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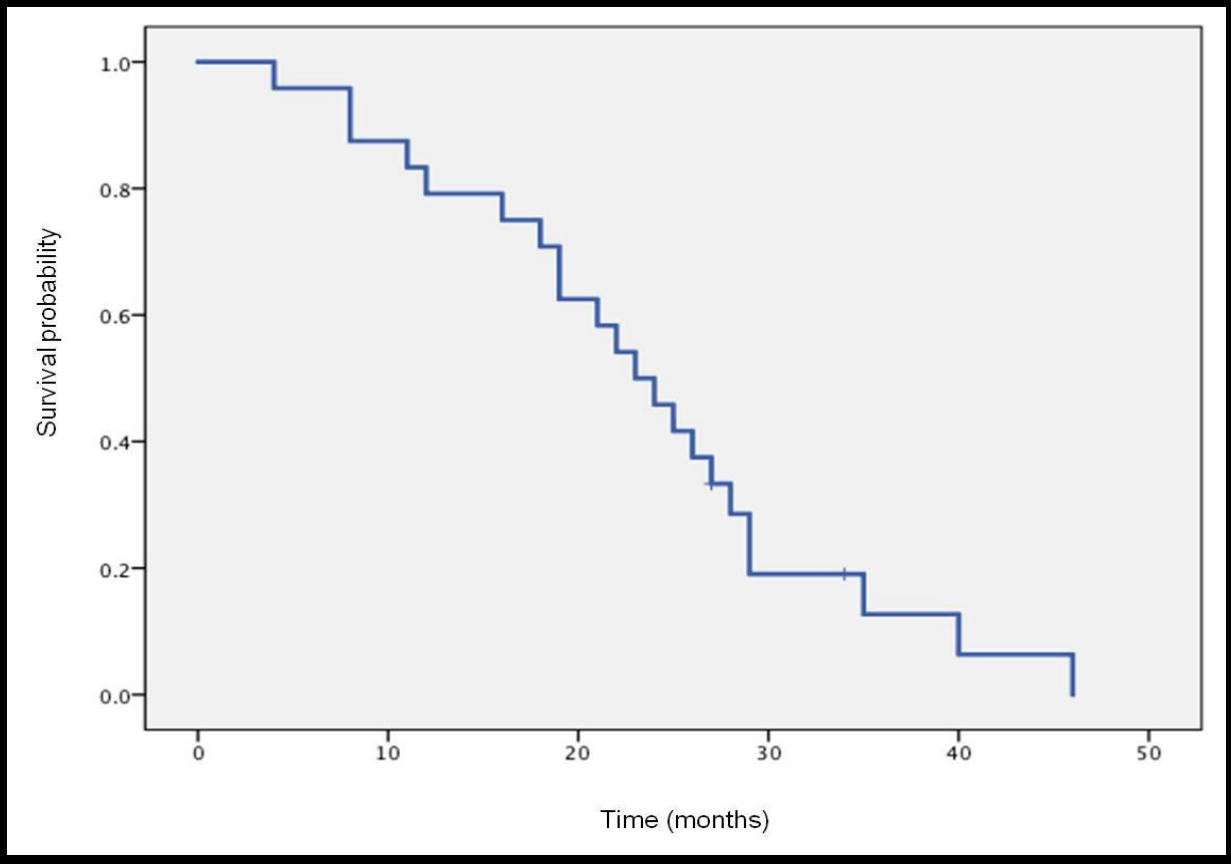

- **Cyberknife re-irradiation for recurrent glioblastoma multiforme The effect of TGN-020 on penicillin induced epileptiform activity in rats**
- **Investigation of antibiotic susceptibility profile and minimal inhibitor concentration changes in Pseudomonas aeruginosa isolates that exposed to subinhibitory concentrations of antibiotic**
- **An effective approach for botulinum toxin injection in patients with stroke for focal spasticity: dual guidance**

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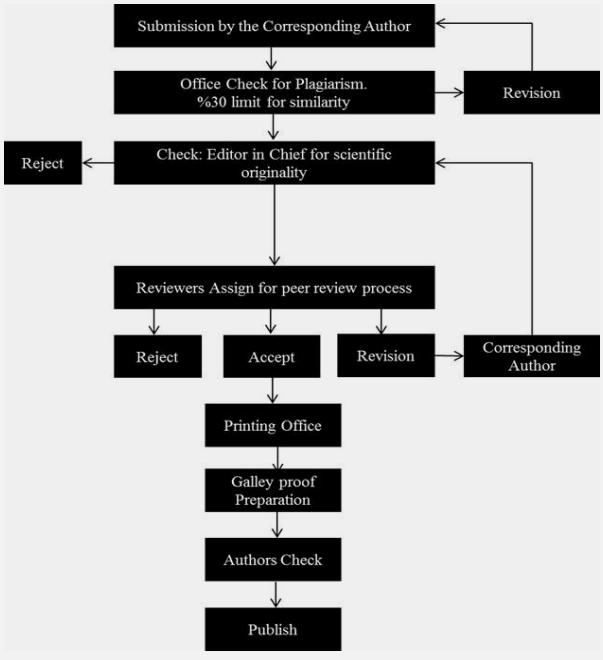

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Contents

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Research Article Doi: 10.17546/msd.452046

Investigation of antibiotic susceptibility profile and minimal inhibitor concentration changes in *Pseudomonas aeruginosa* isolates that exposed to subinhibitory concentrations of antibiotic

Cetin Kilinc[1], Ridvan Guckan[2], Umut Safiye Say Coskun[3]*

Abstract

Objective: During antibiotic use some of the bacteria in our flora can be affected by the used antibiotic in subinhibitory concentrations in addition to pathogenic microorganisms. The aim of this study to investigate in-vitro effects of subinhibitory concentrations antibiotic on antibiotic susceptibility profile of *P.aeruginosa* which can be found in normal flora and be a pathogenic bacteria.

Material and Method: The antibiotic effective concentrations decrease with distance from the antibiotic disc and growth-inhibition zone ends with the effect of the antibiotic falls to subinhibitory concentrations; and growth starts.We accepted this growth starting region as the area in which bacteria exposed to subinhibitory concentrations of antibiotic are located and we developed a model. We separetely exposed the standard *P.aeruginosa* strain to eight different antibiotics (amikacin, gentamicin, imipenem, meropenem, ceftazidime, cefepime, ciprofloxacin, colistin) for seven days in subinhibitory concentrations. *P. aeruginosa* strain is susceptible to these antibiotics and we monitored susceptibility and minimal inhibitor concentration changes. Moreover, we also made these procedures in 20 different clinical *P.aeruginosa* isolates.

Results: We observed that a resistance was developed in the standard *P. aeruginosa* strain starting second day of meropenem exposure, third day of ceftazidime exposure, fifth day of amikacin exposure and sixth day of gentamicin exposure. There was no resistance development after colistin, cefepime, ciprofloxacin, meropenem exposure but significant MIC value increases were detected. This resistance was not only against exposed antibiotic or antibiotic group but also against antibiotics in different antibiotic groups.

Conclusion: It was shown that especially subinhibitory concentrations using carbapenem and aminoglycoside antibiotics triggered resistance development against themselves more than other antibiotic groups. Use of colistin was not shown to cause cross resistance.

Key words: Subinhibitory concentrations, *P. aeruginosa*, antibiotic susceptibility, MIC value changes

Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is found in human body flora, may live in nutrient poor environments (distilled water… etc.) also colonize in hospitals, and cause infections with high mortality and morbidity ratios. Failure to treat infections caused by intrinsic resistance to many antibiotics as well as resistance to antibiotics that are susceptible even during treatment is encountered.Antibiotics that can be used in the treatment of *P. aeruginosa* infections with increasing resistance rates are limited(1-4).

Pathogenic bacteria can expose to nonlethal concentrations of antibiotic (subinhibitory concentrations) for days during the treatment of these infections although they are susceptible to that antibiotic because of using insufficient dose of that antibiotic or reaching of unsufficent concentrations of antibiotic to the area where bacteria locate. During the use of a systemic antibiotic not only infectious bacteria but also all other bacteria in normal body flora can be exposed to inhibitor or subinhibitory concentrations of antibiotics for days.

