

Antibacterial effect of carbon nanotube containing chemical compounds on drug-resistant isolates of *Acinetobacter baumannii*

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ABSTRACT

Background and Objectives: *Acinetobacter baumannii* is recognized as an important pathogen responsible for serious infections causing episodes of hospital infection. Carbon nanotubes (CNTs) have recently emerged as superior materials against antibiotic-resistant bacteria. In this study, a new chemical compound was designed in order to combat *A. baumannii* infections. Subsequently, the effect of this novel carbon nanotube coated with an antibacterial compound on Extensively Drug-Resistant (XDR), Multidrug-Resistant (MDR) and Pan-Drug-Resistance (PDR) strains of *A. baumannii* was investigated.

Materials and Methods: A total of 122 clinical isolates of *A. baumannii* were cultured from burn patients and their susceptibility to antibiotics were checked using disk diffusion method and Minimum inhibitory concentration. Antimicrobial effects of the coated carbon nanotube were evaluated on XDR, MDR and PDR isolates of *A. baumannii*. Cell viability was determined using tetrazolium reduction assay (MTT) on human fibroblast cell line (HDFa). Wound healing processes were assessed by quantitative polymerase chain reaction.

Results: Of the 50 *A. baumannii* isolates, 38 (76%) were found to be MDR and 12 (24%) were XDR. No PDR strains were detected. Results indicated that the carbon nanotube combined with mercury had antibacterial effect against different *A. baumannii* species and it also was able to increase the expression of epidermal growth factor, platelet-derived growth factor and vascular endothelial growth factor A mRNA levels which are involved in wound healing.

Conclusion: The engineered carbon nanotube compound can potentially be used for treatment of burn related infections. This can potentially give clinicians a new tool for treating *A. baumannii* infections.

Keywords: *Acinetobacter baumannii*; Carbon nanotubes; Infections; Anti-bacterial agents; Wound healing; Real time polymerase chain reaction

INTRODUCTION

Over-administration of antibiotics to patients particularly in hospital environment leads to creation of resistant bacteria, which is a major problem worldwide (1). Resistant bacteria are described in different patterns including 1) multidrug-resistant (MDR),

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bacteria that have resistant to several antibiotic, 2) extensively drug-resistant (XDR), is the non-susceptibility of one bacteria species to all antimicrobial agents except in two or less antimicrobial categories. 3) pan-drug-resistant (PDR), the non-susceptibility of bacteria to all antimicrobial agents in all antimicrobial categories (2, 3). *Acinetobacter baumannii* is a Gram-negative bacillus and opportunistic pathogen with high prevalence for immunocompromised patients especially in subject that have been hospitalized for long period (more than 90 days) (4). Another reason for increased resistance of *A. baumannii*, is it's the ability to create biofilms on biotic and abiotic surfaces (5). *A. baumannii* has been implicated in nosocomial infections especially in skin, urinary and respiratory tract infections (6, 7). Controlling of nosocomial infections caused by multidrug-resistant Gram-negative bacteria (MDR) is a serious problem (8). It is thus crucial to develop new alternative remedies to combat unwanted bacteria. Nanoparticles are materials of about one nanometer to several hundred nanometers that offer new opportunities in various medical fields such as biomedicine and pharmaceuticals (9). Their usage in medicine include drug delivery, tumor therapies, and biosensors (10, 11). Carbon nanotubes (CNTs) have received great attention in recent decades with their unique physical, mechanical and chemical properties. Carbon nanotubes are tubular materials with 1-3 nm diameter and made of carbon-carbon covalent bonding. These nanotubes generally have high durability and stability. There are different types of carbon nanotubes, generally classified into single-walled carbon nanotubes (SWNTs) and multi-walled carbon nanotubes (MWNTs) (12). The combination of different chemicals within these nanotubes can lead to increased efficacy and new therapeutic potentials. Although mercury (Hg) is a toxic element, its antibacterial activity combined with it's disability has led to it's widespread use in dentistry (13, 14). The combination of Hg with carbon nanotubes can be used as an effective anti-bacterial tool in treatment of primary infections and prevention of the emergence of new antibiotic resistance. In this study, a combination of Hg and carbon nanotube was designed and its efficacy on resistant *A. baumannii* strains was investigated. It's wound healing potential was also determined by measuring the expression of EGE, PDEGF and VEGFA genes.

