In immunotherapeutic research, over the last two decades, very intensive investigations were carried out of the preparation, experimental and clinical characteristics of a relatively new category of active substances, the so-called immunostimulants. They are products of natural or synthetic origin with different chemical characteristics and mechanisms of action (1). Such an immunostimulating drug is Sterodin®. It is the proprietary name of the medicine produced from bile lipids and bacterial metabolites from *Staphylococcus albus*, *Micrococcus catarrhal*, *E. coli*, *Paratyphoid A and B*, *Staphylococcus aureus*, etc. Each strain has a definite number of cells in each mL of solution. Sterodin® has been claimed to possess a non-specific immunopotentiating activity, particularly in infectious diseases. However, the mechanism of action and the exact parameters that are influenced by Sterodin® are still unknown. Therefore, we performed some toxicological and pharmacological evaluations to get some basic data about Sterodin®.
Drug-induced lipid peroxidation may be considered as a possible mechanism of drug-induced toxicity. Lipid peroxidation is oxidative deterioration of polyunsaturated lipids that occurs through a free radical mediated chain reaction (2). In case of a reduced or impaired defense mechanism and excess generation of free radicals that are not counterbalanced by endogenous antioxidant defense, exogenously administered antioxidants have been proven useful in overcoming oxidative damage (3). The possible role of antioxidants in reducing drug-induced lipid peroxidation provides a scope for considering antioxidants as prospective candidates in therapy aimed at reducing drug-induced toxicity and consequently increasing the therapeutic index. Thus, besides toxicological and pharmacological evaluation of Sterodin®, we also tried to find out whether Sterodin® had a lipid peroxidation induction capacity. As ascorbic acid reduced drug-induced lipid peroxidation in our previous study (4), we also evaluated its antiperoxidative potential in experiments with Sterodin® in vitro.

EXPERIMENTAL

Materials

Some toxicological and pharmacological evaluations were made in vivo and the experimental protocol was approved by the Animal Ethical Committee of Union Drug Company Ltd., India (under the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals, Animal Welfare Division, Government of India). In all in vivo experiments, normal healthy Swiss male albino mice weighing 17–22 g were used. All the animals were housed under normal ambient temperature (25–29°C) and acclimatized in the laboratory for at least 72 h. They were maintained at a standard laboratory diet and water ad libitum. Drug-lipid interaction studies were performed in vitro using goat liver as lipid source. Goat liver was selected because of its easy availability and close similarity to human liver in its lipid profile (5). The liver was collected from a Kolkata Municipal Corporation approved outlet.

Samples of Sterodin® were provided by Union Drugs Ltd., India. Ascorbic acid, thiobarbituric acid (TBA) and trichloroacetic acid (TCA) were purchased from Ranbaxy Fine Chemicals Ltd. India. 5,5’-Dithiobis-(2-nitrobenzoic acid) (DTNB) was purchased from SRL Pvt. Ltd. India. 1,1,3,3-Tetraethoxypropane (TEP) and reduced glutathione were obtained from Sigma Chemicals Co., USA. All other reagents were of analytical grade and locally purchased.

Toxicological tests

Determination of acute toxicity of Sterodin® by the probit analysis method. – A pilot study was conducted to select the dose ranges before performing the acute toxicity study. In the pilot study, there were two groups of mice, of four animals each. In the acute toxicity study, there were seven groups of ten male albino mice each.

The dose ranges of Sterodin® were 0.30, 0.40, 0.50, 0.60, 0.70, 0.80, 1.0 mL per 20 g of body mass. All ten animals in a particular group received the same one dose of Sterodin®. Lethality of Sterodin® was examined by injecting Sterodin® intraperitoneally into
male albino mice. The percentage of mortality at different doses was calculated and the values were transformed to the probit scale and \( LD_{50} \) was calculated after developing the regression equation (6).