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1 Kastamonu State Hospital, Microbiology Laboratory Kastamonu, TR
2 Amasya University Training and Researh Hospital, Department of Medical Microbiology Amasya, TR
3 Tokat Gaziosmanpasa University School of Medicine, Department of Medical Microbiology Tokat, TR
*** Corresponding Author:** Umut Safiye Say Coskun **E-mail:** umut.saycoskun@gop.edu.tr **Phone:** +90 (505) 541 08 56

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In our study we aimed to investigate invitro effect of subinhibitory concentrations of antibiotic exposure on antibiotic susceptibility profile of *P. aeruginosa.*

Material and methods

In the Kirby Bauer disc diffusion method, the antibiotic effective concentrations decrease with distance from the antibiotic disc and growth-inhibition zone ends with the effect of the antibiotic falls to subinhibitory concentrations; and growth starts. We developed a model by accepting this region in which growth started as an area which includes bacteria that exposed to subinhibitory concentrations of antibiotics. In our study we used eight different antibiotic discs (Oxoid, U.K) amikacin (30 µg), gentamicin (10 µg), imipenem (10 µg), meropenem (10 µg), ceftazidime (30 µg), cefepime (30 µg), ciprofloxacin (5 µg), colistin (10 µg) and *P. aeruginosa* ATCC 27853 isolate which are known to be susceptible to these antibiotics and other 20 clinical *P. aeruginosa* isolates. We identified minimal inhibitor concentration (MIC) values of 21 isolates against eight different antibiotics which they were susceptible and we exposed these isolates to subinhibitory concentrations of these antibiotics for seven days. *P. aeruginosa* ATCC 27853 isolate colonies which grow on Eosin Metilene Blue medium homogenized in saline adjusted to the turbidity of 0.5 McFarlandand streaked onto Mueller-Hinton agar (Oxoid, U.K) for Kirby Bauer disc diffussion method. We placed amikacin disc in the middle of medium and after 24 hour incubation at 37^0C and colonies were collected from the region which exposed to subinhibitory concentrations of antibiotic around the disc (Figure 1).

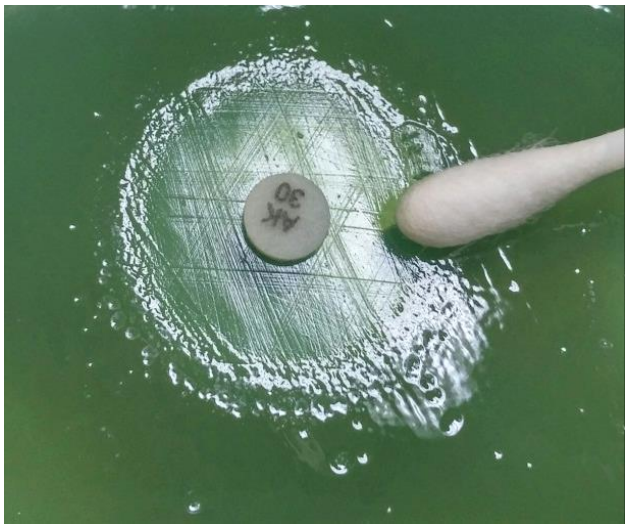

Figure 1: Colony intake from the region which exposed to sub-inhibitor doses of amikacin

Collected colonies were adjusted to 0.5 Mcfarland standard with saline and passaged to Mueller Hinton agar again and incubated for one day after placement of amikacin disc in the middle of passage. This process was repeated for 7 consecutive days.Thus we exposed this bacteria to subinhibitory concentrations of amikacin in vitro for seven consecutive days (Figure 2).

The susceptibility (Kirby Bauer disk diffusion method) and MIC (E test (Oxoid, U.K.)) values of *P. aeruginosa* ATCC 27853 isolate which is known against all antibiotics before amikacin exposure and amikacin susceptibility changes during exposure from day to day were monitored. End of the seventh day it is controlled changes of inhibition zone and MIC values of not only exposed amikacin but also all antibiotics (amikacin, gentamicin, imipenem, meropenem, ceftazidime, cecefepimeime, ciprofloxacin, colistin) which bacteria is susceptible.

Same procedure as above, which we performed with amikacin to *P. aeruginosa* ATCC 27853 isolate, was also applied to other seven antibiotics seperately.

Procedures that we use with *P. aeruginosa* isolate (ATCC 27853) was also applied with 20 different clinical isolates. Furthermore, in order to control whether repeated passages cause any changes for resistance profile of the bacteria; a standard isolate was passaged for seven consecutive days. In our study; antibiotic susceptibilities were controlled according to 2013 Clinical and Laboratory Standards Institute criteria(CLSI) (5). Mid-susceptible isolates were considered resistant.

Results

We detected changes in susceptibility of the *P. aeruginosa* ATCC 27853 isolate against antibiotic which was exposed to subinhibitory concentrations for seven days and MIC values before and after antibiotic exposure. For *P. aeruginosa* ATCC 27853 isolate which exposed to subinhibitory concentrations of antibiotic for seven days, resistance development were not determined after exposure to ciprofloxacin, cefepime, colistin, meropenem antibiotics. But an elevation of MIC values against these oantibiotics was observed. Earliest resistance development according to days was observed as imipenem (second day), ceftazidime (third day), amikacin (fifth day), gentamicin (sixth day) , respectively (Table 1).

P. aeruginosa ATCC 27853 isolate's susceptibility profile was observed not only for the antibiotic that the isolate was exposed but also the exposed antibiotic affects on other antibiotics and MIC values for seven days. (Table 2)

Furthermore, we made these procedures for 20 different clinical isolates in addition to the *P. aeruginosa* ATCC 27853 isolate, we identified susceptibility and MIC value changes of 20 clinical *P. aeruginosa* isolates which were exposed to subinhibitory concentrations of antibiotic for seven days (Table 3).

No changes were detected in antibiotic susceptibility profile of *P. aeruginosa* ATCC 27853 which was passaged for seven consecutive days.

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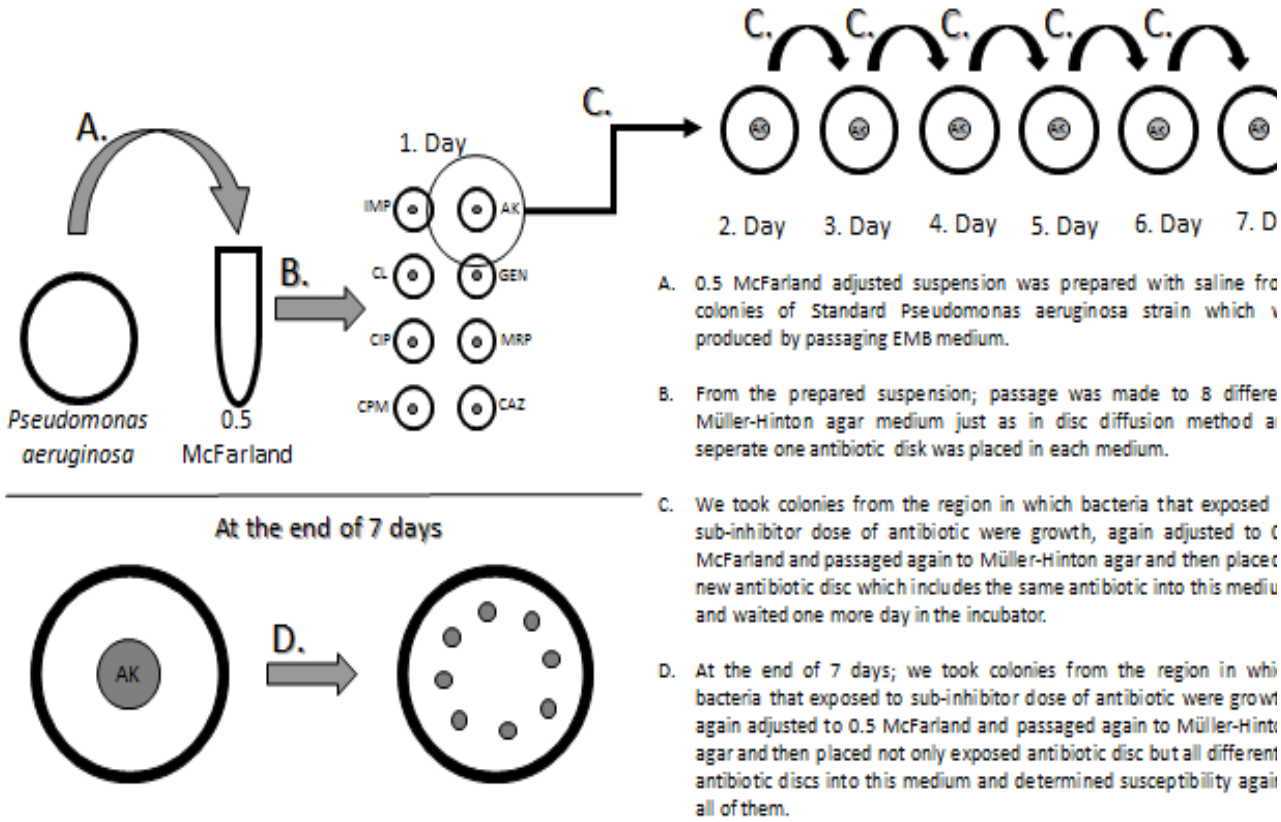

Figure 2: Antibiotic exposure of standard pseudomonas strain for 7 days

Table1: Susceptibility and MIC value changes of ATCC strains from day to day which exposed to sub-inhibitor doses of antibiotic.

