



An In-Vitro Study of Molecular Effects of a Combination Treatment with Antibiotics and Nanofluid Containing Carbon Nanotubes on *Klebsiella pneumoniae*

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Abstract

Background: We aimed to prepare a nanofluid, containing *f*-MWCNTs, and investigate the antibacterial efficacy of *f*-MWCNTs+ ciprofloxacin (cip) on *Klebsiella pneumoniae* by evaluating the virulence gene expression.

Methods: This study was carried out from 2019 to 2020, in the Department of Mycobacteriology and Pulmonary Research, Pasteur Institute of Iran. The nanofluid containing antibiotic and *f*-MWCNTs were prepared by the ultrasonic method. The minimum inhibitory concentrations (MICs) of ciprofloxacin and *f*-MWCNTs were determined using the broth micro dilution MIC tests. For examining the antibacterial effects, the expression level of virulence genes, under the influence of *f*-MWCNTs, was evaluated by a real-time PCR.

Results: The effect of 8 µg/ml ciprofloxacin + 400 µg/ml *f*-MWCNTs, completely inhibited the growth of the resistant isolate of *K. pneumoniae*, while, in the ATCC 700,603 isolate, 2 µg/ml ciprofloxacin with 100 µg/ml *f*-MWCNT could inhibit a bacterial growth. In the resistant *K. pneumoniae* clinical isolate, after *f*-MWCNT+cip treatment, the expression of *fimA*, *fimD*, *wza*, and *wzi* genes was significantly downregulated, compared to the ciprofloxacin treatment, and upregulated, compared to the negative control. For the ATCC 700,603 isolate treated with *f*-MWCNT+cip, the expression of *fimA*, *fimD* and *wza* virulence genes showed upregulation, compared to the negative control and downregulated in comparison with the ciprofloxacin treatment.

Conclusion: Simultaneous treatment of resistant isolate of *K. pneumoniae* with *f*-MWCNTs +antibiotic could improve the effectiveness of antibiotic at lower doses, due to the reduced expression of virulence genes in comparison with antibiotic treatment, besides the increased cell wall permeability to antibiotics.

Keywords: Carbon nanotube; Functionalized MWCNT; *Klebsiella pneumoniae*; Virulence genes

Introduction

The importance of *Klebsiella pneumoniae*, as an opportunistic pathogen in humans, is due to the infections in the hospitalized patients and in-

creased resistance to antibiotics (1). These bacterial species are the main reasons of nosocomial infections (NIs) including respiratory tract infec-



tions, urinary tract infections (UTIs), and blood stream-associated infections (BSIs) especially in patients confined in the intensive care unit (ICU) (2-5). *K. pneumoniae* is responsible for 44.22% of hospital-acquired infections in Iran (6).

Pneumonia is one of the principal causes of childhood death and infection-related fatality in adults in low- and middle-income countries (7). The hospital-acquired pneumonia (HAP), as the second most common nosocomial infection, accounts for 30% of all nosocomial infections (8). A high percentage of patients with HAP, develops the infection after connecting to the ventilator (9). Capsular polysaccharides (CPS), are considered as the major determinant for *K. pneumoniae* involved in pathogenesis through resistance to phagocytosis and serum killing (10-13). Another group of virulence factors of *K. pneumoniae* are fimbrial adhesins (14, 15).

The majority of *K. pneumoniae* clinical isolates usually express type 1 fimbrial adhesins. FimA is the main structural protein of the type 1 fimbrial filament. Ciprofloxacin (cip), from the quinolone antibiotic family, is one of the most widely used antibiotics against *K. pneumoniae*. The major problem in the *K. pneumoniae* treatment is the antibiotic resistance (16). 75% of *K. pneumoniae* clinical strains isolated from HAP specimens in Iran showed resistant to this antibiotic (16, 17). Given the increasing resistance of pathogens to various antibiotics, multidrug-resistant (MDR) pathogens could lead to the death of 10 million people annually by 2050, and surprisingly more than any other life-threatening diseases, including cancer (18). An association between MDR and biofilm genes expression is highly approved in numerous studies (16). Bacteria, from the Enterobacteriaceae family, which are resistant to the third-generation cephalosporins, have been prioritized in the WHO list of pathogens for new research and development of antibiotics (19). According to the WHO announcement, the use of nanomaterials in a post-antibiotic era has the potential to overcome the MDR (multi drug resistant) pathogen issue (20). Carbon nanotube (CNT), a hollow cylindrical shape with high strength and hardness, has unique biological, mechanical,

thermal, and electronic properties that have many diverse applications in biomedicine. Nanofluid containing nanomaterials has been a good candidate in a wide range of applications, including drug delivery, antibacterial applications, cancer treatment, diagnosis, medical imaging (ex. MRI), and wound dressing (17, 21-23).

