The study of oxidative response of V79 cells following cyclodextrins treatment

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ABSTRACT

Cyclodextrins (CDs) are cyclic oligosaccharides with a hydrophobic central cavity, currently used to increase the amount of nonpolar molecules delivery to biological systems. We evaluated cytotoxicity effect of β-, γ- and hydroxypropyl-β-cyclodextrin and oxidative status in V79 cell line with regard to reactive oxygen species generation and macromolecule damages. Cytotoxicity was assessed by MTT test in the range of doses of 0 to 10 mM. The activity of antioxidant enzymes; superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase (CAT), together with oxidative damage biomarkers; malondialdehyde (MDA), dityrosine and 8-OH-deoxyguanosine (8-OH-dG) were measured by spectrometry and HPLC methods. The viability of cells was inhibited by these compounds at concentrations of 5 mM and higher and was considerable for γ- and HP-β-CD respectively at 10 mM. They promoted ROS generation, increased enzyme activities and elevated the levels of MDA and dityrosine in which HP-β-CD was more effective. Treating cells with 30 μM of α-tocopherol in addition to 10 mM of CDs showed significant decrease on the levels of enzyme activities, MDA and dityrosine. As conclusion the present study documents the oxidative radicals forming ability of the studied cyclodextrins and further strengthens the documentation of their cytotoxicity effects through lipid and proteins oxidation damages particularly at levels higher than their ordinary administration levels.

INTRODUCTION

Cyclodextrins are cyclic (toroidal) oligo saccharides, cage molecules with a cone-like cavity. The number of units determines the size of the cavity and the corresponding names of cyclodextrins (CDs). The cavity provides a binding site for hydrophobic molecules of appropriate dimensions. They can encapsulate many compounds in a short time and liberate them in a prolonged time due to hydrophobic interactions between the internal part of CDs and the active molecules. The most common cyclodextrins used are α-, β- and γ-cyclodextrin, with the corresponding of glucose units (α =6, β =7, γ =8). Derivatization of the hydroxyl
groups of these compounds also increases solubility and selectivity compared to the native CDs\textsuperscript{[4]}. The non-toxic nature of CDs and their biocompatibility makes them attractive additives for various biological products\textsuperscript{[5]}. These compounds are useful formulation vehicles, which increase the amount of drug that can be solubilised in aqueous vehicles, thus increasing delivery of many useful medicinal agents to biological systems\textsuperscript{[6,7]}. Accordingly, as xenobiotics, they can interfere with different living systems including normal oxidative metabolism within cells. In aerobic condition, reactive oxygen species (ROS) are generated in cells under normal condition and also under the influence of xenobiotic agents\textsuperscript{[8]}. Oxidative stress results when reactive oxygen species (ROS) are not adequately removed. ROS can oxidize biomolecules such as DNA, proteins and lipids and thus may lead to oxidative injury. Since half-lives of ROS are extremely short, biomarkers of oxidative damage can be used for oxidative stress monitoring\textsuperscript{[9]}. Cells have evolved various antioxidant defenses to protect against the deleterious effects of ROS, which include enzymes and low molecular mass radical scavengers\textsuperscript{[10,11]}. The most important antioxidant enzymes are superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT). Superoxide dismutase catalyses dismutation of the superoxide anion to hydrogen peroxide and molecular oxygen\textsuperscript{[12]}. Glutathione peroxidase protects the membrane lipids from oxidative damage and detoxifies the organic peroxides\textsuperscript{[13]}. Catalase inactivates hydrogen peroxide to oxygen and water\textsuperscript{[14]}. Since no study has not yet been performed on the possibility of cyclodextrins interference with antioxidative enzyme activities and ROS production within cells, we investigated the effect of $\beta$-, $\gamma$- and HP-$\beta$-cyclodextrins on the SOD, CAT and GPX activities at their ordinary administration concentrations and over on V79 cell line. We also evaluated the levels of oxidative damage biomarkers of lipids, proteins and DNA.