Exposed antibiotic	1.day Zone diameter (Susceptibility) /MIC	2. day Zone diameter /Susceptibility	3.day Zone diameter /Susceptibility	4.day Zone diameter /Susceptibility	5. day Zone diameter /Susceptibility	6. day Zone diameter /Susceptibility	7. day Zone diameter (Susceptibility) /MIC
Cefepime	25(S)/<1	24(S)	22(S)	22(S)	20(S)	18(S)	16(S)/8
Ceftazidime	20(S)/2	19(S)	15(R)	13(R)	11(R)	11(R)	10(R)/32
Imipenem	26(S)/2	15(R)	14(R)	14(R)	15(R)	13(R)	12(R)/>16
Meropenem	26(S)/0,5	26(S)	22(S)	20(S)	18(S)	18(S)	17(S)/2
Gentamicin	28(S)/<2	29(S)	24(S)	21(S)	20(S)	14(R)	12(R)/8
Amikacin	26(S)/<2	25(S)	20(S)	18(S)	16(R)	15(R)	13(R)/32
Ciprofloxacin	34(S)/<0,2	33(S)	33(S)	29(S)	30(S)	28(S)	24(S)/1
Colistin	15(S)/<0,5	13(S)	14(S)	12(S)	13(S)	12(S)	11(S)/1

Table 2: Susceptibility and mic value changes status of exposed antibiotic (AK) and other antibiotics after exposure of ATCC strain to sub-inhibitor dose of susceptible antibiotics.

	Antibiotics whose susceptibility status was controlled at the end of seventh day							
	Cefepime	Ceftazidime	Imipenem	Meropenem	Gentamicin	Amikacin	Ciprofloxacin	Colistin
Cefepime	A	R	N	A	N	N	N	N
Ceftazidime	A	R	N	N	N	N	N	N
Imipenem	N	N	R	R	N	N	N	A
Meropenem	N	A	R	A	N	N	A	A
Gentamicin	N	A	N	N	R	R	N	N
Amikacin	N	N	N	N	R	R	N	N
Ciprofloxacin	A	N	R	A	N	N	A	N
Colistin	N	N	N	N	N	N	N	A

N: No changes for susceptibility and mic value, **R**: Resistant, **A**: MIC value increased although susceptibility continues

Table 3: Susceptibility and mic value changes of 20 clinical P.aeuroginosa isolates which were exposed to sub-inhibitor dose of antibiotic.

Exposed antibiotic for 7 days	Number of resistant isolates after antibiotic exposure and antibiotics to which resistance developed (%)	MIC values of isolates increased despite the lack of development of resistance after exposure to antibiotics (%)
Cefepime	In 11 strains cefepime (55%), in 10 strains ceftazidime (50%) , in 5 strains imipenem (25%), in 4 strains meropenem (20%), in 3 strains colistin (15%), in 2 strains ciprofloxacin (10%) resistance developed	Mic value increased in 9 strains against cefepime (45%), in 10 strains against ceftazidime (50%), in 4 strains against imipenem (20%), in 5 strains against meropenem (25%), in 5 strains against ciprofloxacin (25%), in 3 strains against colistin (15%).
Ceftazidime	In 13 strains ceftazidime (65%), in 10 strains cefepime (50%), in 3 strains imipenem (15%), in 2 strains meropenem (10%), in 1 strain colistin (5%) resistance developed	Mic value increased in 7 strains against ceftazidime (35%), in 10 strains against cefepime (50%), in 3 strains against imipenem (15%), in 3 strains against meropenem (15%), in 3 strains against ciprofloxacin (15%), in 2 strains against colistin(10%).
Imipenem	In 17 strains imipenem (85%), in 15 strains meropenem (75%), in 6 strains cefepime (30%), in 4 strains ceftazidime (20%), in 4 strains ciprofloxacin (20%), in 3 strains colistin (15%) resistance developed.	Mic value increased in 3 strains (15%) against imipenem, in 5 strains against meropenem (25%), in 2 strains against cefepime (10%), in 2 strains against ceftazidime (10%), in 3 strains against ciprofloxacin (15%) and in 2 strains against colistin(10%).
Meropenem	In 14 strains meropenem (70%), in 13 strains imipenem (65%), in 6 strains cefepime (30%), in 4 strains ceftazidime (20%), in 4 strains ciprofloxacin (20%), in 3 strains colistin(15%) resistance developed.	Mic value increased in 7 strains against imipenem (20%), in 6 strains against meropenem (30%) , in 2 strains against cefepime (10%) , in 2 strains against ceftazidime (10%) , in 4 strains against ciprofloxacin (20%) , in 4 strains against colistin (20%).
Gentamicin	In 20 strains gentamicin (100%), in 16 strains amikacin (80%), in 4 strains ciprofloxacin (20%), in 3 strains imipenem (15%), in 3 strains meropenem (15%), in 2 strains colistin (10%) resistance developed.	Mic value increased in 3 strains against colistin (15%) , in 5 strains against imipenem, in 2 strains against meropenem (10%) , in 4 strains against ciprofloxacin, and in 4 strains against amikacin (20%).
Amikacin	In 18 strains amikacin (90%), in 14 strains gentamicin (70%), in 4 strains imipenem (20%), in 3 strains meropenem (15%), in 3 strains colistin (15%) resistance developed.	Mic value increased in 3 strains against colistin (15%) , in 5 strains against imipenem (25%) , in 2 strains against meropenem (10%) , in 2 strains against amikacin (10%) , in 6 strains against gentamicin (30%).
Ciprofloxacin	In 7 strains ciprofloxacin (20%), in 3 strains imipenem (15%) , in 2 strains meropenem (10%), in 2 strains colistin (10%) , in 2 strains ceftazidime (10%) and in 2 strains cefepime(10%) resistance developed.	Mic value increased in 13 strains against ciprofloxacin (65%) , in 5 strains against imipenem (25%) , in 2 strains against meropenem (10%) , in 2 strains against colistin (10%) , in 2 strains against ceftazidime (10%) and in 1 strain against cefepime (5%) .
Colistin	In 2 strains colistin (10%) resistance developed.	Mic value increased in 3 strains against colistin (15%) .

Discussion

Antibiotics came into use in the past hundred years and have provided the most significant contribution to human life and make it possible to successfully cure many of deadly infectious diseases. Antibiotics are one of the most important inventions in human history and they have significantly lost their effects because of resistance particularly due to inappropriate and unnecessary use. Microorganisms gain oppositional force, namely resistance, sooner or later against antimicrobials which are used to destroy these microorganisms. Resistance to against antimicrobial agents, today is a very significant problem which will threaten humanity. In a kind of microorganism that has become resistant to an antimicrobial agent; resistance may develop against other antimicrobials which are similar with chemotherapeutic agent in terms of structure or effect (6). Pathogenic bacteria can survive despite exposure to subinhibitory concentrations of antibiotic during treatment for several days although they are susceptible to that antibiotic because of using insufficient amount of the antibiotic or reaching inadequate concentrationss of antibiotic to the area where bacteria locates. Besides bacteria that are members of the normal human flora might be exposed to subinhibitory concentrations of antibiotic during treatment. *P. aeruginosa* is also one of the bacteria which can be exposed to subinhibitory concentrations of antibiotic both as a pathogenic bacteria and member of normal human flora.

Susceptibility against exposed antibiotic and MIC value changes of *P. aeruginosa* ATCC 27853 isolate, which exposed to subinhibitory concentrations of antibiotic, was observed for seven days. It was seen that resistance developed starting from second day of meropeneme exposure, third day of ceftazidime exposure, seventh day of amikacin exposure and sixth day of gentamicin exposure. Although there was no resistance development after colistin, cefepime, ciprofloxacin, meropenem exposures; significant mic value increases were observed (Table1). Resistance development was not only against exposed antibiotic and antibiotic group, but also against antibiotics in different groups. In fact, antibiotics with increased MIC values were observed despite of no change in susceptibility status. In *P. aeruginosa* ATCC 27853 isolate, after ciprofloxacin exposure while imipeneme resistance developed, MIC values against cefepime and meropeneme increased. In the same isolate, amikasin resistance developed after gentamicin exposure, imipeneme resistance developed after meropenem exposure, ceftazidime resistance developed after cefepime exposure, respectively(Table2).

In the treatment of infections caused by *P. aeruginosa* isolates; different groups of antibiotics are used. As carbapenems which are one of the most broad-spectrum b-lactam antibiotics, are resistant against hydrolysis of various beta-lactamase such as extended spectrum beta-lactamases (ESBLs); they can be effectively used for the treatment of infections caused by resistant Gram negative bacteria as *P. aeruginosa* but in the last years increased carbapenem resistance was reported in *Pseudomonas* isolates (7, 8).