Functionalization with a carboxyl group (-COOH) would make MWCNTs binding to bacteria more efficient. Overcoming the antibiotic resistance is one of the advantages of combination therapy. The studies showed that treatment of several tumor cell lines with functionalized multi-wall carbon nanotubes (*f*-MWCNT), led into high percentages of necrosis and apoptosis (24).

This study aimed to prepare nanofluid containing *f*-MWCNTs and evaluate the efficacy of *f*-MWCNTs+cip combination on virulence genes expression of both resistant and ATCC isolates of *K. pneumoniae*.

Methods

The present study was carried out from 2019 to 2020, in the Department of Mycobacteriology and Pulmonary Research, Pasteur Institute of Iran.

Nanofluid (NF) preparation

The MWCNT functionalized with -COOH in powder form, was prepared from United States Research Company. The ingredients including 0.2 g *f*-MWCNTs, 100 ml of deionized water, 6 ml of ethanol 96% and 0.06 g of Arabic gum were mixed and stirred for 20 minutes. Then, the suspension was placed in an ice bucket to the ultrasonic device (Ultrasonic Homogenizer 400 w, 200 kHz), with the power of 200 W for 20 minutes (25).

Bacteria identification

The clinical and ATCC 700,603 isolate of *K. pneumoniae* were prepared from the microbial bank of the mycobacterium and pulmonary research department, Pasteur institute of Iran. The

ATCC 700,603 isolate of *K. pneumoniae* was used as a control in this study. We did the conventional biochemical and molecular validation tests according to the relevant protocols (26, 27).

Drug susceptibility tests

To determine the resistance of both clinical and standard isolates to the conventional antibiotics, the disk diffusion (Kirby–Bauer) testing on Mueller-Hinton agar was carried out and the growth inhibition zone diameters were compared to the clinical and laboratory standards institute (CLSI) guideline (Table 1) (28). The MICs of ciprofloxacin and *f*-MWCNTs, were determined using broth micro dilution MIC tests, according to CLSI 2020 instructions in triplicates (28) and was confirmed by MBC test (Fig. 1).

RNA extraction, cDNA synthesis, Real-Time-PCR

RNA extraction was performed according to RiboEX kit instructions (GeneAll Biotechnology Company, South Korea). The reverse transcriptase PCR (RT-PCR) method was used and the procedure were performed according to the Pars Tous company (Iran) instructions. Relevant primers were designed using the Gene runner software and the primer synthesis order was submitted by Pishgam Company in Tehran to Metabion Company in Germany. Moreover, a real-time PCR assay was performed using SYBR Green 2X master mix (Yekta Tajhiz company, Iran), following the manufacturer's instructions.

TEM imaging

To confirm the internalization of *f*-MWCNTs, the treated and untreated samples were observed under a transmission electron microscope (TEM) (Fig. 2).

Statistical analysis

The data of the studied groups were collected in GraphPad Prism 7.0 and subjected to analysis. The expression levels were also normalized. Data are expressed as mean \pm standard deviation (SD). The one-way ANOVA was used to determine

significance. *P*-values < 0.05 were considered significant.

Results

Nanofluid preparation

By functionalization, the compatibility of *f*-MWCNT improved and dispersion stability was increased up to 6 months with lower toxicity.

Results of microbial tests

The clinical and ATCC 700,603 isolates were identified by differential biochemical tests. They were also genetically confirmed by sequencing, followed by the BLAST technique.

