CHAPTER-II

Mobilization of nuclear copper by plant polyphenols and oxidative breakage of cellular DNA: demonstration using a lysed version of Comet assay.
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Standardization of Lysed version of Comet Assay using H₂O₂:

Lysed version of Comet assay has been used to study the direct interaction of various agents with cell nuclei as it eliminates the barrier presented by cell membrane and the intracellular environment (Szeto et al., 2002). Lysed version of Comet assay was performed as described by Kasamatsu et al. (1996) with some modifications. In this technique, the lymphocytes (embedded in agarose on slides) were lysed and the nuclei on such slides were subsequently treated with the test agent (See "Materials and Methods"). H₂O₂ is a known genotoxic agent and is routinely used in genotoxicity testing. In order to standardize the lysed version of Comet Assay, an experiment was performed with H₂O₂. Photographs of comets (100x) observed after treatment of lymphocyte nuclei with increasing concentrations of H₂O₂ are shown in figure 27. As can be seen, untreated lymphocyte nuclei were not damaged and do not show a tail. However, with increasing concentrations of H₂O₂, a progressive increase in the length of comet tail is observed. In figure 28, the results of the same experiment are plotted as comet tail length (µm) as a function of increasing H₂O₂ concentration.

A comparison of DNA breakage by EGCG in intact lymphocytes and lymphocyte nuclei as measured by Comet Assay:

Since in the lysed version of Comet assay, the membrane and cytoplasmic barrier is eliminated, it would be reasonable to assume that the polyphenol EGCG is able to directly interact with the cell nuclei. Thus, considerably greater DNA breakage should be observed in the lysed version as compared with the standard version where intact lymphocytes are used. Increasing concentrations of EGCG (0-100 µM) were tested for DNA breakage in intact
lymphocytes (standard version of Comet assay) and compared with that observed in lymphocyte nuclei (lysed version). Photographs of comets obtained with 50μM EGCG in the standard and lysed version of Comet assay have been shown in figure 29. In figure 30, the data is plotted as tail length of comets with increasing concentration of EGCG. As can be seen from the figure, the rate of tail length formation is considerably greater in the case of lysed version. The results indicate that EGCG is able to directly interact with nuclei and cause DNA breakage when lysed version of comet assay is used. Similar results have also been reported by Kasamatsu et al. (1996) using H₂O₂ and bleomycin.

A comparison of DNA breakage in lymphocyte nuclei by various polyphenols:

We have previously shown (Azmi et al., 2006) that the presence and position of hydroxyl groups in polyphenols particularly the presence of ortho-dihydroxy groups, is important for cellular DNA cleavage. Thus the parent compounds of various classes of polyphenols that do not possess any hydroxyl group are not cleaving agents. I have therefore compared the relative DNA breakage efficiency of various polyphenols in lymphocyte nuclei. Figure 31 shows comet tail lengths obtained using increasing concentrations of various polyphenols. It is seen that gallic acid (a constituent of tannic acid) is the most effective DNA cleaving agent followed by EGCG, aloin and resveratrol. It may be suggested that the differential DNA cleavage efficiency of various polyphenols is a function of the difference in the number and position of hydroxyl groups as well as copper binding efficiency.
Effect of preincubation of lymphocyte nuclei with polyphenols on polyphenol-Cu(II) mediated DNA breakage:

Nuclear DNA breakage was studied by preincubating the nuclei with polyphenols (resveratrol and EGCG) after which the nuclei were washed with PBS and incubated further in the presence of 5μM CuCl₂. The results given in figure 32 show that the concentrations of polyphenols when used alone (2,5,10 μM) give rise to only a limited degree of nuclear DNA breakage (2-5 μmetres). With either EGCG (figure 32A) or with resveratrol (figure 32B), a progressive and significant DNA breakage could only be seen after incubating the pretreated nuclei further in the presence of CuCl₂. These results indicate that both resveratrol and EGCG are able to enter the nuclei. It is well established that the nuclear pore complex is permeable to small molecules (Mazzanti, 1998). Thus, the nuclear DNA breakage observed in this experiment is presumably the result of direct interaction of polyphenols with chromatin.

