbance was estimated at 530 nm against a TBA blank (prepared by mixing 5 mL of 0.002 M TBA solution and 5 ml of distilled water) using an EC5700B Spectrocolorimeter. The values were determined from the standard curve, which was obtained using 1,1,3,3-tetraethoxypropane (TEP).

### Statistical analysis

Analysis of variance (ANOVA) and multiple comparison were done to check statistical significance of the results. In multiple comparison there are two possible sources of error: the random error associated with the replicate measurements and the other due to animal variations. The variations may be calculated and their effects estimated by a statistical method known as the analysis of variance (ANOVA), where the square of the standard deviation $s^2$ is called the variance $V$. Thus, $F = s_1^2 / s_2^2$, where $s_1^2 > s_2^2$, and may be written as $F = V_1/V_2$, where $V_1 > V_2$. In our study, a degree of freedom (df) between samples is (2,8) and that between animals is (4,8). ANOVA is done to compare the means of more than two treatment groups.

If the F test ($F = \text{mean square between regimens}/\text{mean square within regimens}$) is significant and more than two treatments are included in the experiment, it may not be obvious immediately which treatments are different. Various multiple-comparison procedures have been proposed to solve this problem. The general procedure of multiple-comparison is to list the ranked means from lowest to highest and the means that are not statistically significantly different from each other are placed in a same parenthesis. The procedure is carried out by calculating a 5% allowance, which is defined as the critical difference between means which allows one to reject the null hypothesis and accept the alternative hypothesis for any two sample means at $p = 0.05$.

We have used the least significant difference procedure to solve multiple-comparison analysis. Least significant difference procedure is the least conservative procedure, and this assures that the probability that any one comparison is judged to be significant by chance alone is 5%. However, the probability of one or more comparisons being judged significant would be greater than 5%. Any two means included in the same parenthesis do not differ significantly at $p = 0.05$. Any two means not included in same parenthesis are statistically significantly different at $p = 0.05$.

### RESULTS

In this study we analyzed the MDA content, an index of extent of lipid peroxidation after treatment of liver homogenate with drugs and ascorbic acid. The percent change in MDA content of different samples at different hours of incubation were calculated with respect to the control of the corresponding hours and their averages are shown in Table 1. Analysis of variance (ANOVA) and multiple comparison were done directly from the data obtained by spectrophotometric reading and are shown in Table 2. Interpretation of the results is supported by a statisti-

<table>
<thead>
<tr>
<th>Drugs</th>
<th>MDA content (Mean ± SD)*</th>
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<tbody>
<tr>
<td></td>
<td>2 h of incubation</td>
</tr>
<tr>
<td></td>
<td>D</td>
</tr>
<tr>
<td>Diclofenac sodium</td>
<td>-5.67 (±4.64)</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>-5.36 (±1.79)</td>
</tr>
<tr>
<td>Flurbiprofen</td>
<td>-1.90 (±0.93)</td>
</tr>
<tr>
<td>Paracetamol</td>
<td>-3.92 (±0.84)</td>
</tr>
<tr>
<td>Nimesulide</td>
<td>-7.36 (±0.54)</td>
</tr>
<tr>
<td>Celecoxib</td>
<td>4.58 (±0.09)</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>2.35 (±0.78)</td>
</tr>
</tbody>
</table>

*Average percent changes with respect to controls of corresponding hours [corresponding standard deviation (SD) is within the parenthesis].

D, DA and A indicate drug-treated, drug and antioxidant-treated and antioxidant-treated samples, respectively.
cal multiple comparison analysis using a least significant difference procedure (22, 23).

**DISCUSSION**

From the results for the treatment of liver homogenate with diclofenac sodium (Table 1), it is evident that MDA content significantly decreases after the treatment of sample with drug with respect to control at different hours of incubation. This is possibly due to antioxidant action of the drug because diclofenac sodium has the ability to scavenge the stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) (25), peroxynitrite (26), oxy and lipid radical and also quenches the R-O and ROO species, responsible for the propagation phase (27) but has no radical-trapping ability for superoxide anion (O$_2^-$) or hydroxyl radicals ($\cdot$OH) and no suppressive ability for the NADPH-dependent lipid peroxidation of microsomes (26). These results suggest that diclofenac sodium has a significant cytoprotective effect (28) and may suppress liver injury caused by ischemia-reperfusion through stable radical scavenging and the inhibition of superoxide production in activated phagocytes, both of which may restrain the induction and propagation of oxidative stress. Hepatotoxicity of diclofenac sodium is due to its major metabolite, 5-hydroxy diclofenac (29) and may not be due to its lipid peroxidation activity.