MATERIALS AND METHODS

Sample collection. This prospective observational study was approved by Iran University of Medical Sciences ethics committee (IR.IUMS.REC.1398.1117) and written informed consents were obtained from each participant before enrollment in the study. A total of 122 clinical specimens of burned skin wound infections were collected in Shahid Motahari Hospital, Tehran, Iran. All specimens were transferred to microbiology laboratory for over a period of 1 year from January to December 2018.

Burn wound specimens were collected from patients by swabbing the burned skin area. The sampled swabs were initially placed in Thioglycolate broth tubes and incubated for overnight. Following overnight incubation, the broth was subcultured onto blood agar and Eosin Methylene Blue plates and incubated for any bacterial colonies to appear.

Microbial identification and antibiotic susceptibility testing. After transferring burn wound specimens to the laboratory for bacteriological confirmation, various biochemical tests including catalase, oxidase, Oxidative/Fermentation glucose assay, growth at +42°C and evaluation of pyocyanin pigment production were carried out.

Oxidase-negative bacteria were inoculated into Triple Sugar Iron Agar and lysine decarboxylase media. Negative reactions in the alkaline TSI medium (non-fermented sugar) and lysine decarboxylase test were enough to confirm the bacterium as *Acinetobacter*.

Molecular identification. Bacterial identification was confirmed by polymerase chain reaction (PCR). DNA was extracted from colonies of overnight grown bacteria by boiling method. Fresh colonies were placed in a test tube containing 300 µl of sterile distilled water. Following rapid vortexing, the tubes were placed in boiling water for 10 min. Following centrifugation at 8,000 rpm for 5 min, 5 µl of the supernatant was transferred to sterile tube. PCRs were performed in a final volume of 25 µL which included 5 µL of genomic DNA, 1 µL of each primer, 12.5 µL of PCR Mix Master and the rest was filled with distilled water. Primer sequences were P-Ab-ITSF (5'-CATTATCACGGTAATTAGTG-3') and P-Ab-ITSB (5'-AGAGCACTGTGCACTTAAG-3') which specif-

ically amplified an internal 208-bp fragment of the 16S-23S ribosomal DNA intergenic spacer region of *A. baumannii* (15). Thermal Cycler was programmed to 35 cycles of +94°C for 4 min, +94°C for 30 sec, +55°C for 30 sec, and +72°C for 30 sec. PCR products were electrophoresed in 2% agarose gel and stained with DNA Green Viewer (Parstous Biotechnology, Iran) to visualize the fragments using a UV gel documentation instrument.

Antibiogram testing. Kirby-Bauer disc diffusion method was used to determine drug resistance phenotype according to Clinical and Laboratory Standards Institute criteria (CLSI, 2018) (16). Nine different antibiotic discs (Rosco, Taastrup, Denmark) were used for *A. baumannii*, including gentamicin (10 µg), meropenem (10 µg), colistin (10 µg), levofloxacin (5 µg), tetracycline (30 µg), ampicillin-sulbactam (20 µg), piperacillin-tazobactam (110 µg), cefepime (30 µg) and trimethoprim-sulfamethoxazole (25 µg). The Mueller Hinton agar plates (Merck Co., Darmstadt, Germany) were incubated for 18 to 24 h at +37°C.

Preparation of chemical component. Ethylene diamine (99%), mercury iodide II (99%), dimethyl sulfoxide (99%), methanol (99.6%) was purchased from Merck Co (Darmstadt, Germany). 1-Methyl-1,2,3,4-tetrazole-5-thiol was purchased from Aldrich Co (London, England). The instruments used in this section included: Infrared spectra (4000e250 cm⁻¹) of solid samples were taken as 1% dispersion in CsI pellets using a Shimadzu-470 spectrometer, NMR spectra were recorded on a Bruker AC-300 spectrometer for protons at 300.13 MHz in DMSO-d₆. Melting points were obtained on a Kofler Heizbank Rechart type 7841 melting point apparatus. Elemental analysis was performed using a Heraeus CHNeO Rapid analyzer. Thermal behavior was measured with a STA 503 Bahr apparatus, respectively (17).

