

## Original Article

**Effects of tocotrienols on cell viability and apoptosis in normal murine liver cells (BNL CL.2) and liver cancer cells (BNL 1ME A.7R.1), *in vitro***

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The effects of tocotrienols on murine liver cell viability and their apoptotic events were studied over a dose range of 0–32  $\mu\text{g mL}^{-1}$ . Normal murine liver cells (BNL CL.2) and murine liver cancer cells (BNL 1ME A.7R.1) were treated with tocotrienols ( $T_3$ ), alpha tocopherol ( $\alpha$ -T) and the chemo drug, Doxorubicin (Doxo, as a positive control). Cell viability assay showed that  $T_3$  significantly ( $P \leq 0.05$ ) lowered the percentage of BNL 1ME A.7R.1 cell viability in a dose-responsive manner (8–16  $\mu\text{g mL}^{-1}$ ), whereas T did not show any significant ( $P \geq 0.05$ ) inhibition in cell viability with increasing treatment doses of 0 - 16  $\mu\text{g mL}^{-1}$ . The  $IC_{50}$  for tocotrienols were 9.8, 8.9, 8.1, 9.7, 8.1 and 9.3  $\mu\text{g mL}^{-1}$  at 12, 24, 36, 48, 60 and 72 hours respectively. Early apoptosis was detected 6 hours following  $T_3$  treatment of BNL 1ME A.7R.1 liver cancer cells, using Annexin V-FITC fluorescence microscopy assay for apoptosis, but none were observed for the non-treated liver cancer cells at the average  $IC_{50}$  of 8.98  $\mu\text{g mL}^{-1}$  tocotrienols for liver cancer cells. Several apoptotic bodies were detected in BNL 1ME A.7R.1 liver cancer cells at 6 hours post-treatment with tocotrienols (8.98  $\mu\text{g mL}^{-1}$ ) using Acridine Orange/Propidium Iodide fluorescence assay. However, only a couple of apoptotic bodies were seen in the non-treated liver cancer cells and the BNL CL.2 normal liver cells. Some mitotic bodies were also observed in the  $T_3$ -treated BNL 1ME A.7R.1 liver cancer cells but were not seen in the untreated BNL 1ME A.7R.1 cells and the BNL CL.2 liver cells. Following  $T_3$ -treatment (8.98  $\mu\text{g mL}^{-1}$ ) of the BNL 1ME A.7R.1 liver cancer cells, 24.62%, 25.53% and 44.90% of the cells showed elevated active caspase 3 activity at 9, 12 and 24 hours treatment period, respectively. DNA laddering studies indicated DNA fragmentation occurred in the  $T_3$ -treated liver cancer cells, BNL 1ME A.7R.1 but not in non-treated liver cancer cells and the  $T_3$ -treated and non-treated normal liver cells. These results suggest that tocotrienols were able to reduce the cell viability in the murine liver cancer cells at a dose of 8–32  $\mu\text{g mL}^{-1}$  and that this decrease in percentage cell viability may be due to apoptosis.

**Key Words:** tocotrienols, alpha tocopherol, liver carcinogenesis, liver cancer, apoptosis, Doxorubicin**Introduction**

Tocopherols (T) and tocotrienols ( $T_3$ ), each comprising of 4 different isomers, indicated by alpha, beta, gamma and delta, are 2 classes of compounds with vitamin E activity. Tocopherols are present in most vegetable oils and are more common in the diet than tocotrienols, which are only found at relatively high concentrations, in palm oil and rice bran oil.<sup>1-3</sup>

Experimental and epidemiological studies suggest that tocotrienols, besides their antioxidant activity similar to that of tocopherols as seen in cardiovascular diseases,<sup>4</sup> lipid peroxidation,<sup>5-7</sup> some free radical-related diseases<sup>8-11</sup> and lowering of plasma concentrations of atherogenic LDL cholesterol,<sup>12,13</sup> could also inhibit the proliferation of tumor cells.<sup>10,14-16</sup> However, some of these findings, mostly from experiments conducted either in human cell lines or *in vivo*, in mice and rats, were contradictory. The present *in vitro* study was carried out on the murine non-liver cancer cell line, BNL CL.2 and murine liver cancer cell line, BNL 1ME A.7R.1 to determine whether tocotrienols do indeed decrease cell viability in the murine liver cancer cells and whether any decrease, if detected, may be due to apoptosis rather than necrosis. Doxorubicin, a chemo drug, was used

as a positive control and  $\alpha$ -tocopherol was also studied to compare its effects with that of tocotrienols.