Carbapenem resistance of *P. aeruginosa* can be due to OprD pore loss, MexABOprM active efflux pumping system, permeability mutations, excessive production of chromosomal AmpC beta-lactamase and production of metallo-beta-lactamase enzymes (9). In case of OprD pore loss; meropenem can be susceptile while imipenem is resistant. In MexAB-OprM active efflux pumping system; resistance to develope all beta-lactamases except for imipenem. In togetherness of MexEF-OprN efflux pumping and oprD pore loss; imipenem and quinolone resistant, meropenem susceptible isolates are seen. For development of meropenem resistance during treatment; both pore protein loss and mutation of active efflux pumping system are needed (10, 11).

In our study, among 20 different *Pseudomonas* isolates which exposed to subinhibitory concentrations of imipenem for seven days resistance developed in 17 isolates for imipenem, 15 isolates for meropenem, 6 isolates for cefepime, 4 isolates for ceftazidime, 4 isolates for ciprofloxasin and 3 isolates for colistin, respectively.Besides, although susceptibility resumed in three isolates against imipenem, in five isolates against meropenem, in two isolates against cefepime, in two isolates against ceftazidime, in three isolates against ciprofloxacine and in two isolates against colistin; significant increases for MIC values of these isolates against these antibiotics were observed. Among 20 different *Pseudomonas* isolates which exposed to subinhibitory concentrations of meropenem for seven days resistance developed in 14 isolates for meropenem, 13 isolates for imipenem, 6 isolates for cefefim, 4 isolates for ceftazidime, 3 isolates for ciprofloxacin and 3 isolates for colistin, respectively. Besides, although susceptibility resumed in seven isolates against imipenem, in six isolates against meropenem, in two isolates against cefepime, in two isolates against ceftazidime, in four isolates against ciprofloxacine and in four isolates against colistin; significant increases for mic values of these isolates against these antibiotics were observed (Table 3).

It was suggested that subinhibitory concentrationss of carbapenem exposure in *Pseudomonas* isolates might trigger resistance mechanisms such as pore loss, beta-lactamase activation, permeability mutations, active efflux pumping system and as a result can cause resistance development against both the used antibiotic and different antibiotic groups such as cephalosporin and quinolone.

Another group of antibiotics with activity against *P. aeruginosa* is aminoglycoside. Aminoglycoside resistance can be due to change of affinity against ribosomes (cause resistance in only aminoglycosides), active efflux pump, mutations that can cause membrane permeability changes and aminoglycoside modifying enzyme mutations (6,12,13). Aminoglycoside resistance in *P. aeruginosa* is generally due to aminoglycoside-modifying enzymes and decrease in membrane permeability (14).

Among 20 different *P. aeruginosa* isolates which exposed to subinhibitory concentrations of amikacin for seven days resistance developed in 18 isolates for amikacin, 14 isolates for gentamicin, 4 isolates for imipenem, 3 isolates for meropenem and 3 isolates for colistin, respectively. Besides, although susceptibility resumed in 5 isolates against imipenem, in 2 isolates against meropenem, in 2 isolates against amikacin, in 6 isolates against gentamicin and in 3 isolates against colistin; significant increases for mic values of these isolates against these antibiotics were observed. Among 20 different *P. aeruginosa* isolates which exposed to subinhibitory concentrations of gentamicin for seven days resistance developed in 20 isolates for gentamicin,16 isolates for amikacin, 4 isolates for ciprofloxasin,3 isolates for imipenem, 2 isolates for meropenem and 2 isolates for colistin, respectively. Besides, although susceptibility resumed in six isolates against gentamicin five isolates against imipenem, in two isolates against meropenem, in four isolates against ciprofloxacin, in four isolates against amikacin and in three isolates against colistin; significant increases for MIC values of these isolates against these antibiotics were observed (Table 3).

It is suggested that exposure to subinhibitory concentrationss of aminoglycosides in *P. aeruginosa* isolates can cause aminoglycosides resistance via triggering ribosomal mutations and release of aminoglycosides modifying enzymes and in addition to that aminoglycosides, carbapenems and quinolone resistance via permability mutations and activation of active efflux pump. Main mechanism for resistance to quinolones is mutation of DNA gyrase enzyme and in addition to that the change in outer membrane permeability due to defects of outer membrane proteins such as OmpF, OmpC and active efflux pumping systems can also cause quinolone resistance. Changes in outer membrane porins and efflux pumping systems due to chromosomal mutations can cause resistance to other antimicrobial agents in addition to quinolone resistance (6,15).

Among 20 different *P. aeruginosa* isolates which exposed to subinhibitory concentrations of ciprofloxacine for seven days resistance developed in 7 isolates for ciprofloxacine, 3 isolates for imipenem, 2 isolates for meropenem, 2 isolates for cefepime, 2 isolates for ceftazidime, 2 isolates for colistin, respectively. Besides, although susceptibility resumed in 13 isolates against ciprofloxacin, in five isolates against imipenem, in two isolates against meropenem, in two isolates against ceftazidime, in one isolate against cecefepimeime and in two isolates against colistin; significant increases for MIC values of these isolates against these antibiotics were observed (Table 3).

Ciprofloxacin resistance developed in *P. aeruginosa* isolates due to DNA gyrase mutation caused by exposure to subinhibitory concentrations of quinolone and in addition to that this exposure can cause mutations of outer membrane porins and efflux pumping systems which results with resistance to carbapenems and cephalosporins in adition to quinolone resistance. It has been reported that resistance against cephalosporins in *P.aeruginosa* isolates is increasing. In *P. aeruginosa*, resistance to beta-lactam antibiotics may develop due to AmpC enzyme, ESBL, carbapenemases, efflux, permeability changes (16, 17).

Among 20 different *P. aeruginosa* isolates which exposed to subinhibitory concentrations of ceftazidime for seven days resistance developed in 13 isolates for ceftazidime, 10 isolates for sefepim, 3isolates for imipenem, 2 isolates for meropenem, 1 isolates for colistin,respectively. Besides, although susceptibility resumed in seven isolates against ceftazidime, in ten isolates against cecefepimeime, in three isolates against imipenem, in three isolates against meropenem, in three isolate against ciprofloxacine and in two isolates against colistin; significant increases for MIC values of these isolates against these antibiotics were observed.

Among 20 different *P. aeruginosa* isolates which exposed to subinhibitory concentrations of cefepime for seven days resistance developed in 11 isolates for cefepime, 10 isolates for ceftazidime, 5 isolates for imipenem, 4 isolates for meropenem, 3 isolates for colistin and 2 isolates for ciprofloxasin,respectively. Besides, although susceptibility resumed in nine isolates against cecefepimeime, in ten isolates against ceftazidime, in four isolates against imipenem, in five isolates againstmeropenem, in five isolate against ciprofloxacine and in three isolates against colistin; significant increases for mic values of these isolates against these antibiotics were observed (Table 3).

Subinhibitory concentrations of cephalosporin exposure in *P. aeruginosa* isolates; can trigger resistance mechanisms such as AmpC enzyme, ESBL, carbapenemases, efflux pumping, permability changes and can cause resistance against beta-lactam antibiotics such as cephalosporins and carbapenems due to these resistance mechanisms. In addition to that changes in permability and efflux pump systems can also cause resistance against quinolones. Especially resistance development via various mechainsms against colistin can be seen which are used against multi drug resistant gram negatives. Resistance development is related with decrease of binding points for colistin on cell and decrease of outer membrane polarity. In resistance development PmrA-PmrB and PhoQ-PhoP regularotry systems play role. Besides cross-resistance can be seen between polymyxins (18, 19).

Among 20 different *P. aeruginosa* isolates which exposed to subinhibitory concentrations of colistin for seven days, colistin resistance deveoped in two isolates. Furthermore, although susceptibility resumed in three isolates against colistin; significant increases for MIC values of these isolates against these antibiotics were observed (Table 3).

Exposure to subinhibitory concentrations of colistin in *P. aeruginosa* isolates caused a decrease in binding points of colistin to bacteria and outer membrane polarity. This effect led to colistin resistance with low ratio. Colistin resistance, which develops after exposure to subinhibitory concentrations of other antibiotic groups mentioned above, is related with changes of outer membrane polarity and colistin binding points. Moreover, it was observed that

colistin exposure did not cause any changes of resistance rates of bacteria against other antibiotic groups.

In different studies; it was shown that subinhibitory concentrations of antibiotic can trigger slime formation in *P. aeruginosa* isolates (20-22). This finding suggests that increased resistance after antibiotic exposure can be due to slime formation.

As it was seen in this study; bacteria which can not be killed after exposure to antibiotics can become a much more dangerous infection potential. In studies resistance development of *P.aeruginosa* in a short period was shown in vitro against subinhibitory concentrations of carbapenem or quinolones (23, 24).

