CHAPTER-II

Caffeic acid-Cu(II) induced oxidative DNA breakage in human peripheral lymphocytes.
RESULTS-II
RESULTS

Standardization of alkaline single cell gel electrophoresis/Comet assay

Alkaline single cell gel electrophoresis is a very sensitive technique for detecting DNA single strand breaks at the level of a single cell. In this technique a small number of cells are treated with the test agent, layered on glass slides and sandwiched between layers of agarose. The slides are electrophoresed in alkaline conditions, stained and viewed under a fluorescent microscope for DNA single strand breaks. The technique is called Comet assay because of a comet like appearance of damaged cellular DNA. H$_2$O$_2$ is a known genotoxic agent and is routinely used in genotoxicity testing. In order to standardize Comet assay an experiment was performed with H$_2$O$_2$. Photographs of comets (100X) observed after treatment of lymphocytes with increasing concentrations of H$_2$O$_2$ are shown in figure 17. As can be seen untreated cells are not damaged and without any tail. However, with increasing concentrations of H$_2$O$_2$ a progressive increase in the length of comet tails is observed. In figure 18 the results of the same experiment are plotted as comet tail length (\mu m) as a function of increasing H$_2$O$_2$ concentrations.

DNA breakage by caffeic acid-Cu(II) in lymphocytes as measured by Comet assay

Increasing concentrations of caffeic acid (0-50 \mu M) either alone or in the presence of 20 \mu M CuCl$_2$ were tested for DNA breakage in isolated human peripheral lymphocytes using the Comet assay. Caffeic acid alone at any of the concentrations tested did not damage the lymphocyte DNA whereas, on addition of 20 \mu M Cu(II) DNA damage to increasing degrees was observed. Figure 19A shows photographs of comets (100X) observed with increasing concentrations of caffeic acid in the absence and presence of 20 \mu M copper. The corresponding tail length, % tail DNA and tail moment are plotted as a
function of increasing caffeic acid concentration in figure 20. Untreated lymphocyte controls were identical to caffeic acid alone or Cu(II) alone (figure 19B) and almost no cellular DNA breakage could be observed in all these three category treatments. The results clearly establish that caffeic acid-Cu(II) system is capable of DNA breakage in lymphocytes. A similar experiment with increasing concentrations of Cu(II) (5-20 μM) at fixed concentrations of caffeic acid (50 μM) was also performed. As can be seen from figure 21 an increasing degree of DNA damage with increasing concentrations of Cu(II) is observed.

**Effect of active oxygen scavengers on caffeic acid-Cu(II) induced DNA breakage in lymphocytes**

Previously it has been shown that the polyphenol resveratrol mediated degradation of DNA in the presence of Cu(II) is inhibited to significant degrees by various scavengers of reactive oxygen species (Azmi et al., 2005). Table 1 gives the results of an experiment where the effect of sodium azide, potassium iodide, thiourea, neocuproine, SOD and catalase were tested on caffeic acid-Cu(II) induced DNA breakage in lymphocytes. SOD and catalase remove superoxide and H₂O₂ respectively. Sodium azide is a scavenger of singlet oxygen and potassium iodide and thiourea remove hydroxyl radicals. From the data we conclude that H₂O₂ is an essential component in the pathway that leads to the formation of reactive oxygen species, of which superoxide anion and singlet oxygen are alternate DNA damaging agents. Neocuproine is a Cu(I) specific sequestering agent, which as expected also inhibited DNA breakage. The results therefore, indicate that the lymphocyte DNA breakage by caffeic acid-Cu(II) system is the result of the formation of reactive oxygen species.

