



Evaluation of the Effects of Curcumin Nanoparticles on the Expression of Genes Involved in Biofilm Formation in UPEC

ARTICLE INFO

Article Type

Original Research

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How to cite this article

Shivaee A, Mirshekar M, Sadeghi Kalani B, Bordbar D, Ohadi E, Masjedan Jazi F. Evaluation of the Effects of Curcumin Nanoparticles on the Expression of Genes Involved in Biofilm Formation in UPEC. Infection Epidemiology and Microbiology. 2018;4(4):115-121.

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Article History

Received: January 26, 2018

Accepted: October 06, 2018

ePublished: December 20, 2018

ABSTRACT

Aims Uropathogenic *Escherichia coli* (UPEC) is one of the most important causative agents of urinary tract infection (UTI). UPEC isolates persist in the body through biofilm formation. The successful adhesion is the most important step of biofilm formation. Type 1 pili and P fimbriae are bacterial surface appendices, which play a pivotal role in adhesion of UPEC. The aim of this study was to assess the effect of nanocurcumin on the initial adhesion and *papG* and *fimH* gene expression in UPEC isolates.

Materials & Methods The presence of *papG* and *fimH* genes among 60 UPEC isolates was investigated by PCR; 5 potent biofilm producer UPEC strains from patients with UTI were exposed to the sub-minimum inhibitory concentration of nanocurcumin. Expression of the *papG* and *fimH* genes was evaluated by real-time PCR.

Findings Of the 60 UPEC isolates, biofilm formation was seen in 27 (45%) of isolates, 5 of which produced strong biofilm. The result of PCR assay showed that *papG* was seen in 57 (95%) of the 60 UPEC isolates and *fimH* was seen in 58 (96.6%) of isolates, respectively. Nanocurcumin decreased *papG* and *fimH* expression 7 and 8 fold in all 5 isolates, respectively.

Conclusion Sub-MIC concentrations of nanocurcumin remarkably decreased the expression of the *papG* and *fimH* genes in strong biofilm forming UPEC strains, but nanocurcumin cannot prevent biofilm formation.

Keywords Curcumin; UPEC; Biofilm; Nanoparticle

CITATION LINKS

[1] Biofilm formation by uropathogenic ... [2] Estimating hospital costs of ... [3] Bacterial biofilms: Development, dispersal ... [4] Contamination of abiotic surfaces ... [5] Implant infections: Adhesion ... [6] Insights into pilus assembly and secretion from ... [7] Dynamic restacking of *Escherichia coli* ... [8] Curcumin nanoparticles: Preparation ... [9] Antimicrobial and chemopreventive properties ... [10] Retracted: Design of curcumin ... [11] Bioavailability of curcumin: Problems and ... [12] Protective mechanism of curcumin ... [13] Nanocurcumin effect on drug resistant ... [14] Effects of ZnO nanoparticles on initial ... [15] Initiation of biofilm formation in *Pseudomonas* ... [16] Evaluation of biofilm-forming capabilities ... [17] Flagellin and F4 fimbriae have opposite ... [18] Power comparisons of Shapiro-Wilk ... [19] Phylogenetic group distributions, virulence ... [20] Urinary tract infection *Escherichia coli* ... [21] Distribution of extended-spectrum β -lactam ... [22] Nanoparticles impact the expression of the ... [23] A tale of two pili: Assembly and function ... [24] Virulence characteristics and antibiotic resistance ... [25] Detection of some virulence factors of *Escherichia* ... [26] Frequency of P and type 1 fimbriae ... [27] Prevalence of genes encoding virulence ... [28] Distribution of uropathogenic virulence ... [29] Uropathogenic *Escherichia coli* in ... [30] Detection of uropathogenic *Escherichia* ... [31] The common fimbriae genotyping in uropathogenic ... [32] Risk factors for recurrent urinary ... [33] Genotypic characteristics and biofilm ... [34] Genotypic characterization of virulence ... [35] Photokilling of bacteria by curcumin ... [36] Antibacterial activity of turmeric ... [37] Antibacterial effects of curcumin ... [38] Antibacterial synergy of curcumin with ... [39] Biomolecular mechanisms of *Pseudomonas* ... [40] Inhibition of biofilm development ... [41] Prevention of quorum-sensing ... [42] Curcumin, a known phenolic from ... [43] Combination of silver nanoparticles and ... [44] Trans-cinnamaldehyde decreases attachment ...

