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The Effect of Slime Factor in the Treatment of Spinal Implant Infections

Hasan TURKOGLU[1], Emrullah Cem KESILMEZ[2], Kutsal Devrim SECINTI[2], Ilke Evrim SECINTI[3], Burak KUCUK[4], Murat ARAL[4], Recep EKEN[5], Zafer YUKSEL[2]

[1]Ersin Arslan City Hospital, Department of Neurosurgery, Gaziantep, Türkiye
[2]Kahramanmaras Sutcu Imam University, Faculty of Medicine, Department of Neurosurgery, Kahramanmaras, Türkiye
[3]Hatay Mustafa Kemal University, Faculty of Medicine, Department of Pathology, Hatay, Türkiye
[4]Kahramanmaras Sutcu Imam University, Faculty of Medicine, Department of Microbiology, Kahramanmaras, Türkiye
[5]Adiyaman Research Hospital, Department of Neurosurgery, Adiyaman, Türkiye

Corresponding author: Emrullah Cem KESILMEZ ✉ cemkesilmez@gmail.com

ABSTRACT

AIM: To investigate the effect of the biofilm-forming ability of the bacteria on treatment in rats by using biofilm-forming and non-biofilm-forming strains of Staphylococcus aureus (S. aureus).

MATERIAL and METHODS: Forty rats were divided into four equal groups as Group 1A, 1B, 2A, and 2B. All rats underwent single distance lumbar laminectomy, and titanium implants were introduced. Group 1 rats were inoculated with Slime factor (–) S. aureus, while Group 2 rats were inoculated with biofilm Slime factor (+) S. aureus. None of the rats were given antibiotics. One week later, the surgical field was reopened and microbiological samples were taken. The implants of rats in Groups 1A and 2A were left in place, while the implants of rats in Groups 1B and 2B were removed.

RESULTS: There was no statistically significant difference between the groups inoculated with slime factor (+) S. aureus; although, Groups 1A and 2A showed statistically significant difference. Statistical analysis with respect to bacterial count also showed a statistically significant difference between Groups 1A and 2A. There was a statistically significant difference between Group 1B and 2B.

CONCLUSION: The results obtained in the present study reveal that in case of implant-dependent infection, the first sample taken can be checked for slime factor, and if there is infection with slime factor-negative bacterium, treatment without removing the implant may be recommended. S. aureus was used in the study because it is the most common cause of implant-related infection at surgical sites. Further studies using different bacterial species are needed to reach a definitive conclusion.

KEYWORDS: Biofilm layer, Slime factor, Implant, Rat

ABBREVIATIONS: PNL: Polynuclear Leukocytes, **AELEC:** Animal Experiments Local Ethics Committee, **MRSA:** Methicillin-resistant S. aureus, **PMMA:** Polymethylmethacrylate

INTRODUCTION

With the introduction of spinal instrumentation into surgical practice, operations that could not be performed previously became possible, offering new options to surgeons and patients (15). However, it has been observed that the infection rate in surgical procedures using implants is much higher than similar surgical procedures without implants. Studies have shown that the infection rates

Hasan TURKOGLU : 0000-0002-6813-2064
Emrullah Cem KESILMEZ : 0000-0003-3905-2206
Kutsal Devrim SECINTI : 0000-0003-4345-0805
Ilke Evrim SECINTI : 0000-0002-8614-3971
Burak KUCUK : 0000-0001-5596-3347
Murat ARAL : 0000-0002-3576-4380
Recep EKEN : 0000-0002-2472-4850
Zafer YUKSEL : 0000-0002-9234-5908

of patients undergoing spinal instrumentation vary between 4% and 35% (6). With the increase in implant-related surgical site infections in the literature, many prophylactic methods have been tried and treatment plans have been made with newly developed antibiotics, but sufficient results have not been obtained (14-16,19,20,32,45).

Implant-related surgical site infections are triggered by the adhesion of bacteria to the implant surface and are further progressed by the proliferation of bacteria and the formation of a biofilm layer in the environment. Biofilm is the matrix or extracellular polymeric substances formed by microorganisms adhering either to the implant surface or to each other. Bacteria that produce biofilm exhibit completely different characteristics in terms of genetic structure and protein synthesis according to their origin.

Biofilms are microbial communities that are attached to a surface as a network and consist of a double layer (3,7). The most important characteristics of biofilms are resistance to antimicrobial agents and host defenses (4). This effect is caused by polynuclear leukocytes (PNL) blocking opsonization and phagocytosis by inhibiting chemotaxis (21). Bacteria that can and cannot form biofilms both exist in nature.

In the present study, biofilm-forming and non-biofilm-forming strains of Staphylococcus aureus (S. aureus) were used to investigate the effect of biofilm-forming ability of the bacteria on treatment in rats.

MATERIAL and METHODS

Ethical approval was obtained from Kahramanmaraş Sutcu Imam University Animal Experiments Local Ethics Committee (AELEC) (12.30.2020-36). In order to ensure the adaptation of the rats to the environment, they were fed with regular feed for ten days in the laboratory environment under room temperature (22°C ± 2°C), 60% ± 5% humidity, and periodic white fluorescent light (12 hours dark and 12 hours light). Throughout the experiment, rats were given feed and water ad libitum. In the study, 40 Wistar-albino female rats bred under laboratory conditions were used. The rats were approximately 12 weeks old and weighed approximately 250 ± 50 g. Rats included in the study were randomly selected.

Bacterial suspensions containing S. aureus ATCC 25923 strain (non-biofilm forming, slime factor [–]) and S. aureus ATCC 29213 strain (biofilm forming, slime factor [+]) were cultured in two different sheep blood media. The inoculated sheep blood agar was incubated in an oven with 5% CO_2 for 24–48 hours. The S. aureus colonies were then standardized to 0.5 McFarland in 3 ml tubes containing saline.