In our study cross-resistance development against not only the exposed antibiotic but also various other antibiotics in different groups was shown in most of the isolates which exposed to subinhibitory concentrations of antibiotics and isolates with mult-drug resistance occured. It was shown that especially subinhibitory concentrations use of carbapenem and aminoglycoside antibiotics triggered resistance development against themselves more than other antibiotic groups. This ratio was lower for ciprofloxacin and colistin. Cross-resistance did not develop in isolates which exposed to subinhibitory concentrations of colistin. It was shown that use of different antibiotic groups in subinhibitory concentrations can cause colistin resistance or increase in MIC ratios. *P. aeruginosa* isolates were susceptible against all antibiotics used in our study at the beginning but after exposure of these bacteria to non-lethal concentrationss of these antibiotics; isolates have emerged which are resistant to various antibiotics and the antibiotics used. So, use of appropriate antibiotics with inappropriate amounts can also cause serious problems.

In conclusion, the effect of antibiotics on the bacteria is not limited to just killing them. Subinhibitory concentrations use of antibiotics might change a isolate which is infectious agent into a isolate with multi-drug resistance during treatment and disrupt treatment or some of the bacteria in our flora can turn into a more resistant bacteria after subinhibitory concentrations antibiotic exposure, even become dominant in flora after natural selection and could become a severe infection potantial for the future.

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Ethical issues: All Authors declare, Originality and ethical approval of research. Responsibilities of research, responsibilities against local ethics commission are under the Authors responsibilities. The study was conducted under defined rules by the Local Ethics Commission guidelines and audits.

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Cyberknife re-irradiation for recurrent glioblastoma multiforme

Ozlem Yersal[1*]

Abstract

Objective: Treatment of patients with recurrent glioblastoma multiforme (GBM) is challenging. Treatment alternatives include re-operation, chemotherapy and re-irridation. Stereotactic radiosurgery with cyberknife is a good therapeutic approach to deliver high-dose radiation to a definite target volume with minimizing re-irradiation to nearby healthy tissues. This study, evaluated the efficacy of cyberknife treatment in 24 patients with recurrent GBM.

Methods: Total 24 patients with recurrent GBM who received cyberknife treatment in any line of recurrence between the 2011, 2015 were included in this study. A median dose of 30 Gy was applied to each patient.

Results: Median survival was 10.3 months after cyberknife treatment and 23 months after diagnosis. Patients younger than 60 years (4.8 vs 14.2 month; p:0.05) and patients with primary total tumor excision (9.3 vs 4.9 month; p:0.05) had longer overall survival than other patients in univariate analysis but not in multivariate analysis. In this patient population, any other variables predicting longer overall survival could not be found. Treatment was well-tolerated and no severe toxicities observed.

Conclusion: Although limitations exist, our study demonstrates that SRS in terms of cyberknife for recurrent GBM is feasible and well tolerated by patients with low toxicity.

Key words: Sterotactic radiotherapy, glioma, cyberknife, recurrent, GBM

Introduction

Glioblastoma multiforme (GBM) is the most aggressive primary brain tumor of adults with a median overall survival of around a year (1). Primary treatment modalities consist of maximal safe surgical resection and radiochemotherapy with temozolamide followed by temozolamide chemotherapy (2). Despite multimodality treatment, almost all patients experience recurrence and prognosis remains dismal for these patients (3).

The treatment of recurrent patients is challenging because of, high rates of morbidity and toxicity of treatment in this setting. Second surgery, can be performed in a subset of patients, but it may cause a high risk of neurologic sequele, because of the infiltrative behavior of the tumor (4). Bevacizumab plus irinotecan combination chemotherapy, demonstrated significant antitumor activity in recurrent GBM with a 6 month progression free survival, which resulted with its approval by the US Food and Drug Administration (5-7).

However, treatment options for recurrent patients remain limited and optimal treatment schedules should be established.

Another effective treatment option for recurrent GBM is re-irradiation, which can be achieved with stereotactic radiosurgery (SRS) in the form of cyberknife treatment. Stereotactic radiosurgery is a good therapeutic option to deliver high-dose radiation to a definite target volume with minimizing re-irradiation to nearby healthy tissues (8). The risk of radionecrosis is the primary limitation of this treatment.

This study evaluated the efficacy and tolerability of cyberknife treatment in patients with recurrent GBM. We aimed to define a group of patients who would most benefit from cyberknife treatment.

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1 Adnan Menderes University, School of Medicine, Dept. of Medical Oncology, Aydın, TR
*** Corresponding Author:** Ozlem Yersal **E-mail:** yersal1978@yahoo.com **Phone:** +90 (531) 992 35 53

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Material and Methods

Patients

Patients with GBM who received cyberknife re-irridation as a part of recurrence treatment in any line included in to the study. A total of 24 GBM patients identified from 2011-2015 at our instution. Primary therapy of the included patients after diagnosis mostly consisted of total surgical excision, radiotherapy at a dose of 60 Gy with temozolamide and sequential adjuvant temozolamide chemotherapy. Patients were followed with clinical assessment and magnetic resonance imaging (MRI) scans with diffusion, perfusion and spectroscopic sequences which were performed 6–8 weeks after treatment and at 2-month intervals thereafter. No patient was lost from follow up.

Radiation treatment planning

Treatment planning was performed with Accuray system. The cyberknife include a linear accelerator attached on a robotic arm with six degrees of freedom. It delivers 6 MV photons. All patients undergoing irradiation were immobilized with custom-made thermal plastic masks.

Treatment planning MRI and computed tomography (CT) images were obtained at the same day and fused. All patients had thin cut (1–1.5 mm) axial T1, post-contrast T1 and T2/FLAIR MRI. The gross tumor volume (GTV) was determined on MRI using the gadolinium enhanced T1 weighted sequence. Surrounding edema was not contained in the treatment volume. GTV was the planning target volume the with minimum margin (0–2 mm per the treating physician). Critical normal structures, such as optic nerves, chiasm, and brainstem were also contoured.

Concomittant chemotherapy was not applied. All patients received 1 mg/kg prednisolone therapy during the week of treatment and then decreased doses over a month.

Statistical analysis

Overall survival (OS) after cyberknife treatment was described as the duration between initial cyberknife treatment and death or the last follow-up for surviving patients. Kaplan-Meier curves were used to evaluate the OS. Log-rank test was used for univariate analyses and cox regression hazard modelling was used for multivariate analyses. Age (≤ 51 years and >51 years), gender (female and male), cyberknife fraction (≤5 and >5), cyberknife dose (30 Gy≤ and >30 Gy), tumor size (≤35mm and >35mm), tumor side (left and right), tumor location (frontal and the others), primary surgical procedure (subtotal and total), gross tumor volume (≤10.9 cm3 and >10.9 cm3) were included in univariate analysis. Although tumor location, age and surgical procedure were suitable for multivariate analysis, gender was also included in multivariate analysis since it might have confounding effect. Distributions of continuous variables were controlled with Shapiro-Wilk (SW) test and Histogram. Descriptive statistics were presented as frequency (percentage) for categorical variables and as mean (± standard deviation) for normally distributed continuous variables or median (minimum – maximum) for not normally distributed continuous variables. Statistical analysis was performed with Statistical Package for Social Sciences for MacOS version 24.0 (SPSS Inc; Chicago, IL, USA). Type-1 error (α) was accepted as 0.05.

Results

Patient population and primary treatment parameters

A total of 24 patients who had disease relapse or progression and received cyberknife re-irridation in any line of recurrence treatment included in this study. Pathology was glioblastome multiforme for all patients. Patient characteristics are shown in Table 1. There were 15(62.5%) males and 9(32.5) females. The most common tumor localization was temporal lobe (45.8%). Median age of patients was 51. Primary surgical intervention was total excision for 13(54.2%) patients, subtotal excision for 9(37.5%) patients and biopsy for 2(8.4%) patients. All but three patients had chemoradiotherapy after first operation. Applied total dose of primary radiotherapy was 60 Gy per 2 fractions for all patients. Three patients received radiotherapy without temozolamide because of thrombocytopenia, liver toxicity and patient refuse, concurrently and after radiotherapy.

SRS treatment characteristics

Cyberknife re-irridation treatment was given to 20 patients (83.4%) as the first line treatment, 2(8.3%) patients for second line treatment and 2 (8.3%) patients for the third line treatment after recurrence is confirmed. Median GTV was 10.92 cm3 (2.70-60.84). Lesions were re-irridated with either a median dose of 18Gy in one fraction with a median GTV of 10.98 cm3 (five lesions), 18 Gy in three fractions with an median GTV of 8.03 cm3 (five lesions), and 30 Gy in five fractions with a median GTV of 16.72 cm3 (14 lesions). 3 patients had received cyberknife treatment after reoperation.

Survival

Two patients were alive at the time of survival analysis. All patients died as a result of disease progression. Median survival was 10.3 months after re-irridation with cyberknife and 23 months after diagnosis. Median overall survival from the diagnosis and median overall survival after cyberknife is represented in Figure 1 and Figure 2; respectively.

In univariate analysis; patients younger than 50 years had significantly longer overall survival compared with older patients (4.8 vs 14.2 month; p :0.05). Patients with total resection as primary treatment had also longer OS when compared with subtotal resection (9.3 vs 4.9 month; p :0.05).