**Test for undue toxicity of Sterodin®.** – Safety test was done to determine compliance with the requirements given in individual monographs/specifications. This test is intended to detect any unexpected, unacceptable, biological reactivity in a substance. This is an *in vitro* test designed for safety assessment of biologics and biotechnology derived products.

Five male albino mice each received a single intravenous injection of 0.05 mL of 80 % Sterodin® in water solution per 20 g b. m. The 80 % Sterodin® was injected over a period of about 15–30 s. The substance passed the test if none of the animals died within 24 hours (7).

**Evaluation of behavioral changes induced by Sterodin®.** – The dose level without any toxic symptoms was evaluated on four groups of mice of six animals each. All mice were injected Sterodin® intraperitoneally at one of the following doses: 0.05, 0.10, 0.20, 0.40 mL per 20 g b. m. All animals in a particular group received the same one dose of Sterodin®. Behavioral changes such as the righting reflex, rate of respiration, spasms, convulsions, etc., were observed up to 4 hours.

**Selected pharmacological tests**

**Total and differential white blood cell count (WBC).** – Ten mice were taken for this experiment. Sterodin® was injected intraperitoneally at a dose of 0.05 mL per 20 g b. m. All ten animals received the same dose of Sterodin®. Blood was taken from the tail vein on day 0, 6 and 30 days after the injection. Total WBC and differential white blood cell count (neutrophils and lymphocytes) per cm³, respectively, were recorded using common laboratory techniques in a haemocytometer chamber (8).

**Carbon clearance test for in vivo phagocytosis.** – Control and Sterodin® treated groups, each comprising five animals were administred either 0.2 mL per 20 g b. m. of indian ink (purchased in the form of *i.v.* injection from Rottring, Germany), intravenously only (control group), or 0.05 mL Sterodin® per 20 g b. m. intraperitoneally (Sterodin® treated group) six days before and 30 minutes prior to the experiment. They also received intravenous injection of 0.2 mL per 20 g b. m. of indian ink. Blood samples (20 µL) were withdrawn from the retro orbital plexus of each mouse at 0, 0.5, 2, 5, 15 minutes from the beginning of the experiment. Then 3 mL of distilled water was added to 20 µL of blood to lyse the erythrocytes. At the end, absorbance of the mixed solution (20 µL blood in 3 mL distilled water) was measured using a Shimadzu UV-1700 double beam spectrophotometer (Japan) at 650 nm (9). The carbon clearance rate was expressed relative to the control at respective points of time. The phagocytic index (PI) was calculated by deducing the ratio of the slope of absorbance-time curve of Sterodin®-treated and control group, respectively.
Drug lipid interaction studies

Preparation of tissue homogenate for lipid peroxidation studies. – Goat liver (collected within a few minutes after the death of the animal) perfused with normal saline through the hepatic portal vein was harvested and its lobes were briefly dried between filter papers to remove excess blood and then thinly cut with a heavy-duty blade. The small pieces (5 mm²) were then transferred into a sterile vessel containing phosphate buffer (pH 7.4) solution. After draining the buffer solution from the sterile vessel as completely as possible, the liver was immediately ground to make a tissue homogenate (1 g L⁻¹) using freshly prepared phosphate buffer (pH 7.4). The homogenate was divided into four equal parts, which were then treated differently as mentioned below.

Incubation of tissue homogenate with Sterodin® and/or ascorbic acid. – One goat liver tissue portion was kept as control (C) and another was treated with Sterodin® (S) (1.3 μL g⁻¹ tissue homogenate). The third portion (SA) was treated with Sterodin® (1.3 μL g⁻¹ tissue homogenate) and ascorbic acid (1.874 x 10⁻⁷ mol g⁻¹ tissue homogenate) and the fourth was treated with ascorbic acid (A) alone (1.874 x 10⁻⁷ mol g⁻¹ tissue homogenate). After treatment with Sterodin® and/or ascorbic acid, each portion of liver homogenate was shaken and incubated at 18 ± 2 °C for 24 hours for further processing. This temperature was found suitable for preservation of liver homogenate as well as for subsequent experiments.

