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Abstract

Objective: Tamoxifen (TAM), which is used for the treatment of breast cancer, has another function as an antimicrobial agent. Antibacterial action of TAM against the bacteria of the human oral cavity was investigated.

Materials and Methods: Bacteria of the human oral cavity were isolated from the healthy individuals. Different concentrations of TAM were tested against isolated bacteria. Bactericidal and bacteriostatic effects of TAM were also determined.

Results: Out of 23 isolated bacteria, more Gram-positive bacteria were highly susceptible to low concentration of TAM than Gram-negative. *Kytococcus sedentarius*, as one Gram-positive, and *Pseudomonas stutzeri*, as one Gram-negative, needed high MIC value of TAM (2.5 mg/ml) to inhibit by its bacteriostatic action. Resistance to TAM was also observed in three strains of Gram-positive and in four strains of Gram-negative bacteria.

Conclusion: Tamoxifen has a potential antibacterial effect against the bacteria of the oral cavity, especially against Gram-positive. This effect is mostly bacteriostatic. Bacteria-resistance toward TAM was also found.

Key words: bacteria, oral cavity, tamoxifen, Gram-positive, Gram-negative

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Amaç: Meme kanserinin tedavisinde kullanılan Tamoksifen (TAM), antimikrobiyal bir ajan olarak başka bir işleve sahiptir. TAM'ın insan ağız boşluğunun bakterilerine karşı antibakteriyel etkisi araştırılmıştır.

Gereç ve Yöntem: İnsan ağız boşluğu bakterileri sağlıklı bireylerden izole edildi. İzole edilen bakterilere karşı farklı konsantrasyonlarda TAM test edildi. TAM'ın bakterisidal ve bakteriyostatik etkileri de belirlenmiştir.

Bulgular: İzole edilen 23 bakteriden, Gram pozitif bakterilerin Gram negatifine göre düşük TAM konsantrasyonuna karşı çok hassas olduğu görülmüştür. Bir Gram pozitif olarak *Kytococcus sedanter* ve bir Gram negatif olarak *Pseudomonas stutzeri*, bakteriyostatik etkisiyle inhibe etmek için yüksek TAM değeri (2,5 mg / ml) gerektirmiştir. TAM'ye direnç ayrıca üç Gram-pozitif suşta ve dört Gram-negatif bakteri suşunda gözlemlenmiştir.

Sonuç: Tamoksifenin ağız boşluğundaki bakterilere, özellikle Gram-pozitiflere karşı potansiyel bir antibakteriyel etkisi vardır. Bu etki çoğunlukla bakteriyostatiktir. TAM'a bakteri direnci de bulundu.

Anahtar kelimeler: bakteri, ağız boşluğu, tamoksifen, Gram pozitif, Gram negatif

Introduction

Tamoxifen (TAM) as one member of selective estrogen receptor modulators (SERMs) is mainly used for treatment and prevention of estrogen positive breast cancer [1-2]. It was first introduced by AstraZeneca of UK to become a more effective therapy for estrogen positive breast cancer in women of Pakistan and Australia [3]. After more than 40 years, TAM is considered a golden drug for treatment of breast cancer and to extending the lives of approximately 500,000 women every year worldwide [3-5]. The TAM usage as a chemopreventive is another approach approved by the FDA to protect women from breast cancer for at least 5 years [4, 6]. It also found to have an ability to reduce the mortality due to coronary heart disease (CHD) based on two years of postoperative application [7].

The potential antimicrobial activities of TAM against various pathogenic organisms make this drug repurposing toward another approach of treatment [8]. Direct and indirect antibacterial activity of TAM has been confirmed against different types of bacteria. It is showed direct inhibitory action on the growth of *Staphylococcus aureus* and methicillin-resistant *S. aureus* (MRSA) strains [9] and also against *Mycobacterium tuberculosis* [10-11]. The growth of *Enterococcus faecium* and its pathogenesis was found to be reduced by TAM when applied in a *Galleria mellonella* infection model [12]. Meanwhile, indirect antibacterial action of TAM was observed when it enhanced the activity of immune cells represented by neutrophils against various pathogenic bacteria such as MRSA, *Pseudomonas aeruginosa* and *Escherichia coli* through agonist activity of the G protein-coupled estrogen receptor GPER [13]. Moreover, antibacterial action of standard antibiotics such as polymyxin B is also increased against various bacteria after combination with TAM [14].

