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Effect of biclotymol on mouth cavity mucosal microbiota in children

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Article received: 01.02.2014. Accepted for publication: 24.02.2014**.**

Introduction. Biclotymol [bis(chloro-4-thymol)methyl] is a biphenol derivative used for local treatment of pharyngeal inflammatory diseases. It is an antiseptic drug with bacteriostatic and bactericidal effects. **Objective:** evaluation of the drug's antibacterial activity towards opportunistic and normal oropharyngeal microflora and identification of its minimum bactericidal concentration for the main bacterial pathogens of the upper respiratory tract. **Results:** we revealed that antibacterial effect of biclotymol on mouth cavity microbiota manifests itself with elimination of such species and genera of opportunistic microflora as *Staphylococcus aureus, Streptococcus pyogenes, Haemophilus spp.* and *anginosus* streptococci. We also revealed antibacterial activity of biclotymol against normal microbiota representatives, which included *viridans* streptococci. Out of this group of streptococci, only 1/3 of the initial amount of *Streptococcus salivarius* remained in place. Alongside identification of the minimum inhibitory concentration (MIC) of biclotymol against opportunistic microflora we revealed the most sensitive bacteria requiring the lesser drug concentration for the antibacterial effect to take place – *Streptococcus pneumoniae* and *Haemophilus influenzae*; intermediate – *Moraxella catarrhalis*, *S. pyogenes* and *S. aureus*; and resistant – non-pathogenic *Neisseria* species. Among the strains that survived biclotymol exposure, MIC was the highest in *Neisseria spp.* – 20 mg/ml after a 30-minute-long exposure. **Conclusions:** results of a study of antibacterial effect of a topical antiseptic containing biclotymol demonstrated that the drug's bactericidal activity is primarily aimed at gram-positive cocci represented both by opportunistic and non-pathogenic microbes. Non-pathogenic *Neisseria* species abundantly inhabiting mouth cavity mucosae and gram-negative rod-like microbes, which are uncharacteristic of the biotope under study, appeared to be resistant to bactericidal effect of biclotymol. Preservation of these microbes may somewhat prevent oropharyngeal colonization by undesirable pathogenic species.
Keywords: *biclotymol, antiseptics, antibacterial activity, Streptococcus pneumoniae, Haemophilus influenzae, Moraxella catarrhalis, Streptococcus pyogenes, Staphylococcus aureus, Neisseria spp., treatment.*

INTRODUCTION

Local antiseptic biclotymol [bis(chloro-4-thymol)methyl] is a biphenol derivative widely used in the treatment of infectious inflammatory diseases of mouth cavity and pharynx in children and adults as a component of medical aerosol Hexaspray (Laboratoires Bouchara Recordati, France). The drug produces bacteriostatic and bactericidal effect and may be used of topical monotherapy or in combination with systemic antibiotic therapy. It is practical to analyze antimicrobial activity of local antiseptics due to high frequency of routine use thereof for respiratory infections.

Our previous *in vitro* study demonstrated antibacterial effect of different biclotymol concentrations on opportunistic microbes – the main causative agents of lower and upper

respiratory tract infections [1]. As a result, we revealed dependence of biclotymol's bactericidal effect on the dose thereof, time of exposure and the microbial species. At the same time, local use of the antiseptic drug affects not only the etiologically significant pathogen, but the whole microbial community colonizing mouth cavity mucosa. This study was aimed at assessing antibacterial activity of biclotymol towards opportunistic and normal oropharyngeal microflora and determining the minimum bactericidal concentration of biclotymol for the main upper respiratory tract pathogens.

STUDY MATERIALS AND METHODS

Medical aerosol Hexaspray containing 2.5% (25 mg/ml) biclotymol and biclotymol powder of the same manufacturer was used for assessing antibacterial activity.

Experimental method of the drug's *in vitro* effect on mouth cavity microbiota was developed in order to analyze antibacterial activity of biclotymol towards opportunistic and normal mouth cavity microflora. The aerosol under study was applied to the microbiota obtained in inoculi of oropharyngeal swabs of 30 children hospitalized at departments of the Scientific Center of Children's Health. Swabs were taken from mucosae of mouth cavity and tonsils with tampons fixed in plastic holders; these tampons were further put in transport medium Amies (Copan, Italy).

At the laboratory, tampons with biomaterials were put in the 0.9% sodium chloride (NaCl) solution (10 ml); the obtained microbial suspension was diluted with isotonic NaCl solution 5 times. The obtained suspension was inoculated on 2 Petri dishes with blood agar and 3% horse serum. We deposited 0.1 ml of suspended 50-fold diluted biomaterial on the surface of the growth medium in each dish; the biomaterial was spread over the surface of blood agar with a spatula.