Tail length formation in lymphocyte nuclei and relative H₂O₂ production by various polyphenols in incubation medium of nuclei:

It is well known that polyphenols autooxidize in cell culture media to generate H₂O₂ and quinones that can enter cells/nuclei causing damage to various macromolecules (Long et al., 2000; Clement et al., 2002; Halliwell, 2003). This may lead to extraneous production of reactive oxygen species that could account for cellular DNA breakage. In order to rule out the possible involvement of extraneous H₂O₂, I have compared the DNA breakage efficiency and H₂O₂ production by various polyphenols at a particular concentration. Nuclear DNA tail length formation was determined for a number of polyphenols at a fixed concentration of 50μM. The least efficient among these was found to be tannic acid and the most effective was diadzin. The differential rate of nuclear DNA degradation by the polyphenols tested is possibly the result of differential copper binding and
copper reducing efficiency of various polyphenols (Ahmad et al., 1992) as well as their affinity for DNA binding (Rahman et al., 1990). \( \text{H}_2\text{O}_2 \) production by the polyphenols (in the absence of nuclei, in 0.4 M phosphate buffer, the suspension medium of nuclei) at the same concentration was also determined. As can be seen in table 1, tannic acid is the most efficient generator of \( \text{H}_2\text{O}_2 \) but is least effective as a DNA degrading agent and no correlation exists between the relative \( \text{H}_2\text{O}_2 \) production and the DNA tail length formation by the various polyphenols.

**Effect of neocuproine and bathocuproine on EGCG-induced DNA breakage in lymphocytes and lymphocyte nuclei:**

In a previous study (Azmi et al., 2006), we have shown that resveratrol mediated DNA degradation of lymphocyte DNA is inhibited by neocuproine which is a Cu(I) specific chelating agent and is membrane permeable (Brabouti et al., 2001; Stefan et al., 1995). In the experiment shown in figure 33, I have also used bathocuproine disulphonate, the water soluble membrane impermeable analog of neocuproine to show that whereas neocuproine inhibits EGCG-induced DNA breakage in intact lymphocytes, bathocuproine as expected is ineffective in causing such inhibition (figure 33A). However, when these two copper specific chelators were tested for DNA breakage inhibition in lymphocyte nuclei, both were found to inhibit DNA breakage in a dose dependent manner (figure 33B). Another set of experiment was performed using resveratrol instead of EGCG (figure 34 A&B). In order to get an appreciable degree of cellular DNA breakage that could be measured by Comet assay, the concentration of resveratrol used in lymphocytes (figure 34 A) was 200 \( \mu \text{M} \) as opposed to 50 \( \mu \text{M} \) EGCG (figure 33 A). Also, in lysed version (figure 34 B), 50 \( \mu \text{M} \) resveratrol has been used, as this concentration was sufficient to cause significant DNA breakage. As seen, results similar to those in figure 33 were obtained. These results further confirm that oxidative DNA breakage by plant polyphenols involves
mobilization of chromatin bound copper and that Cu(I) is an intermediate in the pathway that leads to DNA breakage.

**Effect of active oxygen scavengers on resveratrol/EGCG-induced DNA breakage in lymphocyte nuclei:**

We have previously shown (Azmi et al., 2006) that resveratrol-induced DNA breakage in intact lymphocytes is inhibited to significant degrees by various scavengers of reactive oxygen species. Table 2 and table 3 give the results of an experiment where various scavengers (superoxide dismutase (SOD), catalase, thiourea, potassium iodide and sodium azide) were tested for their effect on resveratrol/EGCG-induced DNA breakage in lymphocyte nuclei using the lysed version of Comet assay. SOD and catalase remove superoxide and H$_2$O$_2$ respectively. Sodium azide is a scavenger of singlet oxygen and potassium iodide and thiourea remove hydroxyl radicals. All scavengers caused significant inhibition of DNA breakage as evidenced by decreased tail lengths. It may be mentioned that due to the site specific nature of the reaction of hydroxyl radicals with DNA it is difficult for any trapping molecules to intercept them completely (Czene et al., 1997). The results indicate that superoxide anion and H$_2$O$_2$ are essential components in the pathway that leads to the formation of hydroxyl radical and other species which would be the proximal DNA cleaving agents. It is suggested that a mechanism involving reactive oxygen species is responsible for DNA breakage in lymphocyte nuclei by polyphenols.