**Material and Methods****Cell culture**

Mice cell lines (BNL CL.2 and BNL 1ME A.7R.1) were obtained from America Type Culture Collection (ATCC, Manassas, VA, U.S.A.), cultured in DMEM (high glucose) and serially passaged at sub-confluent cell density. All cell lines were maintained according to ATCC standard protocol with 5% fetal bovine serum.

**Experimental treatments**

Alpha-tocopherol (Sigma Aldrich Chemicals, St. Louis, MO, U.S.A.) and tocotrienols (90%; Hovid (M) Sdn. Bhd., Ipoh, Perak, Malaysia.) were dissolved in absolute ethanol to give

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a stock solution of 3.2 mg mL<sup>-1</sup>. Doxo-rubicin (Pharmachemie, Haarlem, Netherlands) was dissolved in distilled water to prepare a stock solution of 3.2 mg mL<sup>-1</sup>. Treatment solutions were prepared from the stock utilizing the culture media for the various assays.

#### **Cell viability assay**

Cells from both cell lines (BNL CL.2 and BNL 1ME A.7R.1) were seeded into 96-well culture plates at a density of 1 x 10<sup>5</sup> cells mL<sup>-1</sup> per well. The treatment solutions were added after all the cells had attached to the plates (approximately 6 hours). The cells were treated with various doses (32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.0625, 0.03125 and 0 mg mL<sup>-1</sup> medium containing 1% ethanol) of Doxorubicin,  $\alpha$ -tocopherol and tocotrienols for a period of 12, 24, 48, 60 and 72 hours. CellTiter 96 Aqueous One Solution Cell Proliferation Assay Kit (Promega Corporation, Madison, WI, U.S.A.) was used to determine the cell viability.

#### **Annexin V-FITC fluorescence microscopy assay**

The cells were seeded into Labtek II chamber slide at a density of 1 x 10<sup>6</sup> cells chamber<sup>-1</sup>. The attached cells were then treated with 8.98  $\mu$ g mL<sup>-1</sup> medium (IC<sub>50</sub> for tocotrienols on BNL 1ME A.7R.1) of Doxorubicin,  $\alpha$ -tocopherol and tocotrienols for a period of 3, 6, 9 and 12 hours. The cells were then stained with Annexin V-FITC and observed under the fluorescence microscope.

#### **Acridine orange/propidium iodide fluorescence assay**

The cells were again seeded into Labtek II chamber slide at a density of 1 x 10<sup>6</sup> cells chamber<sup>-1</sup>. The attached cells were then treated with 8.98  $\mu$ g mL<sup>-1</sup> medium, of Doxorubicin,  $\alpha$ -tocopherol and tocotrienols for a period 3, 6, 9 and 12 hours. The cells were then stained with Acridine Orange/Propidium Iodide and observed under the fluorescence microscope.

#### **Active caspase 3 activity assay**

The cells were treated with Doxorubicin,  $\alpha$ -tocopherol and tocotrienols at a concentration of 8.98  $\mu$ g mL<sup>-1</sup> medium and incubated for a period of 3, 6, 9, 12 and 24 hours. After incubation, the cells were harvested, washed and incubated with FITC-conjugated active caspase 3 antibodies for 30 minutes. The cells were then washed and resuspended in buffer and analysed by flow cytometry.

#### **DNA fragmentation assay**

The cells were treated with Doxorubicin,  $\alpha$ -tocopherol and tocotrienols at a dose of 8.98  $\mu$ g mL<sup>-1</sup> medium and incubated for a period of 24 hours. After incubation, the cells were harvested and their DNA's were obtained by phenol extraction. Agarose gel electrophoresis and UV imaging were then conducted.

### **Results**

#### **Cell Viability**

The effects of various doses of tocotrienols (90%) on murine non-liver cancer cells (BNL CL.2) and murine liver cancer cells (BNL 1ME A.7R.1) are shown in Figure 1. Tocotrienols significantly ( $P \leq 0.05$ ) lowered the % cell viability of liver cancer cells in a dose-responsive manner (4-16  $\mu$ g mL<sup>-1</sup>) as compared to the negative control.