One inoculated Petri dish was used for control. Surface of the second Petri dish was treated with 2 doses of the aerosol under study. We used a spatula to evenly spread the aerosol's content deposited on an inoculated Petri dish. After that, both Petri dishes were incubated in a thermostat for 24-48 hours at a temperature of 37 ^{o}C.

After the incubation, we performed quantitative count of the colony-forming units (CFUs) and identification of the developed (aerobic and facultative anaerobic) microbes. We used analyzer MALDI-TOF Microflex (Bruker Daltonics, Germany) to identify the microbial species.

Minimum inhibitory concentration (MIC) determination

In order to determine the MIC of biclotymol for opportunistic microflora, we took into consideration its concentration in drugs. The highest initial biclotymol concentration – 20 mg/ml – was selected due to biclotymol concentration in the 2.5% aerosol under study (25 mg/ml).

Operational biclotymol concentrations of 40 mg/ml and lower were prepared using an original (96% ethanol) solution containing 100 mg of biclotymol per 1 ml of alcohol. Biclotymol powder in initial concentrations was completely (until the achievement of transparency) dissolved in 96% ethanol using a plastic stirrer. The initial (100 mg/ml) alcohol biclotymol solution was used to prepare the analyzed drug concentrations.

The analyzed microbial species were *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Moraxella catarrhalis*, *Streptococcus pyogenes*, *Haemophilus influenzae* and 2 Neisseria species – *Neisseria flavescens* and *Neisseria perflava* – extracted from inoculi of oropharyngeal biomaterial swabs.

For purposes of this study, we selected the following parameters on the basis of results of the previous study of biclotymol's antibacterial effect on clinical isolates of opportunistic bacteria [1]:

- biclotymol concentration – 40, 20, 10, 5, 2.5, 1.25, 0.62, 0.31 and 0.015 mg/ml;
- density of the microbial inoculum (turbidity standard) – 0.5 McFarland (0.75 x 10^8 CFU/ml);

- time of exposure of biclotymol with a microbial inoculum in a test tube – 30 minutes in a thermostat at the temperature of 37 ^{o}C.
We added 0.5 ml of a bacterial inoculum to all the test tubes containing different biclotymol concentrations in 5ml of sterile distilled water. For control purposes, we added the same amount of microbial inoculum in a test tube with sterile distilled water as in the test tubes with test biclotymol samples.
We inoculated contents of test tubes on a 5% blood agar surface after a 30-minute-long exposure of different concentrations of biclotymol with a microbial inoculum in a thermostat at a temperature of 37 ^{o}C. Inoculum was delivered to the growth medium with a 10 mcl sampling loop containing 0.75 x 10^5 CFUs of the analyzed microbe.
Antibacterial activity of biclotymol was expressed in the count of the CFUs (colony-forming units), which had grown (survived) in Petri dishes containing blood agar after 24 hours of incubation in a thermostat at a temperature of 37 ^{o}C. The MIC of biclotymol was determined on the basis of the drug's dilution inhibiting further microbial growth.

RESULTS AND DISCUSSION

Species composition and frequency of isolation of certain children's mouth cavity microbiotic specimens determined by means of testing 30 mouth cavity mucus swabs are given in tb.1. 128 microbial isolates (38 microbial species) were isolated from pharyngeal mucus inoculi. The number of microbes observed in a child's pharynx varied from 2 to 8 (4.3 on the average). The dominant mouth cavity microbiotic taxons were *Streptococcaceae* bacteria detected in swabs of 29 (97%) children and gram-negative *Neisseria* cocci detected in 26 (87%) samples.
Opportunistic microflora: frequency of isolation of *S. aureus*, *S. pyogenes*, *Streptococcus anginosus* (*milleri*) and *Streptococcus dysgalactiae* streptococci from oral swabs was 30%, 10% (3 patients) and 20% (6 patients), respectively.
Use of biclotymol resulted in complete disappearance of such opportunistic coccoid bacteria as S. aureus, *S. pyogenes*, *S. anginosus* (*milleri*) and *S. dysgalactiae* streptococci and hemophilic bacteria from microbiota. The only other detected species of oral streptococci was *Streptococcus salivarius*; however, frequency of isolation thereof decreased from 60 to 20%, dish colony density – from 139 to 34 CFUs (tb. 2). Several inconsiderable in amount species/genera of normal microbiota, such as *Gemella spp.*, *Rothia spp.*, *Granulicatella spp.*, *Haemophilus spp.*, disappeared as well.
However, we observed 2 bacterial species not detected initially – *Kingella denitrificans* (2 cases) and *Gracilibacillus dipsosauri* (1 case) – after exposure of mouth cavity microbiota to biclotymol. These microbial species were represented with a small amount of CFUs; that is probably why they were not detected in the initial composition of the diverse microbial community.
The dominant microbiota surviving biclotymol were non-pathogenic *Neisseriaceae* species (see tb. 2). Neisseria species were detected in pharyngeal swabs of 26 (87%) children even before the exposure of microbiota to the antiseptic; 4 patients featured 2 Neisseria species, 1 patient – 3 Neisseria species. Neisseria species were detected in 28 (93%) samples after exposure of mouth cavity microbiota to biclotymol; 7 samples featured 2 Neisseria species, 1 sample – 3 Neisseria species. Initially, we detected 7 *Neisseria* species in pharyngeal swabs; the overwhelming majority of *Neisseria* bacteria belonged to species *Neisseria flavescens* (67% of the children). After biclotymol treatment of inoculi, frequency of isolation of this *Neisseria* species remained at the same level (60%); however, the average count of CFUs was almost twice as high as in the control sample (104.1 and 57.7). The possible explanation for this fact is inhibition of most microbial components of the mouth cavity biocoenosis, which favors a more accurate quantitative assessment of the surviving species. After the biomaterial was treated biclotymol, we detected 5 Neisseria species; 2 Neisseria species (*N. subflava* and *N. lactamica*),