Preparation carbon nanotube. The carbon nanotube was functionalized with carboxylic acid (COOH) group, sulfuric acid / nitric acid compound with 3:2 vol/vol. 0.514 g of carbon nanotubes were dissolved in 100 ml of acetic acid and placed in an ultrasonic bath for 30 min to improve dispersion. Solution was transferred into a 250 ml round bottom flask and heated at +140°C for 3 to 10 h. Finally, the solution was diluted with water until neutral pH was obtained. Fourier-transform infrared spectroscopy (FTIR) was used

to confirm the functionality of the carbon nanotube.

Loading drug in carbon nanotubes. The functionalized carbon nanotube (25 mg) was suspended in 10 ml water. An equal weight of the chemical component (drug) was added and the mixture was placed in an ultrasonic bath with stirring for 24 h. To determine the rate of drug loading on the nanotube, the drug-loaded nanotube was dispersed in a solvent and centrifuged at 1,000 rpm. To determine concentration and loading efficiency, 2 ml of the supernatant was read by spectrophotometer. The effect of nanotubes with and without chemical composition on resistant isolates was investigated by disk diffusion method.

Minimum inhibitory concentration (MIC) assay. To determine the minimal antibiotic concentration that inhibits bacterial growth, minimal inhibitory concentration (MIC) were measured by serial dilutions in accordance with CLSI guidelines (16).

Cell viability assay. The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was used for evaluating the possible cytotoxicity of carbon nano tube and to determine the optimum doses. Human Dermal Fibroblasts cells (HDFa) were seeded in 96-well plates at a density of 1×10^4 cells/well in 100 µl RPMI medium. After twenty-four hours of seeding, the medium was removed and then the cells were incubated for 24 h with RPMI with the absence and/or the presence of various concentration of chemical component and loaded CNT ranging from 5, 10, 20, 50, 75, 150 and 300 µg/ml. After 24 h, MTT solution was added into each well. The plates were incubated again for 4 h in CO₂ incubator at +37°C. Lastly, the optic density of purple formazan absorbance was determined at 570 nm by a microplate reader (Bio-Tek Instruments Inc., Vermont, USA). The cell viability was determined using the formula:

$$\text{Viability \%} = (\text{optical density of sample} / \text{optical density of control}) \times 100$$

Half maximal inhibitory concentration (IC₅₀) values were calculated as the concentrations that showed 50% inhibition of cell line proliferation.

Burned rat model. All animal studies were conducted strictly under the guidelines provided by the university ethical code allocated for this study and were constantly supervised by a veterinarian. The

back of rats was shaved 24 h prior to burning. Rats were anesthetized using a mixture of 100 mg / kg ketamine hydrochloride and 20 mg / kg xylazine in distilled water injected intraperitoneally. Then, the hair-free area was burned by an aluminum sheet in a 1.5 × 1.5-inch with 0.5 ml ethanol for 10 sec. Thus, 12 to 15% of the rats back skin was burned to grade 3. Immediately, 0.5 ml of sterile saline was injected subcutaneously in the burn area to compensate for the lost skin water. Acetaminophen (0.25 mg/ml) injection was used as a post-burn pain reliever.

Determination of gene expression by qPCR in mouse model. RNA was extracted from mouse tissue samples by using the RNeasy Mini Kit (50) (Qiagen, Germany), according to the manufacturer's suggested protocol. RNA integrity, quantity, and quality were determined by agarose gel electrophoresis and the Nano Drop system (Nano Drop Technologies, Inc., Wilmington, DE, USA). RNA was stored at -70°C before use. One-µg samples of RNA were used for complementary DNA (cDNA), which was synthesized by using the total Reverse Transcription Kit (Takara, Japan) after the adjustment of the concentrations, according to the manufacturer's instructions. β-actin gene was used as the reference gene. The sequence of primers for target genes were listed in Table 1. qPCR was carried out on a real-time PCR cyclor (Rotor-Gene Q MDx, Qiagen).