Drug susceptibility tests results

By comparing the inhibition zone diameter, based on the CLSI guidelines 2020 (27), the ES133 clinical isolate of *K. pneumoniae* showed a resistance to all studied antibiotics including, ciprofloxacin (5 μ g), cefepime (30 μ g), ceftazidime (30 μ g), aztreonam (30 μ g), imipenem (10 μ g), meropenem (10 μ g) ertapenem (10 μ g), doripenem (10 μ g) aztreonam (30 μ g), and intermediate sensitivity to gentamicin (10 μ g) and chloramphenicol (30 μ g). The ATCC 700,603 isolate showed resistance to ciprofloxacin, ceftazidime, meropenem and sensitive to cefepime and gentamicin. Considering the inhibition zone diameter, the resistance of ATCC isolate to ciprofloxacin was less than that of the ES133 clinical isolate (Table 1).

MIC results

The broth micro dilution MIC breakpoints were read and compared with the CLSI 2020 guidelines (28). The lowest dose was considered as the minimum inhibitory concentration. By the effect of ciprofloxacin serial dilutions ranging 0.62 -16 μ g/ml on the resistant isolate of *K. pneumoniae*, no growth was observed from the dilution of 1 (16 μ g/ml). In the cases of nanofluids containing *f*-MWCNTs and *p*-MWCNTs, the clearance (growth inhibition) began at a dose of 400 μ g/ml and 600 μ g/ml for *f*-MWCNTs and *p*-MWCNTs respectively.

Table 1: Antibiogram results of resistant and standard *K. pneumoniae* strains to antibiotic disks

Antibiotic disk	Symbol	Disk Content (μg)	Strain	R / I / S	Zone Diameter (mm)	CLSI		
						S	I	R
Cefoxitin	CFX	30	ATCC	R	0	>16	13-15	<12
			133	R	0			
Ceftazidime	CAZ	30	ATCC	-	-	>21	18-20	<17
			133	R	0			
Cefepime	CPM	30	ATCC	S	26	>25	19-24	<18
			133	R	0			
Ciprofloxacin	CIP	5	ATCC	R	20	>26	22-25	<21
			133	R	0			
Imipenem	IMI	10	ATCC	-	-	>23	20-22	<19
			133	R	8			
Ertapenem	ETP	10	ATCC	R	17	>22	19-21	<18
			133	R	0			
Meropenem	MEM	10	ATCC	R	0	>23	20-22	<19
			133	R	0			
Doripenem	DOR	10	ATCC	-	-	>23	20-22	<19
			133	R	8			
Aztreonam	ATM	30	ATCC	-	-	>21	18-20	<17
			133	R	0			
Gentamicin	GM	10	ATCC	S	17	>15	13-14	<12
			133	I	14			
Chloramphenicol	C	30	ATCC	-	-	>18	13-17	<12
			133	I	13			
Colistin	CO	20	ATCC	-	19	-	-	-
			133	-	11			

S: Sensitive, I: Intermediate, R: Resistant, -: Not determined

MBC results

The ciprofloxacin resistant isolate grew under the influence of each 8 $\mu\text{g}/\mu\text{l}$ ciprofloxacin and 400 $\mu\text{g}/\mu\text{l}$ f-MWCNTs alone, but the effect of 8 $\mu\text{g}/\mu\text{l}$ ciprofloxacin in combination with 400 $\mu\text{g}/\mu\text{l}$ f-MWCNTs, completely inhibited the growth of the bacterium. Therefore, 8 $\mu\text{g}/\mu\text{l}$ ciprofloxacin and 400 $\mu\text{g}/\mu\text{l}$ f-MWCNTs were considered as the MICs (Fig. 2). In the ATCC 700,603 isolate of *K. pneumoniae*, doses of 2, 4, 16 $\mu\text{g}/\mu\text{l}$ ciprofloxacin + 100 $\mu\text{g}/\mu\text{l}$ f-MWCNT could inhibit bacterial growth. Therefore, 2

$\mu\text{g}/\mu\text{l}$ ciprofloxacin + 100 $\mu\text{g}/\mu\text{l}$ f-MWCNT were considered as the MICs.

Transmission Electron Microscopy (TEM)

To confirm the internalization of f-MWCNTs and visualize the wall degradation of bacteria, the treated and untreated samples were prepared and visualized under TEM. As a result of the accumulation of f-MWCNTs, a thick layer surrounding the bacterium was observed (Fig. 2-B) and also the particles that managed to internalize into bacteria are illustrated (Fig 2-A, -B).

