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The Role of Ca^{2+} in (-)-Menthol-induced Human Promyelocytic Leukemia HL-60 Cell Death

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Abstract. *A human promyelocytic leukemia HL-60 cell line was selected to examine the effect of (-)-Menthol on cell death. Based on the results from morphological changes and the percentage of viable cells in HL-60 cells after treatment with various concentrations of (-)-Menthol, it was shown that (-)-Menthol induced cell death through necrosis, not apoptosis. No cell cycle arrest was found in HL-60 cells examined by flow cytometry analysis. Also, the DNA gel electrophoresis method showed that (-)-Menthol did not induce apoptosis in HL-60 cells. However, it was found that (-)-Menthol induced the production of Ca^{2+} in these examined cells, dose-dependently. When HL-60 cells were pretreated with the chelator (BAPTA) of Ca^{2+} for 3 hours before addition of (-)-Menthol to the culture, a decrease of Ca^{2+} production was observed. Under the same conditions, the percentage of viable HL-60 cells was increased. Apparently Ca^{2+} production is associated with the induction of (-)-Menthol-induced cell death.*

(-)-Menthol, ([1-α]-5-methyl-2-[1-methylethyl]-cyclohexanol), is an aromatic compound which is an important constituent of four essential oils (eucalyptus, lemongrass, palmarosa and peppermint). It comes from *Mentha haplocalyx* Briq, *Mentha haplocalyx* Briq. Var. *piperascens* and *Mentha piperita* L. and displays antibacterial and antifungal activity (1, 2). (-)-Menthol is a widely used flavoring ingredient present in mouthwash, foods, toothpaste and cigarettes (3). Although the pharmacological activity of (-)-Menthol is not precisely known, it has been demonstrated that (-)-Menthol can inhibit the growth of rat liver epithelial tumors cells (4) and acts as a potent chemopreventive agent during DMBA initiation of rat mammary tumors (5). Recently, it was also reported that (-)-Menthol and synthetic congeners inhibit *in vitro* the microsomal oxidation of nicotine to cotinine and the P450 2A6-mediated 7-hydroxylation of coumarin (3).

Our laboratory has demonstrated that (-)-Menthol inhibits *N*-acetyltransferase activity, based on the amounts of *N*-acetylated 2-aminofluorene and non-acetylated 2-aminofluorene in human hepatoma cells (6), and the distribution and metabolism of 2-aminofluorene in various tissues of Sprague-Dawley rats were affected by (-)-Menthol after oral treatment (7). However, there is no available information on the effect of (-)-Menthol on the cell death of human leukemia cells. In this study, HL-60 human leukemia cells were treated with (-)-Menthol and analyzed for the correlation between cytotoxicity and the production of Ca^{2+}. The effect of (-)-Menthol on cell death associated with different levels of Ca^{2+} was also investigated. Our results demonstrated that (-)-Menthol can induce cell death through the production of Ca^{2+} in human leukemia HL-60 cells.

Materials and Methods

Chemicals and reagents. (-)-Menthol, 2-aminofluorene, *N*-acetyl-2-aminofluorene, ribonuclease A, trypan blue, propidium iodide (PI), Triton X-100 and Tris-HCl were obtained from Sigma Chemical Co. (St. Louis, MO, USA). RNase was obtained from BD Biosciences Clontech (Palo Alto, CA, USA). TE buffer (10 mM Tris-HCl, 1mM EDTA, pH 7.6), potassium phosphates and dimethyl sulfoxide (DMSO) were purchased from Merck Co. (Darmstadt, Germany). RPMI 1640 medium, fetal bovine serum, penicillin-streptomycin and L-glutamine were obtained from Gibco BRL (Grand Island, NY, USA). All of the chemicals used were of reagent grade.

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Key Words: Calcium, (-)-Menthol, promyelocytic leukemia cells, necrosis, apoptosis.

