

Adverse effects of fullerenes on endothelial cells: Fullerenol $C_{60}(OH)_{24}$ induced tissue factor and ICAM-1 membrane expression and apoptosis in vitro

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Abstract: We studied the effects of a C_{60} water suspension at 4 $\mu\text{g}/\text{mL}$ (nC_{60}) and the water soluble fullerenol $C_{60}(OH)_{24}$ at final concentrations of 1–100 $\mu\text{g}/\text{mL}$ on human umbilical vein endothelial cells (HUVECs) in culture. We found that a 24 hr treatment of HUVECs with $C_{60}(OH)_{24}$ at 100 $\mu\text{g}/\text{mL}$ significantly increased cell surface expression of ICAM-1(CD54) ($67 \pm 4\%$ CD54⁺ cells vs. $19 \pm 2\%$ CD54⁺ cells in control; $p < 0.001$). In addition, this treatment induced the expression of tissue factor (CD142) on HUVECs ($54 \pm 20\%$ CD142⁺ cells vs $4 \pm 2\%$ CD142⁺ cells in control; $p = 0.008$) and increased exposure of phosphatidylserine (PS) ($29 \pm 2\%$ PS⁺ cells vs. $12 \pm 5\%$ PS⁺ cells in control; $p < 0.001$). Analysis of cell cycle and DNA fragmentation (TUNEL) showed that both nC_{60} and $C_{60}(OH)_{24}$ caused G1 arrest of HUVECs and $C_{60}(OH)_{24}$ induced significant apoptosis ($21 \pm 2\%$ TUNEL⁺ cells at 100 $\mu\text{g}/\text{mL}$ of $C_{60}(OH)_{24}$ vs. $4 \pm 2\%$ TUNEL⁺ cells in control; $p < 0.001$). We also demonstrated that both nC_{60} and $C_{60}(OH)_{24}$ induced a rapid concentration dependent elevation of intracellular calcium $[Ca^{2+}]_i$. This could be inhibited by EGTA, suggesting that the source of $[Ca^{2+}]_i$ in fullerene stimulated calcium flux is predominantly from the extracellular environment. In conclusion, fullerenol $C_{60}(OH)_{24}$ had both pro-inflammatory and pro-apoptotic effects on HUVECs, indicating possible adverse effects of fullerenes on the endothelium.

Keywords: endothelial cells, fullerenes, tissue factor, apoptosis, ICAM-1, flow cytometry

Introduction

Carbon fullerenes have enormous practical potential in the field of biomedical nanotechnology. Fullerenes and carbon nanotubes have already had a profound impact on the development of diagnostic biosensors, drug delivery nanosystems, and imaging nanoprobe for intravascular use. In addition, fullerenes can be used as components in a variety of plastics, including filtration membranes (Bosi et al 2003; Endo et al 2004; Bianco et al 2005; Sinha and Yeow 2005; Djordjevic et al 2006; Lacerda et al 2006; Polizu et al 2006; Harrison and Atala 2007). It has been reported that hydroxylated fullerene derivatives have potent antioxidant properties (Dugan et al 1996; Tsai et al 1997; Huang et al 1998; Straface et al 1999; Wang et al 1999; Jin et al 2000). Furthermore, the cytoprotective effects of fullerene derivatives in oxidative stress, mostly by free radical scavenging, have been demonstrated in different cell lines in vitro, and also on animal models, in vivo (Lin et al 1999; Tsao et al 1999; Lai et al 2000; Gharbi et al 2005; Daroczi et al 2006). It has been shown that fullerene derivatives have anti-viral and anti-bacterial properties. Also, their photodynamic cytotoxicity has been employed in experimental anti-tumor therapy and pathogen inactivation

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(Kasermann and Kempf 1997; Tabata et al 1997). However, several studies are raising safety concerns by showing possible cytotoxic effects of fullerenes and their derivatives (Sera et al 1996; Chen et al 1998; Kamat et al 1998; Wolff et al 2000; Oberdorster 2004; Sayes et al 2005; Isakovic et al 2006; Zhu et al 2006). Although, it has been demonstrated that residual tetrahydrofuran (THF) used for the C_{60} fullerene solubilization substantially potentiates the toxicity of C_{60} , the recent studies using the THF free water suspensions of fullerenes or water soluble polyhydroxylated fullerene derivatives indicate their cytotoxic effects (Isakovic et al 2006; Zhu et al 2006). Despite the fact that many of the fullerene applications are based on intravascular administration of these compounds, very little is known about the effects of fullerenes on endothelial cells (Yamawaki and Iwai 2006). Here we present a study investigating proapoptotic and pro-inflammatory effects of water soluble fullerene $C_{60}(OH)_{24}$ and a C_{60} fullerene water suspension (nC_{60}) on cultured human umbilical vein endothelial cells.

Materials and methods

Fullerenes

The water soluble poly-hydroxylated fullerene derivative fullerene $C_{60}(OH)_{24}$ (at final concentrations of 1–100 $\mu\text{g}/\text{mL}$) from MER Corp., Tuscon, AZ was used for all experiments. In addition, the water nanocrystalline suspensions of insoluble C_{60} (nC_{60}) (C_{60} of 99.5% purity from SES Res., Houston, TX, USA) was prepared by continuous stirring 400 mg of C_{60} powder in 400 mL of Milli-Q water for 2 weeks at room temperature. After stirring, the suspension was centrifuged twice at 4000 g for 20 minutes to remove larger aggregates of undissolved fullerene. The final supernatant which contained 21 $\mu\text{g}/\text{mL}$ of nC_{60} was collected, and stored at 4 °C. The concentration of nC_{60} was determined by extracting the C_{60} from the suspension by toluene and analyzing the absorbance at 336 nm, as described by Lyon et al (2006). In tissue culture experiments, the supernatant was diluted with the culture medium to a final concentration of 4 $\mu\text{g}/\text{mL}$. As a control, the fullerene vehicle (Milli-Q H_2O) was prepared in parallel by continuous stirring for 2 weeks at room temperature and manipulated the same way as nC_{60} suspension.

