

Original Article



In vitro effect of iron chelators on the growth of *Escherichia coli*, *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Yersinia enterocolitica*, and *Pseudomonas aeruginosa* Strains

Kiavash Fekri¹, Abolfazl Khoshdel^{2*}, Maryam Rasoulynezhad³, Soleiman Kheiri⁴, Afsaneh Malekpour⁵, Behnam Zamanzad⁶

¹Assistant Professor, Department of Pediatrics, Shahrekord University of Medical Sciences, Shahrekord, Iran

²Professor, Clinical Biochemistry Research Center, Shahrekord University of Medical Sciences, Shahrekord, Iran

³MD, Shahrekord University of Medical Sciences, Shahrekord, Iran

⁴Associate Professor, Clinical Biochemistry Research Center, Shahrekord University of Medical Sciences, Shahrekord, Iran

⁵Assistant Professor, Department of Community Medicine, Shahrekord University of Medical Sciences, Shahrekord, Iran

⁶Assistant Professor, Department of Microbiology and Immunology, Shahrekord University of Medical Sciences, Shahrekord, Iran

*Corresponding Author: Abolfazl Khoshdel Department of Pediatrics, Shahrekord University of Medical Sciences, Shahrekord, Iran Tel./Fax: +98 383 3338891 Email: nikakhosh@gmail.com

Abstract

Background and aims:Thalassemia patients are predisposed to septicemia due to iron overload. The iron chelators have a potential role in the treatment of septicemia. This study was conducted to investigate the in vitro effect of iron chelators, deferoxamine (DFO), deferiprone (DFP), and deferasirox (DFS) on the growth of effective bacteria (*Staphylococcus aureus*, *Yersinia enterocolitica*, and *Pseudomonas aeruginosa*) and their synergistic effect with each other and antibiotics.

Materials and Methods: Five bacterial strains were incubated in the presence of seven chelators in Mueller Hinton agar and the number of colonies was recorded for each sample. Antibiotic discs were placed in the plates. Susceptibility to antibiotics was assessed by disc diffusion method (Kirby-Bauer test) and also the zone inhibition around the disc was measured in mm after incubating the relevant plate at 37°C for a day.

Results: The growth of all the studied bacteria increased in the presence of iron and was inhibited in the presence of different chelators. Chelators and antibiotics had synergistic effects on growth inhibition of *Escherichia coli*, *S. aureus*, and *P. aeruginosa* in culture medium, while the growth inhibition of *Y. enterocolitica* was seen in the presence of deferiprone and deferasirox combination.

Conclusion: Chelators in combination with antibiotics exert synergistic effects on growth inhibition of prevalent bacteria (except for *Y. enterocolitica*).

Keywords: *E. coli*, *S. epidermidis*, *S. aureus*, *Y. enterocolitica*, *P. aeruginosa*, Thalassemia, Iron chelators

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Introduction

Thalassemia is a genetic blood disorder which is characterized by abnormality in the synthesis of hemoglobin beta chain and ranges from severe to asymptomatic anemia (1). The prevalence of thalassemia varies worldwide. In Iran, 8000 pregnancies per year are estimated to be at risk of having babies with thalassemia (2). Major Thalassemia is considered as a severe form of thalassemia which is manifested within the first two years of life and the affected patients should regularly undergo red blood cell transfusion (1). Blood transfusion in these patients leads to complications of iron overload with high morbidity and mortality if left untreated (3, 4).

Iron overload in thalassemia patients could cause bone

disorders, endocrine gland disorders (delayed growth, sexual maturation disorders, diabetes mellitus, and failure of thyroid, parathyroid, and adrenal glands), heart problems, and cirrhosis (1,5,6). One of the conditions that could cause death of thalassemia patients is infectious diseases and subsequent septicemia (7-9). In these patients, increase in blood ferritin, repeated venous catheterizations, and splenectomy are considered as major risk factors for septicemia. In addition, these patients are predisposed to infection with gram-positive and -negative bacteria (10, 11).

Although thalassemia declines the quality of life among the affected patients (12), the treatment with different iron chelators enhances life expectancy, decreases iron

overload-associated complications, and promotes health in them (13,4). Some studies have indicated that iron chelators have a potential role in the treatment of septicemia (15). Although iron overload in thalassemia patients is an independent predictive factor for infection, some investigations have demonstrated that bacterial response to iron overload in the body could vary. Accordingly, iron overload causes an increase in the growth of some bacteria and a decrease in the growth of some others. In addition, the type of chelators could contribute greatly to bacterial growth (16,17). It is necessary to investigate new classes of iron chelators recently developed for the treatment of various diseases including thalassemia. It is because their antibacterial activities may be greater than their chelation-related ones (18).