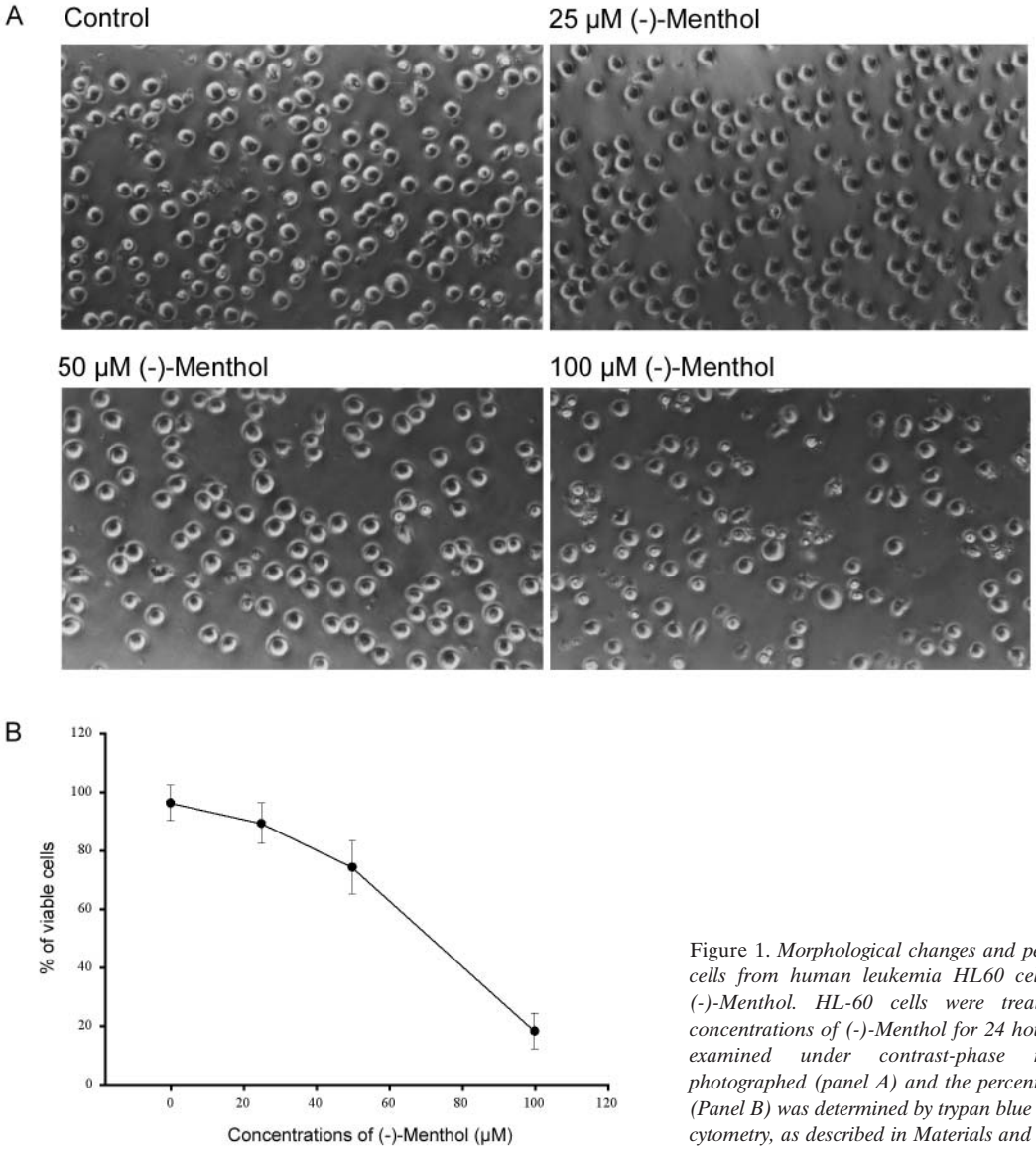


Figure 1. *Morphological changes and percentage of viable cells from human leukemia HL60 cells in response to (-)-Menthol. HL-60 cells were treated with varying concentrations of (-)-Menthol for 24 hours. The cells were examined under contrast-phase microscope and photographed (panel A) and the percentage of viable cells (Panel B) was determined by trypan blue exclusion and flow cytometry, as described in Materials and Methods.*

Cell culture. The human promyelocytic leukemia cells (HL-60) were cultured in RPMI 1640 medium supplemented with 10% FBS, as described previously (8).

