Evaluation of Protective role of Morin on Busulfan-induced Lipid Peroxidation

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Abstract: The study was designed with an aim to evaluate the protective effects of morin on busulfan-induced lipid peroxidation. Goat liver was used as lipid source for this in vitro model of study. Lipid peroxidation study was performed by measuring the malondialdehyde and reduced glutathione content of tissue homogenates. The data presented in this work demonstrate the lipid peroxidation induction potential of busulfan and the antiperoxidative potential of morin on busulfan-induced lipid peroxidation.

Key words: Busulfan; Malondialdehyde; Reduced glutathione; Morin.

INTRODUCTION

Lipid peroxidation is a well known example of oxidative damage in cell membranes, lipoproteins, and other lipid-containing structures (1). This complex process is believed to contribute to human aging and disease by disrupting the structural conformation, the packing of lipid components and, ultimately, the function of biological membranes. The polyunsaturated fatty acids of membrane phospholipids are particularly susceptible to peroxidation and undergo significant modifications, including the rearrangement or loss of double bonds and, in some cases, the reductive degradation of lipid acyl side chains (2, 3). Lipid peroxidation leads to generation of peroxides and hydroperoxide that can decompose to yield a wide range of cytotoxic end products most of which are aldehydes as exemplified by molondialdehyde (MDA), 4-hydroxy-2-nonenal (4-HNE) etc (4). Free radicals are highly reactive molecules with odd number of electrons. They are constantly being generated in the body through various mechanisms and also being removed by endogenous antioxidant defense mechanism that acts by scavenging free radicals, decomposing peroxides and / or binding with pro-oxidant metal ion. Free radical mediated oxidative stress results usually from deficient natural antioxidant defense. In case of reduced or impaired defense mechanism and excess generation of free radicals that are not counter balanced by endogenous antioxidant defense exogenously administered antioxidants have been proven useful to overcome oxidative damage (5).

Busulfan, an alkylating agent used extensively in bone marrow transplantation. But there are evidences that organ toxicity in bone marrow transplantation may in part be due to free radical damage (6).

Plant flavonoids are emerging as potent therapeutic drugs effective against a wide range of free radical mediated diseases. Morin (3, 5, 7, 2’, 4’-pentahydroxyflavone), a member of flavonols, exert antioxidant potential and offer protection against the oxidative stress induced by hydrogen peroxide (7). It was found that there was an enhanced systemic availability of methotrexate in presence of morin in rats (8). Recently study shows that morin could exert a significant chemopreventive effect on colon carcinogenesis induced by 1, 2-dimethylhydrazine (9). Morin increases the bioavailability of tamoxifen and its main metabolite, 4-hydroxytamoxifen in rats (10).
Morin has protective effect on dimethylnitrosamine-induced hepatic fibrosis in rats (11).

The protective effect of various antioxidants on anticancer drug-induced lipid peroxidation had been reported earlier by us (12-14). In continuation of ongoing search for antioxidants, the present work has been carried out in vitro to evaluate the antiperoxidative potential of morin on busulfan-induced lipid peroxidation.

**EXPERIMENTAL**

**Materials**

The drug sample (busulfan) was provided by Elder Pharmaceuticals, Mumbai. Goat liver was used as the lipid source. Chemicals of analytical grade were used for the present study. Thiobarbituric acid (TBA) and trichloroacetic acid (TCA) were purchased from Ranbaxy Fine Chemicals Ltd., New Delhi. 5,5′-dithiobis (2-nitrobenzoic acid) was from SRL Pvt Ltd., Mumbai. 1,1,3,3, tetraethoxypropane, reduced glutathione were from Sigma Chemicals Co. St. Louis, MO, USA. Morin was purchased from Lova Chemie, Mumbai.