**MATERIALS AND METHODS**

**Chemicals**

Methylthiazoldiphenyl-tetrazoliumbromide, $\beta$-cyclodextrin and dihydroethidium were purchased from Sigma Chemical Company (St. Louis, MO, USA). $\gamma$-cyclodextrin was provided from Cerestar USA, Inc. (Hammond, IN, USA) and hydroxypropyl-$\beta$-cyclodextrin was obtained from Aldrich (Steinheim, Germany). Culture medium and fetal bovine serum Gibco Laboratories (Paisley, Scotland). Culture medium was diluted in distilled water. On the day of exposure, the standards of cyclodextrins were dissolved in water, to prepare stock solutions. Stock solution of cyclodextrins were prepared in distilled water and sterilized by filtration through a 0.22-µ filter (Acrodisc, Gelman). Other chemicals were of highest quality commercially available. For each treatment, the stock solution was added to the culture media solution to provide the final favorite treatment concentration.

**Cell cultures**

V79 Chinese hamster cells (V79-UL) were maintained in minimal essential medium (MEM) with Earle’s salts, supplemented with 10% fetal calf serum, 2 mM glutamine and antibiotics. Cells were cultivated in a humidified incubator at 37°C with 5% CO$_2$ at pH 7.2 and harvested with 0.15% trypsin and 0.08% EDTA\textsuperscript{[15]}. Cell culture media were obtained from Biochrom (Berlin, Germany).

**Cytotoxicity assay**

Cytotoxicity was evaluated by the inhibition of cell growth or reduction of cell viability. Amount of viable cells was detected using the colorimetric methylthiazoldiphenyl-tetrazoliumbromide assay (MTT). It was dissolved in phosphate buffer saline (PBS) solution at concentration of 5 mg/ml and filtered through a 0.22 µm filter to sterilize and then stored at 4°C. Cells grown in 96-well tissue culture plates were treated with various doses of cyclodextrins for 24-h and were incubated with the reagent in the Cell Titer 96 Aqueous One Solution Cell Proliferation assay kit (Promega, WI). The absorbance of reduced tetrazolium compound derived from the reagent due to dehydrogenase activities in viable cells was recorded at 490 nm with the subtraction of absorbance of background at 650 nm by a microplate reader. Furthermore, cell numbers in 6-well plates were also counted after a 24 h cyclodextrins treatment at different doses\textsuperscript{[16]}.  

**Spectrophotometric assay of ROS production**

Cells treated with different concentration of cyclodextrins, and untreated control cells were centri-
fuged and incubated with 2 µM of dihydroethidium (λ<sub>ex</sub> =360nm, λ<sub>em</sub> =420 nm) for 10 min, washed with phosphate-buffered saline, and then analysed by spectrophotometry in RPMI-1640 medium without phenol red. In the presence of ROS, dihydroethidium is oxidized to ethidium and fluorescence in red (λ<sub>em</sub> =640 nm). The 640/420 nm fluorescence intensity ratio permit to evaluate the production of ROS in living cells. Raw data were normalized with respect to control value and results expressed as the folds increase of 640/420 fluorescent intensity ratios. Results are given as mean ± SD of three independent experiments<sup>[17]</sup>.

**SOD activity assay**

SOD activity assay was performed according to the Spitz’s method<sup>[18]</sup>. Cells were homogenized in 50 mM potassium phosphate buffer (pH 7.8). Total SOD activity was assayed at 25℃ by the nitroblue tetrazolium (NBT) reduction assay with bathocuproine sulfonate. The rate of reduction of NBT by superoxide, which was generated from xanthine and xanthine oxidase, was monitored spectrophotometrically at 560 nm. One unit of SOD was defined as the amount of protein, which causes a 50% inhibition of the rate of NBT reduction.

