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ABSTRACT

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ANTIMICROBIAL EFFECT OF CITRATE BUFFER WITH ANTIBIOTIC

Antibiotic resistance of pathogens to medications is an essential problem globally. Thus, new medication compositions are one of the ways to solve this problem. This study aimed to investigate the antimicrobial efficacy of the citrate buffer combined with the antibiotic «Amoxiclav» on standard reference strains of microorganisms.

We used standard cultures of *C. albicans* ATCC10231, *E. coli* ATCC25922, *S. aureus* ATCC25923, *E. faecalis* ATCC29213, *M. luteus* ATCC4698, *S. epidermidis* ATCC14990 in the study conducted at the Microbiology, Virology and Immunology Department of the Poltava State Medical University. The sensitivity of standard microorganism strains to the composition was studied with a quantitative method of serial dilutions based on CLSI. ISO/TC 212 «Clinical laboratory testing and in vitro diagnostic test systems. 2021».

Thus, the results of this study clearly showed a synergistic effect of citrate buffer and amoxiclav on all the reference strains of microorganisms. Reference strains of *Staphylococcus aureus* and epidermal staphylococcus showed the highest sensitivity to citrate buffer, which was 4 times ($p < 0.05$) higher than that shown by micrococci, enterococci and fungi, and 8 times ($p < 0.01$) higher than that of *Escherichia coli*, which appeared least sensitive to the test substance. Amoxiclav also inhibited the growth of *S. aureus* ATCC 25923 and *S. epidermidis* ATCC 14990 and had the minimal effect on the reference strain of *E. coli* ATCC 25922, the minimal inhibitory concentration of which was 31.3 times ($p < 0.0001$) higher than that of staphylococci. The proposed citrate buffer-amoxiclav combination significantly increased the antimicrobial efficacy of its components against all the reference strains of microorganisms. Thus, the sensitivity of *E. coli* ATCC 25922 and *M. lysodeikticus* ATCC 4698 to the proposed combination increased 8-fold ($p < 0.05$) compared to their sensitivity to citrate buffer or amoxiclav alone. The sensitivity of *Staphylococcus aureus* and enterococci to the composition increased to the maximum: up to 32-fold ($p < 0.001$) to the buffer and 4-fold to the antibiotic ($p < 0.01$). The effect of a significant increase in the sensitivity of *C. albicans* ATCC10231 strain to the citrate buffer after adding amoxiclav was gone up 128-fold ($p < 0.0001$). It was the evidence of a synergistic fungicidal action of the antibiotic-citrate buffer combination.

Key words: pathogens, endodontic infections, resistance, citrate buffer, antibiotics, sensitivity, minimal inhibitory concentration, minimal fungicidal concentration.

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РЕЗЮМЕ

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ПРОТИМІКРОБНА ДІЯ КОМБІНАЦІЇ ЦИТРАТНОГО БУФЕРУ З АНТИБІОТИКОМ

Стійкість найбільш розповсюджених патогенів, в тому числі збудників ендодонтичних інфекцій, до антибіотиків залишається найважливішою проблемою у всьому світі. Тому, створення нових лікарських композицій є одним із шляхів вирішення цієї проблеми. Метою даного дослідження було визначення ефективності антимікробної дії цитратного буферу в поєднанні з антибіотиком «Амоксиклав» по відношенню до стандартних референтних штамів мікроорганізмів.

Дослідження, в яких використовували стандартні культури *C. albicans* ATCC10231, *E.coli* ATCC25922, *S. aureus* ATCC25923, *E. faecalis* ATCC29213, *M. luteus* ATCC4698, *S. epidermidis* ATCC14990, були проведені на кафедрі мікробіології, вірусології та імунології Полтавського державного медичного університету. Чутливість стандартних штамів мікроорганізмів до складу композиції досліджували кількісним методом серійних розведень відповідно міжнародним стандартам CLSI. ISO/TC 212 «Клінічні лабораторні випробування та тестові системи для діагностики *in vitro*. 2021».