**Determination of TBARS as a measure of oxidative stress in nuclei by EGCG and resveratrol in the presence of neocuproine and thiourea:**

As mentioned in table 2 and table 3, we presume that the DNA breakage observed in lymphocyte nuclei is the result of the generation of hydroxyl radicals and other reactive oxygen species. Oxygen radical damage to
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deoxyribose or DNA is considered to give rise to TBA reactive material (Smith et al., 1992; Quinlan and Gutteridge, 1987). I have therefore determined the formation of TBA reactive substance (TBARS) as a measure of oxidative stress in lymphocyte nuclei with increasing concentrations of EGCG/resveratrol. The effect of preincubating the nuclei with neocuproine and thiourea was also studied. Results given in figure 35A show a dose dependent increase in the formation of TBA reactive substance in lymphocyte nuclei by EGCG. A similar result was seen when resveratrol instead of EGCG was used in the experiment (figure 35B). However, a considerable decrease in the rate of formation of TBARS was observed in the presence of neocuproine and thiourea. The results indicate that DNA breakage in nuclei is inhibited by Cu(I) chelation and scavenging of reactive oxygen. Thus it may be concluded that the oxidative stress induced by polyphenols in lymphocyte nuclei is at least in part mediated by chromatin bound copper.

Effect of desferrioxamine mesylate and histidine on resveratrol-induced DNA breakage in lymphocytes as measured by Comet assay:

It may be mentioned that Fe$^{3+}$ and Cu$^{2+}$ are the most redox active of the metal ions in living cells. Also, copper and zinc are the major metal ions present in the nucleus (Bryan, 1979). To examine the possible involvement of iron and zinc in polyphenol-induced DNA breakage, the effect of desferrioxamine mesylate (a Fe(II) specific chelator) and histidine (a zinc specific chelator) has been seen on resveratrol-induced DNA breakage in lymphocytes. The results of figure 36 (A&B) indicate that desferrioxamine mesylate as well as histidine were unable to inhibit resveratrol-induced DNA breakage in lymphocytes suggesting that polyphenols mobilize endogenous copper ions leading to oxidative DNA breakage (figure 34A).
Effect of desferrioxamine mesylate and histidine on resveratrol-induced DNA breakage in lymphocyte nuclei as measured by Comet assay:

As mentioned earlier, copper and zinc are the major metal ions present in the nucleus (Bryan, 1979). Thus, the effect of desferrioxamine mesylate (a Fe(II) specific chelator) and histidine (a zinc specific chelator) has also been seen on resveratrol-induced DNA breakage in lymphocyte nuclei. The results of figure 37 (A&B) indicate that desferrioxamine mesylate as well as histidine were again unable to inhibit resveratrol-induced DNA breakage in lymphocyte nuclei suggesting that polyphenols mobilize chromatin bound copper leading to oxidative DNA breakage (figure 34B).

Standardization of TBHP-induced oxidative stress in lymphocytes as measured by Comet assay:

TBHP (Tert-butylhydroperoxide) is a well known inducer of ROS mediated oxidative stress that results in DNA damage (Dubuisson et al., 2000; Suzuki et al., 2000). In the work presented here, TBHP has been used to generate ROS mediated oxidative stress in lymphocytes in order to study the antioxidant potential of various polyphenols. Comet assay has been used to measure the degree of DNA breakage induced by TBHP generated ROS. In the experiment shown in figure 38, untreated cells are not damaged and do not show comet tail lengths while increasing concentrations of TBHP lead to a progressive increase in comet tail lengths as a measure of DNA breakage. Another experiment was performed in which inhibition of TBHP-induced DNA breakage was observed using ascorbic acid which is a known antioxidant. As can be seen from figure 39, ascorbic acid is able to inhibit DNA breakage in lymphocytes and hence TBHP-induced oxidative injury.
Antioxidant activity of polyphenols against TBHP-induced oxidative stress in lymphocytes:

Figure 40 and figure 41 shows the experiment where I have evaluated the antioxidant potential of stilbenes and tea catechins in providing protection to lymphocytes against TBHP-induced oxidative injury. Figure 40 compares the antioxidant activities of resveratrol and trans-stilbene. Whereas resveratrol was able to inhibit the TBHP-induced lymphocyte DNA degradation to a considerable degree, its parent compound trans-stilbene was not active to the same extent. The results indicate that resveratrol is a more effective antioxidant as compared to trans-stilbene. In another experiment (figure 41), the antioxidant potential of tea catechins namely epicatechin (EC), epigallocatechin (EGC) and EGCG were compared. It was seen that their antioxidant activities appeared in the following order: EGCG > EGC > EC. The results indicate that EGCG is the most effective and potent antioxidant among the tea catechins.
Figure 27. Standardization of Lysed version of Comet Assay with \( \text{H}_2\text{O}_2 \):

Lymphocyte nuclei were incubated with the reaction mixture (2.0 ml) containing 0.4 M phosphate buffer (pH 7.5) and indicated concentrations of \( \text{H}_2\text{O}_2 \) (0-20 \( \mu \text{M} \)) at 37 °C for 30 min and processed further for lysed version of Comet Assay as described in “Methods”.

Photographs of comets (100x) obtained on incubating lymphocyte nuclei with varying concentrations of \( \text{H}_2\text{O}_2 \).

(A) Untreated lymphocytes
(B) Lymphocytes + 0.5 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \)
(C) Lymphocytes + 10 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \)
(D) Lymphocytes + 20 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \)
Lymphocyte nuclei were incubated with the reaction mixture (2.0 ml) containing 0.4 M phosphate buffer (pH 7.5) and indicated concentrations of H₂O₂ (0-20 μM) at 37 °C for 30 min and processed further for lysed version of Comet Assay as described in "Methods". Comet tail length (μ metres) is plotted as a function of increasing concentrations of H₂O₂ (0-20 μM). All points represent mean of three independent experiments. Error bars denote ± SEM. P value < 0.05 and significant when compared to untreated control.
Figure 29. Comparison of DNA breakage by EGCG in intact lymphocytes and lymphocyte nuclei as measured by Comet assay:

(A) Single cell gel electrophoresis of human peripheral lymphocytes (A) and lymphocyte nuclei (B) showing comets (100x) after treatment with EGCG (50µM). The incubation of lymphocytes and lymphocyte nuclei with EGCG was for 1 hr at 37°C.

(a) Untreated lymphocytes
(b) Lymphocytes + 50 µM EGCG
(a') Untreated lymphocyte nuclei
(b') Lymphocyte nuclei + 50 µM EGCG
Figure 30. Comparison of DNA breakage by EGCG in intact lymphocytes and lymphocyte nuclei as measured by Comet assay:

Lymphocytes / lymphocyte nuclei were incubated with the reaction mixture (2.0 ml) containing PBS (Ca\textsuperscript{2+} and Mg\textsuperscript{2+} free) (standard version) / 0.4 M phosphate buffer (pH 7.5) (lysed version) respectively and indicated concentrations of EGCG (0-100 μM) at 37 °C for 1 hr and processed further for Comet Assay as described in “Methods”. Comet tail length (μ metres) is plotted as a function of increasing concentrations of EGCG (0-100 μM) in lymphocytes (○) and lymphocyte nuclei (■). All points represent mean of three independent experiments. Error bars denote ± SEM. P value < 0.05 and significant when compared to untreated control.
Figure 31. Comparison of various polyphenols on the induction of DNA breakage in lymphocyte nuclei:

Lymphocyte nuclei were incubated with the reaction mixture (2.0 ml) containing 0.4 M phosphate buffer (pH 7.5) and indicated concentrations of different polyphenols, gallic acid (■), EGCG (▲), aloin (○), resveratrol (□) at 37 °C for 1 hr and processed further for lysed version of Comet Assay as described in “Methods”. Values reported are ± SEM of three independent experiments. P value < 0.05 and significant when compared to untreated control.
Figure 32. Effect of preincubating the lymphocyte nuclei with increasing concentrations of EGCG (A) and resveratrol (B) on DNA breakage:

Lymphocyte nuclei were preincubated with reaction mixture (2.0 ml) containing 0.4 M phosphate buffer (pH 7.5) and indicated concentrations of EGCG (A) and resveratrol (B) for 15 min at 37 °C. The slides were then washed twice for 5 min each with PBS and further incubated for 30 min in the presence of 5μM Cu(II) (▲). The other set which served as control was further incubated in PBS alone (□). Values reported are ± S.E.M. of three independent experiments. P value < 0.05 and significant when compared to untreated control.
Table 1. Comet tail length formation in lymphocyte nuclei and relative H$_2$O$_2$ production by various polyphenols in incubation medium of nuclei:

All values represent S.E.M. of three independent experiments.