**CAT activity**

CAT activity was measured by the method of Beers and Sizer with slight modifications<sup>[19]</sup>. For both CAT and GPX activity assays, same preparation of samples was used by homogenizing cells in 50 mM phosphate buffer (pH 7.4). Supernatant from 1000g centrifugation of cell homogenates was used for assays. The assay reaction for CAT consisted of 50 mM potassium phosphate buffer (pH 7.0), 0.02 M H<sub>2</sub>O<sub>2</sub>, and samples in a total volume of 1 ml. The reaction was carried out at 25℃. The rate of absorbance change (ΔA/min) at 240 nm was recorded, which indicated the decomposition of H<sub>2</sub>O<sub>2</sub>. Activities were calculated using the molar extinction coefficient of H<sub>2</sub>O<sub>2</sub> at 240 nm, 43.59 L/mol-cm.

**GPX activity assay**

Activity assays of selenium-dependent GPX were performed as previously described<sup>[20,21]</sup>. The coupling reagent consisted of 50 mM Tris-ClH buffer (pH 7.7), glutathione, glutathione reductase, sodium cyanide, and NADPH. The coupling reagent in 875 and 100 µl of sample was incubated for 2 min at 25C and hydrogen peroxide (final 25 µM) was added to initiate the reaction. ΔA/min at 340 nm was recorded. ΔA /min of blank, in which sample was replaced by Tris-ClH buffer, was also recorded. The net ΔA /min of samples after subtracting the blank rate was used to calculate the GPX activity using the molar extinction coefficient of NADPH at 340 nm, 6220 L/mol-cm.

**High-performance liquid chromatography (HPLC) assay of TBA+MDA**

After crowns incubation the V79 cells were scraped off, using a cell craper, and centrifuged (5 min, 800×g) and were washed twice in nominally calcium and magnesium free MEM. The cells were resuspended in 0.8 ml NaCl, (0.9%, w/v) at 4℃. Aliquots were taken for protein analysis (Bio-Rad)<sup>[22]</sup> and the cells were lysed and proteins precipitated with 40% trichloracetic acid (TCA), w/v. The MDA assay is based on the condensation of one molecule malondialdehyde with two molecules of thiobarbituric acid in the presence of reduced reagent volumes to increase sensitivity<sup>[23]</sup>, generating a chromogen with UV absorbance.

The TBA+MDA complex was analyzed by HPLC essentially as described by Bird B. R. et al.<sup>[24]</sup> Briefly, the HPLC system consisted of a Hewlett + Packard 1050 gradient pump (Avondale, PA) equipped with an automatic injector, a 1050 diode-array absorption detector and a personal computer using Chem Station Software from Hewlett + Packard. Aliquots of the TBA+MDA samples were injected on a 5 mm Supelcosil LC-18 reversed phase column (30 × 4.6 mm). The mobile phase consisted of 15% methanol in double-distilled water degassed by filtering through a 0.5μm filter (Millipore, Bedford, MA). The flow rate was 2 ml/min. MDA+TBA standards were prepared using tetraethoxypropane.

The absorption spectra of standards and samples were identical with a characteristic peak at 540 nm. Measurements were expressed in terms of malondialdehyde (MDA) normalized to the cell protein content.

**Measurement of dityrosine**

Purification of o’, o’dityrosine was accomplished by preparative HPLC. o’, o’Dityrosine was recovered by gradient elution from the C-18 column (Econosil C18, 250mm×10 mm)<sup>[25]</sup>. The composition of eluent varied...
linearly from acetonitrile–water-TFA (1:99:0.02) to acetonitrile–water-TFA (20:80:0.02) over 25 min. The gradient was started 5 min after the injection. A flow rate of 4 ml/min was used. o’, o’-Dityrosine was analyzed by reversed-phase HPLC with simultaneous UV-detection (280 nm) and fluorescence-detection (ex. 280 nm, em. 410 nm). A phenomenex inertsil ODS 2 (150 mm×4.6 mm, 5 µm) HPLC column (Bester, Amsterdam, The Netherlands) equipped with a guard column was used for these analyses. A gradient was formed from 10 mM ammonium acetate, adjusted to pH 4.5 with acetic acid, and methanol, starting with 1% methanol and increasing to 10% over 30 min. The flow rate was 0.8 ml/min. A standard dityrosine sample was prepared according to Ref.[26]. Dityrosine was quantified by assuming that its generation from the reaction of tyrosine with horseradish peroxidase in the presence of H$_2$O$_2$ was quantitative (using the extinction coefficient $\varepsilon_{315} = 4.5$ mM$^{-1}$ cm$^{-1}$ at pH 7.5).