which were represented by 1 bacterium each before biclotymol treatment, were not detected. These Neisseria species might have different threshold of sensitivity to biclotymol.

Microbiota containing 3 gram-negative rod-like species – *Enterobacter cloacae*, *Acinetobacter junii* and *Stenotrophomonas spp.* – was detected in an inoculum of a pharyngeal biomaterial sample. Biclotymol treatment of the inoculum with the mentioned microbes did not produce any bactericidal effect on these species: qualitative and quantitative microbiotic composition regarding these microbial species remained the same.

Our study demonstrated that mouth cavity mucus microbiota after biclotymol treatment differed significantly from the microbiotic composition before biclotymol treatment (pic.). 128 isolates of different bacterial species (4.3 species per swab on the average) were detected in oropharyngeal swabs before biclotymol treatment. The dominant microbes were *Streptococcaceae* (46%). Neisseria species were the second most widely present microbes in oropharyngeal microbiocenosis (25%); *S. aureus* (7%) and hemophilic bacteria (7%) were not as widely present; all the other microbes constituted 15% of microbiota.

Biclotymol treatment of microbial inoculi altered quantitative and qualitative microbiotic composition to a considerable extent: the number of isolates decreased almost 3 times (49 strains). Neisseria species were dominant (76% of the microbial spectrum), the range of streptococci decreased down to 12% (100% - *S. salivarius*); K. denitrificans constituted 4% of the microbial spectrum; 4 other microbial species constituted 2% of the microbial spectrum each.

According to the analysis of *S. aureus*, *S. pneumoniae*, *M. catarrhalis*, *S. pyogenes*, *H. influenzae* and non-pathogenic Neisseria species (*N. flavescens* and *N. perflava*), the most sensitive species regarding the minimum bactericidal biclotymol concentration were *S. pneumoniae* and *H. influenzae*: the MIC therefor was 0.15 mg/ml (tb. 3). The MIC for *S. pyogenes* and *M. catarrhalis* was 0.62 and 1.25 mg/ml, respectively; for *S. aureus* – 5.0 mg/ml. All these microbial species died of exposure to biclotymol.

The highest MIC of biclotymol was registered for *N. flavescens* and *N. perflava* (20 mg/ml). These species of non-pathogenic Neisseria tolerable to such high biclotymol concentrations persisted after treatment with Hexaspray containing biclotymol in the concentration of 25 mg/ml and became dominant in the mouth cavity mucus microbiota of children.

CONCLUSION

Thus, the analysis of antibacterial effect of biclotymol-containing antiseptic demonstrated that the drug's bactericidal activity is primarily aimed at gram-positive cocci represented both by opportunistic and non-pathogenic microbes. Non-pathogenic *Neisseria* species abundantly inhabiting mouth cavity mucosae and gram-negative rod-like microbes, which are uncharacteristic of the biotope under study, appeared to be resistant to bactericidal effect of biclotymol. It is known that non-biclotymol-sensitive *Neisseria* bacteria and the partially surviving *S. salivarius* streptococci are parts of the evolutionarily developed normal symbiotic mouth cavity microbiota. S. salivarius and non-pathogenic Neisseria inhabiting the oral biotope are noted for their antagonism against a range of pathogenic bacteria [2, 3]: apparently, preservation thereof may somewhat prevent oropharyngeal colonization by undesirable pathogenic species.

