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EFFECT of SHORT MULTIWALLED CARBON NANOTUBES on *ESCHERICHIA COLI* K-12 STRAIN

KISA ÇOK DUVARLI KARBON NANOTÜPLERİN ESCHERİCHİA COLİ K-12'YE ETKİSİ

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Abstract

Objective: Multi-walled carbon nanotubes (MWNTs) are obtained by rolling up the graphene structure to form multi-walled cylindrical structures, find wide usage areas in different areas with their large surface areas, hydrophobicity, and high electron conductivity. Evaluation of antibacterial effects has become very important in recent years. In this study, it was aimed to evaluate the antibacterial effect of MWNTs on E. coli K12 strain, which are shorter than most of the MWNTs in the literature, which we have previously applied to different cell lines, nematodes, and which have a very limited toxic effect up to high concentrations.

Methods: Different concentrations ranging from 20 to 100 µg/ml were prepared from MWNTs and applied to E. coli K12 strain. Colony count, optical density measurement, and microscopic images were evaluated.

Results: Compared to control, it was observed that MWNTs affected the proliferation at every concentration from 10 µg/ml to 100 µg/ml, and the antibacterial effect was most pronounced at 50 and 100 µg/ml concentrations. There was no significant difference in inhibition dose between 10 and 20 µg/ml. Growth inhibition was observed to be greater than 20% after 90 minutes at high doses.

Conclusion: Bacteria grown in liquid and solid agar revealed different inhibition properties at low and high concentrations. Depending on the concentration, it was determined that the absorbance and viability values of E. coli cells decreased in both experiments. In addition, decreases in bacterial colonies, and the bacterial activity performed with DAPI staining and image analyzes are quite evident.

Keywords: Multi-walled carbon nanotube, E. coli, inhibition, density, antibacterial effect

Öz

Amaç: Grafen yapının iç içe silindirler oluşturması ile elde edilen çok duvarlı karbon nanotüpler (ÇDNT), geniş yüzey alanları, hidrofobik yapıları, yüksek elektron iletkenlikleri ile geniş kullanım alanı bulmaktadırlar. Son yıllarda antibakteriyel etkilerinin değerlendirilmesi oldukça önemli hale gelmiştir. Buradaki araştırmada daha önce farklı hücre soylarına ve yuvarlak solucanlara uyguladığımız; yüksek konsantrasyonlara kadar oldukça sınırlı bir toksik etkiye sahip olduğunu bildiğimiz, literatürde bulunan ÇDNT çeşitlerinin çoğuna göre daha kısa ÇDNT' lerin, *E. coli* K12 üzerindeki anti bakteriyel etkisi değerlendirilmek istenmiştir.

Yöntem: ÇDNT' lerden 20 - 100 µg/ml aralığında değişen farklı konsantrasyonlar hazırlanmış ve *E. coli* K12'ye uygulanmıştır. Koloni sayımı, bakteriyel konsantrasyon ve mikroskobik görüntüler değerlendirilmiştir.

Bulgular: Kontrol ile karşılaştırıldığında 10 µg/ml'den 100 µg/ml'ye, her konsantrasyonda ÇDNT'lerin bakterilerin çoğalmasını etkilediği, 50 ve 100 µg/ml konsantrasyonlarda en çok antibakteriyel etkinin ortaya çıktığı görülmüştür. 10 ve 20 µg/ml arasında, inhibisyon dozu olarak belirgin bir fark izlenmemiştir. Yüksek konsantrasyonlarda 90. dakikadan sonra çoğalma inhibisyonunun %20'den fazla olduğu görülmüştür.

Sonuç: Sıvı ve katı besiyerinde çoğaltılan bakteriler düşük ve yüksek konsantrasyonlarda farklı inhibisyon özellikleri ortaya çıkarmıştır. Konsantrasyona bağlı olarak *E. coli* K-12'nin absorbans ve canlılık değerlerinde azalma olduğu her iki deneyde de tespit edilmiştir. Ayrıca DAPI boyama ile ÇDNT uygulanan bakteri aktivitesinde ve mikroskopta yapılan görüntü analizlerinde de, nanotüp miktarındaki artışa bağlı olarak bakteri kolonilerindeki azalmalar oldukça belirgindir.