*P-values < 0.05 when compared to control.
Figure 33. Effect of neocuproine/bathocuproine on EGCG-induced DNA breakage in lymphocytes (A) and lymphocyte nuclei (B):

Lymphocyte / lymphocyte nuclei were incubated with the reaction mixture (2.0 ml) containing EGCG (50 µM) and indicated concentrations of neocuproine / bathocuproine (0-50 µM) at 37 °C for 1 hr and processed further for Comet Assay. Comet tail length (µ metres) is plotted as a function of increasing concentrations of neocuproine (□) and bathocuproine (▲) in lymphocytes (A) and lymphocyte nuclei (B). Values reported are ± SEM of three independent experiments. P value < 0.05 and significant when compared to untreated control.
Figure 34. Effect of neocuproine/bathocuproine on resveratrol-induced DNA breakage in lymphocytes (A) and lymphocyte nuclei (B):

Lymphocyte / lymphocyte nuclei were incubated with the reaction mixture (2.0 ml) containing resveratrol (200 μM [A] / 50 μM [B]) and indicated concentrations of neocuproine / bathocuproine at 37 °C for 1 hr and processed further for Comet Assay. Comet tail length (μ metres) is plotted as a function of increasing concentrations of neocuproine (□) and bathocuproine (▲) in lymphocytes (A) and lymphocyte nuclei (B). Values reported are ± SEM of three independent experiments. P value < 0.05 and significant when compared to untreated control.
Table 2. Effect of scavengers of active oxygen species on EGCG-induced DNA breakage in lymphocyte nuclei:

<table>
<thead>
<tr>
<th>Dose</th>
<th>Tail Length (µm)</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>1.12 ± 0.03</td>
<td>-</td>
</tr>
<tr>
<td>EGCG (50µM)</td>
<td>24.91 ± 1.41*</td>
<td>-</td>
</tr>
<tr>
<td>+Sodium Azide (1mM)</td>
<td>5.59 ± 0.32*</td>
<td>77%</td>
</tr>
<tr>
<td>+Catalase (100µg/ml)</td>
<td>7.66 ± 0.22*</td>
<td>69%</td>
</tr>
<tr>
<td>+Potassium Iodide (1mM)</td>
<td>10.34 ± 0.91*</td>
<td>58%</td>
</tr>
<tr>
<td>+Thiourea (1mM)</td>
<td>8.49 ± 0.35*</td>
<td>66%</td>
</tr>
<tr>
<td>+SOD (100µg/ml)</td>
<td>5.04 ± 0.29*</td>
<td>79%</td>
</tr>
</tbody>
</table>

All values represent S.E.M. of three independent experiments.
*P values < 0.05 when compared to control*. 

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Table 3. Effect of scavengers of active oxygen species on resveratrol-induced DNA breakage in lymphocyte nuclei:

<table>
<thead>
<tr>
<th>Dose</th>
<th>Tail Length (µm)</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>1.41 ± 0.03</td>
<td>-</td>
</tr>
<tr>
<td>Resveratrol (50µM)</td>
<td>21.17 ± 1.3&quot;</td>
<td>-</td>
</tr>
<tr>
<td>+Sodium Azide (1mM)</td>
<td>6.02 ± 0.56*</td>
<td>72%</td>
</tr>
<tr>
<td>+Catalase (100µg/ml)</td>
<td>6.54 ± 0.44*</td>
<td>69%</td>
</tr>
<tr>
<td>+Potassium Iodide (1mM)</td>
<td>9.17 ± 0.88*</td>
<td>57%</td>
</tr>
<tr>
<td>+Thiourea (1mM)</td>
<td>7.56 ± 0.74*</td>
<td>64%</td>
</tr>
<tr>
<td>+SOD (100µg/ml)</td>
<td>5.56 ± 0.52*</td>
<td>73%</td>
</tr>
</tbody>
</table>