The findings on the potential viability of iron chelators as therapeutic agents against various bacteria have been inconsistent. Moreover, as bacterial resistance against antibiotics has increased and the synergistic effect of iron chelators and antibiotics has not yet been investigated, this study is conducted to investigate the in vitro effect of iron chelators, deferoxamine (DFO) (Desferal), deferiprone (DFP), and deferasirox (DFS) on the growth of the effective bacteria and their synergistic effect alongside each other and antibiotics in thalassemia patients with septicemia (*Escherichia coli*, *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Yersinia enterocolitica*, and *Pseudomonas aeruginosa*).

Materials and Methods

In this experimental laboratory study, five bacterial samples including *E. coli* ATCC: 27922, *S. aureus* ATCC: 27923, *S. epidermidis* ATCC 12228, *Y. enterocolitica* ATCC 33669, and *P. aeruginosa* ATCC: 27853 were obtained from Scientific and Industrial Research Co, Tehran, Iran. Then, the samples were cultured on iron-containing and non-iron-

containing media using standard bacteriologic methods (19). Iron-containing media were divided into two groups. Then, two sets of chelators were individually added as follows: Group 1 without chelators and group 2 containing 3 plates with only one chelator including DFO, DFS, and DFP (Iran Daru Co), as well as 4 plates with a combination of Desferal + DFS, Desferal + DFP, DFP+DFS and Desferal + DFP + DFS. Then, the sensitivity of cultured bacteria to some common antibiotics in iron-containing media with and without chelators was consequently detected (Figures 1, 2). The details of our procedures were as follows: For the samples with iron, 0.025 g ferric iron chloride (equivalent to 150 μ M ferric chloride) was mixed with Mueller-Hinton agar (Merck), left at 121°C, and autoclaved for 15 minutes. After the temperature declined to 50°C, the chelators of interest at 100 μ M/L concentration were introduced into the medium of interest per molecular mass (g) of each chelator (20,21). Therefore, 0.56 g/L DFO, 0.37 g/L DFS, and 0.14 g/L DFP were added to the culture media.

Bacterial samples in the tubes containing saline and 0.5 McFarland standard media were separated using 10 μ L sampler while being adjacent to flame, cultured in the plates containing 20 cc Mueller-Hinton agar, and incubated at 37°C overnight. The bacterial growth was measured by the number of developed colonies and reported in CFU/mL (19).

Then, the five bacteria (*E. coli*, *S. epidermidis*, *S. aureus*, *Y. enterocolitica*, and *P. aeruginosa*) were inoculated on the plates containing Mueller-Hinton base medium using sterile swab and incubated at 37°C overnight. The bacterial growth was reported in CFU/mL.

In the plates containing Mueller-Hinton base medium and iron, the effective antibiotic discs (Gen Fanavaran Tehran, Iran) at doses of interest were placed (22). In such a way, 30 μ g vancomycin disc (Biomerieux, Sweden) could be used for *S. aureus*, 30 μ g cefazoline disc for *S.*

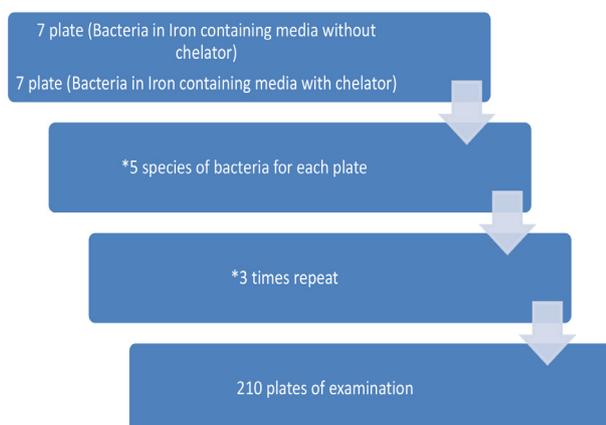


Figure 1. Flowchart of Study Design

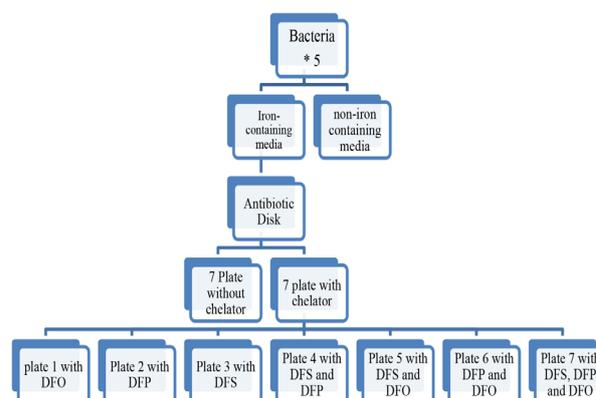


Figure 2. Antibiotic Disc Diffusion (DFO, DFS and DFP). Abbreviations: DFO, deferoxamine; DFS, deferasirox; DFP, deferiprone.