Assay for cytotoxic activity

i) The morphological changes of HL-60 cells after treatment with various concentrations of (-)-Menthol: The HL-60 cells were plated in 24-well plates at a density of $2.5x10^5$ cells/well and grown for 24 hours. They were then added to various concentrations of (-)-Menthol, to final concentrations of 0, 25, 50 and 100 μM and grown at 37˚C in 5% CO_2 and 95% air for different periods of time. In the control regimen, DMSO (solvent) only was added. The morphological changes were examined and photographed (9).

ii) The cell viability of HL-60 cells, treated with or without various concentrations of (-)-Menthol, was determined by trypan blue exclusion and flow cytometry: The HL-60 cells were plated in 24-well plates at a density of $2.5x10^5$ cells/well and grown for 24 hours. They were then added to different concentrations of (-)-Menthol to final concentrations of 0, 25, 50 and 100 μM and grown at 37˚C, in 5% CO_2 and 95% air for different periods of time. In the control regimen, DMSO (solvent) only was added. To determine cell viability, the trypan blue exclusion protocol was used. Briefly, about 10 μl of cell suspensions in phosphate-buffered saline (PBS) were mixed with 40 μl of trypan blue, and the numbers of stained (dead cells) and unstained cells (live cells) were counted using a hemocytometer (9), or for flow cytometric assay as described previously (9).

iii) DNA fragmentation electrophoresis analysis: HL-60 cells were plated in 12-well plates at a density of $5x10^6$ cells/well and grown for 24 hours. They were then treated with 0, 25, 50 and 100 μM (-)-Menthol, while only adding DMSO (solvent) for the control regimen, and grown at 37˚C, in a humidified 5% CO_2 for 72 hours

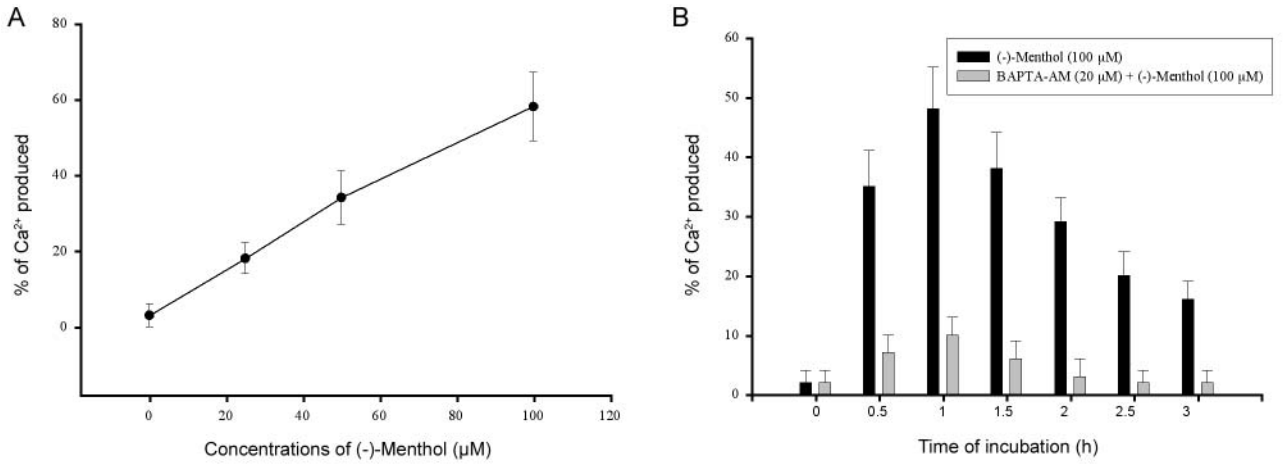


Figure 2. *Flow cytometric analysis of Ca^{2+} concentration in human leukemia HL-60 cells with (-)-Menthol for 24 hours. The HL-60 cells ($5x10^5$ cells/ml) were pretreated with or without BAPTA for 3 hours before various concentrations of (-)-Menthol were added to the culture for various time-periods to detect the changes of Ca^{2+} concentration. The zero concentration was defined as control. The percentage of cells that were stained by Indo-1/AM dye, and the stained cells were determined by flow cytometry, as described in Materials and Methods. *differs between (-)-Menthol and control. $p<0.05$.*

for DNA fragmentation electrophoresis assay, as described previously (10).