Analysis of hydrodynamic size distribution of fullerene particles using Dynamic Light Scattering (DLS)

The Malvern Zetasizer Nano ZS instrument (Southborough, MA) with a back scattering detector was used for measuring the hydrodynamic size (diameter) in batch mode (no

fractionation) at 25 °C in a low volume quartz cuvette. The relative particle volume is used as the size class for histograms of the size distribution (the diameter is plotted on the abscissa and the particle volume on the ordinate). The average diameter (weighted by particle volume) of the largest peak in the volume distribution is reported as the hydrodynamic size, along with its standard deviation and the percentage of the total volume contained in the largest peak. The nC_{60} sample concentration was 21 $\mu\text{g}/\text{mL}$ as described in the stock preparation (see above). The hydroxylated C_{60} derivative ($C_{60}(OH)_{24}$) was weighed and dissolved in 10 mM NaCl to give a final concentration of 2 mg/mL and filtered through a 0.02 μm filter (Anotop 10 Plus, Whatman International Ltd., Maidstone, England). A minimum of twelve measurements were made per sample.

Human umbilical vein endothelial cells (HUVECs)

HUVECs (2nd passage) from Cambrex (Walkersville, MD), were cultured in either 6 or 24 well plates (Becton Dickinson Labware, Franklin Lakes, NJ) with EGM-2 media containing 2% fetal bovine serum and supplements (Cambrex). Cells were used for experiments when they reached 80%–90% confluence (~48 hrs in culture). HUVECs were washed and incubated for 24 hrs at 37 °C with fullerene $C_{60}(OH)_{24}$ or fullerene nC_{60} in water diluted one to five with the EGM-2 medium. After incubation, cells were harvested with 20 mM HEPES buffer with 100 mM NaCl, 0.5% BSA and 10 mM EDTA, pH 7.4. After harvesting, the cells were centrifuged (300 g for 5 min), washed with Hanks' balanced salt solution with 0.35% bovine serum albumin (HBSS/BSA) and used for analysis.

Antibodies

Cy-Chrome conjugated anti-human monoclonal antibody (mAb) to CD54 (ICAM-1, clone HA58), phycoerythrin-conjugated (PE) anti-human mAb to CD142 (tissue factor, clone HTF-1), isotype matched controls, annexin V (FITC-conjugated), and an APO-BRDU kit were purchased from BD Pharmingen (San Diego, CA). All antibodies were titrated to ensure saturating concentrations.

Labeling of endothelial cells

Approximately 10^5 cells were resuspended in 50 μL of HBSS/BSA and incubated with saturating concentration of the mAbs or annexin V. Non-labeled cells and cells incubated with either relevant isotype controls or with annexin V in the presence of 20 mM EDTA were prepared as controls.

After 20 min incubation at room temperature, the suspension of labeled cells was diluted with 2 mL of HBSS/BSA and centrifuged ($300 \times g$ for 5 min). Sedimented cells were resuspended in 0.5 mL of HBSS/BSA and analyzed using flow cytometry.

Flow cytometry of endothelial cells

Cell samples were analyzed as described previously (Simak et al 2002; Simak, Holada, and Vostal et al 2002). Briefly, a FACSCalibur flow cytometer (Becton Dickinson, San Diego, CA, USA) equipped with CELLQuest software, with forward scatter (FSC) and side scatter (SSC) in linear mode was used. Populations of intact cells were gated according to their light-scattering characteristics in order to exclude debris, and 10,000 gated cells were analyzed for each sample. The total percentage (%) of CD54⁺, CD142⁺, and PS⁺ (annexin V-binding) cells was evaluated. Apoptosis (TUNEL) was analyzed using the APO-BRDU kit following the manufacturer's instructions (BD Pharmingen, San Diego, CA). In addition, cell cycle analysis was performed using ModFit software (Verity Software House, Topsham, ME).

Intracellular free Ca²⁺ assay

The acute effect of fullerenes on intracellular free Ca²⁺ concentration was studied in HUVEC loaded with a Ca²⁺-sensitive probe (FURA-2AM). Changes in fluorescence in individual cells ($n = 100$) were monitored at 340 nm and 380 nm excitation (the rate of data capture was 170/min) using a Nikon inverted epi-fluorescence/phase microscope equipped with a low-light level integrating CCD camera with a microphotometer assembly (InCyt I/P-2 TM Imaging and Photometry System, Intracellular Imaging Inc., Cincinnati, OH). Intracellular [Ca²⁺]_i in real time was calculated from the ratio of emission detected at 510 nm at two excitation wavelengths (340 nm and 380 nm) and by comparison to a standard curve established for these settings using buffers of known free [Ca²⁺] with the InCyt Im2 software.

Statistical analyses

Results are presented as means \pm standard deviations (SD). Kruskal-Wallis One Way Analysis of Variance on Ranks and multiple comparisons using Student-Newman-Keuls method or Dunnett's method was used where appropriate (SPSS 12; SPSS, Chicago, IL). A value of $p < 0.05$ was considered statistically significant.

Results

Hydrodynamic size distribution of fullerene particles in nC₆₀ and C₆₀(OH)₂₄ preparations

The mean size distribution by volume and the mean hydrodynamic diameter of nC₆₀ and C₆₀(OH)₂₄ are shown in Figure 1. Based on the size distributions of particles present in the nC₆₀ water suspension (Figure 1A), the major water soluble component is about 224 nm in size. The small peak at about 5000 nm corresponds to approximately 2% of the total particle volume, and may be due to aggregates. Since unmodified nC₆₀ is not immediately water soluble, we stirred C₆₀ in water for two weeks, which resulted in a nanocrystalline suspension of stable aggregates (nC₆₀). nC₆₀ refers to an unknown number 'n' of C₆₀ molecules agglomerating to form the suspension. The formed clusters contain unmodified C₆₀ in their centers, surrounded by partially hydroxylated C₆₀ on the outside (Fortner et al 2005). In contrast, the fullereneol C₆₀(OH)₂₄ is very soluble in water. Based on its size distribution by volume (Figure 1B), only one peak at 4.3 nm was observed, as expected for the hydroxylated C₆₀ derivative.