Anahtar Kelimeler: Çok duvarlı karbon nanotüp, *E. coli*, inhibisyon, yoğunluk, anti bakteriyel etki

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Introduction

Carbon nanotubes (CNTs) are nanomaterials broadly used in different fields due to their large surface areas, hydrophobic structures, high tensile strength, ultra-lightweight, thermal, and chemical stability, and electron conduction rates. Nanotubes are classified as single-walled carbon nanotubes (SWNT) and multi-walled carbon nanotubes (MWNT). The effects of CNTs used in industrial products and biosensors on the biological environment are considered one the important research areas. In many studies in the literature, CNTs have been found to have strong antimicrobial effects. The antibacterial effect of CNTs depends on CNT concentration and length, bacterial species, bacterial concentration, and exposure time.[1] CNT size and surface area are important features for toxicity assessment. As CNTs decrease in size, their specific surface area increases, leading to increased opportunities for interaction and uptake by living cells. It has been shown that single-walled carbon nanotubes exhibit significant cytotoxicity in human and animal cells, while multi-walled carbon nanotubes exhibit relatively less toxicity.[2] On the other hand, biologically functionalized CNTs are more biocompatible due to the organic/biological nature of their functionality. For example, CNTs functionalized with poly-lysine and other biomolecules have shown negligible harm to the environment and the human body, respectively.[3] Additionally, in some studies conducted in recent years, studies on the antibacterial effects of CNTs draw attention. It has been observed that it has antibacterial effects on *E. coli* and Streptococcus and that SWNTs have a more effective antibacterial effect than MWNTs, due to the stronger Van der Waals forces.[4] It has been revealed that CNTs adsorb bacteria, inhibit the growth of *E. coli* through Van der Waals forces and package them into blocks.[5-7] According to the experimental results of Zhang et al., the antibacterial effect of SWNTs is less than that of MWNTs.[8]
In this study, the antibacterial effects of short MWNTs were examined. Since nanotubes are used in biosensors, biomaterials, industrial products, and many different medical materials, examining their antibacterial effects will provide data for the prevention of resistant bacteria and infections.[9]

Methods

Characterization of Carbon Nanotubes

MWNTs were ordered from Nanografi. MWNTs synthesized by the chemical vapor deposition method, have a length of 0.5-2 µm, an outer radius of 8 nm, and purity is greater than 96%. In addition, these CNTs were treated with H_2SO_4 (98%)/HNO_3 (68%) (3/1) for 10 hours at 80 ºC to functionalize, increase biocompatibility and form carboxyl groups on the surface. It was washed with distilled water until a neutral pH was obtained and dried. Before and after these processes, Raman and FTIR spectra were taken, and their characterization was made with TGA and DSC measurements.[10-12] SEM and TEM images were taken from Zeiss® EVO LS 10 (SEM) and Hitachi® HT 7700 (TEM) systems at different resolutions.

Bacteria Strain

E. coli-K12 MG1655 strain was used in the experiments. 5 µl of *E. coli* strains stored at -80 ºC were taken and reproduced by keeping them in 5 ml of LB Broth at 37 ºC at 250 rpm for 16 hours. Afterward, an optical density (OD) measurement was made. It was diluted with LB Broth to an OD of 0.6. Bacterial cells were placed in LB Broth containing MWNT at concentrations of 0, 10, 20, 50 and 100 µg/ml, and OD was measured at 600 nm at intervals of 30 minutes for 2 hours 30 minutes (Shimadzu, UV2450). In addition, CNT was applied to LB solid agar at the same concentrations and dried, and the same number of bacteria was seeded on it. After 24 hours, bacteria were collected from this medium with physiological saline and again subjected to spectrophotometric measurement at 600 nm wavelength.

DAPI Dying and Microscope Images

To visualize dead and viable bacterial cells in MWNT cell samples for 2 hours 30 minutes, the bacterial OD was measured. They were treated with MWNTs after the growth procedure in the previous step (LB Broth at 37 ºC at 250 rpm for 16 hours). It was centrifuged for 5 minutes, at 8000 rpm. After the supernatant was discarded, it was treated with 200 µl PBS, 20 µl of DAPI dye in the dark, dropped onto the glass slide with a cap, and incubated at room conditions for 60 minutes. Fluorescence images were immediately taken with a fluorescent microscope (Olympus CK40/U RFLT 50, Olympus, Japan). Images were taken at 20× and 10x magnifications.
Microscope images without staining were taken using capped microscopic glass (Figure 1). First, bacteria are grown by keeping them at 250 rpm at 37 ºC for 16 hours. OD was measured and diluted to 0.6. Bacteria were added to the capped microscopic glass at 250 µg/ml to cover the entire surface. Then 10, 20, 50, and 100 µg/ml nanotubes were added to the closed plates and incubated at 37 °C for 4 hours. By removing the cover of the plate, a direct image was taken from the same microscope slide.

