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Research Article

The Effect of Nanochitosan Loaded with Antibiotics on Response of Helicobacter Pylori

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ABSTRACT

A study was conducted to identify the effect of the antibiotics tetracycline and ciprofloxacin as free and loaded on nanochitosan on *Helicobacter pylori* bacterium isolates causing acute and hemorrhagic stomach ulcer infection in human. Antibiotics sensitivity tests were done by discs and wells techniques. The virulence and growth of these isolates were measured by ammonia production and optical density (OD 575 nm) respectively, under the conditions of presence of free antibiotics and nanochitosan loaded antibiotics. Sensitivity tests showed that each isolates was sensitive to CIP (5µg) more than T (30µg) discs. Scanning electronic microscope images illustrated the formation of spherical structure of nanochitosan as free and loaded with antibiotics. Results of qualitative tests showed a duplication in inhibition zone in the presence of nanochitosan loaded with ciprofloxacin for each of the two isolates after 48 hours compared with free antibiotics. No negligible effect was found for free tetracycline and nanochitosan. Concentrations of produced ammonia were reduced to less than 5 µg/ml for nanochitosan loaded with ciprofloxacin for each of hemorrhage and acute isolates in comparison with a free ciprofloxacin (28.7 µg/ml). A clear change in the pH and color due to the effect of the antibiotic, nanochitosan and ammonia production.

Keywords : *Helicobacter pylori, Antibiotic sensitivity test, Ammonia, Nanochitosan*

الملخص

هدفت الدراسة إلى التعرف على تأثير المضادات الحيوية التتراسيكلينوالسيبروفلوكساسين الحرة والمحملة علىالكايتوسان النانوي على عزلات بكتيريا *Helicobacter pylori* التي تسبب الإصابة بقرحة المعدة الحادة والنزفية في الإنسان. وقد أجريت اختبارات الحساسية للمضادات الحيوية باستخدام تقنيات الأقراص والحفر. تم قياس ضراوة ونمو هذه العزلات بواسطة إنتاج الأمونيا والكثافة الضوئية(575 nm) على التوالي في وجود المضادات الحيوية الحرة والمحملة. بينت اختبارات أقراص الحساسية أن كلا العزلتان حساسة للمضاد السيبروفلوكساسين (CIP 5µg) أكثر من المضاد تيتراسيكلين (T 30 µg). أظهرت صور المجهرالماسحالإلكتروني نشوء هيكل كروي للكايتوسان الحر النانوي والمحمل بالمضادات الحيوية. أظهرت نتائج الاختبارات النوعيةعن مضاعفة هالةالتثبيط بوجودالكايتوسان النانوي المحمل بالسيبروفلوكساسين لكل العزلتين بعد 48 ساعة مقارنة بالمضادات الحيوية الحرة. لم يتم العثور على تأثير يذكر للتتراسايكلين والكايتوسان

النانوي الحر. انخفضت تركيزات الأمونيا المنتجة إلى أقل من 5 ميكروكرام/مل للكايتوسان النانوي المحمل بسيبروفلوكساسين لكل من العزلتين النزفية والحادة بالمقارنة مع السيبروفلوكساسين الحر (28.7ميكروكرام/مل). يعزى التغيير الواضح في درجة الحموضة ولون المزارع الى تأثير المضادات الحيوية،والكايتوسان النانوي و إنتاج الأمونيا.

الكلمات المفتاحية: الملوية البوابية، فحص الحساية للمضادات الحياتية، الامونيا، الكايتوسان النانوي

Introduction

Helicobacter pylori (*H. pylori*) is the bacteria responsible for stomach and duodenum ulcer in human. It was first discovered in the stomach of patients in 1982 by Marshall and Warren. *H. pylori* is a Gram- negative spiral bacteria measuring 2-4 µm in length, 0.5-1 µm in width and has 2-6 sheathed flagella 3 µm in length. *H. pylori* is a urease positive and has high activity. It is one of the most common bacterial pathogens that infects human around the worldwide which acquired in the early childhood and is carried throughout lifetime if not treated with antimicrobial agents [1].

There are three main gastric phenotypes have been identified. The cases vary between acute and chronic infection and can progress to gastric cancer due to its virulence factors especially the specific urease enzyme [2]. Long-term colonization with *H. pylori* significantly increase the risk of developing gastro-duodenal diseases, peptic ulcer disease, gastric adenocarcinoma and mucosa associated lymphoid tissue (MALT) lymphoma [3].