High dose fullereneol C₆₀(OH)₂₄ induced elevated HUVEC surface expression of ICAM-1 (CD54), tissue factor (CD142), and phosphatidylserine (PS)

HUVECs were cultured for 24 hrs with different concentrations of fullereneol C₆₀(OH)₂₄ (1, 10, 50, and 100 $\mu\text{g}/\text{mL}$), fullerene nC₆₀ at 4 $\mu\text{g}/\text{mL}$, or with the vehicle. Flow cytometry was used to analyze the difference in expression of several surface markers. Figure 2 shows that the 24 hr treatment of HUVECs with 100 $\mu\text{g}/\text{mL}$ C₆₀(OH)₂₄ significantly increased cell surface expression of ICAM-1 (CD54) ($67 \pm 4\%$ CD54⁺ cells vs $19 \pm 2\%$ CD54⁺ cells in control; $p < 0.001$). This treatment also induced expression of tissue factor (CD142) on HUVECs ($54 \pm 20\%$ CD142⁺ cells vs $4 \pm 2\%$ CD142⁺ cells in control; $p = 0.008$). In addition, an increase in exposed phosphatidylserine (PS, detected by Annexin V) ($29 \pm 2\%$ PS⁺ cells vs $12 \pm 5\%$ PS⁺ cells in control; $p < 0.001$) was identified. However, treating the HUVECs with lower concentrations of fullereneol C₆₀(OH)₂₄ or nC₆₀ at 4 $\mu\text{g}/\text{mL}$ did not induce a significant increase of surface expression of CD54, CD142 or PS.

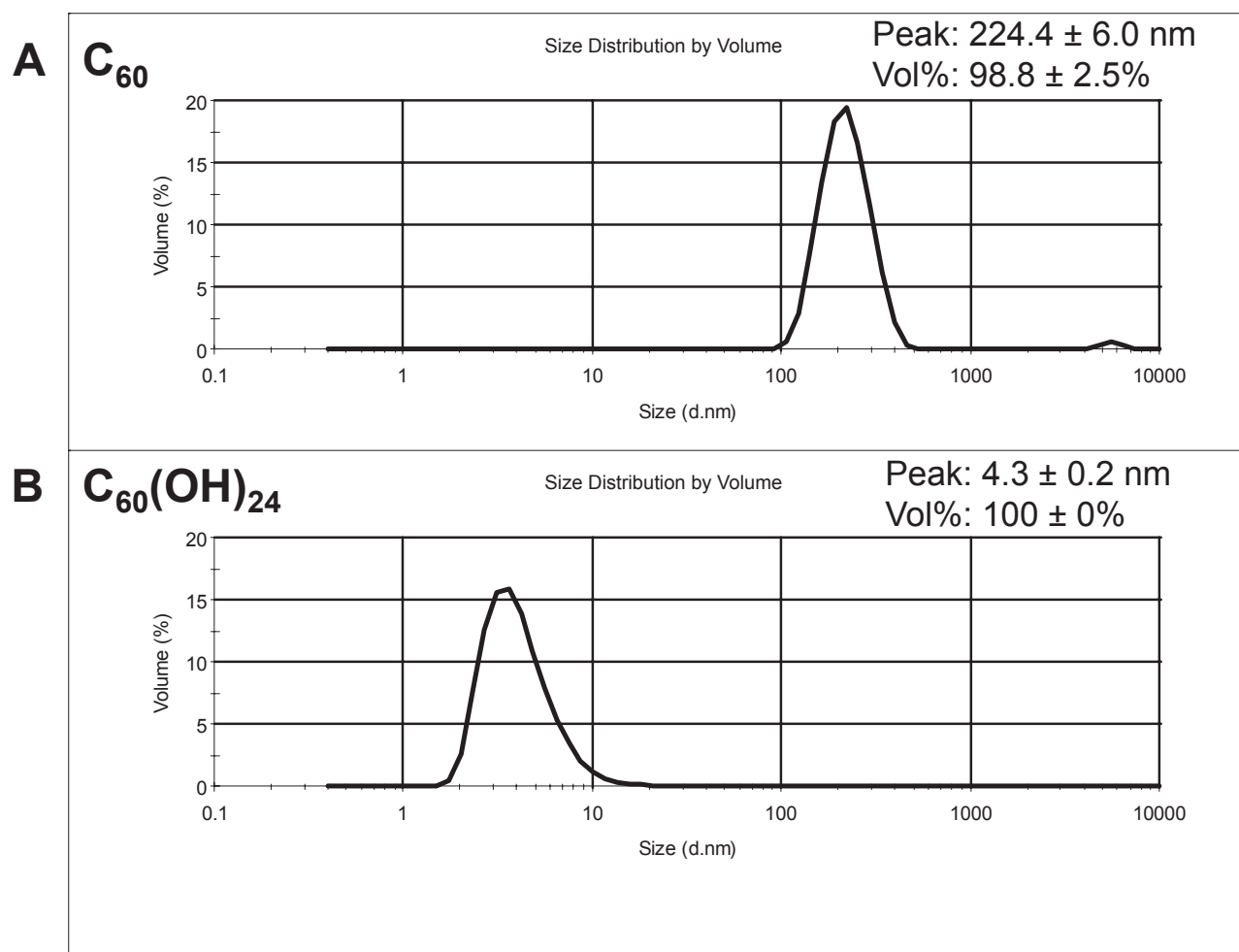


Figure 1 Hydrodynamic size distribution of fullerene particles in nC_{60} and $C_{60}(OH)_{24}$ preparations. Shown are the volume distributions (each line is the average of at least twelve measurements per sample) of the nC_{60} water suspension (**A**), and $C_{60}(OH)_{24}$ in 10mM NaCl (**B**).