Tocotrienols also lowered the percentage cell viability of the non-liver cancer cells, but they were not significant until a dose of 16  $\mu$ g mL<sup>-1</sup>. However,  $\alpha$ -tocopherol did not show any significant inhibitory effect ( $P \geq 0.05$ ) on both BNL CL.2 and BNL 1ME A.7R.1 cells at the range of doses tested. The IC<sub>50</sub>'s of tocotrienols (90%) for BNL CL.2 (Mean: 29.5  $\mu$ g mL<sup>-1</sup>) and that for BNL 1ME A.7R.1 (Mean: 8.98  $\mu$ g mL<sup>-1</sup>) at different incubation periods of 12-72 hours are shown in Figure 2. There are no significant ( $P > 0.05$ ) changes in the IC<sub>50</sub> for tocotrienols, in each of the two cell lines, BNL CL.2 and BNL 1ME A.7R, indicating that the effects of tocotrienols on its IC<sub>50</sub> are not time-dependent over the incubation period tested. Since tocotrienols significantly decreased %cell viability of murine liver cancer cells and to a lesser extent, the murine non-liver cancer cells, in a dose-dependent manner, additional studies were conducted, using the mean IC<sub>50</sub> of 8.98  $\mu$ g mL<sup>-1</sup> for tocotrienols, to determine whether this decrease was due to apoptosis.

#### **Annexin V-FITC fluorescence microscopy assay**

Following treatment with tocotrienols, the Annexin V-FITC Fluorescence Assay showed that the BNL CL.2 cells were left unstained while a large number of the BNL 1ME A.7R.1 cells were stained with Annexin V-FITC (Fig. 3). No fluorescence was detected when the liver cancer cells were treated with  $\alpha$ -tocopherol at the IC<sub>50</sub> for tocotrienols but some were detected when these same cells were treated with Doxorubicin.

#### **Acridine Orange/Propidium Iodide fluorescence assay**

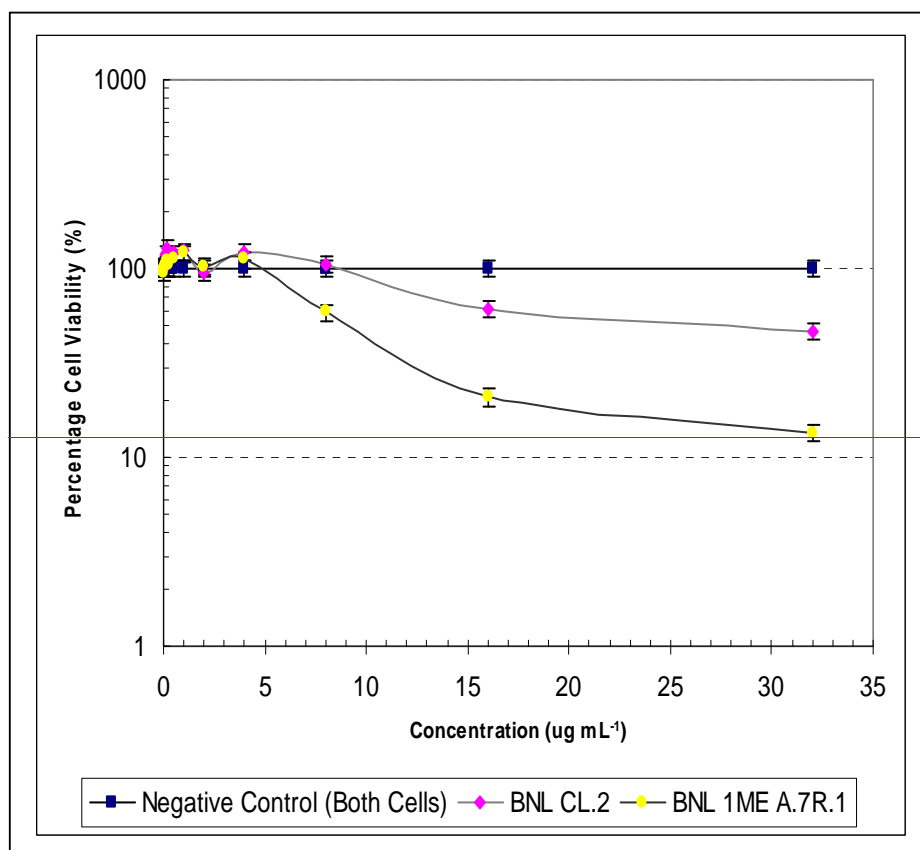
Figure 4 shows that both BNL CL.2 and BNL 1ME A.7R.1 cells were stained with Acridine Orange but not Propidium Iodide, following treatment with tocotrienols for a period of 6 hours indicating the absence of necrosis. Furthermore, numerous BNL 1ME A.7R.1 cells showed condensation of nuclei but these were very few in the BNL CL.2 cells. Condensation of nuclei was not detected in both liver cell lines following treatment with  $\alpha$ -tocopherol. However, both Acridine Orange and Propidium Iodide were detected in the Doxorubicin treatment group indicating necrosis and/or late apoptosis had occurred.