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Table 1. Frequency os isolation of microbiotic species from oropharyngeal mucosa of children before and after biclotymol treatment (n = 30)

Microbes		Before treatment		After treatment	
		Abs.	**%**	**Abs.**	**%**
Staphylococci					
1	*Staphylococcus aureus*	9	30	0	
2	*Streptococcus pyogenes*	3	10	0	
3	*S. dysgalactiae*	1	3	0	
4	*S. anginosus*	2	6	0	
5	*S. intermedius*	3	10	0	
6	*S. constellatus*	1	3	0	
Streptococcus viridans (oral streptococci)					
7	*S. salivarius*	18	60	6	20
8	*S. viridans*	18	60	0	
9	*S. parasanguinis*	4	13	0	
10.	*S. oralis*	3	10	0	
11.	*S. peroris*	3	10	0	
12.	*S. gorgonii*	2	7	0	
13.	*S. cristatus*	1	3	0	
Other streptococci					
14.	*Gemella haemolysans*	4	13	0	
15.	*G. sanguineus*	2	7	0	
Micrococci					
16.	*Rothia mucilaginosa*	3	10	0	
17.	*R. aeria*	1	3	0	
18.	*R. amarae*	1	3	0	
Other gram-positive microbes					
19.	*Granulicatella adiacens*	2	7	0	
20.	*G. elegans*	1	3	0	
21.	*Streptomyces phaeochromogenes*	2	7	0	
22.	*Gracilibacillus dipsosauri*	0	0	1	3
23.	*Agromyces rhizosphera*	1	3	0	0
Gram-positive *Neisseria* cocci					
24.	*N. flavescens*	20	67	18	60
25.	*N. cinerea*	4	13	7	23
26.	*N. perflava*	2	7	4	13
27.	*N. mucosa*	2	7	4	13
28.	*N. macacae*	2	7	4	13
29.	*N. subflava*	1	3	0	
30.	*N. lactamica*	1	3	0	
Hemophilic bacteria					
31.	*Haemophilus haemolyticus*	1	3	0	
32.	*H. parahaemolyticus*	6	20	0	
33.	*H. parainfluenzae*	1	3	0	
34.	*H. influenzae**	1	3	0	

Gram-negative rod-like flora					
35.	*Enterobacter cloacae*	1	3	1	3
36.	*Acinetobacter junii*	1	3	1	3
37.	*Stenotrophomonas spp.*	1	3	1	3
38.	*Kingella denitrificans*	0	0	2	6

Table 2. Comparative frequency of isolation and bacterial growth density (CFUs) before and after biclotymol treatment of mouth cavity microbiota

Microbial species	Before treatment		After treatment	
	Abs.	Average growth density (CFUs)	Abs.	Average growth density (CFUs)
Neisseria species:	32		37	
N. flavescens	20	57.7	18	104.1
N. cinerea	4	47.5	7	36.1
N. perflava	2	13.5	4	95.0
N. mucosa	2	80.0	4	42.5
N. macacae	2	20.0	4	35.7
N. subflava	1	30	0	0
N. lactamica	1	150	0	0
Other microbial species				
S. salivarius	18	139	6	34.0
Enterobacter cloacae	1	55	1	45
Acinetobacter junii	1	40	1	45
Stenotrophomonas spp.	1	50	1	55
Kingella denitrificans	0	0	2	15
Gracilibacillus dipsosauri	0	0	1	50

Pic. 1. Mouth cavity microbiota in children before and after biclotymol treatment

Table 3. Minimum inhibitory concentration (MIC) and CFU count (abs. and %) of the surviving bacteria for different biclotymol concentrations

Microbes	Biclotymol concentration (mg/ml)										MIC (mg/ml)	CFU. control
	40.0	20.0	10.0	5.0	2.5	1.25	0.62	0.31	0.15	0.075		
S. pneumoniae	-	-	-	0	0	0	0	0	0	10 1%	0.15	900
H. influenzae	-	-	-	0	0	0	0	0	0	7 2%	0.15	450
S. pyogenes	-	-	-	0	0	0	0	50 3%	125	-	0.62	1,500
M. catarrhalis	-	-	-	0	0	0	6 0.5%	14	18	125	1.25	1,200
S. aureus	0	0	0	0	25 2%	163	-	-	-	-	5.0	1,700
N. flavescens	0	0	13 0.5%	125	275	-	-	-	-	-	20.0	2,500
N. perflava	0	0	10 0.4%	100	320	-	-	-	-	-	20.0	2,500