Introduction

Urinary tract infection (UTI) is a serious problem with very high recurrence risk. Also, UTI is one of the most urologic problems in humans, which is responsible for more than 150 million cases worldwide [1]. About 11 million new cases of UTIs are reported in the United States each year, whose treatment is costly (about 5 billion dollar per year) [2].

Uropathogenic *Escherichia coli* (UPEC) is the main cause of hospital-acquired and community-acquired UTIs. The capacity of bacteria to persist through forming a complex and stable structure known as biofilm appears to be the main pathogenesis factor and treatment failure.

The formation of biofilm protects UPEC against antimicrobial therapy, host immune system, and the environmental conditions [3]. Biofilm formation is basically specified by 4 steps, including adhesion to the surface, cellular aggregation, the production of an extracellular matrix, and biofilm maturation [4]. A successful adhesion is necessary for the formation of biofilm. The successful adhesion mainly relies on the attendance of cell appendix such as fimbriae, pili, and flagella.

E. coli owns a mannose-specific *fimH* receptor on the tip of their type I pili and are responsible for the persistence state in bacteria. These receptors are also involved in invading cells facilitating adhesion to the surface of host tissue [5]. P pili consist of galabiose specific receptors on a distal *papG* unit encoded by *pap* locus, which permits the bacteria to cause pyelonephritis through colonizing the upper urinary tract via attaching to galabiose specific receptors to the glycolipid galabiose on urinary tract tissue [6]. Actually, the *papG* unit consists of galabiose specific receptors for adhesion to urinary tract tissue [7].

Deletion or control of biofilm has become the main goal of scientists. Because it is a complex structure with low permeability, it is hard to fight with. Today, researchers are turning to traditional medicine to fight against biofilms instead of the chemical agent because of the high levels of side effect of them [8].

Curcumin is a polyphenolic compound divided from *curcuma longa* rhizome that has achieved so many attentions because of lots of biological activities [9]. Previous studies have proved the antimicrobial features of curcumin against fungi, bacteria, malaria, and viruses [10-12]. This traditional medicine, due to the safety of curcumin even at high doses (12g/day) and the variety of antimicrobial activity, was evaluated through clinical trials in humans and it was used as a structural sample in purpose of designing of new antimicrobial agents with improved and increased antimicrobial activity by synthesis of different derivative of curcumin such as nanocurcumin [10]. Previous studies have shown using nanoparticle technology increased the water

solubility and antimicrobial features of curcumin [13].

Objective: This study was designed to evaluate the effect of nanocurcumin on the expression of *fimH* and *papG* genes, as important genes in adhesion of bacteria in biofilm formation.

Materials and Methods

Nanocurcumin preparation: 1024 g of spherical-shaped nanocurcumin with the average size of 70 nm (previously manufactured by Exir Nano Sina Co.) was diluted with 1ml of DMSO 4% according to the manufacturer's protocol.

Sample collection: This is a cross sectional study. 60 urine samples from women with symptomatic UTI with bacterial count (10^5 CFU/ml) were collected between April 2017 and December 2018 from a different hospital in Tehran, Iran. The patients aged between 21-61 years and did not take any antibiotic 1 month before sampling time. 63.3% of the samples from a patient with cystitis and 36.7% from a patient with pyelonephritis were recovered.

Bacterial isolation: Isolated bacteria were identified with the use of standard microbiological and biochemical tests, including Gram staining, oxidase, catalase, methyl red, motility, indole, citrate utilization, and fermentation, utilization of glucose, lactose, and voges-proskauer. All identified isolate was suspended in Luria-Bertani (LB) medium supplemented with 15% glycerol and transferred to the 80°C freezer.

PCR assay: DNA extraction was performed according to QIAamp DNA extraction Kit (Qiagen; Germany). Analysis of purity of the extracted DNA was performed, using NanoDrop spectrophotometer and also 1% agarose gel electrophoresis (Sigma; USA). By using the PCR method, the presence of 3 important genes involved in biofilm formation including *papG*, *fimH*, and *gapA* was examined in a PCR mixture containing 1µl of template DNA 50-100ng/ml, 1µl of Forward, and Reverse primers [Bioneer; South Korea] 9.5 µM DDW and 12.5µl Red Master Mix2X (Ampliqon Company; Denmark) in a total volume of 25µl. Specific primers and PCR condition are shown in (Table 1).