#### **Active caspase 3 activity**

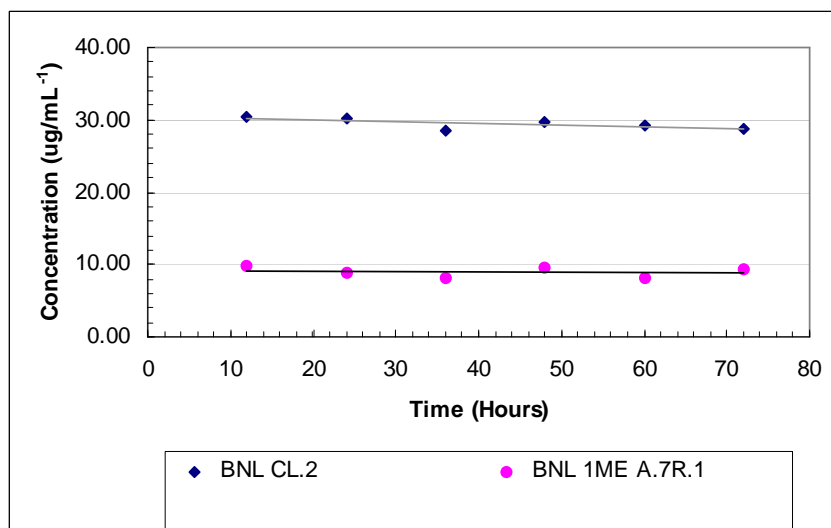
From three- to six-hour incubation period, the % of cells with active caspase 3 were consistently low for both cell lines treated with  $\alpha$ -T, T<sub>3</sub> and Doxorubicin at the IC<sub>50</sub> for T<sub>3</sub>. T<sub>3</sub> treatment showed marked increases in the % of cells with active caspase 3 from the 9-hr to 24-hr incubation period for the liver cancer cells (Table 1). However, this was not detected with  $\alpha$ -T treatment although Doxorubicin showed a much lower increase from 12 to 24 hour incubation period. However, the amount of active caspase 3 in the BNL 1ME A.7R.1 cells began to increase after incubating the cells with tocotrienols for a period of 9 hours and this almost doubled after a 24-hour incubation period.

#### **DNA fragmentation**

BNL 1ME A.7R.1 cells showed DNA fragmentation after treatment with tocotrienols for a 24-hr period, similar to that for the positive control, Doxorubicin (Fig. 5).



**Figure 1.** Effects of various doses of tocotrienols on BNL CL.2 and BNL 1ME A.7R.1 cells, compared with the negative controls. Data points indicate the mean % cell viability for 3 sets of 3 replicates in each treatment group after a treatment period of 24 hours.



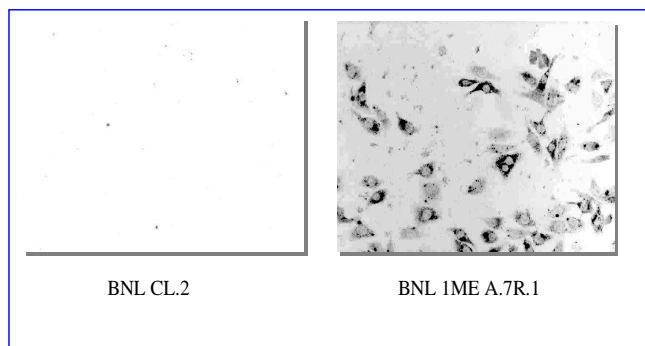
**Figure 2.** Effects of tocotrienols on its IC<sub>50</sub> in BNL CL.2 and BNL 1ME A.7R.1 cells over time. Data points indicate the mean IC<sub>50</sub> for 3 sets of 3 replicates in each treatment-time period.