H. pylori has several adaptations for an acid environment of the stomach, one of them is urease which converts urea that physiologically present in the stomach into ammonia and bicarbonate [4]. *H. pylori* produces large amounts of urease and it has been estimated that up to 10% of the total protein content of *H. pylori* consists of urease[5]. Urease allows short-term survival in the highly acidic gastric lumen and motility is thought to allow rapid movement toward the more neutral pH of the gastric mucosa [6,7]. Ammonia plays a central role in pathogenesis and metabolism of *H. pylori* by contributes to epithelial cell damage and apoptosis [8].

Rapid urease tests enable convenient detection of *H pylori* infection within 3 hours in most cases, although the agar gel–based tests usually require 24 hours of incubation for maximal sensitivity and specificity [9]. Culture also provides a bacterial strain for use in epidemiologic studies to examine associations of virulence characteristics with disease outcome. However, bacterial culture for *H. pylori* is relatively expensive [10]. The phenate method is a very effective method for measuring ammonia concentration in the aqueous solution and in urea fast urea solution [11]. Tetracycline and ciprofloxacin are among antibiotics that inhibited the growth of *H. pylori*[11]. Chitosan was first discovered in 1811 by Henri Braconnot a French chemist and pharmacist, he observed that a certain substance (chitin) found in mushrooms did not dissolve in sulfuric acid [12]. Chitosan nanoparticles are formed spontaneously on the conjunction of polyanion such as tripolyphosphate (TPP) in chitosan solution under continuous stirring condition[13]. Polymeric nanoparticular drug delivery systems of carbohydrate origin such as chitosan have the advantages of cheaper cost, scalability, targeted delivery, biodegradability, biocompatibility, sustainability in release of encapsulated drug and improved efficacy [14]. In common with many cationic polymers, chitosan has obvious antimicrobial effects due to destabilization of the outer membrane of Gram-negative bacteria [15]. Drugs carried by chitosan nanoparticles can be released through degradation and corrosion of chitosan, leading to a clear sustained-release effect. Because of varied degradation rate and time of chitosan of different molecular weight and degree of deacetylation degree, different types of nanoparticles can be used to regulate drug-release rate [16].

Aim of study: The study was aimed to the early detection of *H. pylori* infections by using the growth, ammonia production relationship and treatment with the synthetic nanochitosan loaded with antibiotics.

Materials and methods

***H. pylori* isolates :**Two isolates of *H. pylori* were used in this study , the hemorrhage (H-1) and acute(A-1) according to the cases detection of infection by endoscopy. They activated and propagated in a modified Tryptic Soya Agar [11]. The cultures were Incubated anaerobicaly using gas generating kits at 37 ^{o}C.

Antibiotic sensitivity test: Cip and T are used in our experiment according to the result obtained by Sami *et al*.[11]. A quantify of 10 mg from each antibiotic was dissolved in 100 ml of DW. The final concentration of stock solution was 100 µg / ml. These stocks were prepared as required.

Determination of ammonia concentration: Phenate method [17]was used for ammonia detremination in fast urea solution[18] without phenol red. Standard curve of ammonia was prepared in a range of 0.0 -5µg/ml NH_3 – N and maximum absorption of blue color was observed at 630_{nm}. The color and pH of standard ammonia concentration were measured in fast urea solution with phenol red. Fast urea solution was prepared by filtration through Millipore filter (0.22µm).

Growth and ammonia production: Pure and active colonies of *H. pylori* (H-1 and A-1) were selected from modified TSB agar culture (after incubation for 24 hr at 37ºC under anaerobic condition) and resuspended in sterile normal saline. Suspension density was adjusted to 1 OD_{575nm}. Serial dilutions of suspension were prepared in range 0.1-1 OD using normal saline and inoculated in ten sterile screw cupped tubes contain 9.9 ml of the fast urea solution at pH 6.4 with and without phenol red.

Preparation of nanochitosan : It was prepared according to Ko *et al*.[19].The precipitate was lyophilized and stored in a refrigerator.