Assay for Ca^{2+} concentrations

i) Detection of Ca^{2+} concentrations in HL-60 cells, after treatment with (-)-Menthol, by flow cytometry: The level of Ca^{2+} of the HL-60 cells was determined and quantitated by flow cytometry (Becton Dickinson FACS Calibur), using the Indo 1/AM (Calbiochem, La Jolla, CA, USA). HL-60 cells were treated with or without (-)-Menthol (25, 50 and 100 μM) for 6 hours to detect the changes of Ca^{2+} concentrations. The cells were harvested and washed twice, before being re-suspended in Indo 1/AM (3 μg/ml), incubated at 37˚C for 30 minutes and analyzed by flow cytometry (10).

ii) Detection of Ca^{2+} concentrations in HL-60 cells, after pretreatment with BAPTA and co-treatment with (-)-Menthol by flow cytometry: HL-60 cells were pretreated with BAPTA for 3 hours then treated with or without (-)-Menthol (25, 50 and 100 μM) for 6 hours. The cells were then harvested to examine the changes of Ca^{2+} concentrations, as described above.

iii) The morphological changes of HL-60 cells, after pretreatment with BAPTA then treatment with various concentrations of (-)-Menthol: The HL-60 cells were pretreated with BAPTA for 3 hours then treated with (-)-Menthol (100 μM) for 24 hours. The morphological changes were examined, as described above (9)

iv) The cell viability of HL-60 cells, after pretreatment with BAPTA and then treatment with or without various concentrations of (-)-Menthol, was determined by trypan blue exclusion and flow cytometry: The HL-60 cells were pretreated with BAPTA and then treated with (-)-Menthol (25, 50 and 100 μM) for 24 hours. Cell viability was examined, as described above.

Assay for NAT activity. About $5x10^5$ HL-60 cells/well of medium were incubated with 22.5 μM 2-aminofluorene in individual wells of 24-well cell culture plate with or without (-)-Menthol (25, 50 and 100 μM) for 24 hours. After incubation for 24 hours, the cells and media were removed and centrifuged. The supernatant was immediately extracted with ethyl acetate/ methanol (95:5), the solvent evaporated, and the residue redissolved in methanol and assayed for *N*-acetyl-2-aminofluorene by HPLC, as described previously (11-14).

Results

The morphological changes and percentages of viable HL-60 cells after treatment with various concentrations of (-)-Menthol. The morphological changes of HL-60 cells were increased with increasing concentrations of (-)-Menthol, as presented in Figure 1A. Apparently (-)-Menthol led to cell death, based on the decreased number of cells and the increased debris of cells. The percentage of viable HL-60 cells after treatment with (-)-Menthol decreased with increasing concentrations of (-)-Menthol (Figure 1B).

(-)-Menthol induced the production of Ca^{2+} in HL-60 cells. The percentage of Ca^{2+} was significantly different between the (-)-Menthol-treated group and the control. The effects of (-)-Menthol on HL-60 cells were dose-dependent (Figure 2A). Pretreatment with BAPTA led to a decrease in the production of Ca^{2+} (Figure 2B).

BAPTA pretreatment. The morphological change of HL-60 cells back to normal was increased when the cells were pretreated with BAPTA for 3 hours and then treated with (-)-Menthol, as presented in Figure 3A. The percentage of viable HL-60 cells increased on preatment with BAPTA before (-)-Menthol treratment, as presented in Figure 3B.

DNA fragmentation. DNA from (-)-Menthol-treated and non-treated cells was isolated and examined in 0.8% agarose gel electrophoresis (Figure 4). The assay showed