Biofilm formation assay: According to O'Toole and Kolter protocol [15], the ability of biofilm production of UPEC isolates was checked. In this study, the *E. coli* ATCC 25922 strain was used as a positive control and *Pseudomonas aeruginosa* ATCC 27853 was used as a negative control. UPEC isolates were cultured in LB broth and incubated at 37°C overnight. Next, the bacterial isolates were diluted 1:100 with Tryptic Soy Broth (Merck; Germany); then, 200µl of suspension was added to 96-well microtiter plate (polyvinyl chloride) and incubated at 25°C. After 48h incubation, 150µl of 0.1 % (w/v) aqueous solution of crystal violet was added to each well, which was washed completely with double-

distilled water (DDW). After 15 min incubation, the excessive crystal violet dye was washed with DDW (3 times). After adding 200µl of 33% acetic acid to

each well, at optical density 590nm, the absorbance was measured by using enzyme-linked immunosorbent assay (ELISA) reader.

Table 1 The primers and their target genes used in this study

Primer	Sequence (5' → 3')	Product (bp)	Annealing (°C)	Reference
<i>papGF</i>	TACACAAAGCCTGACCATCG	84	57	This study
<i>papGR</i>	AGCGTTGCTGAACCAGATAG		57	
<i>fimHF</i>	AATGATGTGGTGGTGCCTAC	93	58	This study
<i>fimHR</i>	AGGAATTGGCACTGAACCAG		58	
<i>gapAF</i>	ACTTACGAGCAGATCAAAGC	170	55	[14]
<i>gapAR</i>	AGTTTCACGAAGTTGTCGTT		55	

Antimicrobial susceptibility of UPEC biofilm producer to nanocurcumin: Among 60 UPEC isolates, 5 isolates which produced strong biofilm, were chosen to be used in antimicrobial susceptibility testing. Antimicrobial susceptibility of nanocurcumin against UPEC isolate was performed via minimum inhibitory concentration (MIC) method. For this purpose, 100µl nanocurcumin 1024mg/ml was added to the first well; then, serial dilutions were prepared in 7 wells with concentrations of 512, 256, 128, 64, 32, 16, and 8µg/ml and two wells were negative (including culture media) and positive (including 10µl microbial suspension and 100µl culture media) control. In the following, the 10µl bacterial suspension, which contains 1.5×10^5 CFU/ml of cells was added to wells, which had serially different concentrations of nanocurcumin. At the end, the microplate was incubated for 24h at 37°C. In this assay, *Escherichia coli* ATCC25922 was used as a standard strain [16].

Real-time PCR analysis: The effect of nanocurcumin on the expression of the genes involving in biofilm formation of UPEC isolates including *papG*, *fimH*, and *gapA* was assessed by RT-qPCR method. The sub-MIC concentration was used for RNA extraction. For this purpose, the bacteria in the sub-mic well were collected and pelleted through centrifugation at $2500 \times g$ for 15 min. RNAs were extracted via the RNeasy Plus Mini Kit (Qiagen; USA) according to the protocol of kit.

Thereafter, the purity and quantity of RNAs were analyzed through a NanoDrop and a 260/280nm ratio between 1.9 and 2.1 considered as a high pure RNA. The extracted RNA was treated with DNase at 37°C for 1 h.

500ng-1µg RNA was converted into cDNA, using Easy cDNA reverse transcription (Parstous; Iran). The RT-qPCR was performed in a Rotor-Gene thermal cycler (Corbett 6000; Australia) by SYBR Green method (AccuPower Green Star qPCR Master Mix; Bioneer; Korea). A mixture volume of 20µl reaction containing 2µl of cDNA, 12.5µl SYBR Green master mix, 4.5µl nuclease-free water, and 1µl of each primer (5 pmol) was run according to the following program: an initial activation step at 94 °C for 4 minutes, 35 cycles of denaturation at 94°C for

30 s, annealing at (Table 1 shows annealing temperature for each primer) 30 s and extension at 72°C for 20 s. The *gapA* gene was also analyzed as an internal control to normalize target gene expression measurements [17].

Statistical Analysis: The data of this study were assessed, using SPSS version 19.0 for Windows (IBM, Chicago, IL, USA). A p-value<0.05 was considered as statistically significant.