The change in MDA profile after ibuprofen treatment shows that after both 2 and 4 h of incubation, MDA level is significantly decreased in all cases (D, D, A) compared to the corresponding control value. Surprisingly, ibuprofen, when used along with ascorbic acid, decreased MDA level more than that in case of only ascorbic acid treated samples, which may suggest that ibuprofen has synergistic antioxidant activity with ascorbic acid. The antioxidant activity of ibuprofen may be due to the attenuation of generation of hydroxyl radical or may be due to the formation of iron chelates that lack the free coordination site required for iron to be reactive and thus prevent iron mediated lipid peroxidation (30). Ibuprofen is a potent free radical scavenger, and it could reduce lipid peroxidation and free radical generation and may be a promising new therapeutic avenue for the treatment of neurodegenerative disease such as Alzheimer’s disease (31, 32).

From the changes of MDA content after treatment of liver homogenate with flurbiprofen and ascorbic acid (Table 1), it can be postulated that MDA content decreases to a marginal extent after treatment of sample with flurbiprofen. The marginal difference of MDA value with the corresponding control level suggests weak free radical scavenging activity of flurbiprofen. Surprisingly, when flurbiprofen was used along with ascorbic acid (A), MDA level reduced to lower level than the other two treatments (D and A), which may be due to a potential link towards antioxidant activity of flurbiprofen with ascorbic acid. The results of the present report therefore suggest the suppressive action of flurbiprofen on systemic lipid peroxidation effect (33).

Paracetamol causes significant decrease of MDA level with respect to the control value. The results suggest significant antioxidant activity of paracetamol after 2 and 4 h of incubation. Paracetamol inhibits copper ion-induced, azo com-

### Table 2. Analysis of variance and multiple comparisons

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<td>2 hours</td>
<td>F$_1$</td>
<td>8.92</td>
<td>22.31</td>
<td>16.17</td>
<td>1.22</td>
<td>142.50</td>
<td>27.90</td>
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<td></td>
<td>F$_2$</td>
<td>15.66</td>
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<td>3.05</td>
<td>3.05</td>
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<tr>
<td></td>
<td>S$^2$</td>
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<td>0.46</td>
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<td>25.64</td>
<td>0.33</td>
<td>1.34</td>
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<td></td>
<td>LSD</td>
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<td>0.99</td>
<td>3.07</td>
<td>7.38</td>
<td>0.84</td>
<td>1.69</td>
<td>0.86</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RM</td>
<td>(D,D,A)A</td>
<td>(D,D,A)A</td>
<td>(D,D,A)</td>
<td>(D,D,A)</td>
<td>(D,D,A)</td>
<td>(D,D,A)</td>
<td></td>
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<tr>
<td>4 hours</td>
<td>F$_1$</td>
<td>15.68</td>
<td>17.14</td>
<td>9.4</td>
<td>0.34</td>
<td>109.65</td>
<td>103.60</td>
<td>217.43</td>
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</tr>
<tr>
<td></td>
<td>F$_2$</td>
<td>1.45</td>
<td>20.31</td>
<td>12.13</td>
<td>0.86</td>
<td>1.00</td>
<td>2.70</td>
<td>0.98</td>
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</tr>
<tr>
<td></td>
<td>S$^2$</td>
<td>6.31</td>
<td>0.33</td>
<td>2.09</td>
<td>16.42</td>
<td>0.43</td>
<td>1.03</td>
<td>0.41</td>
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<tr>
<td></td>
<td>LSD</td>
<td>3.66</td>
<td>0.84</td>
<td>2.11</td>
<td>5.91</td>
<td>0.95</td>
<td>1.48</td>
<td>0.93</td>
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<td>RM</td>
<td>(D,D,A)A</td>
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<td>(D,D,A)A</td>
<td>(D,D,A)A</td>
<td>(D,D,A)A</td>
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</tr>
</tbody>
</table>