The treatment of breast cancer by TAM usually takes a long period that could reach to 5 years [3], so the normal bacterial flora of the human body, such as in the oral cavity, could be affected by the potential antibacterial activity of TAM. Thus, *in vitro* antibacterial activity of TAM against the oral flora of the human body was investigated.

Materials and Methods

Chemicals

Tamoxifen citrate was purchased from Ebewe Pharma, Austria. Mueller-Hinton agar (MHA) and Mueller-Hinton broth (MHB) were purchased from HiMedia, India. Cefotaxime was purchased from SDI, Samarra, Iraq.

Isolation of bacteria

Swab samples were collected from the oral cavity of the volunteer healthy individuals by sterilized cotton swabs. Collected samples were cultured on blood agar and MacConkey agar (HiMedia, India). Inoculated media were incubated at 37 °C for 24 hours for growing suspected bacteria. Diagnosis of the isolated bacteria was performed by Vitek® 2 system using Vitek® 2 YST ID diagnostic cards for Gram-positive and Gram-negative bacteria (BioMérieux, France).

Determination of minimum inhibitory concentration (MIC) of TAM

The MIC of tamoxifen was determined based on the methods of dilution antimicrobial susceptibility tests for bacteria that grow aerobically as mentioned by CLSI-M07-A10 (2015) [15]. A broth culture of isolated bacteria was prepared by inoculum selected bacteria in Mueller-Hinton broth (MHB) and incubated at 37° C for 24 hours. The turbidity of growing bacterial cells was adjusted with 0.5 McFarland standards to contain approximately 1×10^8 cfu/ml. Serial concentrations of TAM (5, 2.5, 1.25, and 0.625 mg/ml) were prepared from a stock solution (10 mg/ml). Plastic microdilution plates (96 well) were used to determine the MIC value of TAM. Each well of the plate received 50 µl from standard count of each bacteria suspension and 100 µl from MHB, followed by adding 50 µl of specific concentration of TAM. Several controls were used within a microdilution plate, including MHB with bacteria only, MHB without bacteria, and MHB with TAM only. Cefotaxime was also used as control antibiotic in different concentrations (0.5, 1, 2, 3, and 4 µg/ml). The inoculated plate was incubated at 37 °C for 24 hours. The results were visually read as the presence or absence of bacteria growth.

Bactericides and bacteriostatic effect of TAM was determined by re-culturing of growth inhibited bacteria from the microdilution plate on Mueller-Hinton agar and incubated at 37°C for 24 hours. Growth of inhibited bacteria indicated for bacteriostatic action, while absence of growth showed bactericidal effects.

Results

A total of 23 isolated strains of bacteria were diagnosed after culturing of swab samples from the oral cavity. There were 13 strains of Gram-positive and 10 strains of Gram-negative bacteria. Although more concentrations of TAM were required compared with cefotaxime, most of the Gram-positive bacteria were highly susceptible to low concentration of TAM than the Gram-negative bacteria. They also inhibited in less MIC value of TAM compared with cefotaxime (1-2 than *Kytococcus sedentarius* as one Gram-positive and *Pseudomonas stutzeri* as one Gram-negative needed high concentrations of TAM to inhibit (MIC: 2.5 mg/ml) and the effect of TAM was determined in these two types of bacteria as bacteriostatic action (Table 1 and 2).

The effective low concentrations of TAM on susceptible bacteria which showed MIC at 0.625 mg/ml was mostly determined as bacteriostatic action. This bacteriostatic action was clearly shown in five isolated strains of Gram-positive and in two strains of Gram-negative bacteria, while TAM showed bactericidal action against three strains of Gram-positive and one strain of Gram-negative at the same concentration (Table 1 and 2).

Resistance to TAM was observed in three strains of Gram-positive bacteria, including *Granulicatella elegans*, *Kocuria kristinae* and *Kocuria varians* and in four strains of Gram-negative bacteria, including *Acinetobacter haemolyticus*, *E. coli*, *Enterobacter cloacae* and *Klebsiella pneumonia* (Table 1 and 2).