**Determination of 8-hydroxydeoxyguanosine (8-OH-dG)**

A sensitive analytical technique, described elsewhere$^{[27]}$, was used to measure the amount of 8-OH-dG by HPLC (Unicam; Ultrasphere-ODS; 5µm, 4.6×250 nm) coupled to an electrochemical detector (ESA Coulochem II: guard cell, 0.35 v; detector 1, 0.15 V; and detector 2, 0.30 V). Briefly, the nuclear DNA from cells was extracted using the DNA Extractor WB Kit (Wako Biochemicals, Osaka, Japan). The extracted DNA samples were digested with nuclease P1 (0.8 U, Yamasa, Chiba, Japan) and acid phophatase (1U, Sigma Chemical) in a solution of 1 mM EDTA and 10 mM sodium acetate (pH 4.5). After incubation at 37°C for 30 min, the iron exchange resin Muromac was added to remove the NaI and the mixture was centrifuged at 15,000 rpm for 5 min. The supernatant was transferred to an Ultrafree Probind filter (Milipore, Bedford, MA) and then centrifuged at 10,000 rpm for 2 min. The filtered deoxynucleoside was injected onto the HPLC column. Standard sample of dG (0.5 mg/ml) and 8-OH-dG (5 ng/ml) solutions were used for comparison with the samples of the subjects. The molar ratio of 8-OH-dG to dG was calculated based on the integrated peak area of authentic 8-OH-dG with an electrochemical detector and UV absorbance of dG using a millenium software (Waters, Milford, MA). The titer of 8-OH-dG is shown as the number per 10$^5$ guanine residues.

**Statistical analysis**

Each experiment (n ≥ 3) was run at least in duplicate and the data presented are given as mean ± SD. Statistical analysis of data was performed by analysis of variance (ANOVA) using the SPSS-PC1 version 4.01 (SPSS INC., Chicago, IL). A level of P < 0.05 was considered statistically significant for all experiments.

**RESULTS**

The first biological test, MTT, was carried out to determine the toxicity of all samples, we used β-CD, γ-CD and HP-β-CD over a range of doses (0.5-10 mM) and data are presented in figure 2. Soluble powders of tested compounds exhibited an identical, i.e. excellent biological behavior up to 2.5 mM with percent of corresponding control of 87-100%. At high concentrations (5 mM and over), they showed cytotoxic effect in which around 60% of viability was observed after exposure of cells to 10 mM of HP-β-CD. All CDs revealed a toxic effect with a 50-% lethal concentration of 15, 14 and 12.5 mM for β-, γ- and HP-β-CD respectively. As comparison, HP-β-CD at 10 and then at 5 mM inhibited markedly and significantly the viability after 24 h incubation with respect to β-, γ-CDs.
TABLE 1: Activities of antioxidant enzymes in V79 cells after 24 h treatments