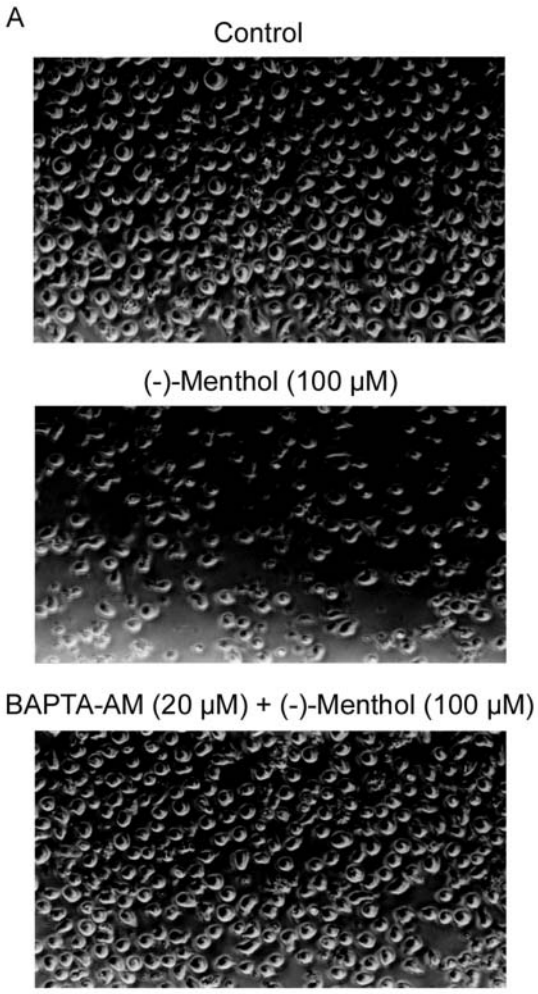


Figure 3. *Morphological changes and percentage of viable cells from human leukemia HL-60 cells that were pretreated with BAPTA in response to (-)-Menthol. HL-60 cells were pretreated with BAPTA then treated with varying concentrations of (-)-Menthol for 24 hours. The cells were examined under contrast-phase microscope and photographed (panel A) and the percentage of viable cells (Panel B) was determined by trypan blue exclusion and flow cytometry, as described in Materials and Methods.*

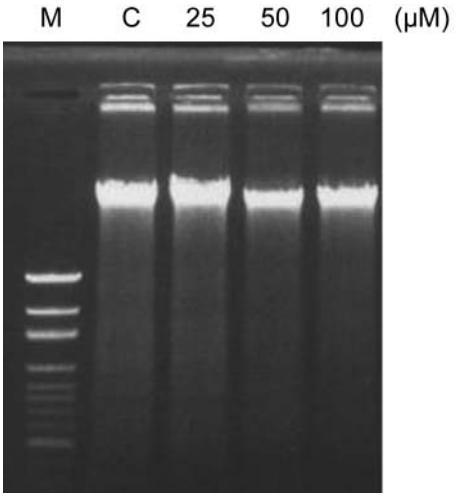


Figure 4. *(-)-Menthol affects DNA damage in HL-60 cells. HL-60 cells were incubated with or without various concentrations of (-)-Menthol for 48 hours, then DNA degradation was analyzed by gel electrophoresis, as described in Materials and Methods.*

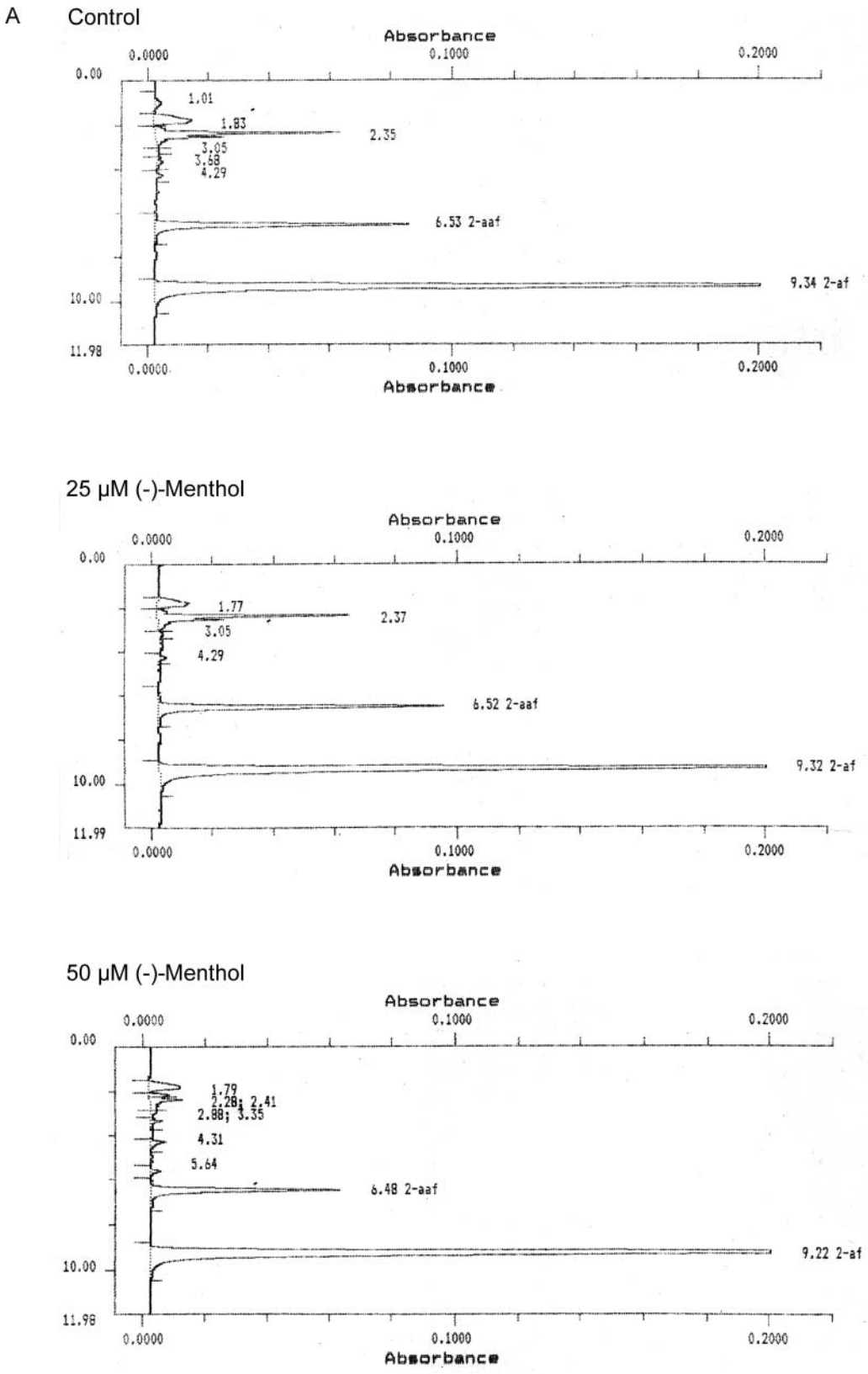
that the DNA from control cells was not degraded, and no increase of DNA laddering occurred in cells treated with (-)-Menthol.