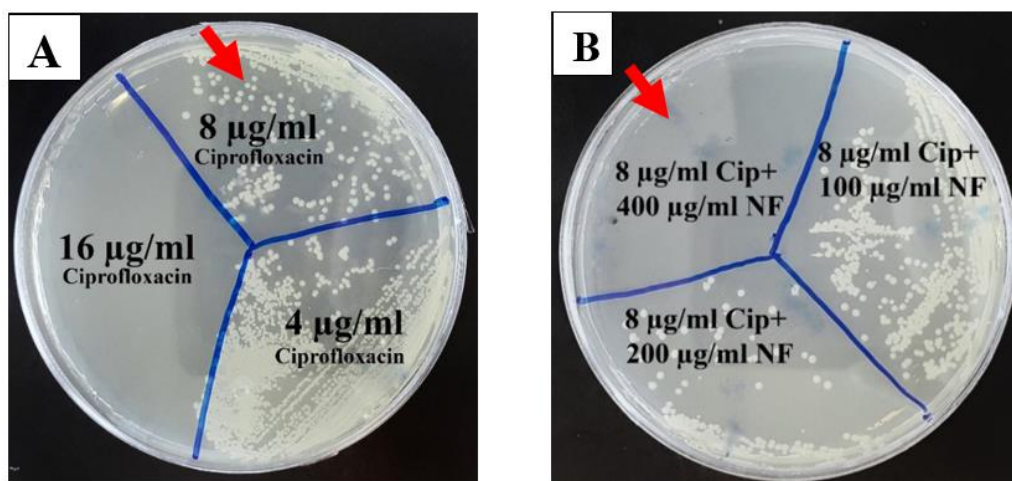


Fig. 1: MBC results of the effect of ciprofloxacin and *f*-MWCNTs on *K. pneumoniae*. A) The effect of 8 µg/ml ciprofloxacin on the *ES133* resistant strain did not inhibit the growth, but 16 µg/ml ciprofloxacin inhibited that. B) The effect of 8 µg/ml ciprofloxacin+ 400 µg/ml *f*-MWCNT completely killed the bacteria. As a conclusion, the effective dose of the antibiotic was reduced when combined with 400 µg/ml *f*-MWCNT

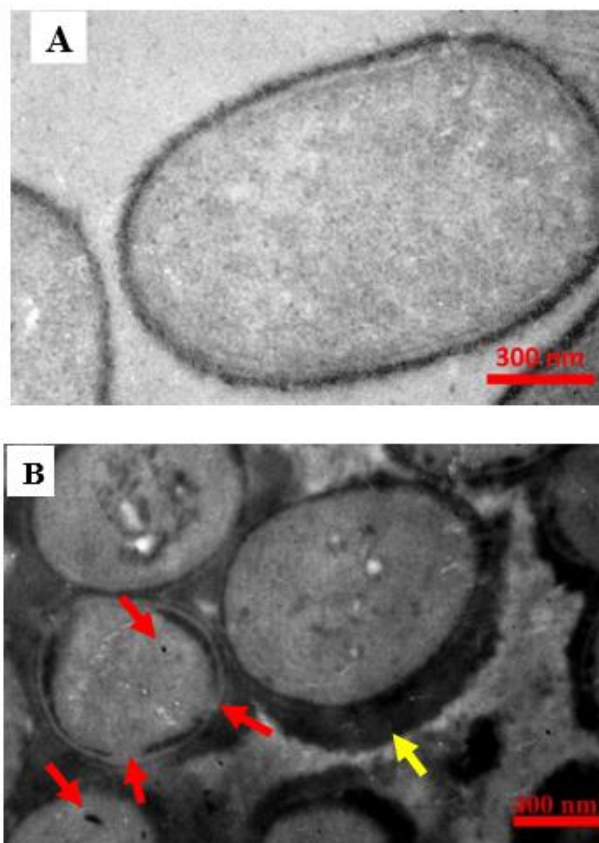


Fig. 2: Transmission Electron Microscopy (TEM) image of *K pneumoniae*. A) Untreated resistant strain (control) B) The same strain treated with 8 µl/ml ciprofloxacin with 100 µg/ml *f*-MWCNTs. Arrows indicate the *f*-MWCNTs particles that have entered the bacteria, as well as the location of the wall destruction. The accumulation of *f*-MWCNTs on the bacterial wall, indicated by a yellow arrow