Discussion

Tamoxifen is an effective drug for the treatment of breast cancer in women and men [3-4]. Based on clinical trial study around the world, TAM can reduce the incidence of breast cancer by 50% in high-risk pre- and postmenopausal women [6]. It also used as adjuvant therapy with more than 5 years efficacy for postmenopausal, node-positive, and estrogen or progesterone receptor positive women since the mid-1980s [3,6]. Thus, TAM is commonly used by more than 7 million patients in a year, saving the lives of approximately 500,000 women [3-4, 6]. In addition to the antagonist binding of TAM with estrogen receptor to prevent the development of breast cancer [4, 6], it also has another mechanism to prevent this type of cancer through stimulating production of TGF-calmodulin and protein kinase C, and also by blocking of the angiogenesis process via lowering of IGF-1 and TGF production [16]. Resistance of bacteria to the most common antibacterial agents has increased progressively within recent times due to massive over-use of this type of agents [17]. Miss use and over-prescription of antibacterial agents is considered the most important factor contributing to the rise of resistant bacterial strains for such type of agents [18]. The bacteria of the oral cavity have also developed drug-resistance toward many common types of antibiotic due to genetic changes in their genomic structure [19]. The resistance of the oral bacteria to erythromycin due to the activity of *mef* and *erm(B)* genes is one example of such antibiotic-resistance [20]. However, the list of antibiotics has hardly changed in 4 decades and most of the pharmaceutical companies left the antibiotic field due to the absence of a new class of antibacterial agents [17]. Thus, antibiotic-resistance is becoming one of the modern crises and the time has come for a global commitment to develop new antibacterial drugs [17, 21]. Repurposing of existing drugs can be introduced as one solution to resolve the limited number of antimicrobial agents and for enhancement of the treatment of most severe bacterial and fungal infections [22]. The repurposing process is usually applied to discovering a new therapeutic action of a specific drug to add to its previously known usage [8]. Tamoxifen is one of those drugs based on many studies which have proved that TAM has a potential antimicrobial effect through introducing promising data to repurpose of its use from anti-cancer drug into antimicrobial agent [9-12, 23]. Low side effect profile and cheap price are other important characters that could encourage continued usage of TAM for treatment of cancer and microbial infections [6].

Out of 23 strains of the oral bacteria isolated in this study, 19 types revealed susceptibility to TAM with variable MIC values. Low concentration of TAM (7.1 µg/ml) exhibited moderate antibacterial effect against *Mycobacterium tuberculosis* [23], while a high concentration of it (MIC₅₀: 5-10 mg/ml) is required to suppress its growth [12]. However, drug-sensitive strains of *M. tuberculosis* could be inhibited by low concentrations of TAM (3.125-6.25 µg/ml) compared with drug-resistance strains (6.25 to 12.5 µg/ml) [10]. Antibacterial effects of TAM were also recorded against various other types of bacteria such as *Enterococcus faecium* and *Staphylococcus aureus* [9, 11] and also against more drug-resistant bacterial strains such as MRSA and *M. tuberculosis* [9-10]. Chemical derivatives of TAM have also shown potent antibacterial action. 4-hydroxytamoxifen, a metabolic derivative of TAM, revealed inhibitory action against *M. tuberculosis* with MIC₅₀ value at 5-10 mg/ml [12]. Newly synthesized triaryl butane, which is an analogue of TAM, exhibited antibacterial activity against Gram-positive and Gram-negative food-borne pathogens, including *Listeria monocytogenes*, *Listeria ivanovii*, *Enterococcus faecalis*, *Staphylococcus aureus* and *E. coli* [24]. Triphenylethylene, which is considered a backbone of TAM, has also shown

antibacterial activity against various types of pathogens including bacteria [8]. Otherwise, TAM was shown to have synergistic action with many known antibiotics to make them more effective against pathogenic bacteria as with polymyxin B against the polymyxin-resistant *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Acinetobacter baumannii* [14, 25], and the three first-line (rifampin, isoniazid, and ethambutol) against *M. tuberculosis* [12]. The activity of chitosan against *E. coli* and *Staphylococcus* spp. was increased in the presence of TAM when they were prepared in nano-fiber PCL structure [26]. Moreover, TAM can play a role to increase the defense ability of the immune cells. This is well proven when the bactericidal activity of neutrophils is increased by TAM against various bacteria such as MRSA, *P. aeruginosa* and *E. coli*, and it can also enhance the bacterial clearance by this immune cell with 2.4–4.2 log reductions in bacterial counts in several types of tissue samples [13]. Intracellular TB in macrophages also decreased after treatment with TAM in a dose-dependent manner [10].

