Isoenzyme Pattern and Activity in Oxidative Stress-Induced Hepatocarcinogenesis: The Protective Role of Selenium and Vitamin E

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Abstract: This work aimed to study the protective effects of selenium and vitamin E on DMN-induced alterations in isoenzyme pattern and activity of some hepatic enzymes, namely aspartate aminotransferase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH) and esterase (EST) in male mice. Thirty five male mice were divided into four groups, Control, DMN, DMNSE and SE. Hepatocarcinogenesis was induced by ip administration of DMN at 4 mg/kg twice a week for 6 weeks. Isoenzyme patterns were determined by PAGE; both total and fractional isoenzyme activities were determined. DMN treatment induced a significant elevation in total hepatic activity of AST, ALP, LDH and EST. In most experimental groups, the fractional isoenzyme activities exhibited the same trend. In case of ALP, none of the specific isoenzyme activities has shown significant change due to DMN hepatotoxicity. Isoenzyme activities of AST$_1$, LDH$_1$, LDH$_3$, EST$_1$, EST$_3$, EST$_5$ and EST$_7$ were feasible to differentiate between the hepatotoxic effect of DMN and/or the protective effect of combined antioxidant administration. In the present study, combined selenium and vitamin E supplementation proved a great ability to counteract almost all DMN-induced changes in total or isoenzyme activities of measured hepatic enzymes. Specific isoenzyme activities might help as additional diagnostic markers during early stages of carcinogenesis.

Key words: isoenzyme pattern, isoenzyme activity, oxidative stress, hepatocarcinogenesis, dimethylnitosamine, hepatic enzymes, selenium and vitamin E.

INTRODUCTION

Recent investigations have shown that free radicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) frequently play a role in the process of carcinogenesis.$^{[4,9,21,27,56,60]}$ Increased incidence of cancer with advancing age has also been attributed, at least in part, to the increasing level of free radical reactions with age, along with the diminishing ability of the immune system to eliminate altered cells.$^{[35]}$

Nutritional antioxidants especially minerals and vitamins show promise as cancer preventing agents, alone and in combination. Among these, selenium and vitamin E have demonstrated a significant role in preventing and treatment of certain cancers in tissue cultures and animal models.$^{[15,40,42,53,60,62]}$

Severe or prolonged exposure to oxidative stress has shown to elicit all stages of carcinogenesis$^{[22]}$. The highly unstable free radicals attack important cellular constituents, including DNA, proteins and other opportune targets, particularly cell membranes.$^{[6,8,17,55]}$. In the initiation stage, DNA is attacked by a carcinogen or free radical causing a mutation in one or more of the genes or chromosomes$^{[59]}$. Under such conditions, early changes in the process of gene expression presumably happens that might affect different enzyme activities as well as isoenzyme pattern and expression.

Isoenzyme patterns and activities have recently drawn a considerable attention in oxidative stress-correlated studies of carcinogenesis, aging and smoking$^{[12,16,29,44,50,64]}$

Upon the above mentioned bases the present study has been suggested with the aim to: (1) explore the specific isoenzyme activity in early stages of induced hepatocarcinogenesis in male mice; (2) test the feasibility of using specific isoenzyme activities as additional diagnostic markers during precancerous stages and (3) explore the protective role of both selenium and vitamin E supplementation on altered isoenzyme activity. For such purpose different classes of hepatic enzymes with various isoenzymes or isoforms, namely aspartate aminotransferase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH) and esterase (EST) have been selected for the present study.