Both fullerene C_{60} and fullereneol $C_{60}(OH)_{24}$ induced G1 arrest and a high dose of fullereneol induced significant apoptosis (TUNEL) in HUVECs

Analysis of the cell cycle by flow cytometry showed that both nC_{60} at $4 \mu\text{g/mL}$ and $C_{60}(OH)_{24}$ at concentrations 10-100 $\mu\text{g/mL}$ caused significant G1 arrest in HUVECs after 24 hours of treatment (Figure 3). In addition, fullereneol $C_{60}(OH)_{24}$ at 100 $\mu\text{g/mL}$ induced significant apoptosis after 24 hours treatment of HUVECs, as compared to control cells treated with the vehicle ($21 \pm 2\%$ TUNEL⁺ cells at 100 $\mu\text{g/mL}$ of $C_{60}(OH)_{24}$ vs. $4 \pm 2\%$ TUNEL⁺ cells in control; $p < 0.001$) (Figure 4). Treating the HUVECs with lower concentrations of fullereneol $C_{60}(OH)_{24}$ or nC_{60} at $4 \mu\text{g/mL}$ did not induce significant increase of TUNEL⁺ cells.

Both fullerene nC_{60} and fullereneol $C_{60}(OH)_{24}$ induced acute Ca^{2+} influx in HUVECs

The acute effect of fullerenes on intracellular free Ca^{2+} concentration $[Ca^{2+}]_i$ was studied, using a ratio fluorometry in HUVECs. Cells were loaded with a Ca^{2+} -sensitive probe FURA-2AM. We demonstrated that both nC_{60} and $C_{60}(OH)_{24}$ induced a rapid concentration dependent elevation of $[Ca^{2+}]_i$. nC_{60} caused a continuous increase in $[Ca^{2+}]_i$ and was about ten times more potent compared to $C_{60}(OH)_{24}$ (Figure 5). This activity of both $C_{60}(OH)_{24}$ and nC_{60} could be inhibited by EGTA, suggesting that the source of $[Ca^{2+}]_i$ in fullerene stimulated calcium flux is predominantly from the extracellular environment.

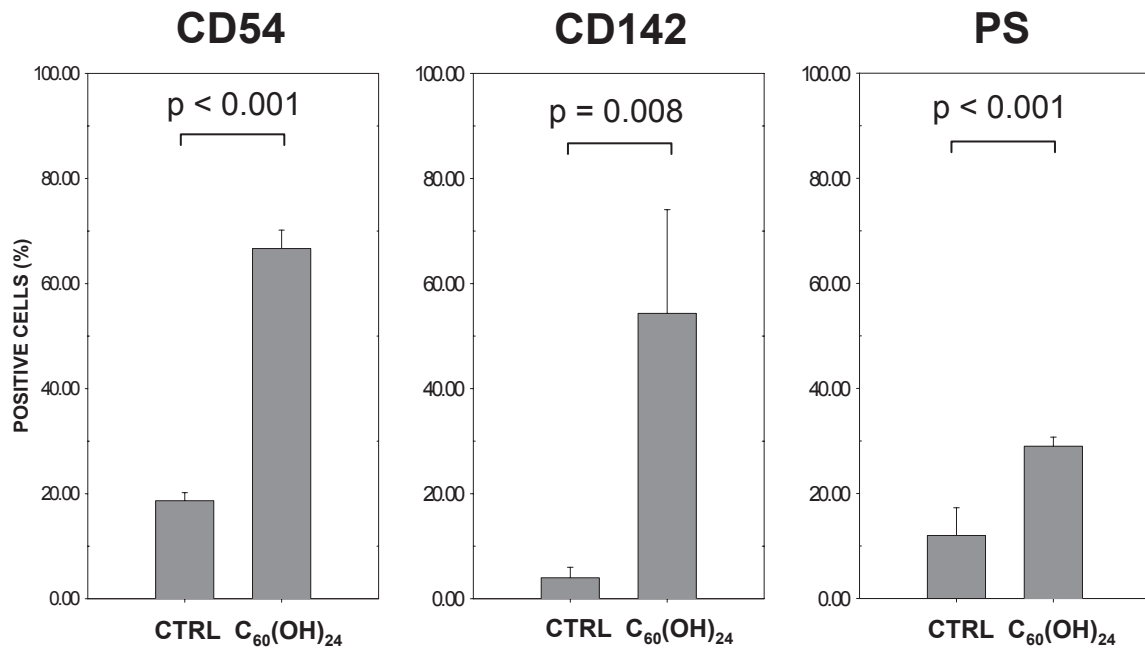


Figure 2 Flow cytometric analysis of HUVECs treated for 24 hrs with fullereneol C₆₀(OH)₂₄ (100 µg/mL) or the vehicle (CTRL). Data are presented as means + SD (n = 3).