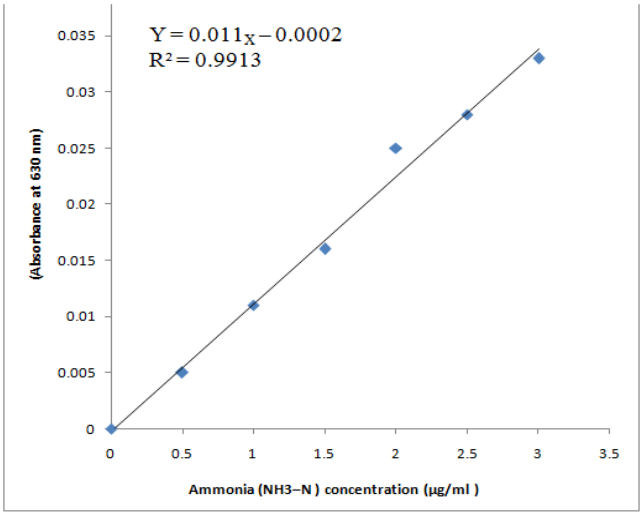
Antibiotic Loaded nanochitosan: Stock antibiotics solution was added to the solution of nanochitosan during the preparation of the later at a concentration ratio 10 : 1. The concentration of free antibiotics was used in qualitative test was 10µg / 0.2ml in agar medium and 10 µg / ml for quantitative test in fast urea solution. Free nanochitosan also added at concentration 1 µg / 0.2ml in the well and 1µg/ml in fast urea solution with and without phenol red. Stock solution of antibiotic loaded nanochitosan was prepared by dissolving appropriate weight of lyophilized loaded nanochitosan and used in the same concentration above.

Five treatments were used in qualitative and quantitative tests .These treatments nanochitosan stock solution**,** nanochitosan and ciprofloxacin, nanochitosan and tetracycline**,** free ciprofloxacin and tetracycline.. In quantitative test the incubation time was 2h. Ammonia,OD_{575nm} and pH were measured and observed in the fast urea solution without phenol red in addition to the color in fast urea solution with phenol red. Inoculum was 1% ml of active culture.

Results

Figures 1 shows the standard curve of ammonia prepared in fast urea solution without phenol red. Figure 2 shows the change in the color of standard curve solutions in presence of phenol red. The change in the color and pH values were increased with the increasing the concentration of ammonia in fast urea solution.

Figure 1: Standard curve of ammonia – nitrogen using phenate method [17].



Standard curve of ammonia – Nitrogen using phenate method.

Figure 2: Color and pH change of standard curve of ammonia in fast urea solution without

Color											
pH	6.4	6.7	7.01	7.25	7.34	7.47	7.53	7.56	7.6	7.62	7.7
Concentrations (µg/ml)	0	0.5	1	1.5	2	2.5	3	3.5	4	4.5	5

Color and pH change in the fast urea solution with phenol red containing ammonia concentration ranged from 0 to 5 µg/ml.

Ammonia production related to growth

Tables 1 and 2 illustrate the production of ammonia in fast urea solution without phenol red for A-1 and H-1 isolates of *H. pylori.* A-1 isolate was more active than H-1 isolate in production the ammonia during 2 hr of incubation. The OD was 0.03 for A-1 with the count of 3×10^4 CFU/ml meanwhile 0.08 and **8×10^4** CFU/ml for H-1, respectively.

OD_{575nm}*	CFU /ml	pH		Ammonia concentration (µg/ml)	
		After 2 h	After 4 h	After 2 h	After 4 h
0.01	1×10^4	7.17	>7.5	1.18	>5
0.02	2×10^4	7.28		2.36	
0.03	**3×10^4**	**7.31**		**3.7**	
0.04	4×10^4	7.02		1	
0.05	5×10^4	7.02		0.9	
0.06	6×10^4	7.0		0.81	
0.07	7×10^4	7.2		1.45	
0.08	8×10^4	7.15		1.18	
0.09	9×10^4	7.03		0.9	
0.1	1×10^5	7.38		4	

* At zero time /1ml of the culture.

Table 1: Ammonia production by acute isolate A-1 related with growth in fast urea solution.