<table>
<thead>
<tr>
<th>Concentration (mM)</th>
<th>SOD (U/mg.protein)</th>
<th>CAT (µM/min/mg)</th>
<th>GPX (µM/min/mg)</th>
<th>MDA (nM/mg.protein)</th>
<th>Dityrosine (nM/mg.protein)</th>
<th>8-OH-dG/10^9dG</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>12.65 ± 2.10</td>
<td>0.84 ± 0.19</td>
<td>6.37 ± 0.56</td>
<td>12.65 ± 2.10</td>
<td>0.84 ± 0.19</td>
<td>6.37 ± 0.56</td>
</tr>
<tr>
<td>0.5</td>
<td>10.41 ± 1.84</td>
<td>0.77 ± 0.21</td>
<td>6.84 ± 0.47</td>
<td>11.37 ± 2.24</td>
<td>0.89 ± 0.22</td>
<td>5.93 ± 0.52</td>
</tr>
<tr>
<td>1</td>
<td>9.73 ± 2.31</td>
<td>0.68 ± 0.10</td>
<td>5.71 ± 0.38</td>
<td>13.22 ± 2.60</td>
<td>0.71 ± 0.19</td>
<td>5.42 ± 0.41</td>
</tr>
<tr>
<td>2.5</td>
<td>12.10 ± 2.18</td>
<td>0.88 ± 0.16</td>
<td>6.91 ± 0.51</td>
<td>10.69 ± 1.81</td>
<td>0.94 ± 0.25</td>
<td>6.62 ± 0.43</td>
</tr>
<tr>
<td>5</td>
<td>14.81 ± 2.72</td>
<td>1.32 ± 0.27</td>
<td>7.42 ± 0.55</td>
<td>17.52 ± 2.84</td>
<td>1.46 ± 0.51</td>
<td>7.73 ± 0.62</td>
</tr>
<tr>
<td>10</td>
<td>21.33 ± 3.45b</td>
<td>2.59 ± 0.41b</td>
<td>7.63 ± 0.76</td>
<td>29.35 ± 4.13b</td>
<td>3.07 ± 1.52b</td>
<td>7.24 ± 0.60</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD (number of replicates = 5). *Significant difference with respect to control (P<0.05).

TABLE 2: The levels of lipid peroxidation, protein and DNA oxidative damage biomarkers of V79 cells after 24 h exposure to cyclodextrins

<table>
<thead>
<tr>
<th>Concentration (mM)</th>
<th>β-CD</th>
<th>γ-CD</th>
<th>HP-β-CD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SOD (U/mg.protein)</td>
<td>CAT (µM/min/mg)</td>
<td>GPX (µM/min/mg)</td>
</tr>
<tr>
<td>0</td>
<td>12.65 ± 2.10</td>
<td>0.84 ± 0.19</td>
<td>6.37 ± 0.56</td>
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<td>5</td>
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<td>7.42 ± 0.55</td>
</tr>
<tr>
<td>10</td>
<td>21.33 ± 3.45b</td>
<td>2.59 ± 0.41b</td>
<td>7.63 ± 0.76</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD (number of replicates = 5). *Significant difference with respect to control (P<0.05).

TABLE 3: The levels of oxidative damage biomarkers and antioxidative enzyme activities after 24 h treatment with 10 mM of each cyclodextrin in the absence and presence of 30 µM alpha-tocopherol