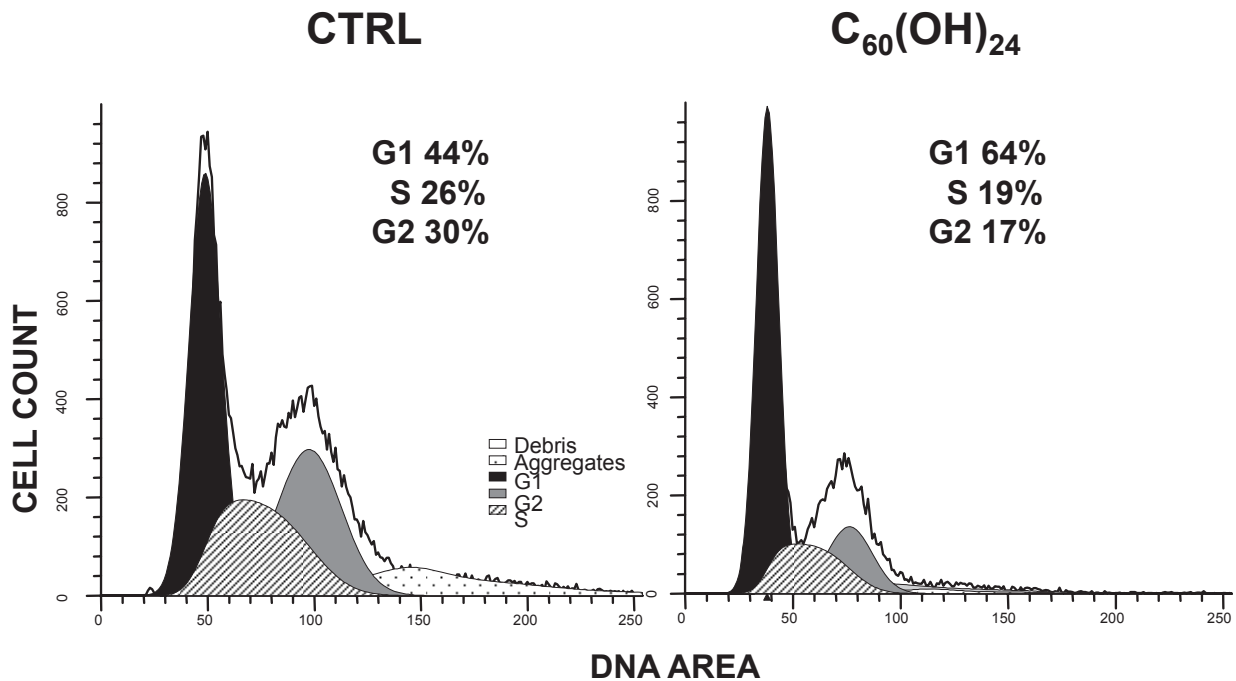


Figure 3 Cell cycle analysis of HUVECs treated 24 hrs with fullereneol C₆₀(OH)₂₄ (100 µg/mL) or the vehicle (CTRL). Representative histograms of 3 experiments are shown.