The results of this study have shown that Gram-positive bacteria are more susceptible to TAM than Gram-negative bacteria. The resistance rate in Gram-negative bacteria was also found higher (36-73%) toward many antibiotics than in Gram-positive [27]. Generally, resistance of Gram-negative bacteria is clearly identified toward various types of antibiotic due to its cell wall components which make them a formidable barrier against any dangerous materials [28]. Inhibition of function of the bacterial cell membrane is the proposed mechanism of TAM action against bacteria [23-24, 29]. This type of antibacterial action is mostly related to the hydrophobicity of TAM due to the presence of alkyl groups attached to the amino group in its structure [9]. Ultrastructural alterations in the components of the cell membrane of *Bacillus stearothermophilus* which lead to bacterial cell killing after treatment with TAM are evidence that TAM is a membrane-active drug [29]. This type of alteration which leads to high K^+ and Na^+ efflux from bacterial cells and causes severe damage in the inner and outer membrane is also recognized after treatment of *E. coli* and *Listeria ivanovii* with an analogue of tamoxifen (triaryl butane)[24]. The mitochondrial membrane of *M. tuberculosis* was also observed collapsing by the ionic protonophore uncouplers of TAM and its lipophilic nature [23].

Seven of the present isolated bacteria, including three types of Gram-positive and four types of Gram-negative showed resistance to TAM. This resistance may related to the modification of the lipid or protein composition of the outer membrane of bacterial cells as observed in most of antibiotic-resistant bacteria [28]. Other membrane modifications may include changing in the action of efflux pumps, expression of various drug deactivating agents, and proteolytic degradation [30].

Acinetobacter haemolyticus as one of four members of Gram-negative bacteria that was resistant to TAM, is also recorded to have resistance against other antibiotics. This bacterium which usually causes nosocomial infections and is frequently isolated from the ICU of the hospitals is emerging to be one of the bacteria-resistant to most of antimicrobial agents [31]. Isolates of *A. haemolyticus* from immunocompromised patients revealed a high level of resistance toward a wide range of antibiotics, including ampicillin–sulbactam, ampicillin, aztreonam, cefuroxime, and ceftazidime [32]. This resistance was also observed among isolated strains from ICU patients toward ciprofloxacin, cefepime, ceftazidime, piperacillin, and amikacin [31, 33]. Like clinical isolates, *A. haemolyticus* isolated from the natural environment also showed resistance to antibiotics such as cefotaxime and ceftazidime [34]. However, resistance of *A. haemolyticus* is mainly dependent on its acquisition of beta-lactamase and cephalosporinase enzymes, while resistance to quinolone is related to the mutations in *gyrA* and/or *parC* genes [35].

A second Gram-negative bacterium resistant to TAM was *E. coli*. This type of bacterium is usually sensitive to almost all relevant antibiotics, but it also has the ability to acquire resistance genes from other species of bacteria through horizontal gene transfer [36]. Another source of multiple- antibiotic- resistance may result from change in amino acids of *mar* locus regulator (MarR) or mutation in operator-promoter region *marO* of the bacterium [37]. A total of 137 *E. coli* isolates from cases of urinary tract infection exhibited high resistance (51.1 % to 94.3 %) to ten types of antibiotic [38], while 17 nonpathogenic *E. coli* strains from different sources revealed multiple-antibiotic-resistance due to the genes carried by class 1 and class 2 integrons [37]. However, transfer of resistance genes acquired by *E. coli* could result from plasmids and from other mobile genetic elements, such as transposons and gene cassettes in class 1 and class 2 integrons [36].

Enterobacter cloacae complex (ECC), which contains common nosocomial bacteria that have the ability to cause various types of infection [39], exhibited resistance to TAM based on the results of this current study. The greatest antibiotic resistance by *E. cloacae* was to penicillin, cephalosporins (cefotaxime, ceftazidime, ceftriaxone), aminoglycosides, colistin and fluoroquinolones [40-41]. An emerging resistance of ECC to a new generation of carbapenems was also recorded [39]. Pathogenic strains of *E. cloacae* that caused bacteremia were found to be resistant to cephalothin and ampicillin and a smaller number resistant to aminoglycosides [42]. However, this bacterium becomes resistant by acquiring resistance genes just like other members of Gram-negative bacteria [39].

Klebsiella pneumoniae, which causes various nosocomial infections, sepsis in neonates and bacteremia [43-44], is another bacterium of Gram-negative group that showed resistance to TAM. Such resistance was also recorded against a wide range of antibiotics as shown by a study from 1998 to 2010 in USA [45]. The emergence of such types of multidrug-resistant bacteria have been increasing nowadays due to their production of extended-spectrum beta-lactamases [43] and a mutation in a *mgrB* regulatory gene which lead to resistance to colistin [46].