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MATERIAL AND METHODS

Animals and Experimental Design: Thirty five male Bulb C mice aged 1-2 months (25-35 g) have been allotted for the present investigation. Animals were provided by The Research Institute of Ophthalmology, Giza, Egypt. They had free access to standard pelleted diet and tape water. After two week of acclimatization period, animals have been classified into 4 group. (1) Control group: given orally 0.5 ml of distilled water using a gastric tube. (2) DMN group: given orally 4 mg/kg B.Wt. of dimethylnitrosamine (DMN) dissolved in distilled water twice a week. (3) DMNSE group: given orally 4 mg/kg B.Wt. of dimethylnitrosamine dissolved in distilled water twice a week and daily oral dose of both selenium (60 µg/kg B.Wt.) and vitamin E (200 mg/kg B.W.) dissolved in distilled water. (4) SE group: given daily an oral dose of both selenium (60 µg/kg B.Wt.) and vitamin E (200 mg/kg B.W.) dissolved in distilled water. This fourth group served as a positive control. The experiment has been extended for 6 weeks. Animals were sacrificed by decapitation after 2 and 6 weeks. Control animals were decapitated only after 4 weeks of the experiment.

Chemicals
Dimethylnitrosamine (DMN): was a product of Sigma, USA.
Selenium: was donated by Arab Company for Pharmaceutical & Medical Plants Mepaco–Egypt in the form of sodium selenite.
Vitamin E: was donated by Arab Company for Pharmaceutical & Medical Plants Mepaco–Egypt in the form a-tocopherol (the water soluble form).

Methods: Livers were excised and washed in normal saline, dried on filter paper, then finally homogenized in ice-cold distilled water using an electric homogenizer (1g/10 ml). Homogenates were kept frozen at −20°C for subsequent isoenzyme analysis of aspartate aminotransferase (AST, 2.6.1.1.), alkaline phosphatase (ALP, 3.1.3.1.), lactic dehydrogenase (LDH, 1.1.1.27.) and esterase (EST, 3.1.1.1.).

Electrophoretic Separation: Frozen tissue homogenates were brought to room temperature one hour prior electrophoretic separation. The isoenzyme fractions of ALP, AST, LDH and EST were separated by polyacrylamide gel electrophoresis (PAGE) using Mini Protein II electrophoretic apparatus, Biorad. Scanning of the isoenzyme fractions was applied using Media Sci Image densitometry 700 Biorad and analyzed using the gel prosoft ware (Ver.3, USA, 1998). Total enzymatic activities were estimated colorimetrically in IU/g according to methods described by Martin and Julian[38]. Fractional isoenzyme activities were calculated as percentage of total activities and presented in the form of IU/g.

Statistical Analysis: Data were statistically analyzed using one-way analysis of variance (ANOVA test) using the statistical software package of SPSS-PC version 11.

RESULTS AND DISCUSSIONS

Total Activity of Hepatic AST: Data presented in Table 1 expose a significant rise in total hepatic AST activity in both DMN and DMNSE groups after 2 and 6 weeks of the experiment as compared to the control group. Comparison of both DMN and DMNSE values, revealed a slight improvement in total AST activity in DMNSE group. On the other side, SE positive control group exhibited almost normal values during the whole experimental period.

Fractional Activity of Hepatic AST Isoenzymes: As demonstrated in Figure 1, electrophoretic pattern of hepatic AST revealed 2 phenotypes in the agarose gel with highly similar electrophoretic mobility in the control group.. In control animals, AST1 was the most expressed isoenzyme (51.48%) while AST2 was the least expressed one (48.52%). Throughout the whole experimental period, the three treated groups (DMN, DMNSE, SE) demonstrated a marked change in the electrophoretic mobility.

After 2 weeks of the experiment, the fractional AST1 and AST2 activities exhibited a significant elevation in DMN and DMNSE groups versus the control group (Table 1). This elevation was less demonstrated in DMNSE group Statistical analysis has also exposed a significant reduction in both AST1 and AST2 in SE group versus DMN and DMNSE.