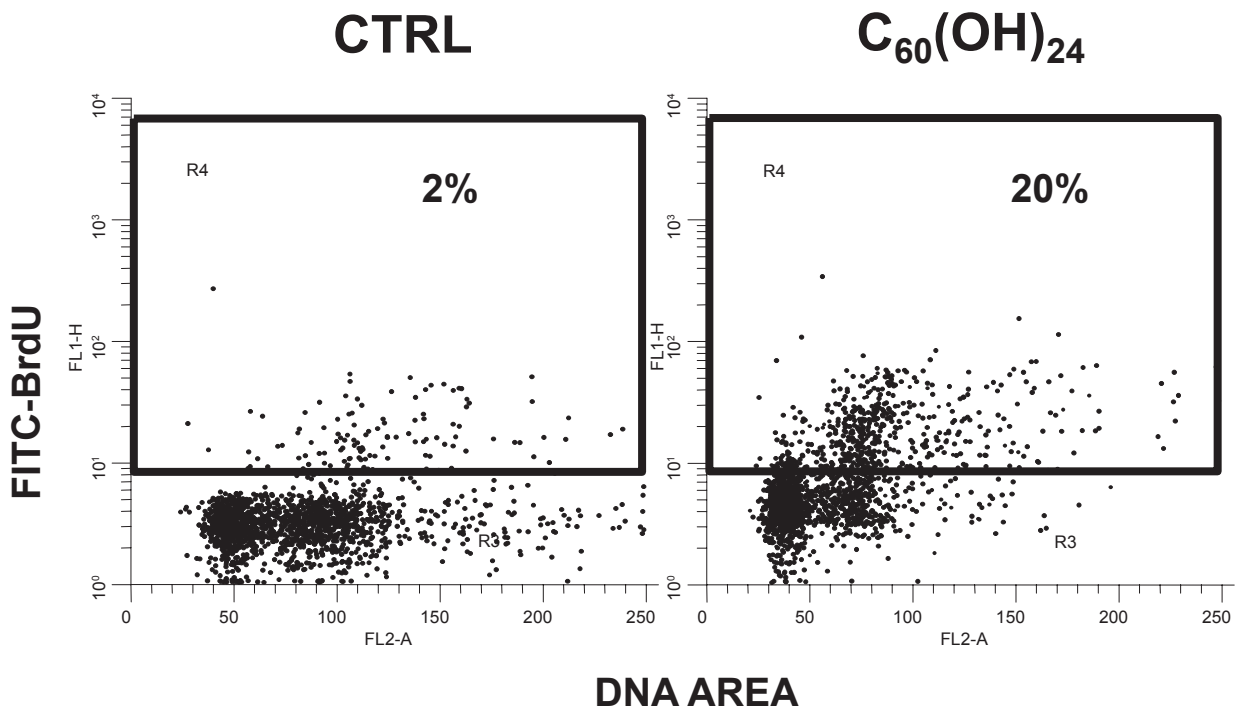
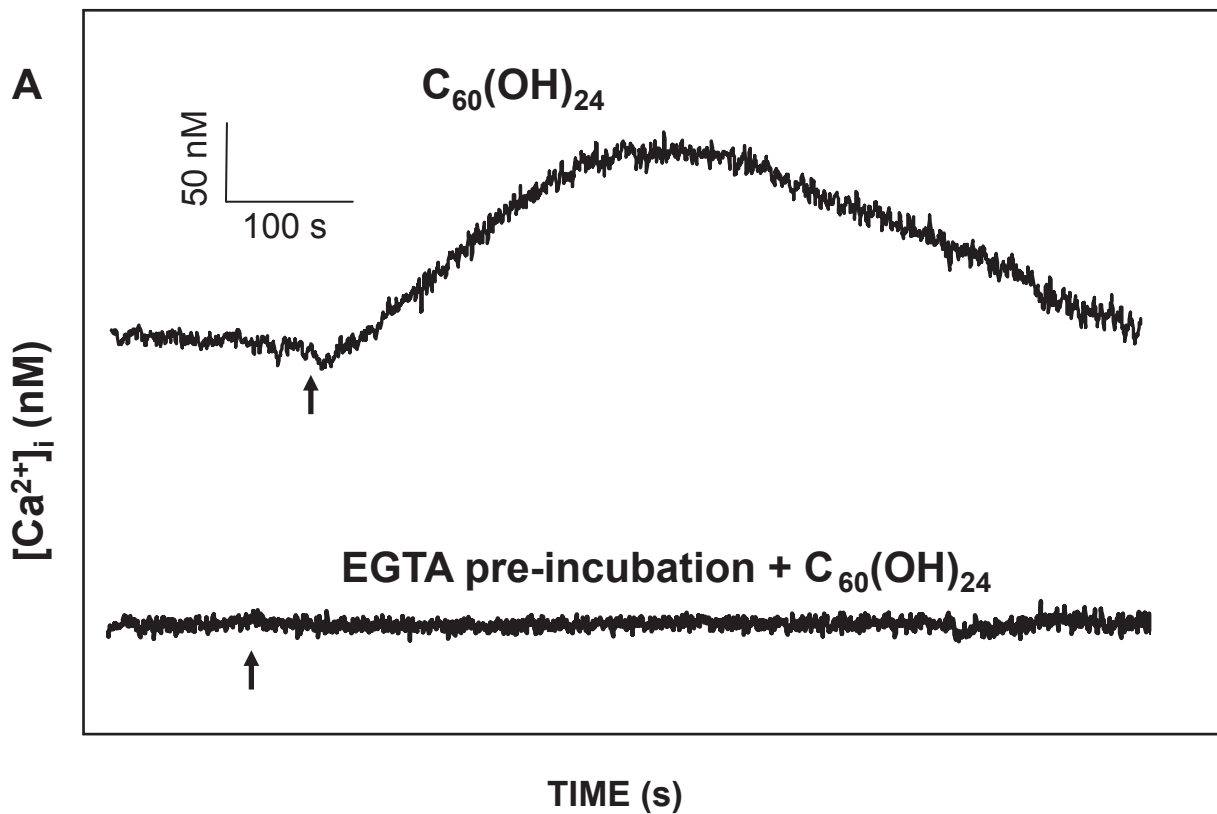