Estimation of lipid peroxidation in liver tissue homogenates (MDA). – The extent of lipid peroxidation was measured in terms of MDA content using the thiobarbituric acid method (10). Estimation was made at 2, 4 and 24 hours of incubation time and was repeated in five animal sets. In each case, three parallel samples of 2.5 mL of the incubation mixture were treated with 2.5 mL of 10 % (m/V) trichloroacetic acid and centrifuged at room temperature at 3000 rpm for 30 minutes to precipitate proteins. Then, 2.5 mL of the supernatant was treated with 5 mL of 2 mmol L⁻¹ TBA solutions and then the volume was made up to 10 mL with distilled water. The mixture was heated on a boiling water bath for 30 minutes. The tubes were cooled to room temperature and the absorbance was measured at 530 nm against a TBA blank (prepared from 5 mL of TBA solution and 5 mL of distilled water). MDA concentration was determined from a standard curve. Different aliquots of standard 1,1,3,3-tetrahydroxypropane (TEP) solution underwent the same procedure as described above. The best-fit equation was A = 0.007171 c, where c is concentration of MDA in nanomoles per 10 mL solution, A is absorbance (R = 0.993).

Estimation of lipid peroxidation in liver homogenates in terms of GSH content. – Reduced glutathione (GSH) was measured by reaction with DTNB (11). Estimation was made at 2, 4 and 24 hours of incubation time and was repeated in five animal sets. In each case three parallel samples of 1 mL of the incubation mixture were treated with 1 mL of 5 % (m/V) TCA in 1 mmol L⁻¹ EDTA and centrifuged at 2000 g for 10 minutes. After that, 1 mL of the filtrate was mixed with 5 mL of 0.1 mol L⁻¹ phosphate buffer (pH 8.0) and 0.4 mL of 0.01 % DTNB in phosphate buffer pH 8.0. Absorbances of the solutions were measured at 412 nm against a blank (prepared from 6.0 mL of phosphate buffer and 0.4 mL of DTNB) (0.01 % in phosphate buffer). The concentration of reduced glutathione was determined from a standard curve. The best-fit equation was A = 0.001487 c, where c is a concentration of reduced glutathione in nanomoles per 10 mL solution, A is absorbance (R = 0.996).
Changes in malondialdehyde and reduced glutathione levels of different samples were expressed as average percent changes with respect to control values at the corresponding hours of incubation along with the corresponding standard error of mean in five sets. Interpretation of the results was supported by the paired-test and by analysis of variance (ANOVA) (12).

RESULTS AND DISCUSSION

Toxicological tests

By probit analysis method (Fig. 1) it was found that, LD$_{50}$ of Sterodin$^\circledR$ injection was 0.63 mL per 20 g b. m. male albino mice (i.e., 31.50 mL kg$^{-1}$ b. m.). The significance of the regression equation is judged from $R = 0.968$; high $F$-value of 75.84 ($F_{\text{theor}} = 6.61$) (df = 1, 5) at $p = 0.05$ speaks on behalf of high variance between groups in respect to variance within groups. In the toxicity study with 80% STERODIN$^\circledR$ injected intravenously none of the animals died within 24 hours and therefore the substance passed the test according to USP 28 (7). The observations of sequential changes in behavioral pattern after administration of Sterodin$^\circledR$ indicate that therapeutic dose to mice of 0.03 to 0.06 mL per 20 g b. m. (intraperitoneally) of the drug did not show any behavioral changes in male albino mice within 4 hours.

![Fig. 1. Estimation of LD$_{50}$ of Sterodin$^\circledR$ by probit analysis.](image-url)
Pharmacological tests

The results on the effect of Sterodin® on the total and differential white blood cell counts are shown in Table I. The final results were expressed as a mean (cm³) with the corresponding standard error of the mean. Maximum increase of 59 and 26 % in mean total and differential white blood cell counts, respectively, was observed on the 6th day after a single Sterodin® injection. After 30 days, the differential white blood cell count came to nearly day 0 level/control value, which indicates that Sterodin® activity can be retained at most for a month. The \( t \)-value was calculated by applying null hypothesis to observe the variability among 10 animals in a particular group.