After 6 weeks of the experiment, almost the same trend of changes has been demonstrated for both AST1 and AST2 isoenzyme activities in all treated groups versus the control group. The only exception was that of AST1 activity which exhibited a normal value in DMNSE group. AST1 activity seemed to be able to differentiate between the hepatoxic effect of DMN and the protective effect of antioxidant administration. In other words, AST2 activity seemed to be more resistant to the protective effect of antioxidant supplementation.
Table 1: Effect of dimethylnitrosamine administration (4 mg/kg b.wt) with or without selenium and vitamin E supplementation on total and fractional isoenzyme activities of aspartate aminotransferase (AST) and alkaline phosphatase (ALP) in liver of male mice

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>C (6W)</th>
<th>DMN (2W)</th>
<th>DMNSE (2W)</th>
<th>SE (2W)</th>
<th>DMN (6W)</th>
<th>DMNSE (6W)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total AST (U/g)</td>
<td>0.784 ± 0.20</td>
<td>1.706 ± 0.38*</td>
<td>1.469 ± 0.38*</td>
<td>0.710 ± 0.18*</td>
<td>1.475 ± 0.74*</td>
<td>1.292 ± 0.17*</td>
</tr>
<tr>
<td>Isoenzyme Activity (U/g)</td>
<td>0.378 ± 0.09</td>
<td>0.819 ± 0.17*</td>
<td>0.677 ± 0.23*</td>
<td>0.311 ± 0.07*</td>
<td>0.830 ± 0.45*</td>
<td>0.724 ± 0.12*</td>
</tr>
<tr>
<td></td>
<td>(48.52%)</td>
<td>(48.16%)</td>
<td>(45.24%)</td>
<td>(44.66%)</td>
<td>(55.24%)</td>
<td>(55.84%)</td>
</tr>
<tr>
<td>AST1</td>
<td>0.406 ± 0.12</td>
<td>0.887 ± 0.22*</td>
<td>0.792 ± 0.16*</td>
<td>0.398 ± 0.14*</td>
<td>0.645 ± 0.32*</td>
<td>0.569 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>(51.48%)</td>
<td>(51.74%)</td>
<td>(54.62%)</td>
<td>(55.42%)</td>
<td>(44.68%)</td>
<td>(44.02%)</td>
</tr>
<tr>
<td>Total %</td>
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<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Total ALP (U/g)</td>
<td>8.404 ± 2.18</td>
<td>10.226 ± 1.61</td>
<td>12.838 ± 3.58</td>
<td>6.125 ± 1.52</td>
<td>14.791 ± 7.97</td>
<td>9.707 ± 1.61*</td>
</tr>
<tr>
<td>Isoenzyme Activity (U/g)</td>
<td>2.889 ± 0.96</td>
<td>3.440 ± 1.16</td>
<td>3.760 ± 1.04</td>
<td>2.116 ± 0.43*</td>
<td>4.127 ± 1.24</td>
<td>2.933 ± 0.56</td>
</tr>
<tr>
<td></td>
<td>(33.82%)</td>
<td>(32.96%)</td>
<td>(34.94%)</td>
<td>(29.30%)</td>
<td>(30.24%)</td>
<td>(34.60%)</td>
</tr>
<tr>
<td>ALP1</td>
<td>2.412 ± 0.60</td>
<td>3.696 ± 0.74</td>
<td>4.243 ± 1.12</td>
<td>2.164 ± 0.51</td>
<td>5.284 ± 4.26</td>
<td>3.249 ± 0.72</td>
</tr>
<tr>
<td></td>
<td>(28.88%)</td>
<td>(37.14%)</td>
<td>(33.58%)</td>
<td>(33.08%)</td>
<td>(33.38%)</td>
<td>(28.84%)</td>
</tr>
<tr>
<td>ALP2</td>
<td>3.103 ± 0.67</td>
<td>3.091 ± 1.06</td>
<td>4.835 ± 1.77</td>
<td>1.845 ± 0.65</td>
<td>5.380 ± 2.58</td>
<td>5.24 ± 0.54</td>
</tr>
<tr>
<td></td>
<td>(37.32%)</td>
<td>(29.88%)</td>
<td>(37.10%)</td>
<td>(36.90%)</td>
<td>(36.38%)</td>
<td>(36.58%)</td>
</tr>
<tr>
<td>Total %</td>
<td>100</td>
<td>100</td>
<td>100</td>
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</tr>
</tbody>
</table>