**Methods**

**Preparation of tissue homogenate**

Goat liver was collected from Drugapur Municipal Corporation (DMC) approved outlet. Goat liver was selected because of its easy availability and close similarity with human liver in its lipid profile (15). Goat liver perfused with normal saline through hepatic portal vein was harvested and its lobes were briefly dried between filter papers to remove excess blood and thin cut with a heavy-duty blade. The small pieces were then transferred in a sterile vessel containing phosphate buffer (pH 7.4) solution. After draining the buffer solution as completely as possible, the liver was immediately grinded to make a tissue homogenate (1 g/ml) using freshly prepared phosphate buffer (pH 7.4). The homogenate was divided into four equal parts, which were then treated differently as mentioned below.

One portion of the homogenate was kept as control (C) while a second portion was treated with the busulfan (D) at a concentration of 0.0013 mg/g tissue homogenate. The third portion was treated with both busulfan at a concentration 0.0013 mg/g tissue homogenate and morin at a concentration of 0.033 mg/g tissue homogenate (DA) and the fourth portion was treated only with morin at a concentration of 0.033 mg/g tissue homogenate (A). After busulfan and/or morin treatment, the liver tissue homogenate samples were shaken for two hours.

**Estimation of malondialdehyde (MDA) level from tissue homogenate**

The extent of lipid peroxidation was measured in terms of malondialdehyde (MDA) content using thiobarbituric acid (TBA) method (16). The estimation was done at 2 hours of incubation and repeated in five animal sets. In each case three samples of 2.5 ml of incubation mixture were treated with 2.5 ml of 10% (w/v) trichloroacetic acid (TCA) and centrifuged at room temperature at 3000 rpm for 30 minutes to precipitate protein. Then 2.5 ml of the supernatant was treated with 5 ml of 0.002 (M) TBA solutions and then volume was made up to 10 ml with distilled water. The mixture was heated on a boiling water bath for 30 minutes. Then tubes were cooled to a 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) using Shimadzu UV-1700 double beam spectrophotometer. The concentrations of MDA were determined from standard curve, which was constructed as follows. Different aliquots from standard 1, 1, 3, 3-tetraethoxypropane (TEP) solution were taken in graduated stoppered test tubes and volume of each solution was made up to 5 ml. To each solution, 5 ml of TBA solution was added and the mixture was heated in a steam bath for 30 minutes. The solutions were cooled to a room temperature and their absorbances were measured at 530 nm against TBA as blank. By plotting absorbances against concentrations a straight line passing through the origin of grid was obtained. The best-fit equation is $A=0.007086M$, where $M=$ nanomoles of MDA, $A=$ absorbance, $r = 0.995$, SEE= 0.006.

**Estimation of reduced glutathione (GSH) level from tissue homogenate:**

Reduced glutathione (GSH) was measured in accordance with Ellman’s method (17). The estimation was done at 2 hours of incubation and repeated in five animal sets. In each case three samples of 1 ml of incubation mixture were treated with 1 ml of 5% (w/v) TCA in 1 mM EDTA centrifuged at 2000 g for 10 minutes. After that 1 ml of the filtrate was mixed with 5 ml of 0.1M phosphate buffer (pH = 8.0) and 0.4 ml of 5, 5′-dithiobis-2-nitrobenzoic acid (0.01% in phosphate buffer pH=8.0) (DTNB) was added to it. The absorbances of the solutions were measured at 412 nm against blank (prepared from 6.0 ml of phosphate buffer and 0.4 ml of DTNB). The concentrations of reduced glutathione were determined from standard curve, which was constructed as follows. Different aliquots of standard reduced
glutathione stock solution were taken in 10 ml volumetric flasks. To each solution 0.4 ml of DTNB solution was added and volume was adjusted up to the mark with phosphate buffer (pH=8.0). The absorbance of each solution was measured at 412 nm against a blank containing 9.6 ml of phosphate buffer (pH=8.0) and 0.4 ml DTNB solution. By plotting absorbances against concentration a straight line passing through the origin of grid was obtained. The best-fit equation was \( A = 0.00151C \), where \( C = \) nanomoles of reduced glutathione, \( A = \) absorbance, \( r = 0.997 \), SEE= 0.008.