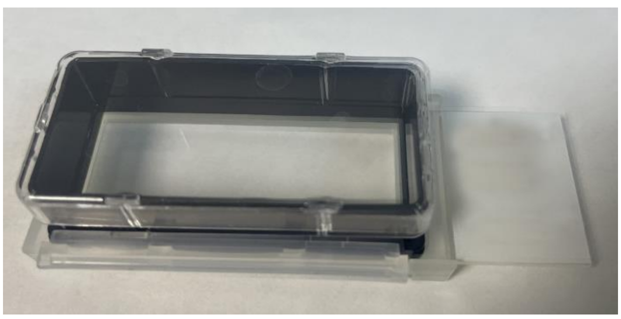

Figure 1. Microscopic glass with lid

Statistical Analysis

Analysis of variance and Bonferroni's t-test were used in the analysis of the data. Data were analyzed according to 5% significance. The evaluation was made by considering the two tests used, concentration, viability, and bacterial density. A value of $p \leq 0.05$ was accepted as a significant difference.

Results

To evaluate the structures, lengths, wall numbers, and radii of carbon nanotubes, TEM images are taken, and the surface structures and bulk densities are evaluated with SEM images. Here, TEM images were taken to confirm the length, radius, and multi-walled structure of the carbon nanotubes. The multi-walled structure of carbon nanotubes, with an inner radius of 2-6 nm and an average outer radius of 8 nm, was confirmed by TEM images (
Figure **2**,
Figure **3**). In addition, tubular structures with a length of 0.5-2 µm are observed from surface SEM images (
Figure **3**).
The multi-walled nanotubes (MWNTs) evaluated here are in the range that can be considered short.[13] Wearable technologies and polymer structures using CNTs whose toxic effects vary according to their physical properties and impurities are quite common.[14,15] While a product that comes into contact with the skin does not have a toxic effect on the cell, it ascribes a new superiority to these structures as they interrupt the communication between bacteria and prevent them from multiplying by wrapping them around.[16]