Diclo. = Diclofenac sodium, Ib. = Ibuprofen, Flur. = Flurbiprofen, Para. = Paracetamol, Nime. = Nimesulide, Cele. = Celecoxib, Indo. = Indomethacin. F$_1$ and F$_2$ correspond to variance ratios between samples [df = (2, 8)] and between animals [df = (4, 8)], respectively. S$^2$ = pooled variance, LSD = Least Square Difference (Ref. 21 and 24). RM = Ranked Means, two means not included within the same parenthesis are statistically significantly different at p < 0.05.
pound-initiated, and mononuclear cell-mediated oxidative modifications of low density lipoprotein (34) which also suggest the antioxidant activity of paracetamol. Ascorbic acid also causes significant decrease of MDA level. It is also evident from the results that the antioxidant activity of paracetamol is weaker than that of ascorbic acid.

The change of MDA profile after nimesulide and ascorbic acid treatments suggests that after 2 h of incubation MDA level is decreased significantly but rises significantly after 4 h of incubation with respect to the control level, which may be due to the antioxidant action of nimesulide at earlier stage of incubation followed by lipid peroxidation induction at the later stage of the incubation period. Nimesulide has potential to protect aging-associated abnormalities (35) and it inhibits lipopolysaccharide-induced production of superoxide anions and nitric oxide in alveolar macrophages (36).

From the results of change of MDA profile after treatment of liver homogenate with celecoxib and ascorbic acid, it is evident that after both periods of incubation celecoxib raises the MDA level with respect to the control value, representing significant peroxidation activity of celecoxib and ascorbic acid is capable of minimizing it to some extent. At 2 h of incubation the average percent change of MDA value of ascorbic acid treated sample with respect to control of corresponding hour is very low and this may be due to the poor generation of MDA in the liver homogenate with respect to the control.

From the change of MDA value after indomethacin and ascorbic acid treatment it is postulated that MDA level of the drug treated sample rises significantly with respect to the control level after both periods of incubation and this is may be due to the fact that indomethacin evokes lipid peroxidation (37, 38). The decreased value of MDA at DA indicates the capability of ascorbic acid to minimize indomethacin-induced peroxidation.

To compare means of more than two samples, multiple comparison analysis along with analysis of variance was performed and Table 2 shows the results. The ranked mean values indicate that there is no significant difference in changes of MDA content in drug-treated samples and those in antioxidant-treated samples except in cases of ibuprofen, flurbiprofen and celecoxib.

CONCLUSION

From the present study it is suggested that diclofenac sodium, ibuprofen, flurbiprofen and paracetamol exert statistically significant antioxidant activity. By contrast, nimesulide at earlier stage of incubation acts as an antioxidant but promotes lipid peroxidation to a little extent at the later stage. Celecoxib and indomethacin are involved in oxidative processes and ascorbic acid shows its antioxidant potential. These observations imply that the adverse effects of diclofenac sodium, ibuprofen, flurbiprofen and paracetamol may not be linked through free radical mediated processes, whereas adverse effects of nimesulide, celecoxib and indomethacin may be directly linked with the free radical generation. The concept of antioxidant co-therapy may also be exploited during future formulation design with an aim of reducing this drug-induced toxicity. Moreover, lipid peroxidation induction capacity of a drug may be tested at the individual level to determine the extent of risk from a drug in case of a particular individual in view of variable in vivo antioxidant defense and accordingly, the decision about safe use of a drug and necessary co-administration of ascorbic acid may be taken. However, further extensive study is required to advance such hypothesis.

Acknowledgement

The authors thank to Nicholas Piramal India Ltd.; Mumbai; India for providing diclofenac sodium and paracetamol, Albert David Limited; Kolkata; India for providing gift samples of ibuprofen and nimesulide and Abbott India Limited; Goa, Wintac Limited; Bangalore and Cipla Ltd.; Mumbai; India for providing flurbiprofen, indomethacin and celecoxib, respectively.

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