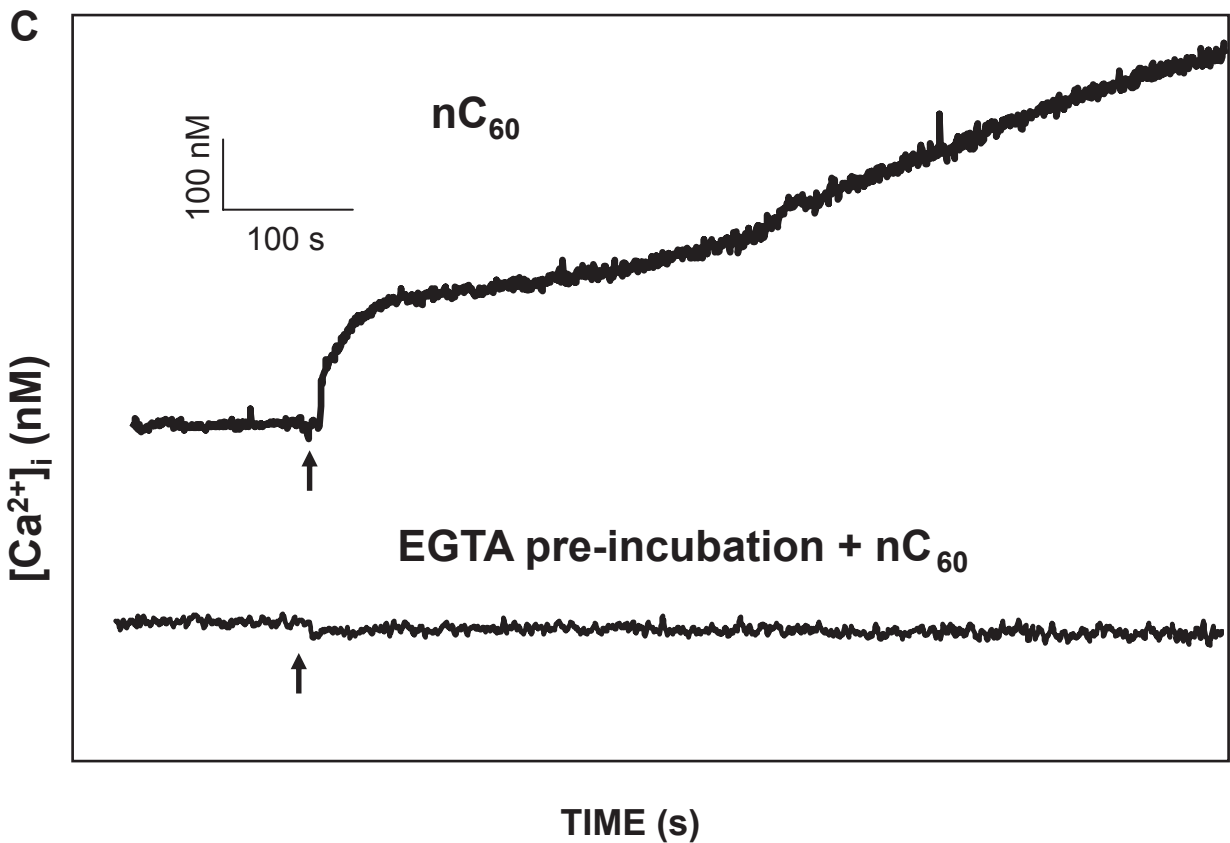
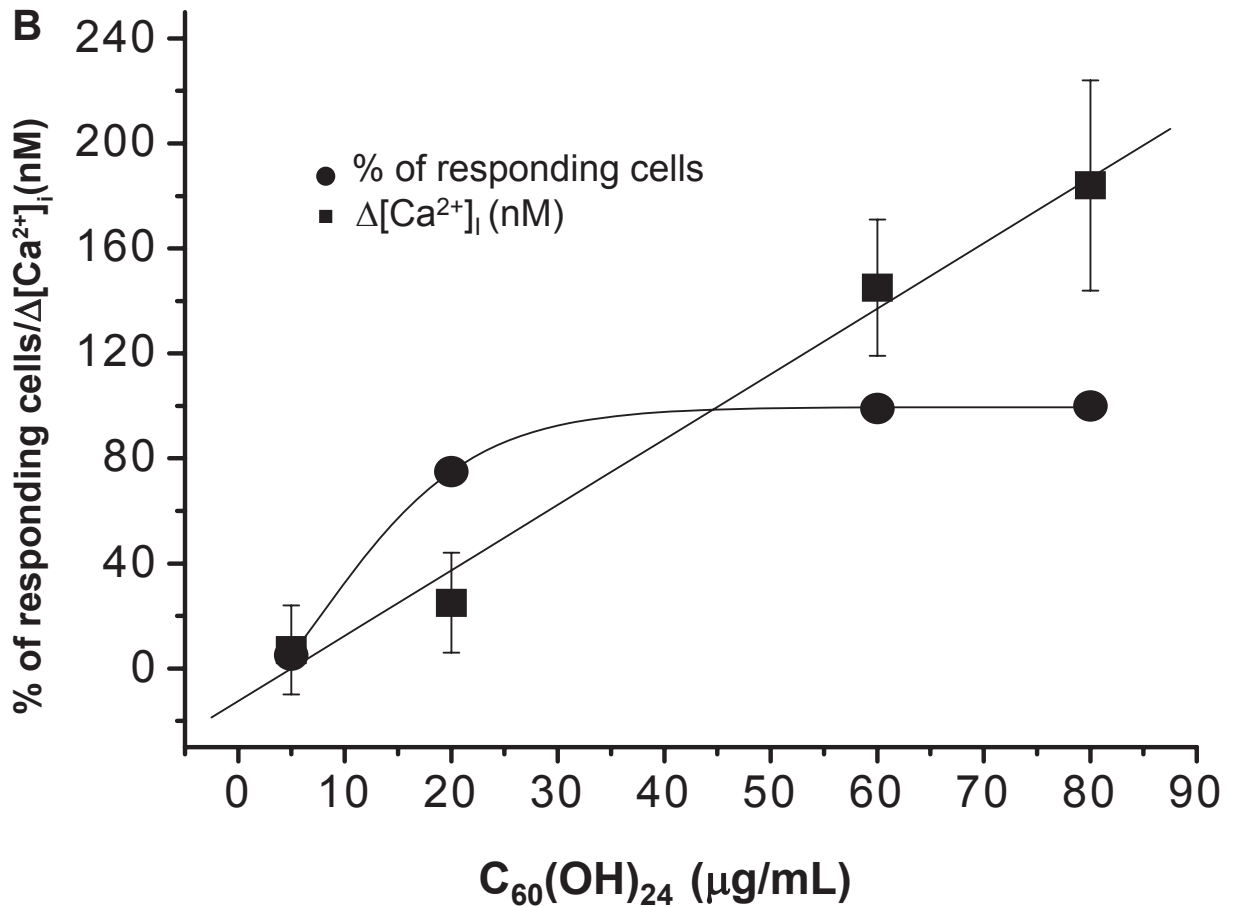


