The antibacterial effect of acidified nitrite on uropathogenic Escherichia coli: In vitro study

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ABSTRACT

Background and Objectives: Urinary acidification induces strong antibacterial effect against different bacterial types involved in urinary tract infections. The aim of this study is to assess in vitro determination of the minimal inhibitory concentration of nitrite alone and acidified nitrite using addition of ascorbic acid in infected urine with different pH of the bacterial media.

Methods: E.coli bacteria have been isolated from the urine of patients with urinary tract infection. Identification of E. coli was achieved using biochemical tests. Determination of bacteriostatic activity of acidified sodium nitrite was carried out using 96- wells microtiter plate. Acidification effect against UTI bacteria was assessed using sequential steps; feeding bacteria with NaNo3, then transferring them to acidified urine after incubation using ascorbic acid.

Results: Ascorbic acid (40 mM) alone and sodium nitrite (200 mM) alone are considered to be a weak antibacterial agents on uropathgenic *E. coli*, while mixing 10 mM ascorbic acid with 625 µM sodium nitrite at pH 5 became a strong antibacterial agent against 14 isolates out of 32. Bacterial death can occur by sequential steps, first feeding bacteria with sodium nitrate and after incubation, transferring to the acidified urine at pH 5 and pH4.6 causing bacterial death.

Conclusions: Strong antibacterial agent can be formed in acidified urine containing nitrite. This antibacterial agent is strongly pH and nitrite dependent and is increased by addition of ascorbic acid. The antibacterial strength is a nitrite-dose dependent in mildly acidified urine.

Key words: Urine acidification, Antibacterial agent, Nitrite. .

INTRODUCTION:

Urinary tract infection (UTI) is one of the most common bacterial infections among human, especially in women [1]. A growing bacterial resistance against several antibiotics used in the treatment of UTIs has been described [2]. Urinary acidification has long been used for treating UTI with the use of various agents to lower urinary pH [3]. Research has shown that urinary acidification with ascorbic acid induce a strong antibacterial agent against *Pesudomonas, E.coli and staphylococcus* [4]. Nitrate is a natural component of many foods, particularly vegetables, fruits, and meats. Dietary nitrate is absorbed in the intestine, transported in the blood, actively concentrated in the salivary glands, and then reduced to nitrite by oral nitrate-reducing bacteria [5]. Studies showed that the activity of stomach acid against food-borne pathogens might be increased by up to 100-fold by physiological salivary concentrations of nitrite. A variety of reactive nitrogen compounds are formed from nitrite in acidic conditions and have been proposed to be the chemical species responsible for killing bacteria in normal human stomach. These compounds include nitrous acid, peroxynitrite, nitrogen dioxide, and, most frequently, nitric oxide (NO). An increase in NO generation would be expected to augment the antimicrobial

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of acidified nitrite. Ascorbic acid (vitamin C) has been shown to increase the formation of NO from nitrite [3, 4, 6].The main problem with UTI that should be taken into consideration is related to the emergence of new strains of pathogens having multi-drug resistance [2]. Nowadays physicians are treating UTI using high doses of antibiotic for long duration, which may impair renal function, particularly in old aged patients.

Research is scant on the use of alternatives to antibiotic for treatment of UTI [7]. This study was designed to assess in vitro determination of the minimal inhibitory concentration of nitrite alone and acidified nitrite with different pH of infected urine. More specifically, this study is aimed at studying the effect of acidified nitrite and vitamin C to inhibit bacterial growth and to evaluate in vitro reduction of nitrate in urine to nitrite using *E.coli* ATCC 25922, followed by acidification of urine to investigate the antibacterial effect.

MATERIALS AND METHODS:

Bacterial collection Thirty two *E.coli* bacteria have been isolated from the urine of patients with UTI attending Rizgari Hospital. Identification of *E. coli* was assessed using biochemical tests.