The current results also exhibited resistance of three isolates of Gram-positive bacteria to TAM, including *Granulicatella elegans*, *Kocuria kristinae* and *Kocuria varians*. *G. elegans* which is related to nutritionally variant streptococci (NVS), is usually found as one of the oral flora with an ability to cause infections and endocarditis under some conditions [47-48]. It is considered the most sensitive species to most antibiotics in comparison to other species of its genus, especially *G. adiacens* [49-50]. Resistance of *G. elegans* to macrolide and beta-lactam antibiotics was recently noticed due to the presence of *erm* and *mef* genes [47, 49, 51]. This resistance could be increased in case of biofilm formation by this bacterium [52].

Our results also showed that two isolates of *Kocuria* spp., including *K. kristinae* and *K. varians*, were resistant to TAM. This Gram-positive cocci bacterium is mostly non-pathogenic, and it mostly causes infection in immunocompromised patients such as with cancer diseases [53-54]. Antibiotic-resistance is hardly recognized in this bacterium due to very limited available data [53]. Thus, the absence of useful guidelines for determining the antibiotic-resistance of *Kocuria* spp. makes the susceptibility test necessary according to the *Staphylococcus* guidelines [54]. However, *K. kristinae* found to be more resistant to antibiotics than *K. varians*. All isolates of *K. varians* from endophthalmitis patients were found sensitive to all tested antibiotics, while *K. kristinae* shown resistance to amikacin and cefazolin [55]. Other isolates of *K. kristinae* from patients with UTI, immunosuppressive conditions, and cancer diseases also exhibited resistance to a wide range of antibiotics [56-57]. Meanwhile, all isolates of *K. varians* from periodontitis and brain abscess exhibited sensitivity to all tested antibiotics [58-59].

Conclusion

The antibacterial action of TAM was clearly observed against oral bacteria, especially against Gram-positive bacteria. The action was mostly a bacteriostatic effect. The repurposing of TAM from cancer therapy into antimicrobial treatment could be encouraged by many factors such as that TAM is a cheap drug with few adverse effects. Although some bacteria show resistance, most known virulent species of isolated bacteria were found to be sensitive to TAM. This will give a promising view to use TAM in the treatment of infections caused by such types of bacteria.

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Table 1: The minimum inhibitory concentration (MIC) of TAM in Gram-positive bacteria

No.	Bacteria	MIC value (mg/ml)	MBC
1	<i>Dermaococcus nishinomiyaensis</i>	0.625	-
2	<i>Gemella sanguinis</i>	0.625	+
3	<i>Granulicatella adiacens</i>	0.625	-
4	<i>Granulicatella elegans</i>	R	+
5	<i>Helcococcus kunzii</i>	0.625	+
6	<i>Kocuria kristinae</i>	R	+
7	<i>Kocuria rosea</i>	1.25	+
8	<i>Kocuria varians</i>	R	+
9	<i>Kytococcus sedentarius</i>	2.5	-
10	<i>Leuconostoc mesenteroides</i> ssp. <i>cremoris</i>	0.625	-
11	<i>Staphylococcus aureus</i>	0.625	+
12	<i>Staphylococcus pseudintermedius</i>	0.625	+
13	<i>Staphylococcus warneri</i>	0.625	+

All tested bacteria were sensitive to 1-2 µg/ml cefotaxime

Table 2: The minimum inhibitory concentration (MIC) of TAM in Gram-negative bacteria

No.	Bacteria	MIC value (mg/ml)	MBC
1	<i>Acinetobacter haemolyticus</i>	R	+
2	<i>Aeromonas salmonicida</i>	1.25	+
3	<i>Burkholderia cepacia</i>	0.625	-
4	<i>E. coli</i>	R	+
5	<i>Enterobacter cloacae</i> complex	R	+
6	<i>Klebsiella pneumoniae</i> ssp. <i>pneumoniae</i>	R	+
7	<i>Pseudomonas fluorescens</i>	1.25	-
8	<i>Pseudomonas stutzeri</i>	2.5	-
9	<i>Ralstonia insidiosa</i>	0.625	+
10	<i>Sphingomonas paucimobilis</i>	0.625	+

All tested bacteria were sensitive to 1-2 µg/ml cefotaxime