Figure 4 Flow cytometric analysis of TUNEL assay of HUVECs treated for 24 hrs with fullerene C₆₀(OH)₂₄ (100 μg/mL) or the vehicle (CTRL). Representative double fluorescence plots of 3 experiments are shown.





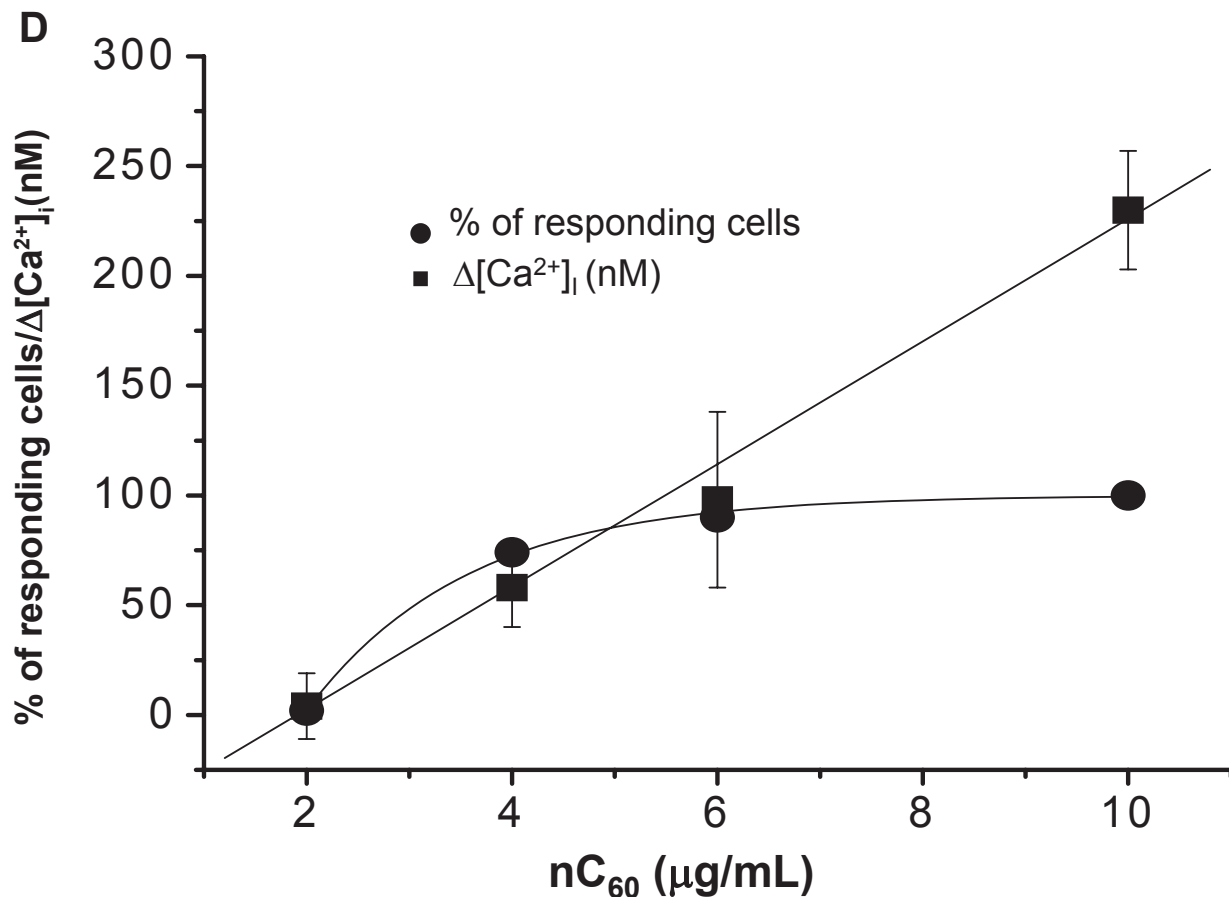


Figure 5 Analysis of $C_{60}(OH)_{24}$ and nC_{60} induced intracellular Ca^{2+} increase ($\Delta[Ca^{2+}]_i$) in HUVECs: **(A)** The upper tracing shows the time course of $C_{60}(OH)_{24}$ -induced $\Delta[Ca^{2+}]_i$ in a representative cell. The lower tracing shows a time course in a representative cell in a Ca^{2+} free solution (containing 1 mM EGTA). Arrows indicate when $C_{60}(OH)_{24}$ (final conc. 80 $\mu\text{g/mL}$) was added. There was no response observed when the fullereneol vehicle was added (not shown). **(B)** A dose-response curves for the effect of $C_{60}(OH)_{24}$ on $\Delta[Ca^{2+}]_i$ and the percentage of cells responded. Mean values of 30 cells \pm SD are shown. **(C)** The upper tracing shows the time course of nC_{60} -induced $\Delta[Ca^{2+}]_i$ in a representative cell. The lower tracing shows a time course in a representative cell in a Ca^{2+} free solution (containing 1 mM EGTA). Arrows indicate when nC_{60} (final conc. 10 $\mu\text{g/mL}$) was added. There was no response observed when the nC_{60} vehicle was added (not shown). **(D)** A dose-response curves for the effect of nC_{60} on $\Delta[Ca^{2+}]_i$ and the percentage of cells responded. Mean values of 30 cells \pm SD are shown.

Discussion

Our study showed that fullereneol induced pro-inflammatory activation of cultured HUVECs, which was indicated by the increase of CD54 (ICAM-1) and CD142 (TF) surface expression. The intercellular adhesive molecule 1 (ICAM-1), also referred to as CD54, is an adhesive receptor of the immunoglobulin gene superfamily. It is constitutively expressed at low levels on most types of vascular endothelial cells, and is highly upregulated on pro-inflammatory cytokine activated endothelium (Mutin et al 1997a). Tissue factor (TF), also referred to as CD142, is a cellular receptor and cofactor of activated factor VIIa. Tissue factor initiates activation of the plasma coagulation system, which leads to the generation of thrombin activity (Steffel et al 2006). Although functional TF is not exposed in significant levels on resting HUVECs, its expression is greatly increased after stimulation with pro-inflammatory cytokines and some

other stimuli, similarly to ICAM-1 (Mutin et al 1997b). In addition, the observed increased exposure of phosphatidylserine (PS) on the plasma membrane of HUVECs indicates a disturbance of the membrane asymmetry, likely due to pro-apoptotic stimulation or other types of cell activation. Although the exposure of PS on cells incubated with the fullereneol vehicle (the Milli-Q water) was higher than expected, the difference in PS-surface exposure between vehicle-treated and fullereneol-treated cells was highly significant ($P < 0.001$). PS is an essential cofactor for activation complexes in the plasma coagulation system. Also, it is a signaling molecule for phagocytes (Zwaal et al 2005). Thus, our results show that the phenotype of endothelial cells treated with fullereneol become pro-inflammatory and pro-coagulant. It is important to further investigate whether fullerenes exhibit similar effects on the endothelium *in vivo* in animal models.

Our cell cycle analysis showed that both nC_{60} and $C_{60}(OH)_{24}$ induced G1 arrest in HUVECs. Polyhydroxylated fullereneol-1 was reported to inhibit the proliferative responses in a variety of cells, including smooth muscle cells and human lymphocytes, in a concentration dependent manner. The tested concentration range was of 10^{-6} to 10^{-2} M, which corresponds to approximately 1 $\mu\text{g}/\text{mL}$ to 10 mg/mL . It was also shown that fullereneol inhibited cytosolic protein kinase C activity. Therefore, it was suggested that the anti-proliferative effect of fullereneol-1 on vascular smooth muscle cells may partly be mediated through the inhibition of protein tyrosine kinase (Lu et al 1998).

We found that $C_{60}(OH)_{24}$ induced apoptosis in HUVECs. This was clearly detected by TUNEL assay, which is a common method for detecting DNA fragmentation. In addition, the observed increase in PS exposure of fullereneol treated cells can be considered as supporting evidence of pro-apoptotic activation of HUVECs. Our finding contradicts the results of a recent study by Yamawaki and Iwai (Yamawaki and Iwai 2006). These authors concluded that fullereneol did not cause apoptosis based on the failure to demonstrate cleavage of caspase 3 and PARP. However, their study showed autophagic cell death in fullereneol treated HUVECs. We may therefore speculate that both apoptotic and autophagic cell death may occur in fullereneol treated endothelium.

To investigate a possible mechanism of fullerene action on endothelium, we demonstrated that both nC_{60} and $C_{60}(OH)_{24}$ induced influx of extracellular Ca^{2+} in HUVECs. This suggests that fullerenes may either activate some of the Ca^{2+} channels on the cell membrane, or that they may create new channels/pores to facilitate Ca^{2+} influx, or both. In either case, it results in an increase in intracellular Ca^{2+} , which may induce activation and/or apoptotic changes of the cells.

In conclusion, the results of this pilot study show possible adverse effects of fullerenes on the endothelium. The hydroxyfullerene $C_{60}(OH)_{24}$, had both pro-inflammatory and pro-apoptotic effects on endothelial cells. In addition, cell cycle arrest and increase of intracellular Ca^{2+} was observed in cells treated with hydroxyfullerene $C_{60}(OH)_{24}$ and also with a low concentration of nC_{60} . With the wide potential applications of fullerenes in mind, the safety hazards of these materials need to be thoroughly evaluated. We suggest that additional in depth studies should be performed to investigate possible adverse effects of fullerenes on the cardiovascular system in exposed populations.

Disclaimer

The findings and conclusions in this article have not been formally disseminated by the Food and Drug Administration

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