Colony Count. One week after treatment, parts of the burned tissue were removed and placed in phosphate buffered saline. Following vigorous vortexing, centrifugation, and serial dilutions of the suspension, bacterial counts were determined by plate culturing.

Statistical analysis. Statistical analyses were performed by descriptive methods (REST 2009, Excel 2019).

Table 1. The listed of qPCR primers sequence.

Gene	Sequence	Length	Reference
EGF	F: GACTGCCTTGCCCTGACTCTAC	22	This study
	R: CGGTGCTGACATCGTTCTCCAA	22	
PDEGF	F: TCTTCCTGCCTCTCTGCTGCTA	22	This study
	R: CCATCTTCGTCTACGGAGTCTCTG	24	
VEGFA	F: GAAGTGGTGAAGTTCATGGAC	21	This study
	R: GCATGATCTGCATAGTGACG	22	

RESULTS

Identification of *Acinetobacter baumannii*: Biochemical tests. Speciation of *A. baumannii* was performed on the basis of TSI, catalase, oxidase, IMViC, urease, oxidative/fermentative tests (OF), growth at +37°C and +42°C tests.

Molecular identification of *Acinetobacter baumannii*. For molecular confirmation of *A. baumannii*, 122 suspected isolates were checked. Out of these only 50 isolates were confirmed. For molecular confirmation of *A. baumannii*, DNA was extracted from the bacteria and specific PCR primer were used for detection of *A. baumannii*. The 200 bp PCR products were visualized on 1% agarose followed by UV irradiation Fig. 1.

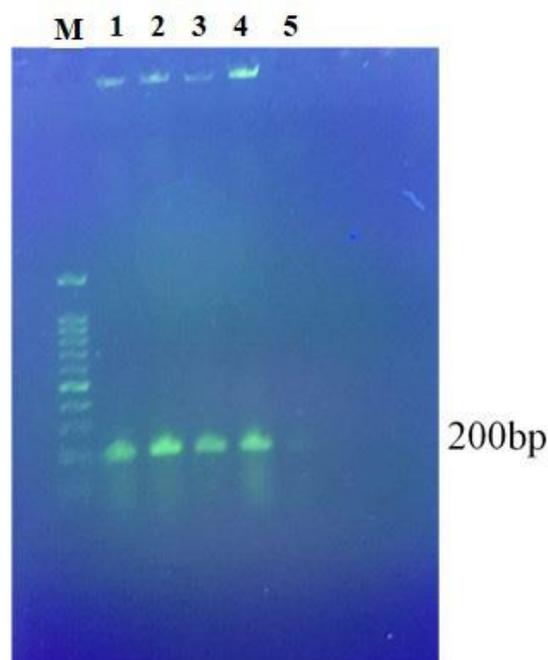


Fig. 1. PCR confirmation of the *Acinetobacter baumannii*, marker 100 bp, amplicon size 200 bp.

Antibiogram. The antibiogram results were divided into resistant (R), intermediate (I), and susceptible (S) phenotypes. The highest antibiotic resistance in *A. baumannii* isolates were shown towards cefepime and trimethoprim-sulfamethoxazole and the lowest sensitivity was related to cefixime. The *A. baumannii* isolates were resistant to three or more antibiotics. Of the 50 *A. baumannii* isolates, 38 (76%) were found to be MDR and 12 (24%) were XDR. Sensitivity range of *A. baumannii* to antibiotic discs is shown in Table 2.

Results of viability of *A. baumannii* after CNT and coated CNT. The percent viability of *A. baumannii* cells after the chemical component and coated CNT treatment were analyzed by MTT staining. The IC₅₀ of the chemical component and its loaded CNT were 54.1482 and 19.379 µg/ml, respectively. Effect of chemical component and its loaded CNT on *A. baumannii* at various concentrations is shown in Fig. 2.