Minimal Inhibatory Concentration (MIC) of Sodium Nitrite Saginur and Gavan & Town methods were used by 96-wells microtiter plate for determination of bacteriostatic activity of acidified sodium nitrite[8, 9]. The bacteriostatic activity of acidified sodium nitrite was determined on disposable, flat-bottom microwell plates (96 wells, each with a volume of 300µl). The two folds sodium nitrite (NaNO2) solutions prepared with final concentrations in urine of (5, 10, 20, 40, 80, 160, 320, 640, 1280, 2560, 5120 and 10240) µM and ascorbic acid (AA) solutions of (5, 10, 20 and 40) mM were prepared using the same urine. These concentrations are mixed and added with total 200 micro litter (µL) to the wells in rows A,B,C and D (Figure 1), the row "E "was specified to NaNo2 concentration Alone which started from 200,000 µM down to 100 µM. Ascorbic acid concentration alone was added to row "F", while the last two rows "G and H" in the plate were prepared for control positive and control negative respectively. The urinary pH was estimated before and after the addition of ascorbic acid using pH meter. The culture was diluted to a bacterial density of 10^6 cfu/ml adjusted with MacFarland tube 0.5 (bioMerieux), the 5µL of bacterial broth added to the all well except control negative. Then the bacterial growth was measured for 20 h at 37°C. The MIC was defined as the lowest concentration at which no visible growth had taken place after 20 hours. The MICs of NaNo2 and AA were determined. The MICs of NaNo2 in combination with a different concentration of AA (2.5, 5, 10 and 20 mM) was also determined. The plates were scanned by ELISA reader at optical density 500nm. The details of this procedure are illustrated in (Figure 1).

Antibacterial effect of acidified nitrite Carlsson method was used to assess the acidification effect against UTI bacteria [4]. *E.coli* ATCC 25922 was obtained from the Medya Medical Center in Erbil/Iraq. Midstream urine was collected from healthy persons, pooled, divided into batches, and immediately frozen (-20°C) until use. The isolate was diluted to around 10^6 cfu/ml adjusted with MacFarland tube 0.5, which inoculated in 10ml urine (pH 6.3) to which different amounts of nitrate (1, 3, 5and 10 mM) were added, and the culture was incubated aerobically for 18 h at 37°C. After incubation, the urinary culture was acidified (pH 5.0 and 4.6) by adding 10 mM and 20 mM AA, respectively and was again incubated for 18 hours at 37°C. Before and after each incubation period, the viable counts were determined. For each urine sample the estimated nitrite that produced from reduction of nitrate was obtained according to standard curve of nitrite as shown in (Figure 2).

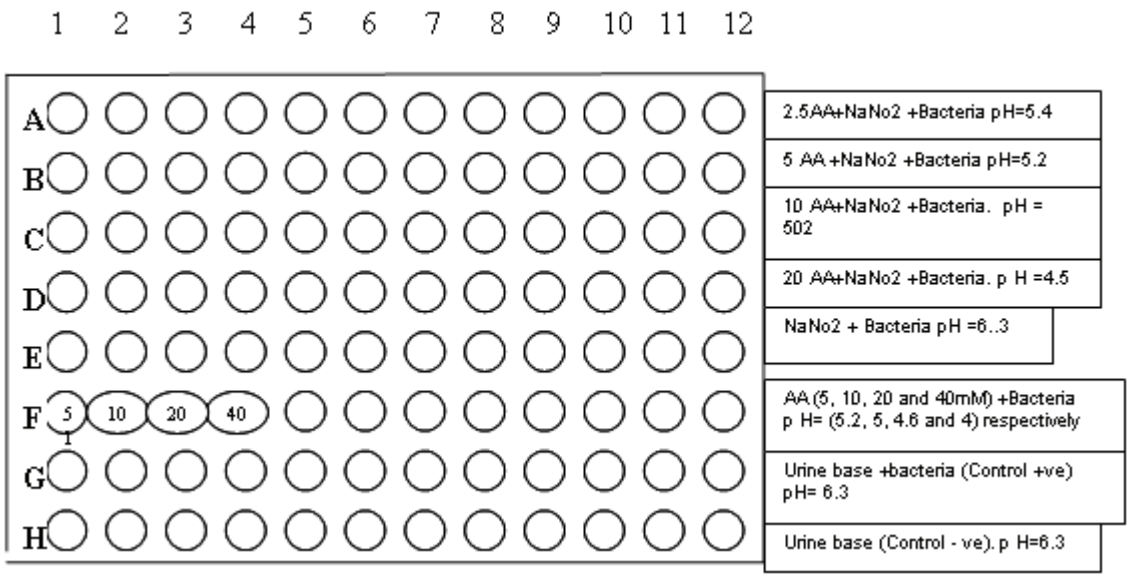

Figure 1: 96-wells Microtiter plate showing the preparation and distribution of different concentrations of ascorbic acid and sodium nitrite.