The virulence genes real time-PCR results

The Real Time-PCR data were analyzed by one-way ANOVA method and graphs were drawn with GraphPad prism 7.0 software. In the resistant *K. pneumoniae* isolate, after *f*-MWCNT+cip

treatment, the expression of *fimA*, *fimD*, *wza*, and *wzi* genes was significantly down regulated compared to the ciprofloxacin treatment and upregulated compared to the control group (Fig. 3).

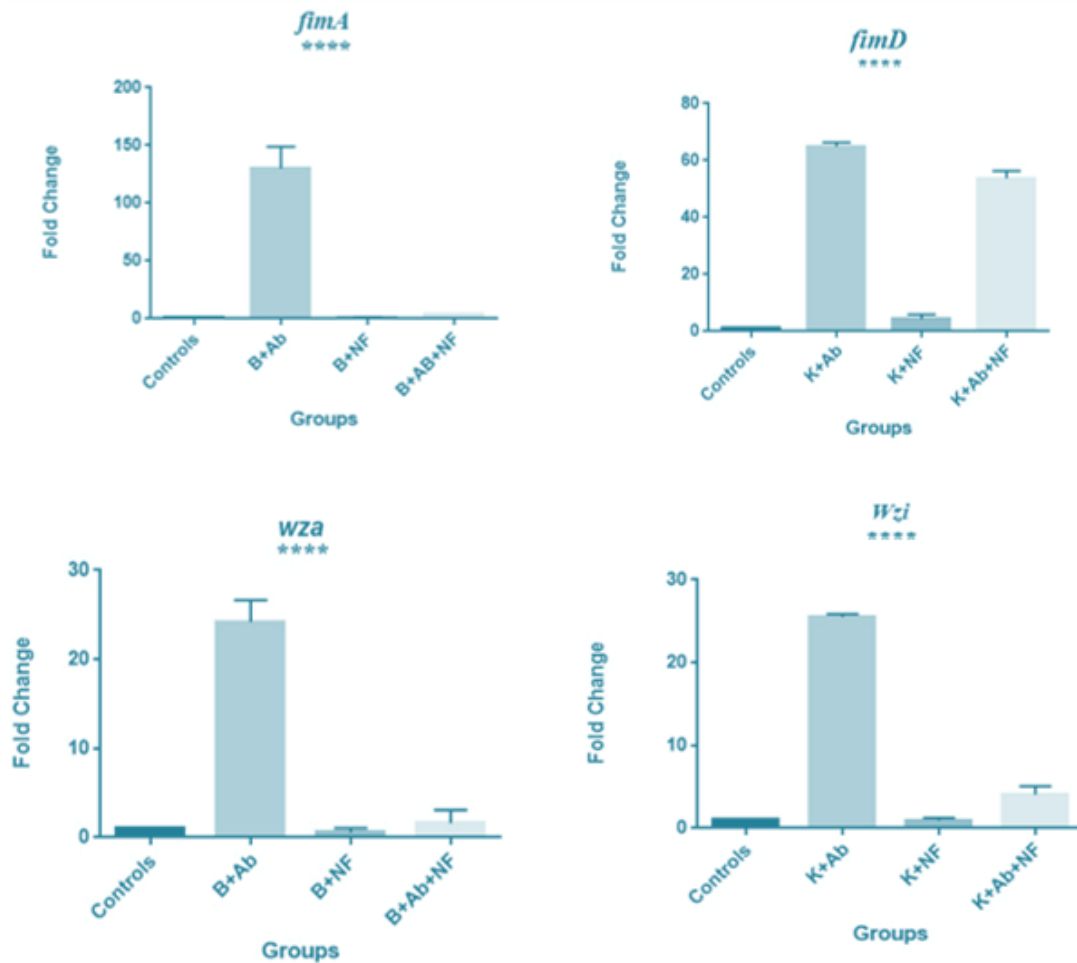


Fig. 3: *fimA*, *fimD*, *wza* and *wzi* genes expression in *K. pneumoniae* resistant strain to antibiotic and *f*-MWCNT. From left to right respectively: the untreated *K. pneumoniae* (negative control), treated with 8 µg/ml antibiotic, 400 µg/ml *f*-MWCNT, 400 µg/ml *f*-MWCNT + 8 µg/ml antibiotics. After treatment with 400 µg/ml *f*-MWCNTs + 8 µg/ml ciprofloxacin in the resistant strain, *fimA*, *fimD*, *wza* and *wzi* genes expression significantly upregulated in comparison with the control, (P value <0.0001, <0.0001, <0.003, <0.0001 respectively) and downregulated compared to the antibiotic alone. Abbreviations: B; Bacteria, K: *K. pneumoniae*; Ab: Antibiotic; NF: Nanofluid. Expression levels were normalized against *16s rRNA* as the reference housekeeping gene

The Real Time-PCR analysis graph of the ATCC 700,603 isolate of *K. pneumoniae* treated with 100 µg/ml *f*-MWCNT with 2 µg/ml ciprofloxacin, showed that the *fimA*, *fimD* and *wza* virulence genes expression were upregulated compared to

the negative control and downregulated in comparison with ciprofloxacin treatment (P values: 0.005, 0.005, 0.06). Though, *wzi* gene expression was downregulated compared to both the control and the ciprofloxacin treatment (P<0.37) (Fig. 4).