Number of samples= 5.
* = Significant difference versus C control group at P<0.05 (ANOVA, LSD).
= Significant difference versus DMN (2w) at P<0.05.
= Significant difference versus DMN-SVE (2w) at P<0.05.
= Significant difference versus DMN (6w) at P<0.05.
= Significant difference versus DMN-SVE (6w) at P<0.05.
Effect of dimethyl nitrosamine administration (4 mg/kg b. wt) with or without selenium and vitamin E supplementation on total and fractional isoenzyme activities of lactic dehydrogenase (LDH) and esterase (EST) in liver of male mice

Table 2: Effect of dimethyl nitrosamine administration (4 mg/kg b. wt) with or without selenium and vitamin E supplementation on total and fractional isoenzyme activities of lactic dehydrogenase (LDH) and esterase (EST) in liver of male mice

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Control (DMN 2W)</th>
<th>DMNSE (2W)</th>
<th>SE (2W)</th>
<th>DMN (6W)</th>
<th>DMNSE (6W)</th>
<th>SE (6W)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total LDH (U/g)</td>
<td>3.190 ± 0.76</td>
<td>4.132 ± 0.86</td>
<td>5.562 ± 1.55*</td>
<td>2.729 ± 0.68*</td>
<td>6.319 ± 3.20*</td>
<td>4.472 ± 0.67</td>
</tr>
<tr>
<td>Isoenzyme Activity (U/g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDH₁</td>
<td>0.755 ± 0.20</td>
<td>0.764 ± 0.17</td>
<td>1.069 ± 0.36</td>
<td>0.546 ± 0.16*</td>
<td>1.265 ± 0.62*</td>
<td>0.911 ± 0.17</td>
</tr>
<tr>
<td></td>
<td>(23.58%)</td>
<td>(18.50%)</td>
<td>(19.04%)</td>
<td>(19.90%)</td>
<td>(20.08%)</td>
<td>(20.44%)</td>
</tr>
<tr>
<td>LDH₂</td>
<td>0.627 ± 0.14</td>
<td>0.976 ± 0.22</td>
<td>1.229 ± 0.35*</td>
<td>0.493 ± 0.15*</td>
<td>1.252 ± 0.71*</td>
<td>0.933 ± 0.13</td>
</tr>
<tr>
<td></td>
<td>(19.78%)</td>
<td>(23.68%)</td>
<td>(22.06%)</td>
<td>(17.96%)</td>
<td>(19.46%)</td>
<td>(20.94%)</td>
</tr>
<tr>
<td>LDH₃</td>
<td>0.617 ± 0.17</td>
<td>0.653 ± 0.14</td>
<td>1.137 ± 0.36*</td>
<td>0.547 ± 0.12*</td>
<td>1.354 ± 0.46*</td>
<td>0.993 ± 0.14*</td>
</tr>
<tr>
<td></td>
<td>(19.22%)</td>
<td>(15.80%)</td>
<td>(20.44%)</td>
<td>(20.26%)</td>
<td>(22.78%)</td>
<td>(20.84%)</td>
</tr>
<tr>
<td>LDH₄</td>
<td>0.596 ± 0.17</td>
<td>0.868 ± 0.20</td>
<td>1.002 ± 0.32</td>
<td>0.617 ± 0.21</td>
<td>1.215 ± 0.64*</td>
<td>0.881 ± 0.18</td>
</tr>
<tr>
<td></td>
<td>(19.04%)</td>
<td>(20.92%)</td>
<td>(17.84%)</td>
<td>(22.48%)</td>
<td>(19.06%)</td>
<td>(19.62%)</td>
</tr>
<tr>
<td>LDH₅</td>
<td>0.596 ± 0.20</td>
<td>0.869 ± 0.23</td>
<td>1.125 ± 0.24*</td>
<td>0.526 ± 0.13*</td>
<td>1.233 ± 0.82*</td>
<td>0.815 ± 0.17</td>
</tr>
<tr>
<td></td>
<td>(18.42%)</td>
<td>(21.02%)</td>
<td>(20.60%)</td>
<td>(19.38%)</td>
<td>(18.64%)</td>
<td>(18.18%)</td>