There was no correlation between survival and fraction (<5 vs >5 fraction), total dose (<30 vs>30 Gy), tumor diameter (<35 vs >35 mm), tumor side (right or left) and primary operation (subtotal or total) type. Univariate analysis of prognostic factors was shown in Table 2.

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Multivariate analysis

Multivariate analysis was performed to investigate whether different variables influenced OS from cyberknife treatment in the study group. These included age at recurrence, localization of recurrence, and primary surgical procedure. None of these variables, demonstrated a statistically significant association with OS. Multivariate analysis of prognostic factors was shown in Table 3.

Toxicity

We did not observe any clinically significant acute toxicity and all patients were able to take the prescribed cyberknife radiation dose without interruption. No patient required hospitalization or surgery for early acute or delayed toxicity.

Table 1: Patient demographic characteristics

		N:24(%)
Gender	Female	9 (37.5)
	Male	15 (62.5)
Primary operation type	Total	13 (54.2)
	Subtotal	9 (37.5)
	Biopsy	2 (8.4)
First line treatment	Chemoradiotherapy	21 (87.5)
	Radiotherapy	3 (12.5)
Side	Left	12 (50)
	Right	12 (50)
Location of recurrence	Temporal	11 (45.8)
	Frontal	8 (33.4)
	Other	9 (20.8)
Age at cyberknife	<50	9 (37.5)
	>50	15 (62.5)
Recurrence treatment	Re-irridation	24 (100)
	Re-resection	3 (12.5)
	Bevacizumab	10 (41.6)
	Temozolamid	3 (12.5)
	Carmustine	1 (4.1)
Mean dose	18 Gy	10 (41.7)
	30 Gy	14 (58.3)
Dose per fraction	6	20 (83.3)
	18	4 (16.7)

Table 2: Univariate Analysis of Prognostic Factors for Survival After Cyberknife Re-irridation

Clinical characteristics	Mean survival	p value
Age ≤51 vs >51	4.8 vs 14.2	0.05
Gender (female vs male)	6.5 vs 10.7	0.51
Cyber fraction <5	9.3 vs 8.5	0.58
Cyber dose <30 or >30	9.3 vs 8.5	0.58
Tumor size <35mm or>35 mm	9.4 vs 8.4	0.90
Tumor side (right vs left)	11.5 vs 7.0	0.96
Frontal vs other location	16.8 vs 6.3	0.08
Total vs subtotal resection	9.3 vs 4.9	0.05
Gross tumor volume (≤10.9 cm3 and >10.9 cm3	8.5 vs 11.5	0.86

Table 3: Multivariate analysis of prognostic factors for survival after cyberknife re-irridation

Variable	Comparison	Hazard ratio	95% CI	p
Age	≤51 >51 years	0.859	0.272-2.719	0.797
Gender	Male Female	1.574	0.563-4.401	0.388
Localization	Others Frontal	1.836	0.551-6.116	0.322
Surgery	Total Subtotal	2.465	0.876-6.938	0.087

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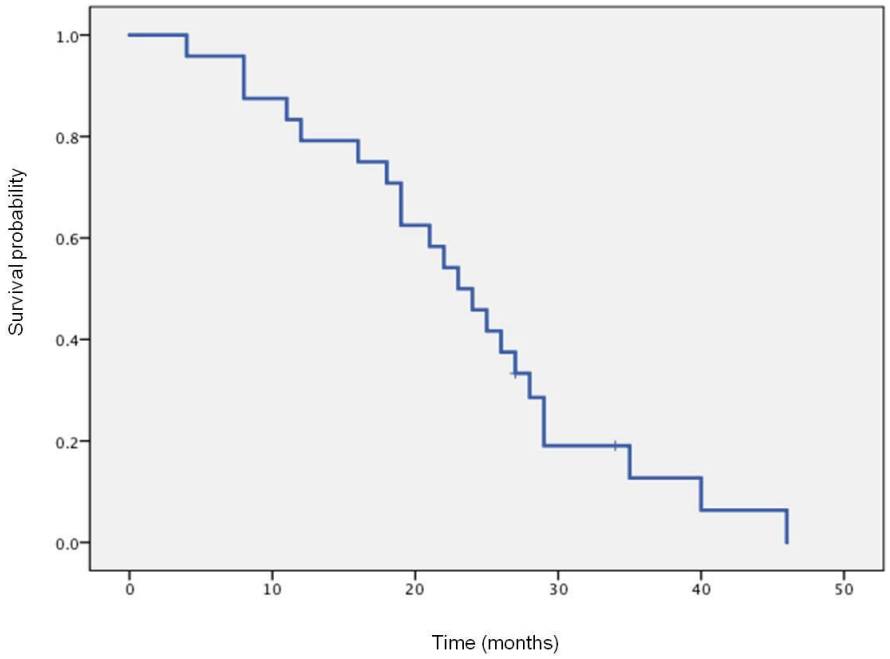

Figure 1: Kaplan–Meier overall survival curve showing OS from the time of initial diagnosis (time in month). A total of 20 patients were included in the survival analysis. Two patients were alive at the time of analysis. Median survival was 23 months from the initial diagnosis.

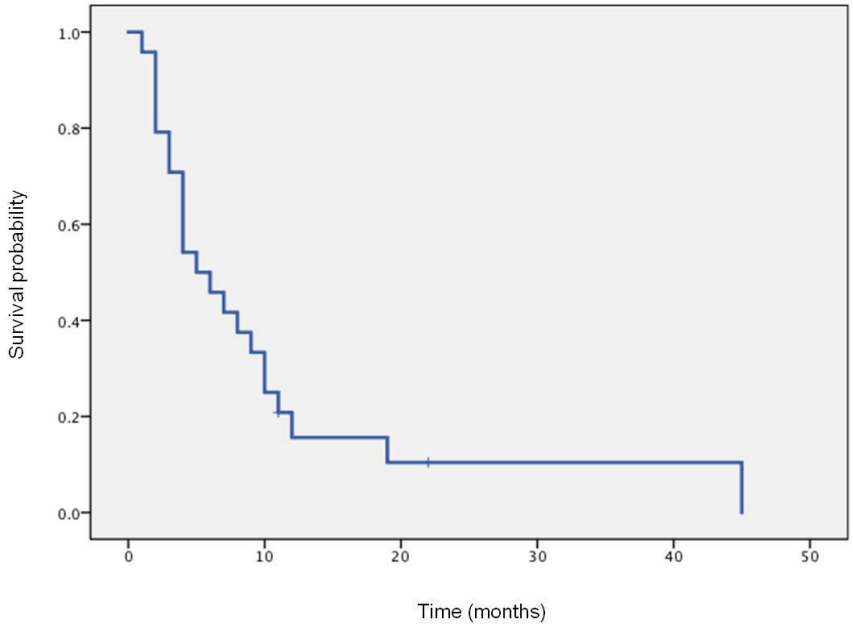

Figure 2: Kaplan–Meier overall survival curve showing OS from the initiation of cyberknife re-irridation (time in month). Median survival was 10.3 months after re-irridation with cyberknife

Discussion

Sterotactic radiosurgery (SRS), is a safe and effective treatment option for the patients with recurrent glioblastoma multiforme. It can be preffered in treating previously irradiated tumors, as it allows deliver the therapeutic dose to tumor area, while minimizing normal tissue toxicity (9). We evaluated sterotactic radiosurgery outcomes of recurrent GBM patients treated in our instution. We observed 10.3 months overall survival after cyberknife treatment. We could not find any prognostic factors for overall survival.

Median survival times of around 11 months for patients with high grade glioma who were treated with fractionated stereotactic radiotherapy has been reported in the literature (10-12). Sutera et al reported salvage SRS results for 55 high-grade glioma patients (13). Overall survival was 23.9 months and survival from SRS was 10.25 months, which is comparable to our results of 10.3 months.

However; overall survival was 23 months which is longer than many historical controls. We could not find any prognostic factors associated with overall survival after sterotactic radiotherapy. Sutera et al evaluated 55 high grade and 21 low grade patients treated with salvage SRS. They did not find any prognostic factors associated with inferior survival on univariate analysis for high grade glioma patients. Also, Combs et al could not find any statistical difference in survival in terms of gender, Karnofsky performance score, presence of neurological symptoms, age or type of primary surgical intervention or size of the lesion (<49 ml vs. >49 ml). Longer overall survival, for our patient cohort may be related with the selection criteria of patients for cyberknife. First, most of the patients received cyberknife treatment after recurrence as first line treatment, so overall survival after SRS might be relatively long; but overall survival in this group of patients was also longer. Second, cyberknife treatment is more effective in low volume tumors, so tumor volumes of the patient cohort are lower which have better prognosis. Third, most of the patients received bevacizumab therapy which was known to reduce radiotherapy related edema and radiation necrosis.