Референтні штами *Staphylococcus aureus* та *Staphylococcus epidermidis* виявили найвищу чутливість до цитратного буферу, яка у 4 рази ($p < 0,05$) перевищувала цей показник по відношенню до мікрококів, ентерококів та дріжджеподібних грибів, і була в 8 разів ($p < 0,01$) вище, ніж чутливість *Escherichia coli*, яка виявилася найменш чутливою до досліджуваної речовини.

Амоксиклав також найкращим чином пригнічував ріст *S. aureus* ATCC 25923 та *S. epidermidis* ATCC 14990 та мінімально впливав на референтний штам *E. coli* ATCC 25922, мінімальна інгібуюча концентрація якого перевищувала цей параметр у стафілококів у 31,3 разів ($p < 0,001$).

Запропонована комбінація цитратний буферу з амоксиклавом значно підвищила антимікробну ефективність окремих її компонентів проти всіх штамів мікроорганізмів. Таким чином, чутливість *E. coli* ATCC 25922 та *M. lysodeisticus* ATCC 4698 до запропонованої комбінації зросла у 8 разів ($p < 0,05$) у порівнянні з цитратним буфером та амоксиклавом окремо. Чутливість золотистого стафілококу та ентерококу до композиції піднялась максимально: до 32 разів ($p < 0,001$) по відношенню до буферу та в 4 рази до антибіотику ($p < 0,01$). Було зафіксовано значне підвищення чутливості штаму *C. albicans* ATCC10231 до цитратного буфера після додавання амоксиклаву: у 128 разів ($p < 0,0001$), що з'явилося доказом синергічної фунгіцидної дії комбінації антибіотик-цитратний буфер.

Ключові слова: патогени, ендодонтичні інфекції, цитратний буфер, антибіотик, чутливість, мінімальна інгібуюча концентрація, мінімальна фунгіцидна концентрація.

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INTRODUCTION/ВСТУП

Endodontic therapy relies mainly on biomechanical preparation, chemical irrigation and intracanal medicaments, which are important in eliminating bacteria in the root canal [1]. Furthermore, adequate obturation is essential to confine any residual bacteria within the root canal and deprive them of nutrients [2]. However, numerous studies showed that complete elimination of bacteria is not achieved due to the complex anatomy of the root canal system [3, 4, 5]. *Enterococcus*, *Staphylococcus* and *Candida* are isolated in cases of endodontic infections and cases of endodontic treatment failure [6]. *E. faecalis* can produce biofilm, invade dentinal tubules, develop a monoinfection form of the biofilm, invade the dentinal tubules, and resist antimicrobial agents [8]. The persistence of microorganisms in the root canal system favours the development and maintenance of apical periodontitis [1]. Antibiotics are an extremely valuable addition to the substances available to health practitioners to manage bacterial infections [9]. Due to the potential risk of adverse systemic effects of systemic applications and the ineffectiveness of systemic antibiotics in the necrotic pulpless tooth and the periradicular tissues, local application of antibiotics may be a more effective model for delivering antibiotics to infected root canals. Therefore, it is important to research and evaluate substances with antimicrobial effects and minimal toxic action on the periapical tissues [10].

Root canal sealers with bioactive properties come in direct contact with the dentin wall and can play a positive role in bacterial elimination and strengthening of the root structure. Citric buffer can be used as a sealer in endodontic treatment [11]. In a previous study, this substance showed low cytotoxicity, without genotoxic effects on fibroblast cells, and had ex vivo antimicrobial activity against *E. faecalis* [12].

Thus, endodontic treatment using citrate buffer compositions with antibiotic may be promising in eliminating microorganisms in the root canal system. The most interesting aspect is the study of the combination of citrate buffer with antibiotic effect on strains most often isolated from root canals of patients with apical periodontitis – *Enterococci*, *Staphylococci* and *Candida* and compares with representatives of the transient (*Escherichia coli*) and normal oral cavity microflora – *Micrococci.*

The aim of this study is to investigate the antimicrobial effect of the citrate buffer combined with the antibiotic "Amoxiclav" on standard reference strains of microorganisms.