OD_{575nm}*	CFU /ml	pH		Ammonia concentration (µg/ml)	
		After 2h	After 4h	After 2 h	After 4 h
0.01	1×10^4	7.0	> 7.5	0.45	> 5
0.02	2×10^4	7.09		1.18	
0.03	3×10^4	7.09		1.27	
0.04	4×10^4	7.19		1.63	
0.05	5×10^4	7.32		2.18	
0.06	6×10^4	7.35		2.27	
0.07	7×10^4	7.39		2.72	
0.08	**8×10^4**	**7.4**		**3.45**	
0.09	9×10^4	7.41		3.45	
0.1	1×10^4	7.1		-1	

* At zero time

Table 2: Ammonia production by hemorrhage isolate H-1 in relation with growth in fast urea solution.*

Nanochitosan synthesis and loaded with antibiotics

Figure (3a, b, c) illustrates the SEM images of chitosan, nanochitosan and antibiotic loaded nanchitosan respectively. Chitosan appeared as hairy smooth structure but after treatment with TPP, it became nano sphere sutructurs (figure-3b) particles according to the method of preparation by [19]. Antibiotic stock solution was added and loaded within and on the surface of nanochitosan as shown in figure-3c.The measuring unit was different in figure 3 according to the size of particles and for 3a,b and c are 40, 5 and 10µm respectively.

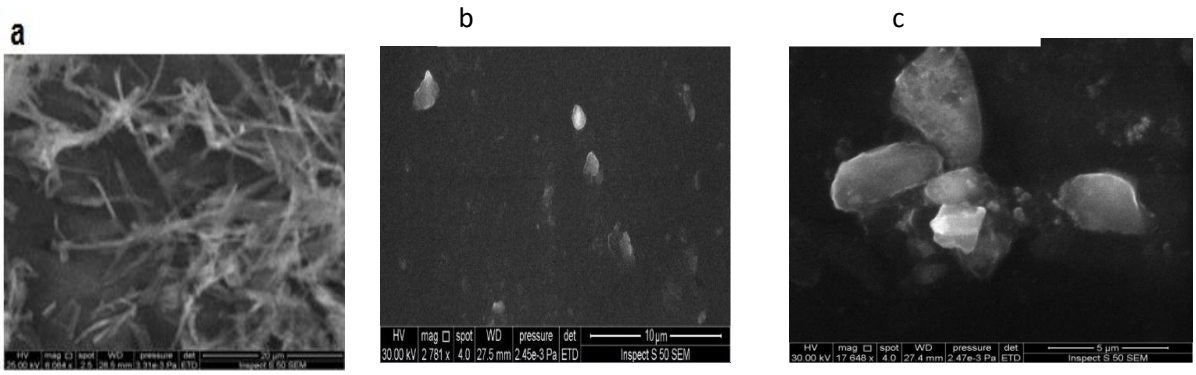


Figure 3: SEM image.(a) Chitosan structur,(b) Nano-chitosan, (c) antibiotic loadedon nano-chitosan.

Quantify of antibiotics used in loading was 10µg/ml which equal to the mean concentration used by human has average weight 75 kg.

Qualitative test: Figure (4) and Tables (3, 4) show the qualitative test of treatments (1: Chitosan, 2:Nanochitosan, 3: NanoCHI + CIP, 4: NanoCHI + T, 5: T, 6: CIP) on sensitivity of *H. pylori* isolates A-1 and H-1. No effect of free chitosan and nanochitisan on A-1 and H-1 growth after 24 and 48 h of incubation was found. H-1 isolate was more sensitive to ciprofloxacin loaded on nanochitosan compared with isolate A-1 and this isolate more resistant to the free antibiotics.

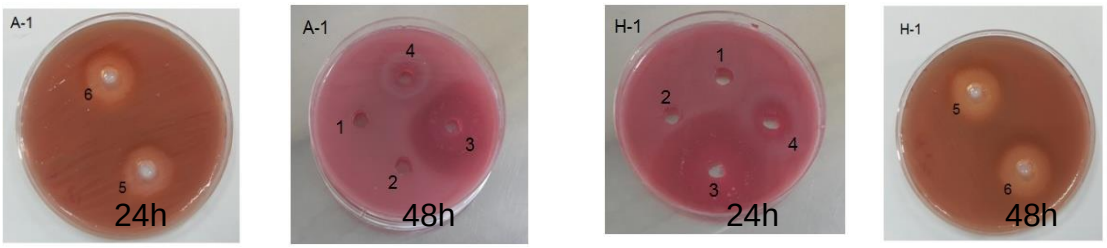


Figure 4: Inhibition zones for tetracycline and ciprofloxacin loaded on nanochitosan for A-1 and H-1 isolates. 1: Chitosan, 2: Nanochitosan, 3: NanoCHI + CIP, 4: NanoCHI + T, 5: T, 6: CIP.