All values represent S.E.M. of three independent experiments.
*P values < 0.05 when compared to control."
Figure 35. Effect of preincubation of lymphocyte nuclei with neocuproine and thiourea on TBARS generated by increasing concentrations of EGCG (A) and resveratrol (B):

The nuclei suspension was preincubated with fixed concentration of neocuproine and thiourea for 30 min at 37 °C after which it was further incubated for 1hr in the presence of increasing concentrations of EGCG (A) and resveratrol (B). Polyphenol alone (■), polyphenol + neocuproine (1 mM) (□), polyphenol + thiourea (1 mM) (▲). Values reported are ± SEM of three independent experiments. P value < 0.05 and significant when compared to control.
Figure 36. Effect of desferrioxamine mesylate (A) and histidine (B) on resveratrol-induced DNA breakage in lymphocytes:

Lymphocytes were incubated with the reaction mixture (2.0 ml) containing PBS (Ca\(^{2+}\) and Mg\(^{2+}\) free), resveratrol (200 \(\mu\)M) and indicated concentrations of desferrioxamine mesylate (A) / histidine (B) (0-100 \(\mu\)M) at 37 \(^\circ\)C for 1 hr. Comet tail length (\(\mu\) metres) is plotted as a function of increasing concentrations of desferrioxamine mesylate (■) and histidine (▲) in lymphocytes. Values reported are ± SEM of three independent experiments. P value < 0.05 and significant when compared to untreated control.
Figure 37. Effect of desferrioxamine mesylate (A) and histidine (B) on resveratrol-induced DNA breakage in lymphocyte nuclei:

Lymphocyte nuclei were incubated with the reaction mixture (2.0 ml) containing 0.4 M phosphate buffer (pH 7.5), resveratrol (50 μM) and indicated concentrations of desferrioxamine mesylate (A) / histidine (B) (0-100 μM) at 37 °C for 1 hr. Comet tail length (μ metres) is plotted as a function of increasing concentrations of desferrioxamine mesylate (■) and histidine (▲) in lymphocyte nuclei. Values reported are ± SEM of three independent experiments. P value < 0.05 and significant when compared to untreated control.
Figure 38. DNA breakage induced by TBHP in lymphocytes as measured by Comet assay:

Reaction mixture (1.0 ml) containing lymphocytes, PBS (Ca$^{2+}$ and Mg$^{2+}$ free) and indicated concentrations of TBHP (0-100 μM) was incubated at 37 °C for 1 hr and processed further for Comet Assay as described in "Methods". Comet tail length (μ metres) is plotted as a function of increasing concentrations of TBHP (0-100 μM). All points represent mean of three independent experiments. Error bars denote ± SEM. P value < 0.05 and significant when compared to control.
Figure 39. Antioxidant activity of ascorbic acid against TBHP-induced DNA breakage in lymphocytes as measured by Comet assay:

Reaction mixture (1.0 ml) containing lymphocytes, PBS (Ca$^{2+}$ and Mg$^{2+}$ free) and indicated concentrations of ascorbic acid (0-50 μM) was incubated at 37 °C for 30 min. After pelleting, the cells were washed twice with PBS (Ca$^{2+}$ and Mg$^{2+}$ free) before resuspension in PBS and further incubated for 30 min in the presence of 50 μM TBHP. Comet tail length (μ metres) is plotted as a function of increasing concentrations of ascorbic acid (0-50 μM). All points represent mean of three independent experiments. Error bars denote ± SEM. P value < 0.05 and significant when compared to control.
Figure 40. Comparison of antioxidant activities of stilbenes against TBHP-induced DNA breakage in lymphocytes as measured by Comet assay:

Reaction mixture (1.0 ml) containing lymphocytes, PBS (Ca$^{2+}$ and Mg$^{2+}$ free) and indicated concentrations of trans-stilbene / resveratrol (0-50 μM) was incubated at 37 °C for 30 min. After pelleting, the cells were washed twice with PBS (Ca$^{2+}$ and Mg$^{2+}$ free) before resuspension in PBS and further incubated for 30 min in the presence of 50 μM TBHP. Comet tail length (μ metres) is plotted as a function of increasing concentrations of trans-stilbene (▲) and resveratrol (■). All points represent mean of three independent experiments. Error bars denote ± SEM. P value < 0.05 and significant when compared to control.
Figure 41. Comparison of antioxidant activities of tea polyphenols against TBHP-induced DNA breakage in lymphocytes as measured by Comet assay:

Reaction mixture (1.0 ml) containing lymphocytes, PBS (Ca$^{2+}$ and Mg$^{2+}$ free) and indicated concentrations of epigallocatechin / epicatechin / EGCG (0-50 µM) was incubated at 37 °C for 30 min. After pelleting, the cells were washed twice with PBS (Ca$^{2+}$ and Mg$^{2+}$ free) before resuspension in PBS and further incubated for 30 min in the presence of 50 µM TBHP. Comet tail length (µ metres) is plotted as a function of increasing concentrations of epicatechin (•), epigallocatechin (▲) and EGCG (■). All points represent mean of three independent experiments. Error bars denote ± SEM. P value < 0.05 and significant when compared to control.
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Over the last several years, this laboratory has extensively characterized a DNA cleavage reaction mediated by a number of plant polyphenols in the presence of copper ions (Rahman et al., 1989; Khan and Hadi, 1998; Ahsan and Hadi, 1998; Ahmad et al., 2000; Azam et al., 2004). Subsequently, using human peripheral lymphocytes and Comet Assay, it has been confirmed that the polyphenol resveratrol in the presence of Cu(II) is indeed capable of DNA degradation in a cellular system (Azmi et al., 2005). Further, it has also been shown that plant polyphenols alone are capable of oxidatively degrading lymphocyte DNA through mobilization of cellular copper (Azmi et al., 2006). The major conclusions of the experiments performed in this chapter are (i) polyphenols are capable of DNA breakage in cell nuclei through mobilization of nuclear copper, (ii) polyphenols are able to directly interact with nuclei and cause DNA degradation (iii) such DNA breakage is caused by the generation of reactive oxygen species (ROS) (iv) polyphenol-induced DNA degradation involves mobilization and redox cycling of chromatin bound copper (v) prooxidant activity of plant polyphenols correlates with its antioxidant potential.

As already mentioned, several classes of plant derived antioxidant polyphenols also exhibit oxidative DNA degradation properties particularly in the presence of transition metal ions such as copper. Evidence in the literature suggests that antioxidant properties of these compounds may not fully explain their anticancer effects (Gali et al., 1992; Ahmad et al., 2000). It is to be noted that the polyphenol gallic acid is highly efficient in DNA degradation as compared with syringic acid (where two of the hydroxyl groups of gallic acid are modified) (Khan and Hadi, 1998). Interestingly, modifications of phenolic hydroxyl groups such as that resulting in the formation of syringic acid abolishes the apoptosis inducing capacity of gallic acid (Inoue et al., 1994). It may also be mentioned that piceatannol which is
a tetra-hydroxy derivative of resveratrol is also a potent inducer of apoptosis in human SK-Mel-28 melanoma cells (Larrosa et al., 2004). Indeed it has been shown that resveratrol is converted to piceatannol by cytochrome P-450 enzyme CYP1B1 from human lymphoblast microsomes (Potter et al., 2002). Also, Dong (2003) has shown that penta-hydroxy derivative of resveratrol was more effective as an inhibitor of EGF-induced cell transformation as compared with resveratrol. Another study with EGCG has shown that alkyl gallate and gallamide derivatives of EGCG were more potent in inducing apoptosis in HL-60 cells than EGCG (Dodo et al., 2008). The result (figure 32) presented in this study also suggests that the DNA cleavage property of polyphenols is dependent on the presence and number of hydroxyl groups. Further, experimental evidence suggests that polyphenols are able to enter the nuclear space and directly interact with the chromatin. A model for the entry and interaction of phenols with chromatin-associated copper has also been described (Li and Trush, 1994). The ability of gallotannins to enter the cell is indicated by the observation that tannic acid prevents formation of the benz(a)pyrene-DNA adduct by inhibiting the binding of the ultimate carcinogen to target tissue DNA rather than by altering the metabolism of benz(a)pyrene (Mukhtar et al., 1998).