Evaluation of wound healing by qRT-PCR. One of the main reasons for non-healing of burn wounds is *Acinetobacter* infections. It has been seen that in-

fection adversely affects the related wound healing expression genes that prevents improvement. Five groups of rats, each group included six rats, were selected. Each group was categorized according to group I (Untreated burns rats), group II (Burned rats treated with the drug), group III (Burned rats contaminated with *A. baumannii* and treated with the drug), group IV (Untreated burned rats that were contaminated with *A. baumannii*) and group V (Burned rats that were contaminated with *A. baumannii* and treated with the drug and antibiotic). To investigate the effect of the synthesized drug on wound healing, we compared the group III with group IV, the results of which showed that VEGFA, EGF and PDEGF mRNA levels significantly were up regulated. Group III was compared with group V in order to evaluate prophylactic property of the synthesized drug. Only EGF and PDEGF expression were significantly up regulated. Comparing group I rats with those of group IV as well as group III with II showed the effect of infection process on wound healing gene expression levels. VEGFA, EGF and PDEGF mRNA levels significantly were down regulated (Fig. 3).

Table 2. Susceptibility range of *A. baumannii* clinical isolates to antibiotic discs

Antibiotics	Resistant	Intermediate	Susceptible	P-value
Gentamicin	49	-	1	0.322
Meropenem	49	1	-	0.322
Colistin	2	-	48	0.000
Levofloxacin	45	5	-	0.025
Tetracycline	16	4	30	0.000
Ampicillin-sulbactam	2	-	48	0.000
Piperacillin - tazobactam	48	2	-	0.48
Cefepime	50	-	-	1.000
Trimethoprim-sulfamethoxazole	50	-	-	1.000

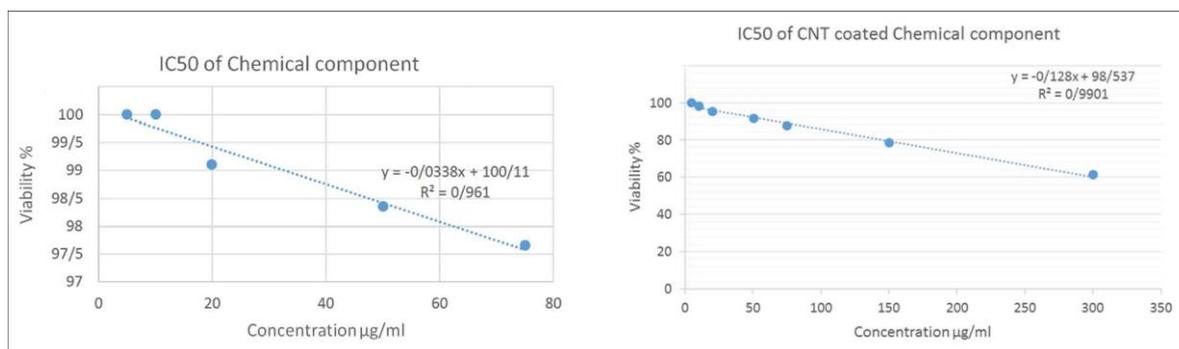


Fig. 2. Various concentrations of the chemical component and its loaded CNT on *A. baumannii*, A. MTT diagram of the chemical compound toxicity, B. MTT diagram of the chemical compound with carbon nanotubes toxicity