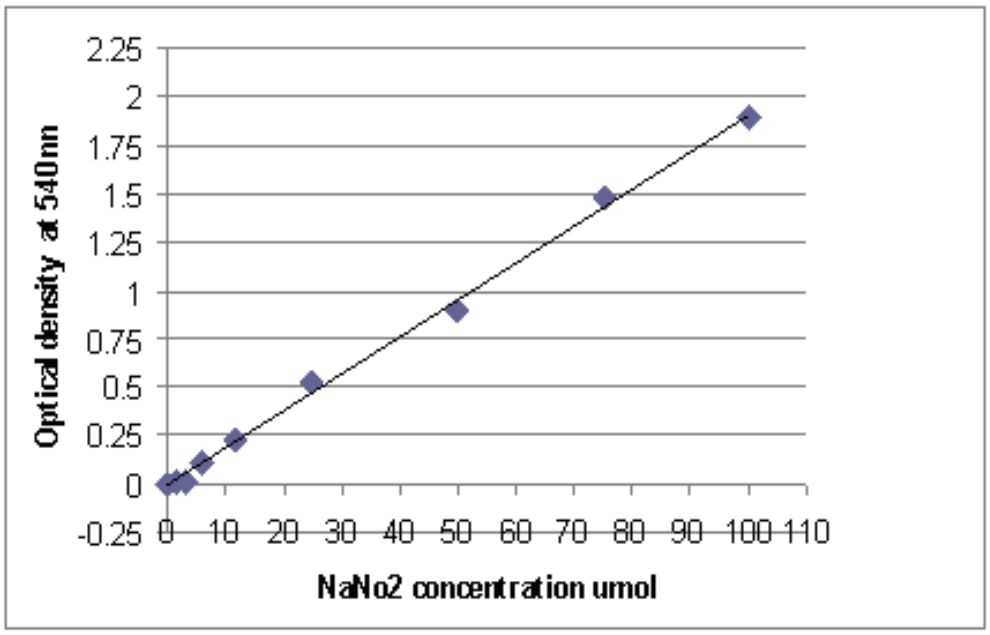

Figure 2: Standard curve for estimation of nitrite in urine sample

RESULT:

The results showed that both of NaNo2 and AA are weak antibacterial agents when are used alone, the MIC of AA alone at pH 4 was 40mM for the total 32 isolates, while MIC of NaNo2 at pH6.3 was ranging between 100,000 and 200,000 µM. Mixing low concentration of NaNo2 and AA at different low pH produced good antibacterial activities as decreasing pH led to stronger antibacterial activity. As shown in table 2, mixing 2.5mM and 5 Mm AA with a serial concentration of NaNo2 at pH 5.4 and pH 5.2 respectively, revealed a weak antibacterial agent, which ranged from (1,280 up to >10,240µM), while addition 10mM and 20 AA to the same NaNo2 concentration at pH 5 and pH 4.5 respectively, showing a strong bacterial inhibition, as it decreased the MIC range (5µM up to 640µM). Moreover, no significant difference of MIC for *E.coli* ATCC 25922 comparing with 32 isolates was observed. The details of the antibacterial activity at different levels of mixing the two agents and at different pH levels are shown in (Table 1).

In the second line of this study, included *E. coli* ATCC 25922 growing in urine with different concentration of NaNo3 (1,3,5 &10 mM) at pH 6.3 result in increasing bacterial growth by measuring the viable count. When it was transferred to acidified urine lby addition of 10 and 20 mM AA to attain pH 5 and 4.6 respectively, caused decreasing viable count or bacterial death occurred. Details in (Table 2)

Table 1: Determination of MIC of different concentrations of acidified nitrite (AA+NaNo2) at different levels of pH.

Ascorbic acid mM	MIC (NaNo2) µM	No. of bacteria	%	MIC µM (NaNo2) *E.coli* ATCC 25922	Final pH
2.5	5,120	4	12.5	> 1,0240	5.4
	> 10,240	28	87.5		
5	320	2	6.3	5,120	5.2
	1,280	1	3.1		
	2,560	2	6.3		
	5,120	16	50		
	> 10,240	11	34.4		
10	10	1	3.1	640	5.02
	20	2	6.3		
	40	1	3.1		
	80	1	3.1		
	160	4	12.5		
	320	7	21.9		
	640	14	43.8		
	1,280	1	3.1		
	2,560	1	3.1		
20	5	4	12.5	20	4.5
	10	9	28.1		
	20	9	28.1		
	40	6	18.8		
	80	4	12.5		