**Comparative study of lymphocyte DNA breakage by hydroxycinnamate phenols**

In order to understand the chemical basis of DNA breakage by caffeic acid-Cu(II) system, the relative efficiency of caffeic acid, p-coumaric acid and o-
coumaric acid for DNA breakage in lymphocytes was compared. Figure 22 shows comet tail lengths obtained as a function of increasing concentrations of the phenols (0-50 μM) in the presence of 20 μM Cu(II). In agreement with the result of figure 12 where the degradation of calf thymus DNA by various hydroxycinnamates was studied, caffeic acid shows the formation of largest comet tail lengths followed by p- and o-coumaric acids. Thus, caffeic acid is a more efficient DNA breaking agent in lymphocytes than the other two hydroxycinnamates. These results demonstrate that the presence of hydroxyl groups, their number and position on the aromatic ring is important for the DNA cleavage activity as well as efficiency.

**Effect of preincubating the lymphocytes with caffeic acid or Cu(II) on caffeic acid-Cu(II) mediated DNA breakage**

Lymphocyte DNA breakage was also studied after preincubating the cells with caffeic acid after which the cells were washed twice with PBS and incubated further in the presence of Cu(II) (20 μM). The results given in figure 23 show that with increasing concentrations of caffeic acid a progressive increase in DNA breakage as indicated by increased tail length of comets is observed. A similar experiment was also performed by initially preincubating lymphocytes with increasing concentrations of Cu(II) and similar results were observed (Figure 24). These results indicate that both caffeic acid and Cu(II) are either able to traverse the cell membrane or bind to it.
Figure 17. **Standardization of Comet assay with H$_2$O$_2$:**

Reaction mixture (1ml) contained $1\times10^5$ cells, RPMI 400 μl, PBS Ca$^{2+}$ and Mg$^{2+}$ free and indicated concentrations of H$_2$O$_2$ (0-50 μM). The reaction mixture was incubated at 37 °C for 30 minutes and processed further for Comet assay as described in “Methods”.

Photographs of comets (100X) obtained on incubating lymphocytes with varying concentrations of H$_2$O$_2$.

(A) untreated lymphocytes
(B) lymphocytes + 10 μM H$_2$O$_2$
(C) lymphocytes + 20 μM H$_2$O$_2$
(D) lymphocytes + 30 μM H$_2$O$_2$
(E) lymphocytes + 40 μM H$_2$O$_2$
(F) lymphocytes + 50 μM H$_2$O$_2$
Figure 18. Standardization of Comet assay with H2O2:

Reaction mixture (1ml) contained $1 \times 10^5$ cells, RPMI 400 µl, PBS Ca$^{2+}$ and Mg$^{2+}$ free and increasing concentrations of H$_2$O$_2$ (0-50 µM). The reaction mixture was incubated at 37 °C for 30 minutes and processed further for Comet assay as described in “Methods”.

Comet tail length (µ meters) plotted as a function of increasing concentrations of H$_2$O$_2$ (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.
Comet tail length (µ meters) vs. H$_2$O$_2$ (µM)
Figure 19A. DNA breakage by caffeic acid-Cu(II) in human peripheral lymphocytes as measured by Comet assay:

Reaction mixture (1 ml) contained $1 \times 10^5$ cells, RPMI 400 μl, PBS Ca$^{2+}$ and Mg$^{2+}$ free, increasing concentrations of caffeic acid (0-50 μM) and 20 μM Cu(II). The reaction mixture was incubated for 30 min at 37 °C. After the incubation the cells were processed further for Comet assay as described in “Methods”.

Photographs of comets (100X) obtained after treatment of lymphocytes with increasing concentrations of caffeic acid and fixed concentrations of Cu(II).

[A] untreated lymphocytes

[B] lymphocytes + caffeic acid 10 μM + Cu(II) 20 μM.

[C] lymphocytes + caffeic acid 20 μM + Cu(II) 20 μM.

[D] lymphocytes + caffeic acid 50 μM + Cu(II) 20 μM.