Material and methods

Studies in which we used standard cultures of *C. albicans* ATCC10231, *E. coli* ATCC25922, *S. aureus* ATCC25923, *E. faecalis* ATCC29213, *M. luteus* ATCC4698, *S. epidermidis* ATCC14990 obtained from the collection of the Museum of the SI "L.V. Gromashevsky Institute of Epidemiology and Infectious Diseases of NAMS of Ukraine (Kyiv), were conducted at the Department of Microbiology of PSMU (Poltava State Medical University). One day cultures of the strains mentioned above were prepared on slant meat peptone agar (MPA) or Saburaud medium ("Pharmactive", Ukraine). The inocula of the cultures were then adjusted to equal turbidity of the 0.5 McFarland standard. The sensitivity of standard strains of microorganisms to the composition was studied with a quantitative method of serial dilutions based on CLSI. ISO/TC 212 «Clinical laboratory testing and in vitro diagnostic test systems. 2021» standard [13].

The study used an extemporaneously prepared antibacterial composition containing citrate

buffer (pH = 7.2) and a complex antibiotic amoxiclav ("Sandoz") corresponding to 10.0 μg/ml of the working concentration of the medication [14]. The combination was added to 10 tubes containing nutrient broth according to standard method, taking into account controls with "positive" and "negative" results. Thus, we obtained tubes with a twofold difference in the concentration of the medication in each adjacent pair of tubes.

According to the method in the study, we used microbial suspension, which was a diluted inoculum and contained 1.5×10^6 CFU/cm^3 (colony-forming units in 1 ml of inoculum). The prepared microbial suspension no later than 15 minutes after its preparation was added to each test tube containing the appropriate amount of nutrient broth and test composition. The tubes were then incubated in a standard atmosphere at 37 °C for 24 hours.

The experimental series contained all the components of the compositions in the appropriate proportions. All the components except the bacterial cultures were included in the control compositions. The control cultures contained nutrient medium and inocula of reference strains.

After incubation, the results were evaluated, and the tested medication's minimal inhibitory concentration (MIC) was determined. Next, the contents of the tubes with no visible growth of microorganisms were inoculated on nutrient agar to determine the minimal bactericidal concentration (MBC) or minimal fungicidal concentration (MFC).

Results and discussion

The determined results of the inhibitory and microbicidal effect of citrate buffer on museum strains of microorganisms are shown in Table 1.

Table 1 – Citrate buffer effect on museum strains of microorganisms

Museum strains	Citrate buffer concentration, μl											
	1:1/500	1:2/ 250	1:4/125	1:8/62,5	1:16/31,3	1:32/15,7	1:64/7,8	1:128/3,9	1:256/1,95	1:512/0,975	C/C	C/CB
E.coli ATCC 25922	-st	+	+	+	+	+	+	+	+	+	+	-
S.aureus ATCC 25923	- st	- st	- st	- st	- st	- st	-	+	+	+	+	-
S.epidermidis ATCC 14990	- st	- st	-	+	+	+	+	+	+	+	+	-
E. faecalis ATCC 29212	- st	- st	+	+	+	+	+	+	+	+	+	-
M.lysodeicticus ATCC 4698	- st	- st	+	+	+	+	+	+	+	+	+	-
C.albicans ATCC10231	- st	- st	+	+	+	+	+	+	+	+	+	-

Notes: – no growth of microorganisms, + growth of microorganisms, C/C – control of culture, C/CB – control of citrate buffer, st – sterility on a solid nutrient medium

MIC of citrate buffer for the reference strain of *E. coli* ATCC 25922 is in 1:1 dilution, which coincided with the MBC of the medication. The citrate buffer has the highest antibacterial effect against *S. aureus* ATCC 25923, which is 1:8; its bactericidal action is 1:4. For *S. epidermidis* ATCC 14990 the citrate buffer MIC is 1:4 and MBC is 1:2. For standard strains of enterococci, micrococci and yeast-like fungi, both concentrations did not differ and were equal to 1:2.