Treatment	Inhibition zone (mm)	
	After 24h	After 48 h
Chitosan	0.0	0.0
Nano CHI	0.0	0.0
NanoCHI + CIP	**30**	**48**
Nano CHI+T	18	24
T	17	17
CIP	15	15

Table 3: Effect of loaded nanochitosan

Treatment	Inhibition zone (mm)	
	After 24 h	After 48 h
Chitosan	0.0	0.0
Nano CHI	0.0	0.0
NanoCHI + CIP	**28**	**42**
Nano CHI+T	15	18
T	18	18
CIP	18	19

Table 4: Effect of loaded nanochitosan on *H.pylori* isolate(H-1).

on *H. pylori* isolate(A-1).

Treatment	Ammonia- N production by isolates (µg/ml)	
	(A-1)	(H-1)
Nano-CHI	33.2	73
Nano-CHI +CIP	5.2	5.3
Nano-CHI +T	20.7	32
CIP	28.7	35.2
T	44.3	40.7

Table 5: Ammonia production by *H. pylori* isolates.

Quantitative test confirmed the results of qualitative test above (Table 5). Tetracycline loaded on nanochitosan was less effective than nano-CHI+CIP but higher than T alone. The most effective treatment in reducing the ammonia production was the Nano-CHI+cipCIP. The high concentrations of ammonia appeared in treatment of Nano-CHI as shown in table (5).

Discussion

Figures 1 and 2 revealed the ability to prepare the standard curve of ammonia in fast urea solution in presences ammonia and also to determine the virulence and severity of infection by *H. pylori* during two hours of culturing the biopsy in fast urea solution through the change in color and pH in comparison with standard. Standard methods[17] not included the determination of ammonia in presence of high concentration of urea and to the change in color.

The results in tables 1 and 2 indicated that the A-1 isolate was more virulence than H-1 in production of ammonia during 2 h. No dilution was used for the cultures to measure the

concentration of ammonia for the reason of simulation the real test of fast urea in the hospital. The virulence of A-1 isolate may due to the high acidity of stomach before bacterial colonized the groves of lining layer to protect themselves and the experiment was performed *in vitro.*

SEM images of chitosan, nanochitosan and antibiotic loaded nanchitosan (figure 3a, b, c, respectively) were illustrated the fragmenation of chitosan by TPP treatment to reach the nanoscale according to the method of preparation by [19]. Chitosan appeared as hairy smooth structure and became nano sphere sutructurs (figure-3b) particles after TPP treatment. After antibiotic stock solution was added and loaded within and on and within the nanochitosan, the shape of particle and size were changed (Figure-3c).Quantify of antibiotics used in loading was 10µg/ml which equal to the dose taken by human has average weight 75 kg.

Qualitative test of the effect the chitosan and nanochitosan treatments on sensitivity of *H. pylori* isolates A-1 and H-1 as shown in figure (4) and Tables (3, 4) showed no effect of free chitosan and nanochitisan on A-1 and H-1 growth after 24 and 48 h of incubation and this is disagrees with the results of Li *et al*.[15]. H-1 isolate was more sensitive to ciprofloxacin loaded on nanochitosan compared with isolate A-1 and this isolate is more resistant to the free antibiotics. In general, it was found the ability of nanochitosan to increase efficiency of antibiotics and maintaining their activity by the hypothesis of slow releasing the antibiotic in the medium or to the specific interactions between nanochitosan and same antibiotics groups.

Quantitative test confirmed the results of qualitative test above (Table 5). It was concluded from the values that the least amounts of ammonia production (5.2 µg/ml) were obtained in Nano-CHI+CIP treatment for (A-1) and (H-1) isolates. High concentrations of ammonia appeared in treatment Nano-CHI in table (5) may be returned to function of nanochitosan as carbon and phosphorous supplement when present as alone in fast urea solution. The low concentration of ammonia produced by isolates may be also expressed asthe interpretation to the synergistic inhibition effect of nano-CHI+CIP on urease activity.

Conclusion

The relationship between the production of ammonia and change the color of phenol red in the cultures of *H. pylori* were guide to the case and virulence of infection. Results of the use chitosan and nanochitosan had no effect on the sensitivity of bacteria but when nanochitosan was loaded with CIP it turns into very inhibition compared with Tetracycline. The results of qualitative and quantitative tests were indicated to the high inhibitory effect of Nano-CHI+CIP on growth of acute and hemorrhage isolates of *H. pylori* and reducing the ammonia production to the lower concentrations compared with another treatments.

Recommendation

Application of the results *in vivo* by using laboratory animals and analytical study for the structure and interaction of nanochitosan with ciprofloxacin.

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