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<tr>
<td>Total %</td>
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<td>100</td>
<td>100</td>
<td>100</td>
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</tr>
<tr>
<td>Total EST (U/g)</td>
<td>2.647 ± 0.63</td>
<td>3.101 ± 0.64</td>
<td>3.330 ± 0.93</td>
<td>1.634 ± 0.41*</td>
<td>5.704 ± 2.87*</td>
<td>3.204 ± 0.46</td>
</tr>
<tr>
<td>Isoenzyme Activity (U/g)</td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>EST₁</td>
<td>0.390 ± 0.11</td>
<td>0.623 ± 0.15</td>
<td>0.491 ± 0.19</td>
<td>0.220 ± 0.06*</td>
<td>0.870 ± 0.42*</td>
<td>0.421 ± 0.06*</td>
</tr>
<tr>
<td></td>
<td>(14.68%)</td>
<td>(19.96%)</td>
<td>(14.52%)</td>
<td>(13.64%)</td>
<td>(15.54%)</td>
<td>(13.14%)</td>
</tr>
<tr>
<td>EST₂</td>
<td>0.422 ± 0.11</td>
<td>0.432 ± 0.09</td>
<td>0.388 ± 0.12</td>
<td>0.204 ± 0.09</td>
<td>0.812 ± 0.52*</td>
<td>0.530 ± 0.20</td>
</tr>
<tr>
<td></td>
<td>(15.88%)</td>
<td>(13.94%)</td>
<td>(11.58%)</td>
<td>(12.12%)</td>
<td>(13.54%)</td>
<td>(16.46%)</td>
</tr>
<tr>
<td>EST₃</td>
<td>0.416 ± 0.09</td>
<td>0.509 ± 0.12</td>
<td>0.346 ± 0.11</td>
<td>0.169 ± 0.04*</td>
<td>0.818 ± 0.41*</td>
<td>0.309 ± 0.13*</td>
</tr>
<tr>
<td></td>
<td>(15.82%)</td>
<td>(16.40%)</td>
<td>(10.33%)</td>
<td>(10.43%)</td>
<td>(14.26%)</td>
<td>(09.57%)</td>
</tr>
<tr>
<td>EST₄</td>
<td>0.330 ± 0.08</td>
<td>0.376 ± 0.08</td>
<td>0.435 ± 0.20</td>
<td>0.206 ± 0.08</td>
<td>0.990 ± 0.58*</td>
<td>0.557 ± 0.09*</td>
</tr>
<tr>
<td></td>
<td>(12.46%)</td>
<td>(12.13%)</td>
<td>(12.76%)</td>
<td>(12.35%)</td>
<td>(16.80%)</td>
<td>(17.48%)</td>
</tr>
<tr>
<td>EST₅</td>
<td>0.366 ± 0.09</td>
<td>0.280 ± 0.06</td>
<td>0.815 ± 0.26*</td>
<td>0.427 ± 0.10*</td>
<td>0.780 ± 0.24*</td>
<td>0.400 ± 0.06*</td>
</tr>
<tr>
<td></td>
<td>(13.86%)</td>
<td>(99.04%)</td>
<td>(24.34%)</td>
<td>(26.34%)</td>
<td>(14.76%)</td>
<td>(12.52%)</td>
</tr>
<tr>
<td>EST₆</td>
<td>0.392 ± 0.09</td>
<td>0.449 ± 0.08</td>
<td>0.464 ± 0.14</td>
<td>0.244 ± 0.05</td>
<td>0.617 ± 0.28</td>
<td>0.421 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>(14.80%)</td>
<td>(14.56%)</td>
<td>(14.65%)</td>
<td>(15.08%)</td>
<td>(10.95%)</td>
<td>(13.00%)</td>
</tr>
<tr>
<td>EST₇</td>
<td>0.332 ± 0.08</td>
<td>0.432 ± 0.10</td>
<td>0.391 ± 0.12</td>
<td>0.165 ± 0.05</td>
<td>0.816 ± 0.47*</td>
<td>0.566 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>(12.56%)</td>
<td>(13.84%)</td>
<td>(11.77%)</td>
<td>(10.01%)</td>
<td>(13.96%)</td>
<td>(17.70%)</td>
</tr>
<tr>
<td>Total %</td>
<td>100</td>
<td>100</td>
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</tr>
</tbody>
</table>

Number of samples= 5.