However, no DNA fragmentation was found in the negative control using non-treated cells and the  $\alpha$ -T-treated cells in both cell lines and also the T<sub>3</sub>-treated BNL CL.2 cells.

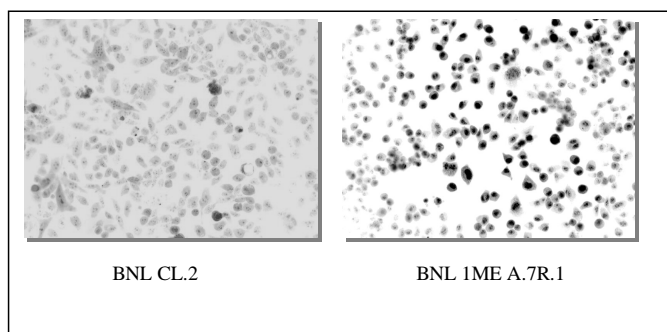
### Discussion

Results from the present studies demonstrated that the murine liver cancer cells, BNL 1ME A.7R.1 were more responsive to tocotrienols with respect to cell viability

(Fig.1) and apoptosis (Figs. 3-5; Table 1), than the murine non-liver cancer, BNL CL.2 cells. The transformed liver cancer cells may have altered membrane characteristics towards tocotrienols. Furthermore, at the dose range of 0–32  $\mu\text{g mL}^{-1}$ ,  $\alpha$ -tocopherol did not decrease the % cell viability of both of these murine liver cell lines although it had been reported to exert anti-proliferative and cytotoxic effects on other cell lines at higher doses.<sup>17</sup> However,  $\alpha$ -tocopherol may have inhibitory effects on these



**Figure 3.** Cells stained with Annexin V-FITC: BNL CL.2 cells were not stained, while BNL 1ME A.7R.1 cells were, showing the incorporation of Annexin V-FITC to the translocated phosphatidylserine (PS) in the outer leaflet of the plasma membrane following 6 hours of treatment with tocotrienols. (400X)



**Figure 4.** Cells stained with Acridine Orange / Propidium Iodide (AO/PI) following tocotrienol treatment. Both BNL CL.2 and BNL 1ME A.7R.1 cells were stained with only Acridine Orange. BNL 1ME A.7R.1 cells but not those of BNL CL.2 showed prominent condensed nuclei. (400X)

**Table 1.** Percentage of cells stained for active caspase 3 following incubation over various time periods of murine non-liver cancer (BNL CL.2) and murine liver cancer (BNL 1ME A.7R.1) cells with  $\alpha$ -T, T<sub>3</sub> and Doxorubicin (positive control)

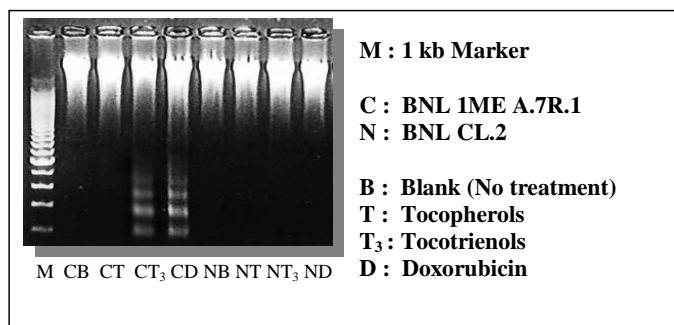
Incubation Time (hr)	Liver Cell Line	% Cell with caspase 3 activity			
		Negative Control	$\alpha$ -T	Doxo	T3
3	BNL CL.2	0.43	0.42	0.29	0.11
	BNL 1ME A.7R.1	0.06	0.07	0.50	0.48
6	BNL CL.2	0.49	0.55	0.45	0.17
	BNL 1ME A.7R.1	0.09	0.12	0.54	0.62
9	BNL CL.2	0.21	0.32	1.17	1.69
	BNL 1ME A.7R.1	0.17	0.19	0.69	24.62
12	BNL CL.2	3.57	8.81	6.83	2.02
	BNL 1ME A.7R.1	3.99	1.76	10.72	25.53
24	BNL CL.2	3.57	9.13	14.24	28.39
	BNL 1ME A.7R.1	0.30	1.76	13.22	44.90

these murine liver cells, but at much higher doses. Direct comparisons between these two vitamin E sub-classes indicated that tocotrienols were significantly more potent in decreasing % cell viability than  $\alpha$ -tocopherol.