**STATISTICAL ANALYSIS**

Interpretation of the result is supported by student “\( t \)” test. Analysis of variance (ANOVA) and multiple comparison analysis using least significant different procedure (18, 19) were also performed on the percent changes data of various groups such as busulfan-treated (D), busulfan and morin (DA) and only morin-treated (A) with respect to control group of corresponding time.

### Table 1: Effects of Morin on Busulfan-induced lipid peroxidation: changes in MDA profile

<table>
<thead>
<tr>
<th>Name of the antioxidant</th>
<th>Name of the drug</th>
<th>Time of incubation (h)</th>
<th>Animal sets</th>
<th>% Changes in MDA content (with respect to corresponding control) due to treatment with drug and or antioxidant</th>
<th>Analysis of variance and multiple comparison</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morin</td>
<td>Busulfan</td>
<td>2</td>
<td>An 1</td>
<td>12.52(^a) -8.87(^b) -5.52(^a)</td>
<td>F1=146.32 [df=(2,8)] F2=3.50 [df=(4,8)]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>An 2</td>
<td>13.85(^a) -4.55(^b) -5.87(^a)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>An 3</td>
<td>14.15(^a) -7.71(^d) -8.80(^a)</td>
<td>Pooled variance</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>An 4</td>
<td>18.58(^a) -1.58(^b) -4.36(^a)</td>
<td>((S^2)^*)=4.85</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>An 5</td>
<td>11.52(^a) -4.52(^a) -12.6(^a)</td>
<td>Critical difference</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Av.</td>
<td>14.12(^b) -5.45(^b) -7.43(^b)</td>
<td>((p=0.05)^)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(±1.21)</td>
<td>Ranked means (^\text{**})</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(±1.29)</td>
<td>(D) (DA, A)</td>
</tr>
</tbody>
</table>

Percent changes with respect to controls of corresponding hours are shown. C, D, DA & A indicate control (not treated with busulfan or morin), only busulfan -treated, busulfan and morin -treated and only morin-treated samples respectively; Av. = Averages of five animal sets; SEM = Standard error of estimate (df=4); Significant of ‘\( t \)’ values of the changes of MDA content (df=2) are shown as: a>99%; b=97.5-99%; c=95-97.5%; d=90-95%; e=80-90%; f=70-80%; g=60-70%; h<60%; Theoretical values of F: p=0.05 level F1=4.46 [df=(2,8)], F2=3.84 [df=(4,8)] P=0.01 level F1=8.65 [df=(2,8)], F2=7.01 [df=(4,8)], F1 and F2 corresponding to variance ratio between groups and within groups respectively; * Error mean square, # Critical difference according to least significant procedure (18, 19) **Two means not included within same parenthesis are statistically significantly different at p=0.05 level.
Table 2: Effects of Morin on Busulfan-induced lipid peroxidation: changes in GSH profile

<table>
<thead>
<tr>
<th>Name of the antioxidant</th>
<th>Name of the drug</th>
<th>Time of incubation (h)</th>
<th>Animal sets</th>
<th>% Changes in GSH content (with respect to corresponding control) due to treatment with drug and or antioxidant</th>
<th>Analysis of variance and multiple comparison</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Samples</td>
<td></td>
</tr>
<tr>
<td>Morin</td>
<td>Busulfan</td>
<td>2</td>
<td>An 1</td>
<td>D</td>
<td>-2.24&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>An 2</td>
<td>DA</td>
<td>-4.54&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>An 3</td>
<td>A</td>
<td>-3.21&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>An 4</td>
<td></td>
<td>-2.12&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>An 5</td>
<td></td>
<td>-2.42&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Av.</td>
<td></td>
<td>-2.91&lt;sup&gt;a&lt;/sup&gt;</td>
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<td></td>
<td></td>
<td></td>
<td>(+SEM)</td>
<td></td>
<td>(+0.45)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Av.</td>
<td></td>
</tr>
</tbody>
</table>