Findings

Detection of *papG*, *fimH*, and *gapA* genes using PCR: Evaluation of the presence of *papG*, *fimH*, and *gapA* in the UPEC isolates showed that *papG* was seen in 57 (95%), *fimH* 58 (96.6%), and *gapA* 60 (100%) of the 60 UPEC isolates (Figure 1).

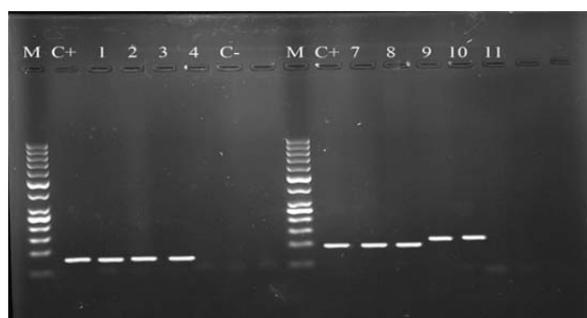


Figure 1 PCR result for *papG*, *fimH*, and *gapA* genes. *papG* gene results for UPEC isolates (1-4). M: marker, C+: positive control, C-: negative control. *fimH* gene result for UPEC isolates (7-9). M: marker, C+: positive control

Biofilm formation of UPEC isolate: In this study, 60 UPEC were isolated from inpatient and outpatients. The capacity of biofilm production of isolate according to previous studies was categorized in 4 classes. Among 60 UPEC isolates, 5 (8.03%) isolates produced strong biofilm, 12 (20%) isolates were moderate biofilm producers, 10 (16.6%) isolates were weak biofilm producers, and 33 (55%) isolates were non-biofilm producers. Among 27 biofilm producer isolates, 17 (62%) were isolated from pyelonephritis patients and 10 (37%) were isolated from cystitis patients. Strong biofilm producer was used for further analysis.

MIC: Minimum inhibitory concentration (MIC) of nanocurcumin was carried out for 5 isolates, which

produced strong biofilm. MIC of nanocurcumin against 4 isolates was 64µg/ml and 1 isolate was 128µg/ml.

Real-time PCR: The expression of genes involving in biofilm formation was examined by RT- qPCR method with the help of Rotor gene and Rest software. All sample were recovered from 64µg/ml for RNA extraction. The RT-qPCR result showed an increase in critical threshold in all of the samples treated with 64µg/ml concentration of nanocurcumin in comparison with an untreated sample. Analysis of the result of real-time PCR indicated that nanocurcumin remarkably decreased the 7 expression of *papG* and *fimH*.01, 8.01 folds, respectively.

Statistical analysis: In order to compare *papG* and *fimH* among the control and treatment groups, normalization of the data for *fimH* and *papG* in the treatment group was studied by Shapiro Wilk test [18]. The results showed normal distribution of the data (p>0.05), and one sample T-test was used to compare the treatment group and the control group since the control group is considered as constant one. Results were shown in (Table 2).

According to the results, difference between the expression levels of *papG* and *fimH* is significant among the control and treatment group (p<0.05; Diagram 1).

Table 2) The result of statistical analysis using T-test

Variables and Groups	Mean± SD	Test type	Results
<i>fimH</i>			
Treatment	0.12391±0.02731	T-test	T=-51.92; df=4; p< 0.001
control	1.00±0.00		
<i>papG</i>			
Treatment	0.142651±0.0369	T-test	T=-51.92; df=4; p<0.001
control	1.00±0.00		

*Total number of samples with treatment equals 5.

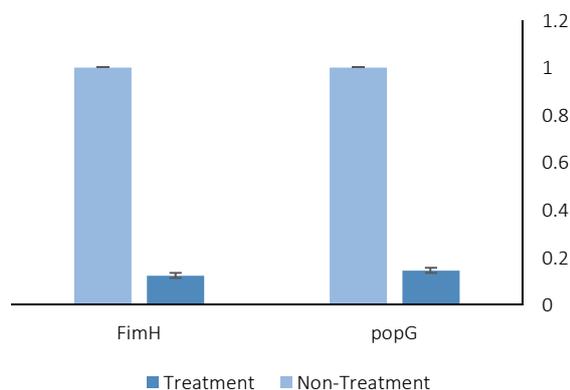


Diagram 1) Histogram of *fimH* and *PapG* expression levels in treatment and non-treatment groups

Discussion

UTI is one of the most infectious diseases influencing both outpatients and inpatients at all ages [19]. 80% of UTIs are caused by UPEC. UPEC potently creates a

complex structure containing multicellular communities known as a biofilm [20].