Table 2 displays the results on the growth inhibition of microorganism reference strains by the amoxiclav antibiotic. Amoxiclav inhibits most of all the growth of staphylococci at the dose of 0.08 μg/ml, have no fungicidal effect, the least sensitive to it have *Escherichia coli* and *Micrococcus lysodeicticus*, the MIC of those are 2.5 μg/ml, and MBC is equal to 5 μg/ml. On the other hand, the growth of *Enterococcus faecalis* is inhibited by the antibiotic at the dose of 0.16 μg/ml, which coincided with the bactericidal effect.

The results of the reference strains sensitivity to the combination of citrate buffer with the antibiotic "Amoxiclav" detecting are presented in Table 3.

Table 2 – Amoxiclav effect on museum strains of microorganisms (μg/ml)

Museum strains	5	2,5	1,25	0,63	0,31	0,16	0,08	0,04	0,02	0,01	C/C	C/A
E.coli ATCC 25922	- st	-	+	+	+	+	+	+	+	+	+	-
S.aureus ATCC 25923	- st	- st	- st	- st	- st	-	-	+	+	+	+	-
S.epidermidis ATCC 14990	- st	- st	- st	- st	- st	- st	-	+	+	+	+	-
E. faecalis ATCC 29212	- st	- st	- st	- st	- st	- st	+	+	+	-	+	-
M.lysodeicticus ATCC 4698	- st	-	+	+	+	+	+	+	+	-	+	-
C.albicans ATCC10231	+	+	+	+	+	+	+	+	+	+	+	-

Notes: – no growth of microorganisms, + growth of microorganisms, C/C – control of culture, C/CA – control of Amoxiclav, st – sterility on a solid nutrient medium

As shown in table 3, the citrate buffer-amoxiclav composition inhibits all microorganisms' growth to a much greater extent than each solution separately. Though *Escherichia coli* and micrococci still were the least sensitive to the medication mixture, the MIC of *E. coli* ATCC 25922 corresponded to a 1:64 dilution and was 0.08 μg/ml. The sensitivity of *E. coli* ATCC 25922 to the proposed combination increases 8-fold ($p < 0.05$) compared with citrate buffer. The MIC of the composition against *M. lysodeikticus* ATCC 4698 shows a minimum buffer-antibiotic effect of 1:16/0.31 μg/ml, but it also 8-fold ($p < 0.05$) exceeds the activity of its components.

Table 3 – The effect of citrate buffer with Amoxiclav composition on museum strains of microorganisms (μg/ml)

Museum strains	Dilution, composition concentration, μg/ml											
	1:1/5	1:2/ 2,5	1:4/1,25	1:8/0,63	1:16/0,31	1:32/0,16	1:64/0,08	1:128/0,04	1:256/0,02	1:512/0,01	C/C	C/CB + A
E.coli ATCC 25922	- st	- st	-	-	-	-	-	+	+	+	+	-
S.aureus ATCC 25923	- st	- st	- st	- st	- st	- st	- st	- st	-	+	+	-
S.epidermidis ATCC 14990	- st	- st	- st	- st	- st	- st	-	-	+	+	+	-
E. faecalis ATCC 29212	- st	- st	- st	- st	- st	- st	-	+	+	+	+	-
M.lysodeicticus ATCC 4698	- st	- st	- st	-	-	+	+	+	+	+	+	-
C.albicans ATCC10231	- st	- st	- st	- st	- st	- st	- st	- st	- st	+	+	-

Notes: – no growth of microorganisms, + growth of microorganisms, C/C – control of culture, C/CB + A – control of citrate buffer with amoxiclav, st – sterility on a solid nutrient medium

Staphylococcus aureus and *Candida albicans* with a MIC of 1:256/0.02 μg/ml are most sensitive to the composition, and its fungicidal effect coincided with the fungiostatic one. The MBC of citrate buffer-amoxiclav composition for *Staphylococcus epidermidis* ATCC 14990 and *Enterococcus faecalis* ATCC 29212 is the same and equal to 1:32/0.16 μg/ml. However, the MIC of the composition for epidermal staphylococci is 1:128/0.04. So they appear more sensitive to it than enterococci.