<table>
<thead>
<tr>
<th>Treatment</th>
<th>SOD (U/mg.protein)</th>
<th>CAT (µM/min/mg)</th>
<th>GPX (µM/min/mg)</th>
<th>MDA (nM/mg.protein)</th>
<th>Dityrosine (nM/mg.protein)</th>
<th>8-OH-dG/10^9dG</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>18.21 ± 2.7</td>
<td>6.82 ± 1.5</td>
<td>21.13 ± 2.5</td>
<td>12.65 ± 2.10</td>
<td>0.84 ± 0.19</td>
<td>6.37 ± 0.56</td>
</tr>
<tr>
<td>β-CD</td>
<td>43.38 ± 5.2</td>
<td>28.53 ± 3.8</td>
<td>49.17 ± 6.1</td>
<td>21.33 ± 3.45</td>
<td>2.59 ± 0.41</td>
<td>7.63 ± 0.76</td>
</tr>
<tr>
<td>β-CD + α-TCP</td>
<td>24.35 ± 3.1</td>
<td>11.74 ± 1.7</td>
<td>28.07 ± 3.2</td>
<td>15.82 ± 2.62</td>
<td>1.12 ± 0.23</td>
<td>5.14 ± 0.42</td>
</tr>
<tr>
<td>γ-CD</td>
<td>48.15 ± 5.6</td>
<td>31.46 ± 3.5</td>
<td>51.42 ± 6.2</td>
<td>29.35 ± 4.13</td>
<td>3.07 ± 1.52</td>
<td>7.24 ± 0.60</td>
</tr>
<tr>
<td>γ-CD + α-TCP</td>
<td>30.27 ± 3.4</td>
<td>17.39 ± 2.2</td>
<td>38.56 ± 4.1</td>
<td>19.70 ± 2.84</td>
<td>2.16 ± 0.48</td>
<td>6.02 ± 0.41</td>
</tr>
<tr>
<td>HP-β-CD</td>
<td>57.81 ± 5.9</td>
<td>37.42 ± 3.7</td>
<td>59.62 ± 6.4</td>
<td>45.38 ± 5.41</td>
<td>6.34 ± 1.87</td>
<td>8.83 ± 0.68</td>
</tr>
<tr>
<td>HP-β-CD + α-TCP</td>
<td>28.65 ± 3.8</td>
<td>25.14 ± 3.2</td>
<td>36.47 ± 4.6</td>
<td>27.18 ± 4.02</td>
<td>2.73 ± 1.41</td>
<td>5.64 ± 0.47</td>
</tr>
<tr>
<td>α-TCP</td>
<td>12.81 ± 1.47</td>
<td>4.12 ± 1.3</td>
<td>15.30 ± 2.2</td>
<td>9.11 ± 1.81</td>
<td>0.68 ± 0.16</td>
<td>4.72 ± 0.38</td>
</tr>
</tbody>
</table>

*Significant difference from control. **Significant difference from control and from treatment in the presence of α-TCP (P<0.05).
There were insignificant increases in enzyme activities from β-CD to HP-β-CD for each concentration in the range of treatment doses. On the other hand, cells incubated with 10 mM HP-β-CD revealed significantly increase in enzyme activities with respect to β-CD.

TABLE 2 showed that the formation of 8-OH-dG, dityrosine and MDA as DNA, proteins and lipids damage biomarkers occurred in control condition and varied insignificantly at treatment doses of 0.5, 1 and 2.5 mM for each CDs with respect to control. The level of MDA and dityrosine elevated significantly at 5mM and higher concentration of HP-β-CD in comparison with control. Incubation of cells with 10 mM of β- and/or γ-CDs caused significantly increase in the levels of MDA and dityrosine. There were insignificantly variations in the levels of 8-OH-dG in cells exposed to β- and/or γ-CDs with respect to control in the range of doses of 0.5 to 10 mM. In addition, HP-β-CD caused significantly elevation in MDA and dityrosine at 5 mM and in all tested oxidative biomarkers at 10 mM with respect to β-CD. Antioxidative enzyme activities along with the levels of MDA, dityrosine and 8-OH-dG in treated cells with CDs in the presence and absence of alpha-tocopherol (α-TCP) are presented in TABLE 3. These parameters decreased significantly in each cyclodextrin (10 mM) treated cells in the presence of α-TCP (30 μM) as compared with treated cells in the absence of this antioxidant. In spite of these decrements, the difference between control and treated cells with γ-, β- and/or HP-β-CD in presence of α-TCP were significant for the levels of antioxidative enzyme activities. In presence of α-TCP, there was also significant increase in MDA and dityrosine levels in treated cells with γ-, and/or HP-β-CD with respect to control. Alpha-TCP alone induced significant decrease in the levels of SOD, GPX and caused an insignificant decrease in other studied parameters in this table as compared with control.