Glioblastoma multiforme recurrences, mostly develop within or in close proximity of the primary tumor site, which require tolerable and effective recurrence treatment (14). There are a number of radiotherapeutic approaches for recurrent gliomas. Conventional external-beam radiotherapy is often associated with only small benefit for the patients, with mostly unacceptable toxicity and total dose is limited by normal tissue tolerance (15). Cyberknife reduces this concern with minimal tissue exposure.

Our patients have not received any chemotherapy or immunotherapy during cyberknife treatment. The role of chemotherapy combined with SRS for recurrent glioma patients are unclear and prospective trials are needed. Stereotactic reirradiation in combination with temozolomide or bevacizumab reported to yield longer overall survival compared with radiation treatment alone (16). Minniti et al evaluated the efficacy of hypofractionated stereotactic radiotherapy (HSRT) combinationed with fotemustine or bevacizumab in patients with recurrent malignant glioma as salvage treatment. They reported longer overall survival after HSRT with bevacizumab than fotemustine combination (11 vs 8.3 months). The treatment was well tolerated (17).

In our study, patients younger than 50 years had longer overall survival than patients older than 50 years after SRS in univariate analysis, but not in multivariate analysis. Age is reported to be a prognostic factor in some studies however some studies did not find an association between young age and better prognosis. Fogh et al reported that younger age was associated with better overall survival (18) . Conversely, Veninga et al did not find overall survival difference between patients under 40 years and others (19).

This study has limitations, in terms of; the small sample size and retrospective nature of the cohort. Additionally treatment modalities before and after SRS are heterogenous as a result of physician choice and experience. Radiation toxicity was difficult to evaluate because of limited reporting and unclear documentation. Although limitations exist, our study demonstrates that salvage SRS for recurrent GBM is feasible and well tolerated by patients with observed low toxicity

In conclusion, this study demonstrated the efficacy and tolerability of salvage SRS for recurrent glioma and contributed new data to the growing body of research. A group of patient benefit from first line cyberknife treatment after recurrence. Prospective randomized trials are necesseray to identify these patients.

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Ethical issues: All Authors declare, Originality and ethical approval of research. Responsibilities of research, responsibilities against local ethics commission are under the Authors responsibilities. The study was conducted under defined rules by the Local Ethics Commission guidelines and audits.

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An effective approach for botulinum toxin injection in patients with stroke for focal spasticity: dual guidance

Emre Ata[1*], Murat Kosem[1]

Abstract

Objective: There are several studies in the literature focusing the guided botulinum toxin injections into the spastic muscle. However, these guides were applied separately and their effectiveness was compared among themselves. We could not find any study investigating the effectiveness of combined 2 guides in the literature. This study aimed to compare the efficacy of botulinum toxin injections, applied to the upper limb muscles of the stroke patients in our clinic who have being diagnosed with focal spasticity, that are performed via ultrasonography and ultrasonography + electrical muscle stimulator guidance.

Materials and Methods: Electronic data on 62 hemiplegic stroke patients with grade 2 and 3 focal spasticity who had received botulinum toxin injections into their upper limb muscles by the same physician, who used similar protocol and recorded the results, were scanned retrospectively. The spasticity of the patients in both groups was assessed with the Modified Ashworth Scale at the end of two weeks and three months.

Results: A statistically significant difference was found between the Modified Ashworth Scale values of both groups in terms of all muscles, compared to the values seen in the pre-treatment period ($p<0.05$). The Modified Ashworth Scale values at 3 months posttreatment in ultrasonography + electrical muscle stimulator group were not statistically different from those at 2 weeks posttreatment, with respect to wrist flexion and finger flexion. In intergroup comparison, there was no statistically significant difference between the Modified Ashworth Scale values at pretreatment and 2 weeks posttreatment. However, statistically significant difference in all muscle groups was found in favor of the ultrasonography + electrical muscle stimulator group at 3 months posttreatment controls ($p<0.05$).

Conclusion: Upper limb spasticity due to stroke can be substantially recovered with botulinum toxin injections that are applied via only ultrasonography guidance or via ultrasonography + electrical muscle stimulator guidance. According to data from the assessment at 3 months posttreatment, the botulinum toxin injection performed via ultrasonography + electrical muscle stimulator guidance had more positive effects.

Key words: stroke, muscle spasticity, botulinum toxin, injections, electrical muscle stimulation

Introduction

Stroke is a medical condition that most leads to disability and dependency (1). Spasticity is known to be among the complications most frequently seen following a stroke. Upper limb spasticity, a common complication after stroke, results in a decrease in the quality of life by impairing the functions of the limbs (2; 3). In spasticity treatment, non-invasive methods should be applied first before turning to invasive methods (4). In cases where the spasticity affects a specific muscle group, local treatments should be preferred. The most common local treatment for focal spasticity is botulinum toxin (BTX) injection. (5). In clinical practice, intramuscular BTX injection can be applied using several types of guidance, including manual needle placement (MNP), electromyography (EMG), electrical muscle stimulation (EMS), and ultrasonography (USG). Today, many clinics frequently perform BTX applications together with the MNP technique, considering that the anatomic points of muscles are known very well (6). On the other hand, BTX applications under the guidance have several advantages, such as being able to identify the proper localization of the muscle and the target point in the muscle and not causing harm to surrounding structures (7). This study aimed to compare the efficacy of BTX injections, applied to the upper limb muscles of the patients in our clinic who have being diagnosed with focal spasticity, that are performed via USG and USG+EMS guidance by scanning the data of the patients retrospectively.

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1 University of Health Sciences, Sultan Abdulhamid Han Research Hospital, Dept of Physical Medicine and Rehabilitation, Istanbul, TR
*** Corresponding Author:** Emre Ata **E-mail:** emreata.ftr@gmail.com **Phone:** +90 (533) 615 21 62

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Material and Methods

In this study, electronic data on 62 hemiplegic stroke patients with grade 2 and 3 focal spasticity who presented to our clinic between May 01, 2013 and May 01, 2018 and had received BTX injections into their upper limb muscles by the same physician, who used similar protocol and recorded the results, were scanned retrospectively. As this was a retrospective study, approval from an ethics committee was not required.

Inclusion Criteria

1. Male and female patients, between the ages of 18 and 80 with stroke-driven focal spasticity, who were administered BTX injection to m. biceps brachii (BB), pronator teres (PT), m. flexor carpi radialis (FCR), m. flexor carpi ulnaris (FCU), m. flexor digitorum superficialis (FDS) and m. flexor digitorum profundus (FDP) muscles, via USG guidance.
2. Male and female patients, between the ages of 18 and 80 with stroke-driven focal spasticity, who were administered BTX injection to BB, PT, FCR, FCU, FDS and FDP muscles, via USG+EMS guidance.
3. The patients who had level 2 or level 3 spasticity according to the Modified Ashworth Scale (MAS) before the treatment.

Exclusion Criteria

1. Male and female patients, between the ages of 18 and 80 with stroke-driven focal spasticity, who were administered BTX injection to BB, PT, FCR, FCU, FDS and FDP muscles, applied by MNP technique.
2. The patients who were unable to come to the two-week and/or three-month control check-up following BTX, or whose examination records on these dates could not be found.
3. The patients who had level 1 and level +1 spasticity in the specified muscles according to the MAS before the treatment.

Protocol

BTX injections were administered by the same physician to the patients of both groups under sterile conditions as they were in supine position. During the applications, all of the patients were administered Botulinum Toxin Type A (Dysport) diluted with saline solution, which contained 2.5 ml of 0.9 percent sodium chloride. The patients for whom only USG, and USG+EMS were applied as the guide procedure over the course of BTX administration are defined as USG group, and USG+EMS group, respectively.

The spasticity of the patients in both groups was assessed with the MAS at 2 weeks and 3 months posttreatment.

Statistical analysis

Statistical analyses were performed using commercially available statistical software (SPSS, version 22.0; SPSS, Inc., Chicago, IL). The Kolmogorov Smirnov test was applied to determine whether the data were in accord with normal distribution or not, the results of which showed that the data did not have normal distribution. The chi-square test and Mann-Whitney U test were used for discrete data and continuous data to determine whether there were any statistically significant differences between the demographic data and the initial assessments (MAS), respectively. The existence/absence of any statistically significant differences between assessments at baseline, and at 2 weeks and 3 months post treatment within the group was determined by applying the Friedman test. Statistical significance was accepted at $p<0.05$. After the determination of a statistically significant difference, post-hoc (paired comparisons) analysis was performed using the Wilcoxon test. Bonferroni correction was also applied, and $p<0.005$ was taken as the significance coefficient. Mann-Whitney U test was applied to see whether there was any statistical intergroup difference between assessments at baseline, and at 2 weeks and 3 months posttreatment.

Results

The demographic data of the patients included in the present study are given in Table 1. Although the groups were homogeneous with respect to age, gender, duration of disease, hemiplegic side, the presence of hypertension, and the presence of hyperlipidemia, homogeneity was not observed in terms of the presence of diabetes mellitus and history of smoking. No statistically significant difference was detected between the MAS values of the groups before the treatment ($p>0.05$).