epidermidis, 10 µg gentamicin disc for *Y. enterocolitica*, 10 µg gentamicin disc for *E. coli*, and 30 µg amikacin as well as 30 µg ceftazidime discs separately for *P. aeruginosa*. The zone of growth inhibition (in mm) was recorded after the incubation of the relevant plate at 37°C overnight (19).

Afterwards, the bacteria and the Mueller-Hinton base medium alongside the iron were added to the culture media and the chelators, both as single and combined, which were also added to antibiotic discs incubated at 37°C overnight. The zone of growth inhibition was reported in mm and classified as susceptible, intermediate, and resistant with reference to the Clinical and Laboratory Standards Institute (CLSI) (22) (Table 1).

Results

The growth of all bacteria increased in Mueller-Hinton agar in the presence of iron. The growth of *E. coli*, *S. aureus*, *S. epidermidis*, *Y. enterocolitica*, and *P. aeruginosa* increased in the plate containing Mueller-Hinton agar and antibiotic disc with addition of iron (Table 2). Overall, the growth of all bacteria was inhibited in the presence of the chelators. The growth inhibition was found different only for *E. coli*. In the presence of various chelators (both alone and combined), the growth of *E. coli* (in CFU) was inhibited differently. The lowest positive effect of the chelators on *E. coli* growth inhibition was derived for DFS+DFP as compared with others (Table 2). No difference was seen in the number of grown colonies of *E. coli* between the plates with chelators and the ones without them. In addition, increase in the zone of growth inhibition of *E. coli* was seen in the presence of the antibiotics and chelators, that is, they had a synergistic effect on the growth inhibition of *E. coli* (Table 2).

According to the guidelines of CLSI, *E. coli* was susceptible to gentamicin with 14-mm zone of growth inhibition. In the presence of various chelators (both single and combined), *E. coli* showed high susceptibility to gentamicin. The growth of *S. aureus* was inhibited in the presence of different chelators, with no difference in inhibition among the chelators (Table 2). No difference was

seen in the number of grown colonies of *S. aureus* between the plates with chelators and the ones without them. The increase in the zone of growth inhibition of *S. aureus* was seen in presence of antibiotics and chelators (Table 2). Based on CLSI guidelines, *S. aureus* was susceptible to the used antibiotic with 13-mm zone of growth inhibition. In the presence of various chelators and antibiotics (both single and combined) in the culture medium, the zone of growth inhibition increased.

The growth of *S. epidermidis* was inhibited in presence of different chelators, with no significant difference in inhibition among the chelators (Table 2). No difference was seen in the number of grown colonies of *S. epidermidis* between the plates with chelators and the ones without them. The increase in the zone of growth inhibition of *S. epidermidis* means the inhibition of bacterial growth in presence of chelators and antibiotics (Table 2).

According to CLSI guidelines, *S. epidermidis* was susceptible to cefazolin with 18-mm zone of growth inhibition. In the presence of various chelators (single and combined), the zone of growth inhibition increased, and therefore *S. epidermidis* growth was inhibited. The growth of *Y. enterocolitica* was inhibited in the presence of different chelators, with the highest inhibition in the presence of DFP and DFS (Table 2). No difference was seen in the number of grown colonies of *Y. enterocolitica* between the plates containing chelators and the ones without them.

The growth of *Y. enterocolitica* was inhibited mostly in the presence of DFP+DFS+ gentamicin (the highest zone of growth inhibition). The growth of *Y. enterocolitica* did not change in the presence of DFS and gentamicin disc, and the zone of growth inhibition of *Y. enterocolitica* decreased in the presence of gentamicin and other chelators (Desferal, DFP, Desferal + DFS, Desferal + DFP, and Desferal + DFP+DFS), which represents the increased growth of *Y. enterocolitica* (Table 2).