This study has also shown that tocotrienol-induced cell death was due to apoptosis, i.e. programmed cell death, as indicated by the Annexin V-FITC Fluorescence Microscopy Assay, Acridine Orange/Propidium Iodide Fluorescence Assay, Active Caspase 3 Activity Assay and DNA Fragmentation Assay. At the IC<sub>50</sub> of 8.98  $\mu\text{g mL}^{-1}$ , obtained for tocotrienols on BNL 1ME A.7R.1 cells, Annexin V-FITC fluorescence was detected substantially, as early as 6 hours post treatment of the liver cancer cells with tocotrienols. This showed that the plasma membrane of BNL 1ME A.7R.1 cells had incorporated Annexin V-FITC, indicating that phosphatidylserine (PS) had been

translocated from the inner to the outer leaflet of the plasma membrane (early step in apoptosis), following treatment with tocotrienols. This suggests that tocotrienols might have initiated apoptosis in these liver cancer cells.

Studies with Acridine Orange and Propidium Iodide (AO/PI) showed that both BNL CL.2 and BNL 1ME A.7R.1 cells were stained with Acridine Orange but not with Propidium Iodide, indicating that hardly any necrosis occurred in these cells after tocotrienol treatment for a period of 6 hours (Fig. 4). Hence, this cell death, as reflected in the decrease in % cell viability, is probably due to apoptosis rather than necrosis. However, staining of the liver cells with both Acridine Orange and Propidium Iodide were detected following treatment with the positive control, Doxorubicin at the same dose. This suggested the presence of some necrosis or late apoptosis with this chemo drug.



**Figure 5.** Electrophoretograms showing DNA fragmentation in tocotrienols-treated and Doxorubicin-treated (positive control) BNL 1ME A.7R.1 cells after incubation for a 24-hr period.

Six hours after incubation of BNL 1ME A.7R.1 cells with tocotrienols at its  $IC_{50}$  of  $8.98\text{-}\mu\text{g mL}^{-1}$ , numerous numbers of cells with nuclei condensation were observed but very few were detected in the BNL CL.2 cells (Fig. 4). This suggested that apoptosis was more prominent in these liver cancer cells when compared to the non-liver cancer BNL CL.2 cells. This may partly explain the reason for the greater decrease in cell viability of the BNL 1ME A.7R.1 cells when compared to the non-liver cancer BNL CL.2 cells following treatment with tocotrienols.

Another useful method to determine apoptosis is to assay for active caspase 3 in the cells. Cells, which undergo apoptosis, will cause pro-caspase 3 to cleave thus producing active caspase 3. In the assay, the active caspase 3 will bind to FITC-conjugated anti-active caspase 3 antibody and this fluorescence was easily measured by flow cytometry. In the present study, an increase in the number of BNL 1ME A.7R.1 cells containing active caspase 3, were observed, after incubation with tocotrienols for a time period of 9–24 hours. At 24 hours, this was 3.4 times higher than that obtained for the positive control Doxorubicin.

DNA fragmentation as an indication of late apoptosis was detected when BNL 1ME A.7R.1 cells were incubated with Doxorubicin and tocotrienols separately, at the  $IC_{50}$  for tocotrienols, for a period of 24 hours. However, no DNA fragmentation was observed with BNL CL.2 cells (Fig. 5). There was hardly any evidence that the BNL CL.2 cells undergo extensive apoptotic reactions after treatment with tocotrienols at its  $IC_{50}$  for liver cancer cells. This is expected as the % cell viability of BNL CL.2 cells in the region of the  $IC_{50}$  for tocotrienols obtained on the liver cancer, BNL 1ME A.7R.1 cells, were not significantly different from that of the negative controls.

One possible explanation for the apparent higher biopotency of tocotrienols compared to  $\alpha$ -tocopherol is that tocotrienols are more easily or is preferentially taken up by BNL 1ME A.7R.1 cells and to a lesser extent by the BNL CL.2 cells. Since tocotrienols differ from  $\alpha$ -tocopherol in possessing an unsaturated side chain, the presence of these three double bonds might result in a less planar molecular configuration that facilitates less restricted trans-membrane passage of tocotrienols into the cell, as compared with  $\alpha$ -tocopherol. Although tocotrienols display greater biopotency than  $\alpha$ -tocopherol *in*