* = Significant difference versus C control group at P<0.05. † = Significant difference versus DMN (2w) at P<0.05.

** = Significant difference versus DMN (6w) at P<0.05. *** = Significant difference versus DMN-SVE (2w) at P<0.05.

** Total Activity of Hepatic ALP:** As shown in Table (1), total hepatic ALP activity has revealed a significant rise in DMN-treated group versus the control value only after 6 weeks, and a significant rise versus SE group throughout the whole experimental period. Combined antioxidant supplementation restored the normal values in DMNSE group after 6 weeks.

**Fractional Activity of Hepatic ALP Isoenzymes:** As demonstrated in Figure 2, electrophoretic pattern of hepatic ALP revealed 3 distinct isoenzymes in all
In control animals, ALP was the most expressed isoenzyme (37.32%) and ALP2 was the least expressed one (28.88%). The three treated groups exhibited marked changes in electrophoretic mobility versus the control group.

After 2 weeks of the experiment, only ALP2 and ALP3 activities exhibited a remarkable but insignificant elevation in DMN group versus the comparable control values (Table 1). A similar elevation has been demonstrated in DMNSE group for all ALP isoenzymes versus the control values. In contrast, significantly reduced activities of ALP1 and ALP3 could be detected in SE group versus the comparable values in DMNSE group. However, this significant reduction could not be detected versus the control values or those of DMN group (P>0.05).

**Fractional Activity of Hepatic LDH Isoenzymes:** As demonstrated in Figure 3, electrophoretic separation of hepatic AST revealed 5 distinct isoenzymes in all experimental groups. In control animals, LDH3 was the most expressed isoenzyme (23.58%) and LDH1 was the least expressed one (18.42%). The three treated groups exhibited some changes in electrophoretic mobility, which were less demonstrable at the end of the experiment versus the control pattern.

After 2 weeks of the experiment, the activity of LDH1, LDH2 and LDH4 exhibited a nonsignificant elevation in DMN group versus the comparable control values (Table 2). Despite the significant rise recorded for total hepatic LDH activity in DMNSE group versus the control one, statistical analysis has only proved a significant rise in isoenzyme activity of LDH1, LDH3 and LDH4. LDH2 and LDH4 seemed to be more resistant. On the other hand, SE group exhibited almost normal activity of all hepatic LDH isoenzymes.

After 6 weeks, DMN group exhibited a significant rise in all hepatic LDH isoenzymes versus the control group, which has been corrected almost completely by combined selenium and vitamin E supplementation. LDH1 isoenzyme activity exhibited the least protective response to antioxidant supplementation; the value being significantly lower than that of the comparable DMN (6W) group. On the other side, SE group animals exhibited normal LDH isoenzyme activity versus the control values. Only LDH3, LDH4 and LDH5 exhibited significantly lower activity versus DMN group (6W).

**Total Activity of Hepatic EST:** After 2 weeks of the experiment, the three treated groups exhibited an insignificant variation in total hepatic esterase activity versus the control group (Table 2). Meanwhile, statistical analysis revealed a significant reduction in SE group versus DMNSE group (P<0.05). After 6 weeks of the experiment, DMN-intoxication induced a
significant rise in total hepatic EST activity versus the control value. Combined antioxidant supplementation seemed to counteract this induction. On the other hand, SE positive control group exhibited normal hepatic activity during the whole experimental period.

**Fractional Activity of Hepatic EST Isoenzymes:** As demonstrated in Figure 4, electrophoretic separation of hepatic AST revealed 7 distinct isoenzymes in all experimental groups. In control animals, EST$_6$ was the most expressed isoenzyme (15.88%) and EST$_1$ was the least expressed one. Some changes in the electrophoretic mobility could be demonstrated in the three treated groups versus the control pattern. These changes were more pronounced in DMNSE and SE groups after 2 weeks.