Most of the plant polyphenols possess both antioxidant as well as prooxidant properties (Inoue et al., 1994, Ahmad et al., 1992). Therefore, I have also examined the antioxidant potential of resveratrol and EGCG along with their prooxidant action. The results indicate that resveratrol is a more effective antioxidant than its parent compound trans-stilbene. Earlier studies in this laboratory have already shown that resveratrol is also a more potent DNA cleaving agent than trans-stilbene (Azmi et al., 2005). Similar results were obtained with tea catechins where EGCG has the highest antioxidant activity when compared with EGC and EC. Also, EGCG exhibits greater prooxidant activity among the catechins (Azam et al., 2004). Thus, it can be concluded
that the properties that confer antioxidant activity to polyphenols are also the ones responsible for their prooxidant action.

Based on our own observations and those of others, we have earlier proposed a mechanism for the cytotoxic action of plant polyphenols against cancer cells that involves mobilization of endogenous copper and the consequent prooxidant action (Hadi et al., 2000). This idea is strengthened by a number of other observations in literature. Copper is a major metal ion present in the nucleus (Bryan, 1979; Ebadi and Swanson, 1988) and serum (Yoshida et al., 1993) and tissue (Nazulewis et al., 2004) concentrations of copper are greatly increased in various malignancies. Copper ions from chromatin can be mobilized by metal chelating agents giving rise to internucleosomal DNA fragmentation, a hallmark property of cells undergoing apoptosis (Burkitt et al., 1996). Further, it has been proposed that most clinically used anticancer drugs can activate late events of apoptosis (DNA degradation and morphological changes) and the essential signaling pathways differ between pharmacological cell death and physiological induction of cell death (Smets, 1994). The major conclusion drawn from this chapter is that plant polyphenols mobilize nuclear copper leading to oxidatively generated DNA breakage. There are a number of observations which suggest that this is indeed the case. The generation of hydroxyl radicals in the proximity of DNA is well established as a cause of strand scission. It is generally recognized that such reaction with DNA is preceded by the association of a ligand with DNA followed by the formation of hydroxyl radicals at that site. Among the oxygen radicals, the hydroxyl radical is the most electrophilic with high reactivity and therefore possesses a small diffusion radius. Thus, in order to cleave DNA it must be produced in the vicinity of the DNA (Pryor, 1988). The location of the redox-active metals is of utmost importance for the ultimate effect, because the hydroxyl radical, due to its extreme reactivity, interacts exclusively in the vicinity of the bound metal (Chevion, 1988). Indeed, it has already been shown that
flavonoids are able to form a ternary complex with DNA and Cu(II) where Cu(II) is reduced to Cu(I) (Rahman et al., 1989). Polyphenols are known to reduce molecular oxygen to superoxide anion leading to the formation of H$_2$O$_2$ (Ahmad et al., 1992). Superoxide can also be formed by reoxidation of Cu(I) to Cu(II) in the ternary complex (Rahman et al., 1989). Chromatin bound copper is understood to be present in the reduced form (Cu(I)) (Lewis and Laemelli, 1982) and thus would be available for reoxidation to Cu(II) by H$_2$O$_2$ in the Fenton type reaction and binding to polyphenols and recycling.

It is well known that polyphenols autooxidize in cell culture media to generate H$_2$O$_2$ and quinones that could enter cells/nuclei causing damage to various macromolecules (Long et al., 2000; Halliwell, 2003; Clement et al., 2002). This may lead to extracellular production of reactive oxygen species that could account for lymphocyte DNA breakage. However, this does not appear to be the case in our system since I have shown that DNA breakage is not observed in lymphocyte nuclei on preincubating them with EGCG/resveratrol alone up to a concentration of 10 μM. DNA breakage could only be seen after incubating the pre-treated cells further in the presence of Cu(II) (figure 33 A&B). Further, the results in table 1 indicate that there is no correlation between the relative H$_2$O$_2$ production and the DNA tail length formation by the various polyphenols.

Fe$^{3+}$ and Cu$^{2+}$ are the most redox-active of the metal ions in living cells. Although iron is considerably more abundant in biological systems, the major ions in the nucleus are copper and zinc (Bryan, 1979). The polyphenol-induced DNA breakage is inhibited by copper specific chelators (neocuproine and bathocuproine) whereas iron and zinc chelators (desferrioxamine mesylate and histidine respectively) were unable to do so thereby strongly suggesting that polyphenol-induced cellular DNA breakage involves nuclear copper.