**DISCUSSION**

Natural cyclodextrins are cyclic amylose-derived oligomers composed of a varying number of α-1,4-linked glucose units that are formed by the action of bacterial enzymes on starch. Because of their ability to form stable inclusion complexes with organic molecules, they have received considerable attention. β-, γ- and HP-β-CDs are all used successfully to incorporate drugs into aqueous vehicles[28,29]. Accordingly, different living systems can be exposed to these compounds as xenobiots. Toxicity profile of these exogenous chemicals has been investigated extensively[30], however, our study was the first in its kind that searched cytotoxicity effects of three CDs following V79 cell treatment with regard to ROS generation process and antioxidative enzymes responses. V79 cell line was considered as a living system model that has been frequently used to clarify the mechanism of cytotoxicity and ROS effects in response to various compounds[31]. Our data demonstrated that β- and γ-cyclodextrins did not inhibit the proliferation of cultured cell in the range of doses of 0.5-5 mM and there was slightly inhibition on viability test by HP-β-CD at 5 mM. Non-toxic effect of these compounds was also reported by many authors that used CDs for therapeutic administration and toxicity

![Figure 2](image-url)  
**Figure 2:** Cytotoxicity of different cyclodextrins in V79 cells as assessed by MTT test after 24 h treatments. Results are presented as percent of corresponding control and representative of at least three duplicated independent experiments.

![Figure 3](image-url)  
**Figure 3:** ROS production levels in V79 cells treated for 24 h with different concentration of cyclodextrins. Results are expressed as mean ± SD of at least three replicated independent experiments.
study\cite{32-34}. They concluded that HP-β-Cd and then β- and γ-CD were well tolerated in the animal species tested with limited and reversible toxicity. The low toxic effect of CDs and their water solubility make these host compounds particularly amenable for the design of drug carriers to increase their bioavailability\cite{35}. Due to these documents, cyclodextrin complexation with antimycotic drugs revealed more toxic effects on human TR146 buccal cell culture model with respect to native antifungal drug administration. The toxicity did not arise from CD treatment even at 4 mg/ml and was due to drug super-saturation, thereby increasing the bioavailability of antimycotics\cite{32}. We found considerable cytotoxicity of tested compounds at 10 mM in which HP-β-CD showed around 40% inhibition on MTT assay. In agreement with our finding, Ulloth et al., reported that exposure of NGFDPC12 cells to 0.12% methyl beta cyclodextrin dose not affect cell viability, but 0.18% or higher concentrations trigger massive loss of cell viability and apoptotic cell death\cite{36}.

Hydroxypropil-derivatives of β-CD have a much higher water solubility and stability than the native β-CD and may be slightly more toxicologically benign. In addition, the HP-β-CD derivatives give rise to fewer concerns about safety than the native β-CD with regard to parental administration\cite{2,37}. It is our conviction that the difference in observed cytotoxicity intensity of CDs at 10 mM may be a function of their hydrophobic cavity properties or the ability of CDs to induce perturbations in the cell membrane in which cholesterol play important role\cite{41}. It is suggested that the negative effects of CDs on cell viability may be attribute to the role of these molecules in depleting cholesterol from the cell membrane. Cyclodextrins have different ability in removing cholesterol from cell membrane. The efficiency by which cyclodextrins mediated cholesterol transfer is related to their ability to reduce the activation energy for cholesterol incorporation into their hydrophobic cavity when they interact directly\cite{38}. In addition, the differences in the ability of cells to metabolize CDs could be considered as a main factor on their living effects. These cyclodextrin molecules, although similar in their unit make up, possess slightly different absorption rates, possibly due to differences in degradation processes\cite{28}. On the other hand, it has been confirmed that variation in viability of cell culture is usually associated with alteration in oxidative process within treated cells\cite{39,40}. Accordingly, Chinese hamster V79 cell culture exhibited considerable elevation in ROS level after 24 h incubation with 5 and/or 10 mM of CDs particularly with HP-β-CD.