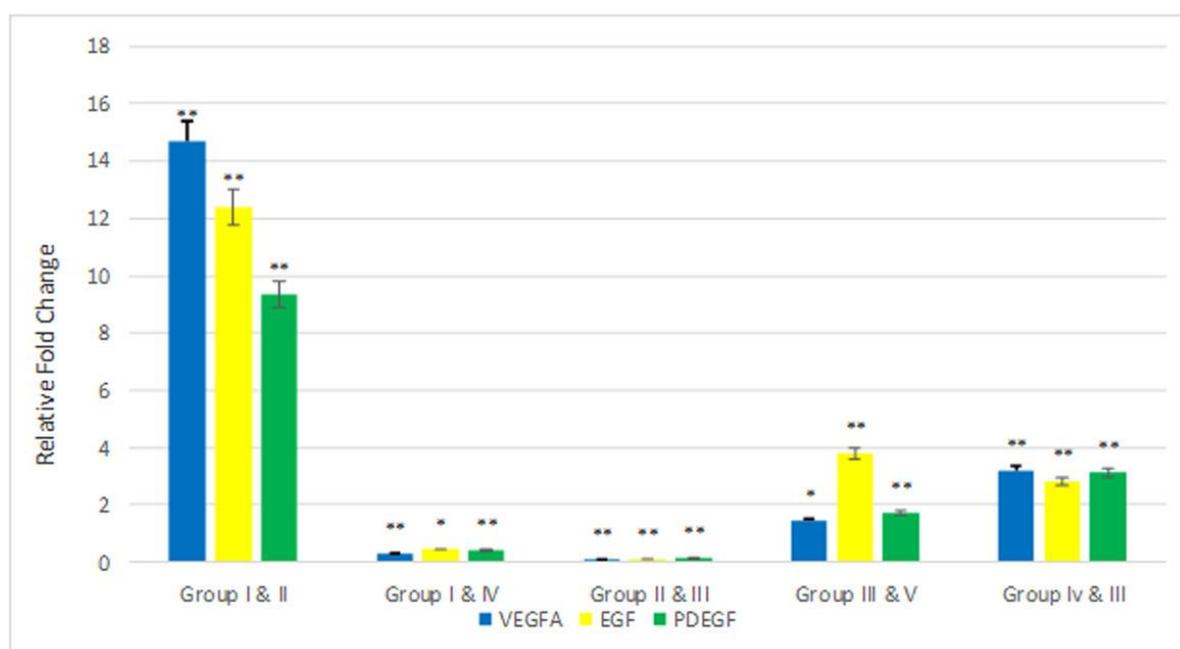


Fig. 3. Relative expression of VEGFA, EGF and PDEGF levels in rats' tissues compared with categorized group including, group II (Burned rats treated with the drug), group III (Burned rats contaminated with *A. baumannii* and treated with the drug), group IV (Untreated burned rats that were contaminated with *A. baumannii*) and group V (Burned rats that were contaminated with *A. baumannii* and treated with the drug and antibiotic). * $p < 0.05$, ** $p < 0.001$.

Effect of nanotube on *Acinetobacter baumannii* infection. Rats were burned and categorized according to the previous section. 100 μ l of *A. baumannii* suspension was injected subcutaneously. Colony counts were performed in all groups. More detail is shown in Table 3.

DISCUSSION

One of the most promising solutions to overcome bacterial resistance is the use of metal nanoparticles. Due to their small size, high surface-to-volume ratio, these nanoparticles have a high level of contact with the environment and microorganisms, which can lead to their biological and chemical activity. These

Table 3. Results of colony counts in *Acinetobacter baumannii* mouse infected groups.

Group	<i>A. baumannii</i>
I	254 CFU/gr (total count)
II	No growth
III	16 CFU/gr (<i>A. baumannii</i>)
IV	6.1×10^7 CFU/gr (<i>A. baumannii</i>)
V	No growth

nanoparticles can disrupt the normal function of the cell membrane, including its selective permeability and cellular respiration. Once in the bacterial cell, they block the function of sulfur-containing proteins and phosphorus-containing molecules, such as DNA, and destroy their function.