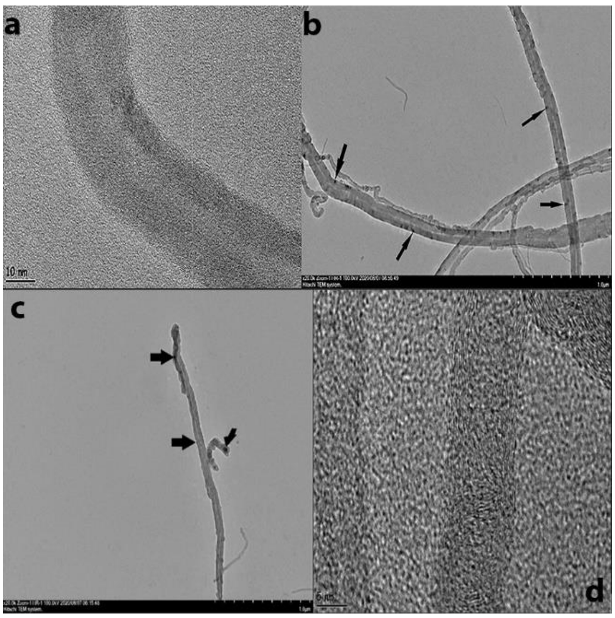

Figure 2. TEM images of CNTs at 10 nm and 1 µm resolutions

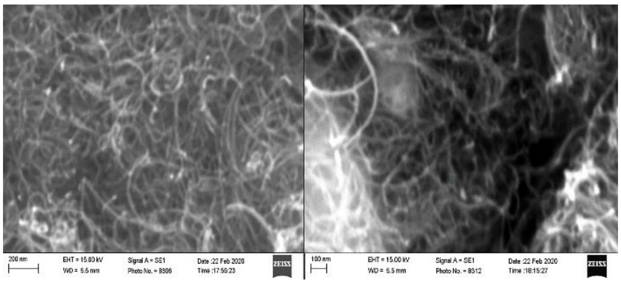

Figure 3. SEM images of CNTs

Depending on the size, radius, and functional groups formed on the surface, different antimicrobial effects can be observed. Although there are shorter ones in the literature, MWNTs whose antibacterial effects are investigated here can be evaluated as short and narrow. The activity, proliferation, and viability of *E. coli* K-12 strain applied with increasing concentrations of CNT were assessed. It was determined that there was a significant decrease in the measurements made by measuring the optical density from the liquid bacterial medium, especially in the measurements made after 90 minutes. Compared with the control, it was observed that it affected the proliferation at every concentration from 10 µg/ml to 100 µg/ml, and the antibacterial effect was most pronounced at 50 and 100 µg/ml concentrations. There was no significant difference in inhibition dose between 10 and 20 µg/ml. At higher doses, more than 20% growth inhibition was observed after 90 minutes. Concentration-independent changes in growth rates are evident between 60 and 90 minutes (
Figure **4**).

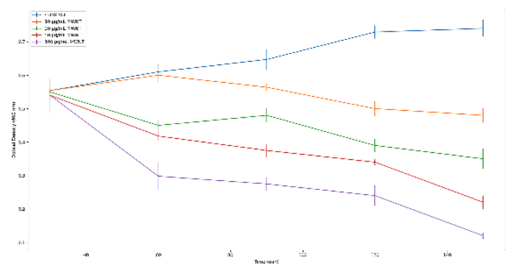

Figure 4. OD measurements of bacteria in medium with CNT

When the bacterial density obtained from MWNTs at 10, 20, 50, and 100 µg/ml concentrations added to solid bacterial culture plates was evaluated, it was observed that the inhibition effect was quite pronounced after 24 hours. According to these results, which are evaluated as the viability value after colony counting, concentration-dependent inhibition is evident (
Figure **5**).

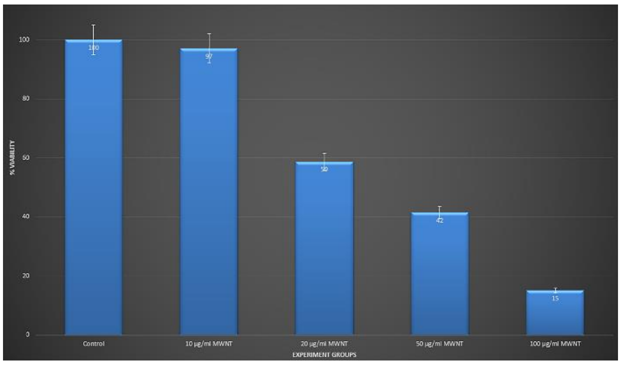

Figure 5. Viability of K-12 cells in solid, MWNT-containing medium ($p<0.05$)

Unlike the optical density measurement in liquid culture, the difference between 10 and 20 µg/ml, 50 and 100 µg/ml is greater. When the results of these two experiments were examined together, no increased inhibition was found directly at increasing concentrations in the first experiment. In the first experiment, MWNTs were applied after 16 hours, and OD measurements were completed in the next 4 hours. The results which are shown in Figure 4, and the decrease in bacterial colonies depending on the concentration, were compared with the bacteria that were kept at 37 ºC for 24 hours after 10, 20, 50, and 100 µg/ml MWNT was applied to the solid agar. Microscopic examinations showed that MWNTs inhibited bacterial growth depending on the concentration. Here, it was observed that CNTs did not allow bacterial colonization and caused more diffuse and gapped proliferation (
Figure **6**). DAPI staining was performed for the control and 100 µg/ml concentration, and it was seen that there was a distinct decrease in cell activity at this concentration compared to the control (Figure 7).