![Figure 4: Isoenzyme pattern of hepatic esterase (EST) of male mice as affected by dimethylnitrosamine treatment and/or antioxidant administration for 2 or 6 weeks. Individual lanes represent individual sample per group. C= control; DMN= Dimethylnitrosamine treated group; DMNSE= DMN and selenium and vitamin E treated group; SE= selenium and vitamin E treated group. 2W = 2 weeks; 6W = 6 weeks.](image)

After 6 weeks of the experiment, the fractional activity of almost all EST isoenzymes exhibited a significant elevation versus the control and DMN (6W) groups, EST$_3$ was the only exception. On the other hand, DMNSE group exhibited normal activity of almost all EST isoenzymes. Meanwhile, fractional activities of EST$_3$, EST$_4$, EST$_5$, EST$_7$ have shown a significant reduction in DMNSE group versus the comparable values in DMN (6W) group. Almost the same trend of changes has been encountered in SE group versus DMN (6W) groups. However, fractional activities of EST$_1$, EST$_2$, EST$_3$, and EST$_7$ were significantly lower in comparison with the comparable activities in DMN (6W) group.

**Discussion:** Nitrosamines like dimethylnitrosamine (DMN) are N-nitroso alkyl compounds, categorized as potent hepatotoxins and hepatocarcinogens in experimental animals, producing reproducible tumors after repeated administration$^{[30]}$. Nitrosamines are reported to undergo metabolic activation by cytochrome p450 enzymes to form reactive electrophiles, which cause nitrosative stress leading to cytotoxicity, mutagenicity and carcinogenicity$^{[3]}$.

DMN is known to induce nitrosative stress that leads to both necrotic and apoptotic cell death$^{[10,41,45,46,48,51]}$. DNA damage has been suggested to contribute to DMN-induced necrosis$^{[31]}$ and may be an important action of this alkylating agent$^{[48]}$.

The administration of a carcinogenic may bring about changes in enzymatic activity arising from clonic proliferation, so it is of great importance to analyze quantitative variation in the enzyme activity in order to understand the process involved$^{[33,34]}$. The mutagenic effect of reactive nitrogen species induced by nitrosamines seems to affect not only the total activity of many hepatic enzymes but also the rate of expression of their isoenzymes.

The present study highlights the effect of DMN on the total activity as well as isoenzyme pattern and activity of hepatic AST, ALP, LDH and EST in mice liver. It also explores the protective effect of combined selenium and vitamin E supplementation. First of all, the present data have demonstrated a significant rise in total hepatic AST, ALP, LDH and EST activity in
DMN-treated group versus the control group especially during the last stage of the experiment. In most cases combined selenium and vitamin E has shown to play a protective effect against DMN-induced hepatotoxicity. In this context previous studies have also reported a significant rise in total serum or tissue-specific activity of ALT, AST, GGT, G-6-PDH, LDH, β-glucuronidase and hexokinase due to nitrosamine treatment [11,20,23,32,49].

Previous studies have also proved the protective effect of vitamin E pre-treatment against N-nitrosodiethylamine-induced changes in enzyme activities especially on total serum activities of AST, ALT, ALP and LDH [9]. On the other hand, selenium-enriched malt showed a better chemopreventive efficiency in decreasing the content of ALT, ALP in plasma of diethylnitrosamine-treated rats [77].

As regards the isoenzyme pattern and activity of hepatic enzymes, the present study has exposed some important findings: (1) In the four isoenzyme patterns described, changes in electrophoretic mobility were recorded in the three treated groups versus the control group. In case of AST and ALP, these changes could be improved to a considerable degree in DMN-treated group by combined antioxidant supplementation for 6 weeks. (2) In most experimental groups, the fractional isoenzyme activities exhibited the same trend recorded for the total enzyme activity; (3) In rare cases, like that encountered in ALP in DMN group, none of the specific isoenzyme activities has reached the significant rise seen for total activity throughout the whole experimental period; (4) In some cases, isoenzyme activity instead of total activity proved the ability to differentiate between the hepatotoxic effect of DMN and the protective effect of combined antioxidant administration or even their interaction. Among these are activity of AST1, LDH1 and LDH3, as well as activity of EST1, EST2, EST3 and EST4; (5) Combined selenium and vitamin E supplementation proved a great ability to counteract almost all DMN-induced changes in activity of hepatic AST, ALP, LDH and EST isoenzymes.