[E-H] lymphocytes + caffeic acid (10-50 μM) in the absence of Cu(II).
Figure 19B. DNA breakage by increasing concentrations of Cu(II) in human peripheral lymphocytes as measured by Comet assay:

Reaction mixture contained $1 \times 10^5$ cells, RPMI 400 µl, PBS Ca$^{2+}$ and Mg$^{2+}$ free and increasing concentrations of Cu(II) (0-50 µM). The reaction mixture was incubated at 37 °C for 30 minutes and the cells were processed further for Comet assay as described in "Methods".

Photographs of comets (100X) obtained after treatment of lymphocytes with increasing concentrations of Cu(II).

[A] untreated lymphocytes

[B] lymphocytes + Cu(II) 10 µM.

[C] lymphocytes + Cu(II) 20 µM.

[D] lymphocytes + Cu(II) 50 µM.
Figure 20. DNA breakage by caffeic acid-Cu(II) in human peripheral lymphocytes as measured by Comet assay:

Reaction mixture (1 ml) contained $1 \times 10^5$ cells, RPMI 400 μl, PBS Ca$^{2+}$ and Mg$^{2+}$ free, increasing concentrations of caffeic acid (0-50 μM) and Cu(II) 20 μM. The reaction mixture was incubated for 30 min at 37 °C. After the incubation the cells were processed further for Comet assay as described in “Methods”.

[A] Comet tail length (μ meters) plotted as a function of increasing concentrations of caffeic acid (0-50 μM) in the presence of 20 μM Cu(II).

[B] Comet tail DNA (%) plotted as a function of increasing concentrations of caffeic acid (0-50 μM) in the presence of 20 μM Cu(II).

[C] Tail Moment (arbitrary units) plotted as a function of increasing concentrations of caffeic acid (0-50 μM) in the presence of 20 μM Cu(II).

All points represent mean of three independent experiments. Error bars denote ±SEM. P value < 0.05 and significant when compared to control.
Figure 21. DNA breakage by caffeic acid-Cu(II) in human peripheral lymphocytes as measured by Comet assay:

Reaction mixture (1ml) contained $1 \times 10^5$ cells, RPMI 400 µl, PBS Ca$^{2+}$ and Mg$^{2+}$ free and increasing concentrations of Cu(II) (5-20 µM) and caffeic acid 50 µM. The reaction mixture was incubated at 37 °C for 30 minutes and processed further for Comet assay as described in “Methods”.

Comet tail length (µ meters) plotted as a function of increasing concentrations of Cu(II) (0-20 µM) in the presence of 50 µM caffeic acid.

All points represent mean of three independent experiments. Error bars denote ±SEM. P value < 0.05 and significant when compared to control.
Figure 22. A comparison of the DNA breakage in human lymphocytes by caffeic acid, \( p \)-coumaric acid and \( o \)-coumaric acid by Comet assay:

Reaction mixture (1ml) contained \( 1 \times 10^5 \) cells, RPMI 400 \( \mu l \), PBS \( \text{Ca}^{2+} \) and \( \text{Mg}^{2+} \) free, increasing concentrations of the three hydroxycinnamate phenols (0-50 \( \mu \text{M} \)) and \( \text{Cu(II)} \) 20 \( \mu \text{M} \). The reaction mixture was incubated at 37 °C for 30 minute and the cells were processed further for Comet assay as described in “Methods”. (●) caffeic acid; (■) \( p \)-coumaric acid and (□) \( o \)-coumaric acid.

All points represent mean of three independent experiments. Error bars denote ±SEM. P value < 0.05 and significant when compared to control.
Figure 23. Effect of preincubating the lymphocytes with increasing concentrations of caffeic acid on DNA breakage:

Isolated lymphocytes suspended in RPMI 1640 were pre-incubated with the indicated concentrations of caffeic acid for 30 minutes at 37 °C. After pelleting, the cells were washed twice with PBS Ca\(^{2+}\) and Mg\(^{2+}\) free twice before re-suspension in RPMI 1640 and further incubation for 30 minutes in the presence of 20 \(\mu\)M Cu(II).