The results on the effect of Sterodin® on carbon particle clearance from the blood are reported in Table II. Carbon clearance after 15 minutes of incubation suggested a significant enhancement (43.2 % with respect to the control) in the phagocytic function of the macrophages, and thus contributing to nonspecific immunity. The phagocytic index of 1.314 indicates stimulation by Sterodin® of both the reticuloendothelial system and the activity of macrophages. The two-tailed \( t \)-values (df = 4) in Table II indicate the pronounced effects of Sterodin® on carbon clearance from 2 min onwards.

### Table I. Effect of Sterodin® on total and differential white blood cell counts in mice

<table>
<thead>
<tr>
<th>No. of animals</th>
<th>Dose of Sterodin® (mL per 20 g)</th>
<th>Time of incubation (days)</th>
<th>Total WBC count (cm³)(^a)</th>
<th>Differential WBC count (cm³)(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0.05</td>
<td>0</td>
<td>5882 ± 181(^b)</td>
<td>61 ± 1.5(^b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>9345 ± 805(^b)</td>
<td>77 ± 1.6(^b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30</td>
<td>6875 ± 190(^b)</td>
<td>67 ± 2.04(^b)</td>
</tr>
</tbody>
</table>

N, L – neutrophils and lymphocytes, respectively.
\(^a\) Mean ± SEM (\(n = 10\)).
\(^b\) Significant difference between animals in a group: \(p < 0.005\).

### Table II. Effect of Sterodin® injection on carbon clearance and the phagocytic index

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Carbon clearance (%) ((t)-value, df = 4)(^a)</th>
<th>Phagocytic index ((PI)(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>8.0 (13.79)</td>
<td>1.314</td>
</tr>
<tr>
<td>0.5</td>
<td>1.5 (6.17)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>7.8 (300.56)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>21.6 (280.74)</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>43.2 (156.47)</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Percent change with respect to control at respective time points. The two tail \(t\)-value at the \(p = 0.01\) is 4.604 (df = 4).
\(^b\) PI = K (Sterodin®-treated)/K (control), where K is the slope of regression line.
Drub-lipid interaction studies. – Incubation of the liver homogenates with Sterodin® caused an increase in MDA content with respect to the control such as 8.6, 8.9 and 5.2 % at 2, 4 and 24 hours of incubation, respectively (Table III). These observations suggest that Sterodin® has the ability to induce the lipid peroxidation process. It was further found that MDA content was significantly reduced, by –15.7, –15.9 and –10.2 % after different hours of incubations, when the liver tissue homogenate was treated with both Sterodin® and ascorbic acid compared to the Sterodin®-treated group. This implies that ascorbic acid could reduce the extent of Sterodin®-induced lipid peroxidation.

Incubation of the tissue homogenate with ascorbic acid only also increased the MDA content with respect to the control at the 2nd hour of incubation (4.9 %). This implies the pro-oxidant effect of ascorbic acid. It was postulated that ascorbic acid could reduce Fe3+ to Fe2+, which promotes generation of hydroxyl radicals and other reactive oxygen species through Fenton’s reaction (13). Many vitamins have been reported to act as pro-oxidants in the presence of transition metals (14).

Drub-lipid interaction studies. – Incubation of the liver homogenates with Sterodin® caused an increase in MDA content with respect to the control such as 8.6, 8.9 and 5.2 % at 2, 4 and 24 hours of incubation, respectively (Table III). These observations suggest that Sterodin® has the ability to induce the lipid peroxidation process. It was further found that MDA content was significantly reduced, by –15.7, –15.9 and –10.2 % after different hours of incubations, when the liver tissue homogenate was treated with both Sterodin® and ascorbic acid compared to the Sterodin®-treated group. This implies that ascorbic acid could reduce the extent of Sterodin®-induced lipid peroxidation. Treatment of the tissue homogenate with ascorbic acid only also increased the MDA content with respect to the control at the 2nd hour of incubation (4.9 %). This implies the pro-oxidant effect of ascorbic acid. It was postulated that ascorbic acid could reduce Fe3+ to Fe2+, which promotes generation of hydroxyl radicals and other reactive oxygen species through Fenton’s reaction (13). Many vitamins have been reported to act as pro-oxidants in the presence of transition metals (14).