According to CLSI, *Y. enterocolitica* was susceptible to gentamicin with 25-mm zone of growth inhibition. In the presence of various chelators and antibiotics (both single and combined), the zone of growth inhibition increased, and therefore *Y. enterocolitica* growth was inhibited (Table 2). No difference was seen in the number of grown colonies of *S. epidermidis* between the plates with chelators and the ones without them. The growth of *P. aeruginosa* was inhibited in the presence of chelators (except for DFP) and amikacin, which was illustrated as increase in zone of growth inhibition in Table 2. The zone of growth inhibition or inhibition of *P. aeruginosa* growth was highest in the presence of various combinations of chelators. Based on CLSI, *P. aeruginosa* was susceptible to the used antibiotic with 30-mm zone of growth inhibition. In the presence of various chelators (both single and combined), the zone of growth inhibition increased, and therefore *P. aeruginosa* growth was inhibited in the plate with the antibiotic, chelator, and iron.

Table 1. Susceptibility of bacteria to antibiotics by zone of growth inhibition

Bacteria and Antibiotic	Resistance	Intermediate	Susceptible
<i>Escherichia coli</i> -gentamicin	12 ≤	13-14	15 ≥
<i>Staphylococcus aureus</i> -vancomycin	6 ≤	6-7	7 ≥
<i>Staphylococcus epidermidis</i> -cefazoline	14 ≤	15-17	18 ≥
<i>Yersinia enterocolitica</i> -gentamicin	12 ≤	13-14	15 ≥
<i>Pseudomonas aeruginosa</i> -amikacin	14 ≤	15-16	17 ≥
<i>Pseudomonas aeruginosa</i> -ceftazidime	14 ≤	15-17	17 ≥

Table 2. Comparison of number of colony-forming units and zone of growth inhibition for different bacteria in medium with and without iron

	Without iron		With iron	
	Bacterial growth (CFU/mL)	Zone of growth inhibition with antibiotic (mm)	Bacterial growth (CFU/mL)	Zone of growth inhibition with antibiotic (mm)
<i>Escherichia coli</i>	30×10 ³	20	45×10 ³	14
<i>Staphylococcus aureus</i>	24×10 ³	15	32×10 ³	13
<i>Staphylococcus epidermidis</i>	2.3×10 ⁴	30 ^a	3×10 ⁴	25 ^a
<i>Yersinia enterocolitica</i>	9×10 ³	28	14.5×10 ³	25
<i>Pseudomonas aeruginosa</i> (with amikacin)	18×10 ³	31 ^a	23×10 ³	30 ^a
<i>Pseudomonas aeruginosa</i> (with ceftazidime)	18×10 ³	32 ^a	23×10 ³	28 ^a

^a susceptible (according to the Clinical and Laboratory Standards Institute).

Pseudomonas aeruginosa zone of growth inhibition increased in the presence of ceftazidime and different chelators, with the highest increase in the presence of Desferal and the lowest zone of growth inhibition or the highest bacterial growth in the presence of Desferal + DFS (Table 2). Based on CLSI, *P. aeruginosa* was susceptible to ceftazidime with 28-mm zone of growth inhibition. In the presence of various chelators plus ceftazidime, the zone of growth inhibition increased, and therefore bacterial growth was inhibited.

Discussion

In the present study, the growth of all bacteria in the culture medium and also antibiotic disc increased as iron was introduced. Clearly, studies have demonstrated that many pathogenic bacteria need external sources of iron for growth and survival (23-30). Iron as a cofactor contributes mainly to many of the reactions of oxygen transmission, metabolism, and production of energy and nucleic acid. Therefore, the growth of bacteria is likely to increase as iron is added. This study indicated that the growth of all bacteria was inhibited in the presence of different chelators, but the inhibition was different only for *E. coli*.

Thompson et al conducted a study on the antibacterial effect of iron chelators on nosocomial pathogens and demonstrated that DFP in tissue culture medium inhibited the growth of *E. coli* and other bacteria under study, while DFO did not do so (26). Because DFO is likely to provide iron to the bacteria by the receptor of their cognate siderophore, these findings are predictable. On the other hand, other mutant species of bacteria need 0.05 mm iron for growth (31), and therefore resistant species should be further studied.

For *S. aureus*, the growth was inhibited in the presence of various chelators in the present study. In a study on the effect of DFP on in vitro growth of staphylococci, the growth of *S. aureus* was inhibited in the presence of

DFP, which could be considered for the patients with iron overload (32). The results of the study conducted by Thompson et al were consistent with the ones of the present study. Moreover, DFO had no inhibitory effect on *S. aureus* growth because it facilitates the transmission of iron for bacterial growth (26). *S. aureus* is actively involved in the absorption of iron in iron-limited environments by producing siderophore (33), which probably leads to inconsistency of the response to DFO. The findings of another study indicated that the reason for the increased growth of *S. aureus* in the presence of Desferal is the use of DFO by the bacteria in ATP-binding cassette transport system for further use of environmental iron (34).