(-)-Menthol inhibited the N-acetylation of 2-aminofluorene in HL-60 cells. The levels of *N*-acetylation of 2-aminofluorene was significantly different between the (-)-Menthol-treated group and the control. The representative profiles of *N*-acetylation of 2-aminofluorene are presented in Figure 5A. The effects of (-)-Menthol on the *N*-acetylation of 2-aminofluorene in HL-60 cells were dose-dependent (Figure 5B).

Discussion

Although (-)-Menthol affects cancer cells, the mechanisms are not well established and do not show anticancer action. The present study demonstrated that (-)-Menthol induced morphological changes and decreased the percentage of viable cells in human leukemia HL-60 cells. However, the results

Figure 5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Control
Absorbance
0.0000
0.1000
0.2000
0.00
1.01
1.83
2.35
3.05
3.68
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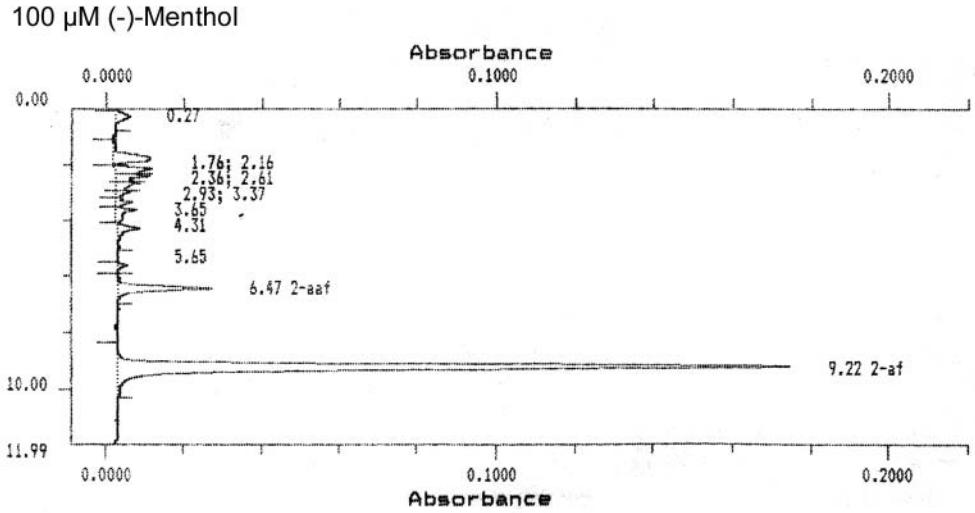
6.53 2-aaf
9.34 2-af
10.00
11.98
25 μM (-)-Menthol
1.77
2.37
3.05
4.29
6.52 2-aaf
9.32 2-af
11.99
50 μM (-)-Menthol
1.79
2.28; 2.41
2.88; 3.35
4.31
5.64
6.48 2-aaf
9.22 2-af