Thus, the results of this study clearly show a synergistic effect of citrate buffer and amoxiclav on all the reference strains of microorganisms. Moreover, if the increased antibacterial effect of a complex beta-lactam antibiotic with clavulanic acid caused by the citrate buffer can easily be explained through the increased permeability of the bacteria cell wall, how can we explain the significantly increased effect of citrate buffer on fungi of the genus *Candida* caused by amoxiclav? After all, this is not an antifungal antibiotic. According to the

literature, citric acid inhibits the growth of yeast-like fungi and even of the biofilm formed by them [7]. Amoxicillin, especially amoxiclav, as a broad-spectrum antibiotic, can cause candidiasis in the body, but clavulanic acid-related substances – clavams – show antifungal effects during *in-vitro* experiments [8]. In this case, the acidic environment probably induces certain degradation products of clavulanate having similar activity.

Thus, the effect of the experimental composition is based on a set of clearly coordinated mechanisms, which create a precedent for mutually reinforced pharmacological effect of optimally selected components on the principle of potentiating synergism. Every combination of two substances – of citrate buffer, which has little antimicrobial activity but significantly enhances the effect of antibiotics, and amoxiclav, a complex medication containing amoxicillin and clavulanic acid – provide a versatile and high pharmacological effect. The antifungal effect of the experimental composition determines the prospects for the use of a mixture of its components in the treatment of pathological processes in the oral cavity, where candidiasis is usually the first manifestation of dysbacteriosis associated with the use of broad-spectrum antibiotics.

CONCLUSIONS/ВИСНОВКИ

1. Reference strains of *Staphylococcus aureus* and epidermal staphylococcus show the highest sensitivity to citrate buffer, which was 4 times ($p < 0.05$) higher than the sensitivity to it shown by micrococci, enterococci and fungi, and 8 times ($p < 0.01$) higher than the sensitivity of *Escherichia coli*, which appears least sensitive to the test substance.

2. Amoxiclav also best inhibits the growth of *S. aureus* ATCC 25923 and *S. epidermidis* ATCC 14990 and has the minimal effect on the reference strain of *E. coli* ATCC 25922, the MIC of which exceeds this parameter in staphylococci 31.3 times ($p < 0.0001$).

3. The proposed citrate buffer-amoxiclav combination significantly increases its individual components' antimicrobial effect against all the reference strains of microorganisms. Thus, the sensitivity of *E. coli* ATCC 25922 and *M. lysodeisticus* ATCC 4698 to the proposed combination increased 8-fold ($p < 0.05$) compared to their sensitivity to citrate buffer and amoxiclav separately. The sensitivity of *Staphylococcus aureus* and enterococci to the composition increase to the maximum: up to 32-fold ($p < 0.001$) to the buffer and 4-fold to the antibiotic ($p < 0.01$).

4. The sensitivity of *C. albicans* ATCC10231 strain to the citrate buffer after the amoxiclav addition significantly increases 128-fold ($p < 0.0001$). It is evidence of a synergistic fungicidal action of the antibiotic-citrate buffer combination.

PROSPECTS FOR FUTURE RESEARCH/ПЕРСПЕКТИВИ ПОДАЛЬШИХ ДОСЛІДЖЕНЬ

Further studies will be focused on the effect on clinical strains of endodontic pathogens.

CONFLICT OF INTEREST/КОНФЛІКТ ІНТЕРЕСІВ

The authors declare no conflict of interest.

COMPLIANCE WITH ETHICS REQUIREMENTS/ДОТРИМАННЯ ЕТИЧНИХ ВИМОГ

The authors declare that all procedures of this research respect the ethical standards in the Helsinki Declaration of 1975, as revised in 2008.

FUNDING/ДЖЕРЕЛА ФІНАНСУВАННЯ

None.

AUTHOR CONTRIBUTIONS/ВКЛАД АВТОРІВ

All authors substantively contributed to the drafting of the initial and revised versions of this paper. They take full responsibility for the integrity of all aspects of the work.

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