All points represent mean of three independent experiments. Error bars denote ±SEM. P value < 0.05 and significant when compared to control.
Figure 24. Effect of preincubating the lymphocytes with increasing concentrations of Cu(II) on DNA breakage:

Isolated lymphocytes suspended in RPMI 1640 were pre-incubated with the indicated concentrations of Cu(II) for 30 minutes at 37 °C. After pelleting, the cells were washed twice with PBS Ca$^{2+}$ and Mg$^{2+}$ free before resuspension in RPMI 1640 and further incubation for 30 minutes in the presence of 50 μM caffeic acid.

All points represent mean of three independent experiments. Error bars denote ±SEM. P value < 0.05 and significant when compared to control.
Table 2
Effect of scavengers of reactive oxygen species on caffeic acid induced lymphocyte DNA breakage

<table>
<thead>
<tr>
<th>Dose</th>
<th>Tail length (µ meters)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>1.66 ±0.12</td>
<td>-</td>
</tr>
<tr>
<td>Caffeic acid (200 µM)</td>
<td>14.88 ±1.33</td>
<td>-</td>
</tr>
<tr>
<td>+ Thiourea (1mM)</td>
<td>4.33 ±0.89*</td>
<td>70.9.46</td>
</tr>
<tr>
<td>+ Catalase (1µg/ml)</td>
<td>5.85 ±1.01*</td>
<td>60.68</td>
</tr>
<tr>
<td>+ SOD (1 µg/ml)</td>
<td>5.22 ±0.97*</td>
<td>64.91</td>
</tr>
</tbody>
</table>

*All represent ±SEM of three independent experiments. *P value < 0.05 when compared to control.
DISCUSSION-II
DISCUSSION

The major conclusions of the experiments described in this chapter are: (i) caffeic acid-Cu(II) system is capable of oxidative DNA cleavage in whole cells, (ii) the cellular DNA damage by caffeic acid-Cu(II) involves the formation of Cu(I) and reactive oxygen species, and (iii) the cellular DNA breakage induced by this system depends on the number and the relative position of hydroxyl group on the aromatic ring of hydroxycinnamates.

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). Further, we have previously shown 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 & 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). As mentioned in the ‘Introduction’ caffeic acid and p-coumaric acid studied here have been shown to possess anticancer properties. In addition, caffeic acid has also been shown to be a potent inducer of apoptosis in cancer cell lines. It is interesting to note that the apoptosis inducing activity of caffeic acid was enhanced in the presence of CuCl₂. Based on our own observation and those of others, we have proposed a mechanism for the cytotoxic action of plant polyphenolics against cancer cells that involves mobilization of endogenous copper and the consequent prooxidant action (Hadi et al., 2000). Thus, the major conclusion of the present study is that caffeic acid-Cu(II) mediated chemical cleavage of DNA is a physiologically feasible reaction and may be of biological significance. The idea of mobilizing endogenous copper and consequent prooxidant action by plant derived polyphenols is strengthened by a
number of other observations mentioned earlier (Hadi et al., 2000). More significantly 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 pathway differs between pharmacological cell death and physiological induction of cell death (Smets et al., 1994). Cu$^{2+}$ and Zn$^{2+}$ are the major metal ions present in the nucleus (Bryan, 1979) and that serum (Ebadi & Swanson, 1988) and tissue (Nazulewis, et al. 2004; Yoshida et al., 1993) 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 property which is the hallmark of cells undergoing apoptosis (Burkitt et al., 1996). It has been recently shown that the polyphenol curcumin mediated apoptosis in HL60 cells is closely related to the increase in the concentrations of reactive oxygen species possibly generated through the reduction of transition metals in cells (Yoshino et al., 2004). Thus, it is possible that cellular DNA fragmentation by plant polyphenolics that involves mobilization of intra-cellular and extra-cellular copper could be one of the important mechanisms involved in the chemopreventive properties of these compounds.