Figure 5 *continued*



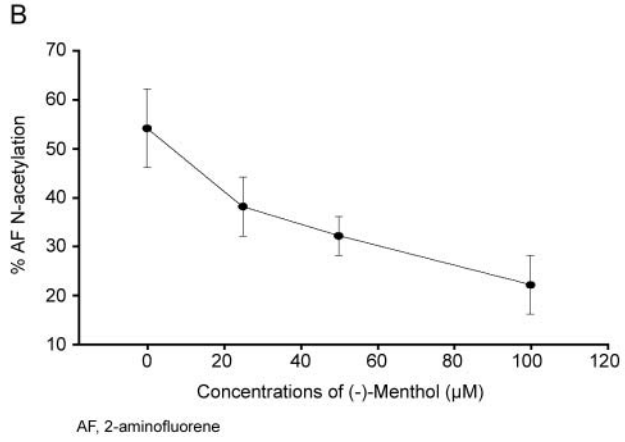


Figure 5. *Representative profiles from HL-60 cells after treatment with (-)-Menthol on the production of N-acetyl-2-aminofluorene. The cells were incubated with various concentrations of (-)-Menthol (0, 25, 50 and 100 µM) and 25 µM 2-aminofluorene for 24 hours. Both N-acetyl-2-aminofluorene and 2-aminofluorene were determined by HPLC, as described in Materials and Methods. Each point represents the mean of triplicate assays. The values are mean±SD (n=3). *Difference between wogonin and control. $p<0.05$.*

from our studies demonstrated that (-)-Menthol-induced cell death is associated with the production of Ca^{2+} in the HL-60 cells. On pretreatment of HL-60 cells with BAPTA followed by the addition of (-)-Menthol, the viable cell number was increased. This is in agreement with another report which demonstrated that (-)-Menthol specifically increased cytosolic Ca^{2+} in airway epithelium, which may be derived from intracellular Ca^{2+} stores (15). However, (-)-Menthol did not induce reactive oxygen species (ROS) in these examined cells (data not shown). (-)-Menthol did not induce apoptosis in HL-60 cells, while our earlier reports showed that (-)-Menthol also did not induce apoptosis in human gastric cancer SNU-5 cells (16). However, our earlier studies demonstrated that (-)-Menthol affected topoisomerase I and II gene expressions and protein levels (16).

In the present study, DNA damage was shown by gel electrophoresis. DNA isolated from (-)-Menthol-treated HL-60 cells indicated the occurrence of necrosis and not apoptosis (Figure 4). This was also confirmed by flow cytometry analysis, which indicated that (-)-Menthol did not induce the sub-G1-phase in the cell cycle analysis of HL-60 cells (data not shown), but only an increase of cell debris indicating cell death through necrosis. Many anticancer agents have been shown to induce cell apoptosis and cell cycle arrest in cancer cells. Our results from cell cycle analysis showed no cell phase or (-)-Menthol-induced apoptosis in the examined cells. However, the production of Ca^{2+} did increase after cotreatment with (-)-Menthol dose-dependently for 6 hours after treatment, but, increased time led to decreased production of Ca^{2+}; after 12 hours treatment, the production of Ca^{2+} was reduced to 35% and after 24 hours it was normal. Whether or not (-)-Menthol can increase the health risk of smoking menthol-containing cigarettes is not known. It was reported that (-)-Menthol and

synthetic congeners inhibit the microsomal oxidation of nicotine to cotinine and the P450 2A6-mediated 7-hydroxylation of coumarin (3). It was also reported that pure menthol and menthol in food or beverages have a similar systemic bioavailability and that menthol has a small cardio-accelerating effect (17).

Our results also showed that (-)-Menthol decreased the *N*-acetylation of 2-aminofluorene, but no effects of (-)-Menthol on *NAT* gene expression (mRNA NAT) and the levels of NAT protein in HL-60 cells were observed. By studying viable cells, it was concluded that the decrease of *N*-acetylation of 2-aminofluorene was due to the decrease of cell number of HL-60, because (-)-Menthol induced cell death through necrosis that leads to the decrease of *N*-acetylation of 2-aminofluorene. This was also confirmed by our earlier studies in human hepatoma cells (6).

Acknowledgements

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