Table 2: Viable count of *E.coli* ATCC 25922 in different NaNo3 concentration in urine base (1,3,5 and 10mM).After incubation for 20 hours the cultures transferred to acidified urine pH 5 and pH4.6 and bacterial count has done.

	pH 6.3 cfu/ml	NaNo2 µmol production	After 20 h. the cultures transferred to acidified urine	+ 10 AA pH 5.0 cfu/ml	+ 20 AA pH4.6 cfu/ml
Base urine (control)	$4x10^7$	2		$5x10^5$	$7x10^3$
Base urine with 1 mM NaNo3	$5x10^7$	800		$16x10^3$	0
Base urine with 3mM NaNo3	$11x10^6$	2650		$1x10^3$	0
Base urine with 5mM NaNo3	$7x10^6$	3850		0	0
Base urine with 10mM NaNo3	$15x10^6$	5000		0	0

DISCUSSION:

Amount of nitrite may exist in infected urine as a result of bacterial nitrate reductase activity. This study revealed that nitrite alone is a weak antibacterial agent when the MIC ranged between (100,000 - 200,000 µM). Such amount never accumulate in the bladder of patients having UTI, since the nitrate range in human bladder is around (0.6 –0.7mM), which may produce 2 µM nitrite in case of UTI [5]. On the other hand, when the nitrite is acidified and transferred to be a strong antibacterial agent, as causing bacterial inhibition by NaNo2 in acidified urine in dose dependent. This study showed that the MIC of NaNo2 was started from 10 µM up to 640 µM, when10 mM AA added. The rate of MIC depended on increasing nitrite dose and acidity of urine (Low pH) Potent antibacterial effect of the two concentrations that mentioned above 10µM (approximately 0.69 µg/ml) and 640 µM (approximately 44 µg/ml) is greater than invitro standard breakpoint MIC of some commonly used antibiotics for treating UTI, for example 0.69 µg/ml is more potent than the breakpoint MIC of gatifluxacin, cefotaxime, ceftriaxone and cefixime, comparing with their cut-off value ≤ 1 µg/ than breakpoint MIC of nitrofuraton with a cut-off value ≤ 64 µg/ml [10]. Furthermore table (1) showed that the 32 *E.coli* isolates have MIC rates close to each other even of *E.coli* ATCC 25922, which showed the same MIC, the reason might be due to the newly exposure these isolates to this type of antibacterial compound. The fact that ascorbic acid potentiates the bactericidal effects of nitrite in urine, suggests that the production of nitric oxide (NO) at some stage formation is important to achieve the antibacterial effects observed. Thus, ascorbic acid greatly increases the level of production of NO from nitrite [11, 12] Furthermore, when the bacterial isolates exposed to a low pH, ascorbate alone at different concentrations, or NaNo2 alone, no effect on growth was observed. Thus, effective killing required a sequential procedure. Table (2) showed the growth of *E. coli* ATCC 25922 was enhanced by NaNo3 in urine in a dose dependent manner. Addition of ascorbic acid (10 mM) after incubation for 24 h. enhanced the inhibition of bacterial growth by NaNo2, which produced from nitrate reduction, depending on nitrite dose and the pH. The results showed that the *E. coli* is

transferred from nitrate-rich urine to acidified urine pH 5 and 4.6 respectively. Larger amounts of nitrate in the first medium resulted in more nitrite accumulation and more effective killing. Notably, the nitrite that accumulated in the first medium was sufficient to kill the bacteria. Nitrite is rapidly converted to toxic nitrogen oxides when the pH is lowered [13]. The exact chemical nature of the toxic nitrite derived compound is not known, nor is the exact mechanism by which killing occurs. The chemistry of acidified nitrite is very complex, and a variety of nitrogen oxides are generated directly or after reactions with other compounds. These include NO, N2O3, N2O4, NO, HNO2, NO2, $ONOO^-$, and S-nitrosothiols, many of which have antimicrobial activities [14, 15].

CONCLUSION:

Strong antibacterial agent can be formed in acidified urine containing nitrite. This antibacterial agent is strongly pH dependent and is increased by addition of ascorbic acid. The antibacterial strength is a nitrite-dose dependent in mildly acidified urine, which is stronger than breakpoint MIC of some antibiotics. ATCC 29522 *E.coli* can be killed by a two step procedure including growing the bacteria in nitrate, followed by acidification of urine.

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