Percent changes with respect to controls of corresponding hours are shown. C, D, DA & A indicate control (not treated with busulfan or morin), only busulfan -treated, busulfan and morin -treated and only morin-treated samples respectively; Av. = Averages of five animal sets; SEM = Standard error of estimate (df=4); Significant of 't' values of the changes of GSH content (df=2) are shown as: a>99%; b=97.5-99%; c=95-97.5%; d=90-95%; e=80-90%; f=70-80%; g=60-70%; h<60%; Theoretical values of F: p=0.05 level F<sub>1</sub>=4.46 [df=(2,8)], F<sub>2</sub>=3.84 [df=(4,8)] P=0.01 level F<sub>1</sub>=8.65 [df=(2,8)], F<sub>2</sub>=7.01 [df=(4,8)], F<sub>1</sub> and F<sub>2</sub> corresponding to variance ratio between groups and within groups respectively; * Error mean square, # Critical difference according to least significant procedure (18, 19) **Two means not included within same parenthesis are statistically significantly different at p=0.05 level.

RESULTS & DISCUSSION

The percent changes in MDA and GSH content of different samples at two hours of incubation were calculated with respect to the control of the corresponding time of incubation and was considered as indicator of the extent of lipid peroxidation. The results of the studies on busulfan-induced lipid peroxidation and its inhibition with morin were shown in Tables 1-2.

From Table 1 it was evident that tissue homogenates treated with busulfan showed an increase in MDA (14.12 %) content in samples with respect to control to a significant extent. The observations suggest that busulfan could significantly induce the lipid peroxidation process. MDA is a highly reactive three-carbon dialdehyde produced as a byproduct of polyunsaturated fatty acid peroxidation and arachidonic acid metabolism (20). But the MDA (-5.45%) content were significantly reduced in comparison to busulfan-treated group as well as control group when tissue homogenates were treated with busulfan in combination with morin. Again the tissue homogenates were treated only with the morin then the MDA (-7.43%) level were reduced in comparison to the control and the busulfan treated group. This decrease may be due to the free radical scavenging property of the morin.

It was evident from Table 2 that tissue homogenates treated with busulfan caused a decrease in GSH (-2.91%) content with respect control to a significant extent. The decrease in GSH content was associated with an increase in lipid peroxidation. When tissue homogenates were treated both with busulfan and morin then the GSH (4.95%) levels increased in comparison to busulfan treated group. Tissue homogenates treated only with morin also increase the GSH (4.37%) contents in comparison to the control samples. The increase in GSH level suggests the antiperoxidative potential of morin. Glutathione is an important antioxidant and plays a very important role in the defense mechanism for tissue against the reactive oxygen species (21). The depletion of GSH is associated with increase in lipid peroxidation. The decrease in GSH level may be a
consequence of enhanced utilization of this compound by the antioxidant enzymes glutathione peroxidase and glutathione-S-transferase.

To compare means of more than two samples, multiple comparison analysis along with analysis of variance was performed on the percent changes data with respect to control of corresponding hours. It is seen that there is significant differences among various groups (F1) such as busulfan-treated, busulfan and morin -treated and only morin -treated. But within a particular group, differences (F2) are insignificant which shows that there is no statistical difference in animals in a particular group (Tables 1-2). The Tables also indicate that the level of MDA / GSH in busulfan -treated group is only statistically significantly different from the busulfan and morin-treated group as well as only morin -treated group. But there is no statistically significantly difference among the busulfan and morin -treated group and only morin -treated group.

CONCLUSION
The results also suggest the antiperoxidative effects of morin and demonstrate its potential to reduce busulfan-induced lipid peroxidation and thus to increase therapeutic index of the drug by way of reducing toxicity that may be mediated through free radical mechanisms. However a detailed study is required to conclude such hypothesis.

REFERENCES
16. Ohkawa H., Ohishi N. and Yagi K., Assay of lipid peroxidation in animal tissue by


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