*vitro*, absorption and transportation of individual tocopherol and tocotrienol isoforms *in vivo* may be influenced by selectivity and binding of specific transport proteins and transport mechanisms that exhibit significant preference for  $\alpha$ -tocopherol.<sup>18</sup>

Additional studies are required to determine the *in vitro* effects of tocotrienols on cell proliferation *per se*, to give an overall picture of the effects of tocotrienols in these cell lines since the % cell viability obtained is the net result of the actual cell proliferation and apoptosis. Studies on characterizing the intracellular mechanisms and pathways responsible for mediating the anti-proliferative and apoptotic effects of tocotrienols could also provide important information necessary to the understanding of the action of tocotrienols in the cells, in particular, cancer cells. Further studies on whether tocotrienols act on the membrane receptors or have to be transported into the cells for its action would also be of interest.

In conclusion, tocotrienols have a greater biopotency on the cell viability of BNL 1ME A.7R.1 liver cancer cells ( $IC_{50}$  of  $8.98\text{ }\mu\text{g mL}^{-1}$ ) than that of the non-liver cancer cells of BNL CL.2 ( $IC_{50}$  of  $29.5\text{ }\mu\text{g mL}^{-1}$ ). It is possible that the growth-inhibitory effects of tocotrienols on the murine liver cancer cells reflect an increase in the numbers of cells undergoing apoptosis, and do not reflect necrosis. Tocotrienols may act initially at the plasma membrane to initiate apoptosis, which progressed to nuclei condensation followed by activation of Caspase 3, which ultimately leads to DNA fragmentation and cell death.

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**生育三烯酚对正常鼠肝细胞 (BNL CL.2) 和肝癌细胞 (BNL 1ME A.7R.1) 发育及凋亡的影响, 离体试验**

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生育三烯酚对鼠肝细胞发育及凋亡影响的研究是在 0-32  $\mu\text{g mL}^{-1}$  的剂量范围进行的。分别用生育三烯酚 (T3)、 $\alpha$ -生育酚 ( $\alpha$ -T)、化学药物阿霉素 (Doxo) 对小鼠肝细胞 (BNL CL.2) 和肝癌细胞 (BNL 1ME A.7R.1) 进行处理, 其中阿霉素处理组作为阳性对照组。结果显示: 生育三烯酚 (8-16  $\mu\text{g mL}^{-1}$ ) 能够以剂量效应的方式, 显著降低鼠肝癌细胞发育的比例 ( $P \leq 0.05$ )。然而在 0-16  $\mu\text{g mL}^{-1}$  范围内, 随着处理浓度的增加, 生育酚对肝癌细胞的发育能力无显著抑制作用 ( $P \geq 0.05$ )。生育三烯酚处理 12、24、36、48、60 和 72 h 的半数有效浓度分别为 9.8、8.9、8.1、9.7、8.1  $\mu\text{g mL}^{-1}$ 。用荧光显微镜 (膜联蛋白 V-FITC) 来检测, 生育三烯酚 (8.98  $\mu\text{g mL}^{-1}$ ) 处理肝癌细胞 6 h 后, 出现了早期的凋亡, 而未用生育三烯酚处理的肝癌细胞并未出现凋亡。用丫啶橙/典化丙啶荧光测定法, 在接受生育三烯酚 (8.98  $\mu\text{g mL}^{-1}$ ) 处理 6 h 的肝癌细胞中观测到几个凋亡的细胞, 而在未处理的肝癌细胞和正常的肝细胞中, 仅观测到两个凋亡的细胞。在生育三烯酚处理的鼠肝癌细胞中, 还发现了一些有丝分裂的个体, 而在未处理的肝癌细胞和正常的肝细胞中均未发现。接受生育三烯酚 (8.98  $\mu\text{g mL}^{-1}$ ) 处理 9、12 和 24 h 后, 分别有 24.62%、25.53% 和 44.90% 的肝癌细胞活性卡斯帕酶 3 的活力得到提高。DNA 条带研究显示: 生育三烯酚处理过的肝癌细胞中出现了 DNA 片断, 而在未接受处理的肝癌细胞、接受和未接受处理的正常肝细胞中均为出现。以上结果显示: 生育三烯酚在 8-32  $\mu\text{g mL}^{-1}$  的剂量范围内能够降低鼠肝癌细胞的发育能力, 这种抑制作用可能是通过提高其细胞凋亡来实现的。

关键词: 生育三烯酚, 肝癌, 鼠肝细胞, 凋亡