Isoenzyme patterns and expression have been previously studied for exposing the impact of free radicals, many xenobiotics including carcinogens and that of protective antioxidants [1,2,26,36,44,47,56,63,64]. Redox imbalance is known to induce lipid peroxidation, oxidation of proteins, DNA damage and interference of reactive oxygen species (ROS) or reactive nitrogen species (RNS) with signal transduction pathways. These consequences become even more harmful when genetic variations impair protein expression. Therefore, therapeutic strategies often aimed at reducing free-radical formation and scavenging free-radicals by the help of antioxidant supplementation.

As regards changes in electrophoretic mobility demonstrated in the present study, it seemed that free radicals affect the integrity of the polypeptide chain in the protein molecule causing fragmentation of the polypeptide chain due to sulphydryl-mediated cross linking of the labile amino acids as claimed by Bedwell et al. [7]. The changes in the fractional activity of different isoenzymes seemed to be correlated with changes in the rate of protein expression secondary to DNA damage initiated by free radicals. Free radical induction by DMN is well documented in the literature [10,46,48,51,41].

The present data provide an additional evidence about the free radical induction theory of hepatocarcinogenesis by DMN. It has demonstrated the protective role of combined selenium and vitamin E supplementation against the DMN-induced changes in total or specific hepatic isoenzyme activities of AST, ALP, LDH and EST. The protective role of selenium and/or vitamin E in prevention and treatment of cancer has been intensively reviewed by Valko et al. [60].

Several mechanisms by which selenium and/or vitamin E may protect against cancer have been postulated. Selenium has shown to play an important role in the inhibition of carcinogen-induced covalent DNA adduct formation and retardation of oxidative damage to DNA, lipids and proteins and for modulating cellular and molecular events that are critical in cell growth inhibition and in the multi-step carcinogenesis process [15]. Selenium, in addition to its inclusion in the selenoenzyme glutathione peroxidase, which deactivates free radicals, enhances the immune response, resulting in potentially increased resistance to cancer initiation or growth [15]. On the other hand, vitamin E is known to enhance the body’s immune response, inhibit nitrosamine formation, enhances cell communication and inhibit the metabolic activation of carcinogen [61]. Vitamin E can effectively counteract ROS generated by toxicant or carcinogen induced oxidative damage by trapping reactive oxyradicals and preserve membrane integrity [54].

A combination of selenium and vitamin E showed a greater ability than either nutrient alone to inhibit transformation of normal mouse embryo cells to cancerous cells after exposure to radiation and chemicals [25]. Selenium and vitamin E appear to function cooperatively and often have interrelated and synergistic effects in cancer studies [28,12,24,39,43]. However, several studies have demonstrated an inverse association between vitamin E and/or selenium supplementation and the subsequent risk of cancer at
various sites.[40,53,52,19,18,57,13,14].

From the other side, the present data have demonstrated that combined selenium and vitamin E supplementation to normal mice did not affect the total hepatic enzyme activities as compared to the control group. However, it might affect the fractional isoenzyme activity. This might reflect the ability of controlled application of combined supplementation of selenium and vitamin E in preventive medicine.

The present study could provides also a considerable evidence about the feasibility of using isoenzyme activity of AST, LDH and EST beside total enzyme activity as diagnostic markers during early stages of hepatocarcinogenesis. It could also help to differentiate between the nitrosamine-induced hepatotoxic effect and/or the protective effect of combined antioxidant administration. Nevertheless, further investigations seem necessary to provide substantial evidence before using specific isoenzyme activities as biomarkers in different stages and types of cancer.

REFERENCES