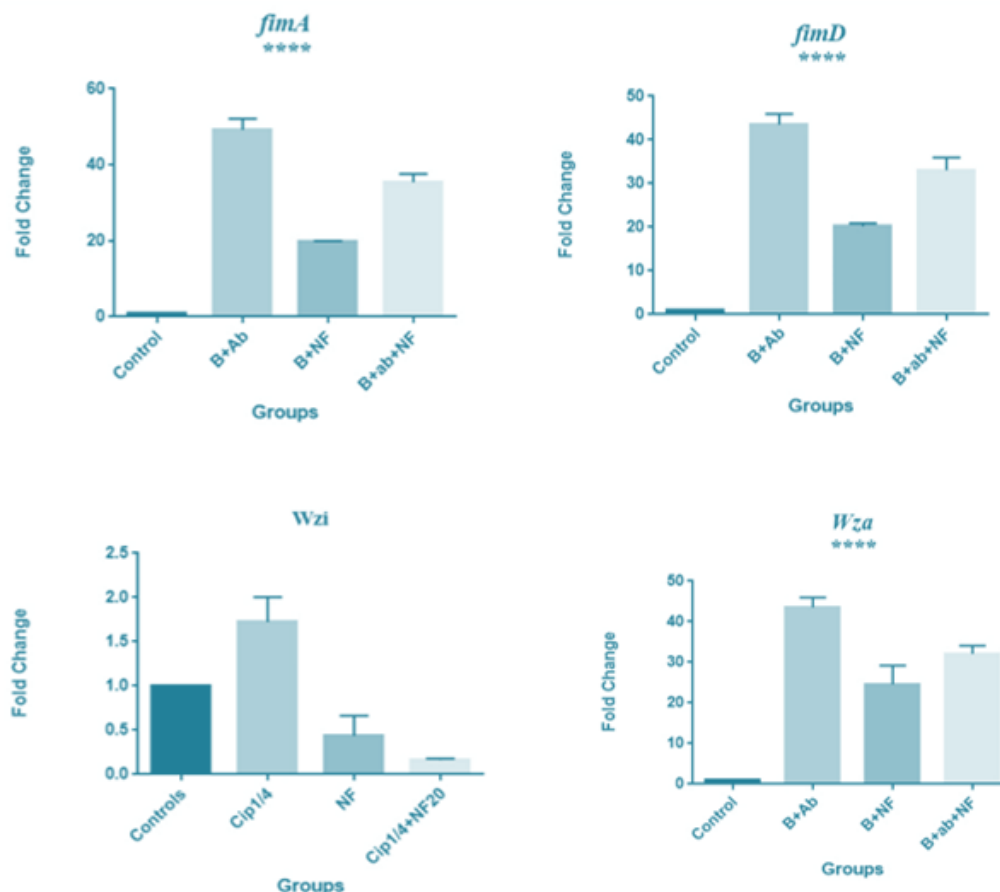


Fig. 4: *fimA*, *fimD*, *wza* and *wzi* genes expression in *K. pneumoniae* ATCC strain to antibiotic and *f*-MWCNT. From left to right respectively: untreated *K. pneumoniae* (negative control), treated with 2 µg/ml ciprofloxacin, 100 µg/ml *f*-MWCNT, 2 µg/ml ciprofloxacin + 100 µg/ml *f*-MWCNT. In the ATCC strain treated with 2 µg/ml ciprofloxacin + 100 µg/ml *f*-MWCNT, the expression of *fimA*, *fimD* and *wza* genes showed an upregulation compared to the control and showed a downregulation in comparison with ciprofloxacin treatment (P value <0.005, 0.005, 0.06), while *wzi* gene expression was downregulation compared to both the control and the ciprofloxacin treatment, although the rate of reduction was lower than the significant level (P value <0.37). Abbreviations: B: Bacteria; Ab: Antibiotic; NF: Nanofluid. Expression levels were normalized against *16s rRNA* as the reference housekeeping gene

Agarose gel electrophoresis

The electrophoresis of Real Time-PCR products showed sharp bands, formed exactly where they were expected on 2% agarose gel and matched the product length of the designed primers.

Discussion

Owing to the increasing emergence of antibiotic resistance as a public health concern, an efficient drug delivery system can improve the therapeutic

effects of antibiotics against human pathogens. This study aimed to prepare a nanofluid containing *f*-MWCNTs and to evaluate the efficacy of *f*-MWCNT+cip co-administration on the virulence gene expression of both resistant and standard *K. pneumoniae* isolates. Since unique physicochemical properties, conjugation capabilities as well as targeting capacity, CNTs can facilitate antimicrobial drugs delivery and overcome some of the limitations of traditional antibiotic treatments. New methods using *f*-MWCNTs, such as combination

of *f*-MWCNTs with antibiotics, can improve their effectiveness at lower doses (25).

The combination of AgNPs with various antibiotics were evaluated for antibacterial activity, and the greatest increase, obtained by the ciprofloxacin-AgNPs combination (29). After exposure to *f*-MWCNT+ antibiotic, the expression profile of each bacterial virulence gene, might change in a different pattern (24, 30). Similarly, in this study, the expression pattern of *wzi* gene was different from other studied genes. Thus, in *f*-MWCNT+cip treated bacteria compared to both the control and ciprofloxacin-treated, *wzi* expression showed a decrease, though it was below the significance level of < 0.05 for *P*-value ($P < 0.37$) (Fig. 4).