A statistically significant difference was found between the MAS values of both the USG group and the USG+EMS group in terms of all muscle groups, compared to the values seen at baseline.($p<0.05$).

Results from the intragroup paired comparison according to MAS parameters in the USG group showed that the decrease in MAS values measured at 2 weeks and at 3 months posttreatment was statistically significant compared to baseline ($p<0.005$). Moreover, there was a statistically significant difference between the MAS values at 2 weeks and 3 months posttreatment; that is, there was an increase in MAS values (Table 2).

Results of the intragroup paired comparison of the change in the MAS parameters in the USG+EMS group showed that the decrease of the MAS values at 2 weeks and 3 months posttreatment were statistically significant compared to baseline($p<0.005$). The MAS values at 3 months posttreatment were not statistically different from the values at 2 weeks posttreatment, with respect to wrist flexion and finger flexion. However, the increase in the MAS values of elbow flexion and forearm pronation at 3 months posttreatment compared to 2 weeks posttreatment was found to be statistically significant (Table 3).

In intergroup comparison, there was no statistically significant difference between the MAS values at baseline and 2 weeks posttreatment. However, statistically significant difference in all muscle groups was found in favor of the USG+EMS at 3 months posttreatment ($p<0.05$) (Table 4).

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Table 1. Patients Demographic characteristics

Parameters	USG Group (n=22)	USG+EMS Group (n=22)	p
Age (yrs) (mean ± sd)	59,81±11,54	60,13±10,87	>0,05
Sex (male / female)	14/8	14/8	>0,05
Duration of stroke (month) (mean ± sd)	43,09±36,19	44,31±68,91	>0,05
Type of stroke (ischemic / hemorrhagic)	19/3	14/8	**<0,05**
Hemiplegic side (right / left)	12/10	15/7	>0,05
Diabetes Mellitus (+/-)	8/14	1/21	**<0,05**
Hypertension (+/-)	13/9	13/9	>0,05
Hyperlipidemia (+/-)	9/13	8/14	>0,05
History of smoking (+/-)	5/17	10/12	**<0,05**

sd: standard deviation, yrs: years

Table 2. Intragroup Comparisons of USG group. (PT: Post-Treatment)

Modified Ashworth Scale	Baseline	PT 2 weeks (median)(min/max)	PT 3 months	p
Elbow flexion	2 (2-3)	1 (1-2)	2 (1-3)	**<0,05**
Wrist flexion	3 (2-3)	1 (0-1+)	1+ (1-2)	**<0,05**
Hand flexion	3 (2-3)	1+ (1-1+)	1+ (1-2)	**<0,05**
Forearm pronation	3 (2-3)	1+ (0-1+)	1+ (1-2)	**<0,05**

Table 3. Intragroup Comparisons of USG+EMS group

Modified Ashworth Scale	Baseline	PT 2 weeks (median) (min/max)	PT 3 months	p
Elbow flexion	2 (2-3)	1 (0-1+)	1 (0-2)	**<0,05**
Wrist flexion	3 (2-3)	1 (0-1+)	1 (0-2)	**<0,05**
Hand flexion	3 (2-3)	1 (0-1+)	1 (0-2)	**<0,05**
Forearm pronation	3 (2-3)	1 (0-1)	1 (0-1+)	**<0,05**

Tablo 4. Intergroup Comparisons

Modified Ashworth Scale		USG median (min/max)	USG+EMS median (min/max)	p
Baseline	**Elbow flexion**	2 (2-3)	2 (2-3)	>0,05
	Wrist flexion	3 (2-3)	3	>0,05
	Hand flexion	3 (2-3)	3 (2-3)	>0,05
	Forearm pronation	3 (2-3)	3	>0,05
PT 2 weeks	**Elbow flexion**	1 (1-2)	1 (0-1+)	>0,05
	Wrist flexion	1 (0-1+)	1 (0-1+)	>0,05
	Hand flexion	1+ (1-1+)	1 (0-1+)	>0,05
	Forearm pronation	1+ (0-1+)	1 (0-1)	>0,05
PT 3 months	**Elbow flexion**	2 (1-3)	1 (0-2)	**<0,05**
	Wrist flexion	1+ (1-2)	1 (0-2)	**<0,05**
	Hand flexion	1+ (1-2)	1 (0-2)	**<0,05**
	Forearm pronation	1+ (1-2)	1 (0-1+)	**<0,05**

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Discussion

In the present study evaluating the efficacy of BTX injections on focal spasticity, applied to upper limb muscles combined with USG or USG+EMS, statistically significant decrease in spasticity was observed in both groups compared to baseline. BTX injection applied via USG+EMS guidance was found to be a superior treatment method for reducing spasticity in the long term.

BTX injection is an effective, safe and local treatment method for stroke patients with focal or multifocal spasticity. BTX injection can be performed either through the MNP technique without using any guide, or together with guides, such as EMS, USG, and EMG (7; 8; 9; 10).

Although the MNP technique is commonly used on superficial and large muscles, it requires good knowledge of anatomy. When performing BTX injection under EMG guidance it can be ensured that the needle is in a spastic muscle, but it is difficult to know if the injection is applied to the targeted muscle (11). Various studies which controlled the accuracy of the MNP technique reported that the accuracy rates for the gastrocnemius medialis muscle, gastrocnemius lateralis, hip adductors, medial hamstring, tibialis posterior, BB, PT, adductor pollicis, FCR and FCU were 92.6%, 64.7%, 67%, 46%, 11%, 62%, 35%, 22%, 13% and 16%, respectively (8; 10) Therefore BTX applications under the guidance of USG or EMS, for deeply located and small muscles, is recommended (7; 12; 13).

In the literature, there are numerous studies comparing BTX applications performed with different guides. Kwon et al. compared the efficacy of BTX injections applied via USG and EMS guidance on children with cerebral palsy who had equine deformity secondary to m. gastrocnemius spasticity. They observed a significant decrease in spasticity levels in both groups at 1 month posttreatment according to the MAS and Tardieu Scale (TS); however, they also reported that the significant decreases in spasticity persisted at 3 months posttreatment only in the USG group (9).

Picelli et al. compared the data obtained by applying BTX to the forearm muscles for the spasticity in stroke patients using three different injection techniques; MNP, EMS and USG (7). The patients were evaluated based on MAS, TS and the level of passive joint range of motion, with respect to the spasticity of the wrist and finger, at 4 weeks after the treatment. The EMS and USG groups had better results than the MNP group in terms of all parameters. No statistically significant difference was reported between the EMS and USG groups.

In another study by Picelli et al. which compared the accuracy of BTX applications under the guidance of MNP and EMS in stroke patients with equine deformity due to ankle plantar flexor spasticity, the accuracy of EMS method was found to be higher than MNP. However, they identified the EMS as a blind method just like the MNP method (12).

It was stated that BTX injection applied via USG guidance is superior than the other guide methods, with regard to protection of neurovascular structures, as well as application to the right muscle. However, BTX penetrates to the cell membrane with receptor mediated endocytosis and cannot enter the nerve cytosol by directly passing the cell membrane. Therefore, the effect of the toxin injection on a hyperactive muscle is directly related to the amount of toxins in the neuromuscular product. Thus, injections that target motor endplates are important for achieving optimum therapeutic effect with lower doses and less side effects. In this sense, the injection to be performed via EMS guidance is seen as the most appropriate method (14-15).

In the present study, we evaluated the effect of BTX injections under the guidance of USG or USG+EMS on spasticity according to only the MAS values at 2 weeks and 3 months posttreatment. The decrease in spasticity for both groups was statistically significant. It was further found that the decrease in the spasticity of wrist flexors and finger flexors that were observed in the USG+EMS group at 2 weeks posttreatment continued at 3 months posttreatment. Moreover, the data obtained from USG+EMS group at 3months posttreatment was found to be statistically better than USG group. We believe that this difference obtained not only is emerged as a result of the BTX injection to the correct muscle via USG guidance, but also as a result of the injection to the region with the densest motor endplates via EMS guidance.

Additionally, there were no complications in either group during the applications. Both methods are considered to be reliable.

Conclusion

There are several studies in the literature focusing the guided BTX injections into the spastic muscle. However, these guides were applied separately and their effectiveness was compared among themselves. We could not find any study investigating the effectiveness of combined 2 guides. In addition, one of the guiding methods (USG) we used in our study aims to find the right muscle for the injection while the other (EMS) aims to find the correct point in the right muscle.

In the present study, upper limb spasticity due to stroke can be substantially recovered with BTX injections that are applied via only USG guidance or via USG+EMS guidance. According to data from the assessment at 3 months posttreatment, the BTX injections performed via USG+EMS guidance had more positive effects. Randomized, controlled and prospective studies with larger patient groups would bring greater understanding to this subject.

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responsibilities against local ethics commission are under the Authors responsibilities. The study was conducted under defined rules by the Local Ethics Commission guidelines and audits.

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