Incubation of the liver tissue homogenate with Sterodin® decreased the GSH level with respect to the corresponding controls such as –10.9, –9.9 and –8.5 % after 2, 4 and 24 hours of incubation, resp. (Table IV). Depletion of the GSH content was associated with an increase in lipid peroxidation. The decreased GSH level may be a consequence of enhanced utilization of this compound by the antioxidant enzymes glutathione peroxidase and glutathione-S-transferase. When the tissue homogenate was treated both with Sterodin® and ascorbic acid, the GSH level increased in comparison with the Sterodin®-treated group by 22.0, 19.5 and 22.8 % at different hours of incubation. Incuba-
tion of tissue homogenates with ascorbic acid only also enhanced the GSH level by 7.7, 10.0 and 14.3 % after different hours of incubation suggesting that increase in the GSH level may be due to the antioxidant property of ascorbic acid.

In both MDA and GSH estimations, there are significant differences (F1) among groups, such as Sterodin®-treated, Sterodin® plus ascorbic acid-treated as well as only ascorbic acid-treated group, but differences within a particular group (F2) are not significant, which shows that there is no statistical difference in goat liver obtained from different animal sets. The calculated t-values for the above mentioned groups with respect to the corresponding controls at specific times of incubation are higher than the theoretical value pointing to the fact that pharmacological effects have taken place in those groups (Tables III and IV).

**CONCLUSIONS**

Sterodin®, composed of bacterial metabolites and bile lipids, has been claimed to possess immunopotentiating activity, particularly in infectious diseases. The experimental data reported here indicates that Sterodin® increased the total WBC count, neutrophils count and stimulated the phagocytic activity in vivo. All of these effects can increase the efficacy of the immune system. Our findings of drug-lipid interaction studies suggest that ascorbic acid prevented lipid peroxidation induced by Sterodin®. The con-
cept of antioxidant co-therapy may also be exploited in the future formulation design of
drugs to reduce drug-induced toxicity. However, further extensive study is required to
advance this hypothesis.

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providing the facility and support to this work.

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SAŽETAK

Sterodin®, novi imunostimulator: neka toksikološka i farmakološka vrednovanja in vivo i interakcija lijek-lipid in vitro

SARBANI DEY RAY, PARTHA PRATIM ROY i SUPRATIM RAY

Sterodin® je novi nespecifični imunostimulator koji sadrži žučne lipide i bakterijske metabolite. U radu su opisana neka njegova toksikološka i farmakološka svojstva in vivo, te interakcija lijek-lipid in vitro. Nadalje, proučavana je moguća peroksidacija lipida inducirana Sterodinom® te učinak askorbinske kiseline na tu peroksidaciju. LD$_{50}$ Sterodina® bio je 0,63 mL u mužjacima albino miševa mase 20 g (31,50 mL kg$^{-1}$ tjelesne mase). U istim životinjama Sterodin® je povećao ukupan broj leukocita i neutrofila (59 odnosno 26 % mjereno 6 dana nakon injekcije Sterodina®) i stimulirao aktivnost fagocita in vivo. U ispitivanjima interakcije lijek-lipid in vitro korištena je jetra koze kao izvor lipida. Rezultati ukazuju da Sterodin® inducira peroksidaciju lipida, koja se može spriječiti askorbinskom kiselinom.

Ključne riječi: Sterodin®, imunostimulator, askorbinska kiselina, peroksidacija lipida, malondialdehid, reducirani glutation

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