The findings of the present study are consistent with our recent research, as *f*-MWCNT+ cefepime could inhibit bacterial growth in resistant isolate of *K. pneumoniae* at lower doses compared to cefepime alone. Our previous investigations on *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Staphylococcus aureus* antibiotic resistant isolates, showed that simultaneous treatment with *f*-MWCNTs and antibiotics could increase the permeability of the bacteria and improve the effectiveness of antibiotics at lower doses (25). Furthermore, based on the data obtained in our other research, *f*-MWCNT conjugated with isoniazid nano-drug, resulted in optimal drug delivery, at lower concentrations than in the free state (17). The remarkable synergistic effect of ciprofloxacin and *f*-MWCNTs offer their potential in nanomedicine for clinical application as a combined therapy in the future.

Conclusion

By accompanying the *f*-MWCNTs, the drug delivery efficiency of ciprofloxacin, that *K. pneumoniae* is resistant to that, was increased. Due to the high durability of *K. pneumoniae* on surfaces and also the antibacterial effects of carbon nanotubes, the CNTs can be used as surface disinfectants to prevent infection, though our studies in this area are ongoing. For further comprehension

the *f*-MWCNT's mechanism of action, and the immune system response to them, further cell signaling pathways and in vivo studies are also required. Although functionalization of MWCNTs with carboxyl group could reduce the toxicity and improve biocompatibility, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay is proposed prior to the clinical usage of them. Conjugation of ciprofloxacin with *f*-MWCNT's is recommended in future studies for optimal drug delivery to *K. pneumoniae* as well.

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflict of interest

The authors have no conflict of interest.

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