This structure can protect bacteria against two of the most important tools to combat bacterial infection, host immune response, and antibiotic therapy [1]. Therefore, biofilm formation can cause the increasing rate of antibiotic resistance among the UPEC isolates and will complicate their therapeutic management in the future [21]. Recently, nanotechnology is considered as a beneficial technology in nano-antibiotics field. Nanotechnology improves the scale and size of the particles. The ratio of surface to the volume of nanoparticles is high and the particle in nanoscales has better efficacy compared to its larger scales [22]. Curcumin nanoparticles are the product of nanotechnology, which remarkably showed antimicrobial features.

Type 1 fimbriae are one of the most important virulence factors of UPEC. Type 1-mediated adherence has been believed to play a pivotal role in the formation of biofilm, induction of inflammation, and enhancing *E. coli* virulence for the urinary tract. P fimbriae, known to participate in pathogenesis by developing colonization of bacteria and by provoking an injurious host inflammatory response [23]. Since the attachment is the first phase of biofilm formation, and *fimH* and *papG* genes are one of the most important genes involved in bacterial attachment to the surface, *E. coli* isolates were examined for *papG* and *fimH* genotype and specific primers were used to detect them.

In this study, the prevalence of *fimH* and *papG* genes that are responsible for the adhesion of bacteria was 95% and 96.6%, respectively. We found high prevalence of *fimH* and *papG* genes in our isolates. The results of this study for the prevalence of *fimH* gene was different with the studies conducted by Derakhshandeh *et al.* in Shiraz, Bahalo *et al.* in Shahrekord and Mahdikhani *et al.* in Karaj, which reported a prevalence of *fimH* gene 34.1%, 30%, and 33.3%, respectively [24-26].

The reason for the difference in the frequency of *fimH* gene in *E. coli* isolates in this study with other studies can be due to differences in geographical area, type, method of sampling, number of samples, and especially genetic variation in the phylogenetic group of isolates. The results of this study are consistent with the results of a study conducted by Kaczmarek *et al* [27].

In Poland, Tarchouna *et al.*, in Tunisia, Momtaz *et al.*, in Tehran, Karimian *et al.*, and in Tonekabon, Arabi *et al.* reported the prevalence of *fimH* gene (more than 68%) [28-31]. Among the acute factors in the pathogens of *E. coli* isolated from the cases of UTI, fimbriae are of particular importance. P fimbriae is one of the mannose-resistant fimbriae, which has had the greatest role in the development of UTI. Considering the presence of this fimbria as one of the most important factors in manifestation UTI, there are many reports in various countries [32]. A

study conducted by Agarwal *et al.* in 2013 showed the prevalence of *papG* 90% [33]. In the study conducted by Tiba *et al.*, the prevalence of *papG* was reported 40% [34]. The results of their study were consistent with ours.

This result confirms the incidence presented in the studies, where *fimH* was found in associations with P fimbriae UPEC.

In comparison with other antimicrobial agents, nanoparticles have the lowest toxicity to the ecosystem; thus, they can be a wise choice to use against pathogens. Curcumin is a naturally occurring yellow pigment known as turmeric, which many studies have proven its potentially antimicrobial activity against gram positive and negative bacteria [35, 36].