Acinetobacter baumannii is a major pathogen responsible for hospital infections and epidemics, especially in older and hospitalized patients causing skin disorders, respiratory tract infection, and wound infections (18). *A. baumannii* have inherent tendency to acquire multiple antibiotics resistance (MAR) and long-term survival in hospital environments (19-26). The most effective antibiotic against *A. baumannii* infections are carbapenems. Carbapenems are subset of β -lactam antibiotics. They can bind to penicillin-binding proteins causing inhibition of cell wall synthesis and consequently cell death (27). Unfortunately, carbapenem resistant strains of *A. baumannii* are rapidly on the rise (28). Although, newer therapies such as colistin has been used, but these antibiotics can cause severe side effects, including neurotoxicity or nephrotoxicity (29). Treatment of infections caused by multiple-resistant *Acinetobacter* has become a global concern (23, 30). Reports of strains of *A. baumannii* that are resistant to all known an-

tibiotics have highlighted an alarm for international health organizations (20, 21).

In this study, a new carbon nanotube chemical was synthesized in order to eliminate resistant *A. baumannii*. Most *A. baumannii* isolates in this study displayed multiple antibiotic resistance especially towards tetracycline, trimethoprim and calcitonin.

In a study by Ayan et al. in 2003, a total of 52 *A. baumannii* strains were studied (31). All isolates were resistant to piperacillin, piperacillin-tazobactam, ticarcillin-clavulanate, cefepime, cefotaxime, ceftazidime, ceftriaxone, gentamicin, and aztreonam but were susceptible to imipenem and meropenem. Resistance to tobramycin, ciprofloxacin, ampicillin-sulbactam, trimethoprim-sulfamethoxazole and amikacin were found in 5%, 8%, 55%, 66%, and 74% of the isolates, respectively (31).

In a study by Adams and colleagues in 2008, 142 *A. baumannii* isolates were studied (32). 65 of the isolates (45.8%) had multiple MDR resistance and the tested antibiotics were ciprofloxacin (96%), ceftazidime (88%), cefepime (80%), ampicillin-sulbactam (41%), imipenem (18.4%), meropenem (22.4%), amikacin (37%), tobramycin (61.2%), gentamicin (78%) and tetracycline (80%). Eight of these isolates were resistant to 6 classes of the tested antibiotics and no resistance to colistin or tigecycline were observed (32).

Carbon nanotubes, through their unique properties, including strong bonds between atoms and carbon-to-carbon covalent bonds provide an effective weapon against multidrug-resistant bacteria (33). In addition, mercury has been used as a drug for a wide range of treatments. In veterinary medicine, mercury is used as an ointment to treat blisters (34). By combining mercury and carbon nanotubes, we aimed to combat burn-related infections.

In a study by Sobouti et al. in 2020, 115 clinical *A. baumannii* strains were isolated from burnt children in Tehran, Iran which were examined for antibiotic resistance. Of those, 36 (58%) were indicated as MDR, 17 (27.5%) as XDR, and nine (14.5%) isolates were reported as PDR (35). Our findings show that 24% of *A. baumannii* infections have a XDR phenotype and 76% have a MDR phenotype. No PDR strains were detected in our study.

In a study conducted by Zeynali Aghdam et al. in 2019, they investigated the antibacterial effects of a mixture of silver nanoparticles combined with shallot and nettle alcoholic extracts against resistant *A.*

baumannii isolates. They concluded that this combination nanobiotechnology product is highly effective against resistant bacteria (36). In our study, tetazole derivatives showed substantial therapeutic potential. The chemical component ($\{[H_2en][Hg_2(m-tz)_4(\mu-I)]\}$) which was coated on CNT was potentially inhibit glutamic acid gamma-carboxylation in bacterial cell wall peptidoglycan.

Banihashemi et al. in 2020 investigated the antibacterial properties of this same chemical compounds coated onto the same carbon nanotubes on 30 burned Wistar mice which were artificially infected with *Pseudomonas aeruginosa*. They concluded that simultaneous treatment of the compound and nanotubes was highly effective for treatment (37). Limitation of the current study was that we have not yet tested the chemical compound directly on human burn wounds.

In conclusion, by synthesis of carbon nanotube combined with mercury, a novel chemical component has been developed which was effective against burn-related infections. This can potentially give clinicians a new tool for treating *A. baumannii* infections. It can be concluded that the combined use of nanoparticles and antibiotics makes it possible to reduce the unwanted toxicity on human cells and is a good way to overcome the problem of microbial resistance.

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