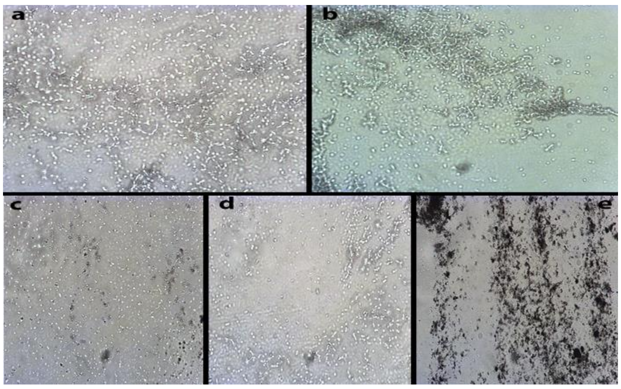

Figure 6. (a) Control, (b) 20, (c) 50, (d)100 and (e) 1000 µg/ml concentrations of nanotube applied bacteria

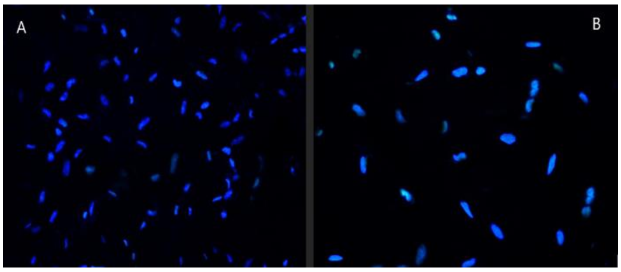

Figure 7. (A) 0 µg/ml and (B) 1000 µg/ml MWNT treated bacteria. Distribution of active bacteria seen by DAPI staining.

Conclusion

The spread of antibiotic-resistant bacteria complicates the treatment of diseases. It was determined that 2.6 million people were affected by resistant antibiotics and 35,000 people died.[17] The increase in death rates and the prolongation of hospital stay increase the burden on the health system.

It is very important to develop new materials and strategies that can prevent biofilm formation on surfaces in hospitals. Nanotechnology-based materials appear as a new option here as well. Studies are showing that carbon nanotubes have antimicrobial effects depending on their size and surface area.[18,19]

While performing the characterization of carbon nanotubes, the multi-walled structure and radius are valued with TEM images, and all morphological assessments of radius, dimensions, and purity of the tubes are made with SEM images.[20] Tubular structure and length are confirmed in SEM images (Figure 3). The amount of metal particles that may form impurities was observed in the images at ratios confirming the purity ratio (Figure 2-C). In the TEM images, the -COOH groups on the surface in the marked parts in Figure 2-B, their lengths by following the values in the given range are shown, and the multi-walled structure and inner-outer radii in Figures 1-A and 1-D are confirmed.

Toxicity assessments of MWNTs, whose antibacterial effects were evaluated here, were applied before to 3 different cell lines and roundworms for toxicity assessment, and their molecular binding capacities were investigated. Certain concentrations showed no toxic effects in cells (up to 50 µg/ml) and roundworms (up to 100 µg/ml), while limited toxic effects were observed at high concentrations.[11,21] It was perceived that it caused more toxic effects in *E. coli* K-12 than cells and nematodes and provided inhibition depending on the concentration.

Studies have been conducted showing that CNTs induce oxidative stress in bacteria and thus cause damage to the bacterial membrane.[22-24] In some other reports, it was concluded that CNTs showed antibacterial effects as a result of mechanical interaction with bacteria.[25-30] The most noticeable effect in the studies carried out so far is that it captures bacteria and damages the cell wall.[31] Due to the high surface absorbance capacity, the use of biologically active carbon in the removal of bacteria from organic pollutants began after the 1970s.[32] The

antibacterial effects of carbon nanotubes were first revealed by Kang et al. in 2007.[30]
Later, research on the effects of functional groups on CNT surface, concentration, shape, and size on antibacterial properties emerged. The results and properties of CNTs used in these studies are different and they revealed bacterial inhibition results that may be due to many factors.[16,25,33]
The inhibitory effect of MWNTs which have -COOH groups on their surface was investigated, but more research and data are needed on exactly at which stages this effect occurs in a bacterial cell. Membrane integrity is impaired in bacteria that encounter CNT. Proteins in the membrane of *E. coli* interact with -COOH groups of CNTs, leading to disruption of the membrane structure. Short multi-walled CNTs with carboxyl groups formed on the surface revealed antibacterial effects. For this reason, when biofilm formation is needed to be prevented, they can be used with different types of materials, as well as bacteria catchers in filters used for cleaning dirty water.

Ethical Approval
No ethics committee decision is required for the study.

Conflicts of Interests
No competing interests have been declared by the authors.

Author Contribution
All authors contributed equally to this work.

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