This study showed that antimicrobial resistant UPEC strains were highly sensitive to curcumin nanoparticles. The MIC of curcumin in this study was 64µg/ml. However, in a study conducted by Gunes *et al.*, the amount of curcumin MIC against *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Escherichia coli*, *Staphylococcus aureus* was 175µg/ml, 129µg/ml, 219µg/ml, 217µg/ml, 163µg/ml, and 293µg/ml, respectively [37]. In another study, which was conducted by Kali *et al.*, the minimum inhibitory concentration of curcumin against *E. coli* biofilm producer strains were reported 117.4mg/l [38]. This suggests that the potential use of nanoparticle technology improves the properties of curcumin, so that the mic obtained in this study was much lower than the other studies mentioned above, indicating an increase in antibacterial properties of curcumin due to the use of nanoparticle technology. Biofilm formation of gram-negative bacteria is basically determined via a series of steps, including adhesion of bacteria to the surface, cellular aggregation, and the production of an extracellular matrix. Adhesion is the most important step, as without a successful adhesion to the surface biofilm will not be able to form. The successful adhesion to the surfaces mainly relies on the attendance of cell appendices, such as fimbriae, pili, and flagella. UPEC owns a mannose-specific *fimH* receptor on the tip of its type I pili and a galabiose specific PapG receptors on the tip of its p fimbria, which is responsible for the persistence of bacteria in target cells and also adhesion to host tissue surfaces [39]. Therefore, efforts to reduce the expression of these genes can be effective in counteracting the formation of biofilms in the UPEC isolates. In this study, we evaluated the effects of nano-curcumin on the expression *fimH* and *papG* genes. The results showed that nano-curcumin remarkably reduced the expression of *fimH* and *papG* genes to 7 and 8 fold, respectively.

Formation and development of biofilms are based on the signal-mediated QS system [40]. Thus, an interference with QS can prevent the biofilm development of uropathogenic bacterial. The result

of biofilm biomass assay showed a reduced production of biofilm biomass in uropathogens when treated with curcumin [41]. Curcumin not only decreased the biofilm biomass, but also decreased the microcolony formation. This compound has been shown previously to prevent the biofilm of PAO1 without affecting the planktonic growth [42]. Furthermore, as shown in this study, curcumin can reduce the attachment of bacteria to the surface by decreasing the expression of the genes, which are responsible for attachment as a first step of biofilm formation. So, this result indirectly confirmed that curcumin exerts its antibiofilm activities via the prevention of biofilm formation process itself rather than destroying bacteria [43].

Amalaradjou *et al.* have recently conducted a study, which showed that cinnamon essential oil reduces the expression of genes associated with bacterial adhesion and invasion (*fimH*, *sfaS* *papG*, and *focA*). They derived in that the reduction of the expression of the mentioned genes also reduces adhesion [44]. Shakerimoghaddam *et al.* evaluated the effect of ZnO nanoparticles on UPEC biofilm producer. The MIC concentration of ZnO was 1250µg/ml, while the minimum inhibitory concentration of nanocurcumin in this study was 64µg/ml. furthermore, the sub-mic concentration of ZnO decreased the expression of *fimH* to 4 fold [14], while in this study, the expression of *fimH* decreased to 8 fold. This nanocurcumin may be a better option to fight against biofilm producer bacteria than ZnO. Meanwhile, another study performed by Loo *et al.* which showed the combination therapy with ZnO and nanocurcumin, was the most potent to eliminate biofilm compared to mono-drug therapy [43].

The results of our study indicated that sub-MIC concentrations of nanocurcumin decrease the expression of *papG* and *fimH* genes, which in turn, leads to a reduction of biofilm formation; however, more studies are needed on adhesion of bacteria to surfaces and gene expression to distinguish whether nanocurcumin inhibit UPEC adhesion and biofilm formation.

Conclusion

The result of this study indicated that Sub-MIC concentrations of nanocurcumin significantly decrease the expression of *papG* and *fimH* genes in strong biofilm producer UPEC strains, not being able to prevent biofilm formation. Moreover, nanoparticles could be highly effective on reduction of biofilm formation and these compounds can be an option to fight against highly resistant bacteria.

Acknowledgements: The authors would like to extend their sincere appreciation to all the staff in microbiology Department of Iran University of Medical Science.

Ethical Permissions: Since we did not use any animal models and we used isolates, which were

previously obtained from clinical samples, we have no ethical code for our study.

Conflict of Interests: The authors declare that there is no conflict of interest.

Authors' Contribution: Shivaee A (First author), Original researcher/ Statistical analyst/ Discussion author (10%); Mirshekar M (Second author), Methodologist/ Original researcher/ Discussion author (10%); Sadeghi Kalani B (Third author), Introduction author/ Methodologist/ Original researcher/ Discussion author (25%); Bordbar D (Fourth author), Introduction author/ Original researcher (25%); Ohadi E (Fifth author), Original researcher/ Discussion author (10%); Masjedani Jazi F (Sixth author), Statistical analyst/ Discussion author (20%).

Funding: This research was supported by Iran University of Medical Science.

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