For *S. epidermidis*, the growth was inhibited in the presence of various types of chelators, with no remarkable difference among them. In a study on the effects of DFP and DFO on the in vitro growth of various staphylococci, Kim and Shin demonstrated that *S. epidermidis* was not able to absorb iron and growth in the medium containing the transferrin-binding iron (non-free iron). Moreover, the growth of *S. epidermidis* was inhibited in the presence of Desferal. *S. epidermidis* also responded to DFP in the culture medium which acted as an inhibitory agent for its growth (32). In another study, the growth of *S. epidermidis* was inhibited in the presence of the chelator and the inhibitory effect was removed when iron was introduced (35). Anyway, *S. epidermidis* exhibits a better growth in the patients with increased blood iron (36), and therefore this finding could not be considered surprising. On the other hand, *S. epidermidis* could be susceptible to the chelator and gene variations probably have not yet been developed to give this property to *S. epidermidis*.

For *Y. enterocolitica*, the growth was inhibited when different types of chelators were added to Mueller Hinton agar and iron. In addition, *Y. enterocolitica* showed different responses to growth inhibition in different media.

The zone of growth inhibition of *Y. enterocolitica*

decreased in the presence of gentamicin and other chelators (Desferal, DFP, Desferal + DFS, Desferal + DFP, and Desferal + DFP + DFS). This means that the growth of *Y. enterocolitica* increased, which could be explained by the factors associated with doses of chelators, the type of antibiotic, or virulent properties of the used strain. DFP inhibited the growth of *Y. enterocolitica* by decreasing iron availability, which is consistent with other studies (35). The study conducted by Chan et al demonstrated that DFO could increase the risk of infection with *Y. enterocolitica*, while DFP did not contribute positively to both in vivo and *in vitro* growth of *Y. enterocolitica* (17). These findings on *Y. enterocolitica* in different media could be explained by the variety of doses of chelators and siderophore-producing property of *Y. enterocolitica* (37). Yersiniabactin is a siderophore which has been initially detached from *Y. enterocolitica*. In addition to having the ability to absorb iron, this siderophore can bind to copper to prevent bacterial death (38).

In the present study, the growth of *P. aeruginosa* was inhibited in the presence of chelators (except for DFP) and amikacin. The study conducted by van Asbeck et al also indicated that DFO exerted inhibitory effect on *P. aeruginosa* growth in the absence of iron (35). Moreover, the study conducted by Thompson et al indicated that DFP had an inhibitory effect on the growth of *P. aeruginosa*, but DFO did not (26). The siderophore in *P. aeruginosa*, called pyoverdine, could help to explain the difference in response to chelators (39).

In the present study, the growth of *E. coli*, *S. epidermidis*, *S. aureus*, and *P. aeruginosa* was inhibited in the presence of used antibiotic discs and different chelators. The growth of *Y. enterocolitica* was inhibited in the presence of gentamicin and combination of DFP and DFS. van Asbeck et al and Hartzen et al also found that DFO and antibiotics could have a synergistic effect on growth inhibition of bacteria (35,40). Notably, the *in vivo* combination of chelators and antibiotics should be used cautiously, which deserves detailed in-depth studies.

Overall, iron chelators prevent bacterial growth by preventing iron absorption, but the difference in the effect on different bacteria among the chelators could be due to the difference in the interaction of the type of siderophore in the cell wall, the type of chelating drug, and the mutant strains of the bacteria.

Conclusion

Due to the affinity of iron with various bacteria and the positive effect of chelators through the decrease in iron availability and the synergistic effect with antibiotics, it seems logical to recommend that chelators should be used for thalassemia patients with bacterial septicemia. However, as the growth of *Y. enterocolitica* increased in the presence of Desferal, DFP should be prescribed instead of Desferal. Future studies are recommended to investigate various

bacteria and antibiotic-resistant strains as well as chelators at various doses with regard to bacterial growth.

Conflict of Interests

The authors have no conflict of interests.

Ethical considerations

This project was approved by the Ethics Committee of Research and Technology Department, affiliated with Shahrekord University of Medical Sciences (IR.SKUMS.REC.1393.9.13).

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