Apart from these documents, it has not been indicated whether increase in ROS formation is implicated in cell damage and toxicity after exposure to CDs. To clarify and monitor the involvement of oxidative damage in the toxicity of xenobiotics, biomarkers of ROS damages on macromolecules can be used\cite{41}. The abundant presence of membrane phospholipids at sites where ROS are formed rapidly affected them and leading to lipid peroxidation. This degenerative propagation reaction is accompanied by the formation of MDA, the most widely used index of lipid peroxidation\cite{42}. To better characterize whether bulk proteins were damaged by ROS, we measured the levels of o, o'-dityrosine. One electron oxidation of L-tyrosine generates long-lived tyrosyl radicals, can react with each other and form dityrosine that is considered as a novel biomarker of protein oxidation damage\cite{43}. With regard to the importance of these biomarkers, we found detectable levels of o, o'-dityrosine and also MDA not only in controls but also in treated cells with 0.5 to 2.5 mM CDs that were not cytotoxic levels. These findings suggested that a base line level of bulk cell proteins and lipids oxidation damage exist in normal condition and in nontoxic treatments that rise from normal oxidative process without perturbation\cite{44}. However, considerable and significant elevation in the levels of oxidative biomarkers in treated cells with 10 mM of CDs provided direct evidence that CDs exposure was a relevant source of oxidative stress in which local production of oxidative radicals played important roles. In particular, evidence exist that decrease in cell viability after exposure to ROS inducer is accompanied by an increased formation of MDA and dityrosine\cite{39,45}. Another major macromolecule target for ROS attack is DNA that oxidative modification of its bases may release modified bases among which 8-OH-dG is considered as biomarker of DNA oxidative damage. The level of this parameter did not varied significantly in β- and/or γ-CDs treated cells in the range of studied concentrations with respect to control, suggested that the rate of oxidative DNA damage and repair were approximately

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balanced\textsuperscript{46}. On the other hand, treated cells with 10 mM HP-β-CD caused significant increase in this biomarker as compared with control that may resulted from highest ROS level appeared among treatments, exceeded DNA oxidative damage from the level of repair. Continuous exposure of aerobic organisms to proxidant challenges has endows living cells with efficient and sophisticated antioxidants systems. As the most important members of the enzymatic defense system including SOD, CAT and GPX have been distinguished. Accordingly, our evaluation revealed a base line level of these enzymes in controls and in treated cells with 0.5 and 1 mM of CDs leading to limitation of ROS elevation with respect to control. In addition, enhanced enzyme activities of treatments with 2.5 mM and higher in our study consisted with other reports that have shown these enzymes triggered by ROS\textsuperscript{47,48}.

Another layer of protective system consisted of non-enzymatic defense, including α-tocopherol and its derivatives. With regard to this layer importance, we along with other authors, observed considerable decrease effect of α-tocopherol on biomarkers levels of key cellular macromolecules oxidative damage; MDA, dityrosine, and also on enzymatic defense system\textsuperscript{42,49}. This effect might be ascribed to the scavenging activity of α-tocopherol on ROS and particularly due to its inhibitory effect on lipid peroxidation\textsuperscript{50}. The observed protecting effect of α-tocopherol on induced ROS toxicity have been shown mainly is associated with plasma membrane\textsuperscript{51} although cyclodextrins could notably improve the migration time of α-tocopherol as a lipophilic compound into the cell membrane. CDs enhance the solubility of nonpolar substances by non-covalent incorporation of the lipophilic portion of the molecule into their hydrophobic cavity\textsuperscript{52}. Our findings that α-tocopherol decreases the yield of proteins oxidative damage, must therefore be appreciated within the context of an oxidizing tone in which more than one oxidant was involved and α-tocopherol probably acted on more than one free radical species. Collectively, as the interest to use these cyclodextrins intensifies, the present study documents the radical forming ability of the studied cyclodextrins at high concentrations and further strengthens the documentation of their cytotoxicity effects through lipids and proteins oxidative damages. The involvement and initiation pattern of our cyclodextrins in these processes may possibly be attributed to their cavity size and depth and to their ring substituents that are known as important factors in their biological activities. It is also our conviction that cyclodextrins interactions with lipids and proteins within membrane and cells may be suggested as a part of mechanism for ROS generation. However, the precise mechanisms in detail through which cyclodextrins increase cellular ROS level and the pattern of their oxidative effects in our treated cells are unclear and under investigation.

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**REFERENCES**

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