For sedation, 50 mg/kg ketamine hydrochloride (ketamine 50 mg/ml 10 ml vial, Pfizer Istanbul) and 5 mg/kg Xylasine Hydrocloride (Rompun 2% solution, 50 cc. Vial, Bayer, Istanbul) was administered intraperitoneally. All rats were shaved with a razor to clear the surgical site. Each rat was given a code to avoid confusion during the operation and taken to the operating room. The spinal regions of the rats were cleaned with povidone iodine (MEDICA brush; 4% chlorhexidine soap, MEDICA BV, Holland) for 10 min for antisepsis and stained with povidone iodine (POVIOD; 10% polyvinylpyrrolidone-iodine complex, Saba, Turkey). The surgical field was covered with sterile sheets before the operation. Gloves were changed with fresh sterile pairs after every operation. The rats were operated on by a single surgeon. At the beginning of the operation, rats were placed in prone position and the skin and subcutaneous tissue were crossed with an incision of approximately 3 cm in the midline. The paraspinal muscles at this distance were separated by blunt dissection. The L4 lamina was exposed. L4 total laminectomy was performed. Two titanium implants (1 mm in diameter and 20 mm long titanium wire) were placed. The rats were divided into four equal groups of ten rats each. Group 1A and 1B rats were inoculated with (slime factor [–]) S. aureus (ATCC 25923) which is not capable of biofilm formation. Group 2A and 2B rats were inoculated with (slime factor [+]) S. aureus (ATCC 29213) strain capable of biofilm formation. Seven days later, swab cultures were taken from the rats in all groups for microbiological analysis for bacterial growth, and the implants of Group 1A and Group 2A rats were removed, while the implants of Group 1B and Group 2B were kept in place. Antibiotics (14 mg/kg vancomycin) were given to all rats for fourteen days. On the 21st day of the study, washing fluid and histopathological samples were taken from all rats and the rats were sacrificed. Rats that died before the sacrifice were not replaced. Histological examination was performed blindly. Bacterial count was determined from the "wound washing fluid" obtained from the rats. The results were evaluated statistically and $p<0.05$ was considered significant in all analyses (Table I).

Microbiological Examination

Swab cultures obtained on the seventh day of the study were sent to Kahramanmaraş Sutcu Imam University Faculty of Medicine Medical Microbiology Laboratory. The samples received by the laboratory were inoculated on sheep blood medium. Samples were kept in an incubator with 5% CO_2 for 24–48 hours and bacterial growth was evaluated. Colonies that formed beta hemolysis were included in the evaluation. Other colonies were considered as contaminants. Pure growths of beta-hemolytic colonies were examined for colony morphology, Gram staining, and biochemical characteristics without passaging and their species were determined by BD Phoenix™-100 automated system (Becton Dickinson, Sparks, Maryland, USA). Samples without pure growth were passaged and processed.

Histopathological Method

Tissue samples taken for histopathological examination were sampled after fixation in 10% buffered formalin solution for 24 hours. After routine follow-up procedures, 5 µm thick sections were taken and stained with Hematoxylin and Eosin. All sections were evaluated under a light microscope (Olympus BX53, Tokyo, Japan). Samples were examined by an experienced pathologist blinded to the procedure. Histopathological evaluation was made by modifying the method of Cetinkaya, and the degree of inflammation was scored semiquantitatively as follows (Table II) (5).

Score 1: Activity-free chronic inflammation, granulation tissue formation, and early fibrosis

Score 2: Minimally active chronic inflammation (with or without foci of suppurative inflammation).

Score 3: Severe active chronic inflammation with diffused suppurative inflammation (with or without abscess formation).

Statistical Analysis

All data were analyzed using the SPSS version 21.0 package program (IBM Corp, Armonk, NY). In the comparison of quantitative and ordinal data between independent groups, normality tests were not applied due to the low number of subjects and nonparametric one-way analysis of variance (Kruskal–Wallis) was applied. In cases where the difference between independent groups was found to be statistically significant, Mann–Whitney U test with Bonforreni correction was used to determine the groups that caused the difference. Fisher's exact test was used to analyze qualitative data. Results are summarized in the relevant tables using median, minimum, and maximum values or percentage values. $P<0.05$ was accepted as statistically significant in all analyses.

Table I: Microbiological Analysis Results of Wound Site Washing Fluid

Bacteria	**S. aureus (10^6 CFU, 1 mL)**		**S. aureus (10^6 CFU, 1 mL)**	
	Slime factor (–)		**Slime factor (+)**	
	Implant (+)	**Implant (–)**	**Implant (+)**	**Implant (–)**
Subject No	**Group 1A**	**Group 1B**	**Group 2A**	**Group 2B**
1	1 x 10^6 CFU	No reproduction	>1 x 10^8 CFU	88 x 10^6 CFU
2	35 x 10^6 CFU	No reproduction	64 x 10^6 CFU	No reproduction
3	Exitus	No reproduction	98 x 10^6 CFU	>1 x 10^8 CFU
4	No reproduction	2 x 10^6 CFU	56 x 10^6 CFU	>1 x 10^8 CFU
5	Exitus	Exitus	34 x 10^6 CFU	80 x 10^6 CFU
6	No reproduction	58 x 10^6 CFU	56 x 10^6 CFU	6 x 10^6 CFU
7	1 x 10^6 CFU	No reproduction	78 x 10^6 CFU	50 x 10^6 CFU
8	No reproduction	No reproduction	36 x 10^6 CFU	No reproduction
9	>1 x 10^8 CFU	80 x 10^6 CFU	44 x 10^6 CFU	1 x 10^8 CFU
10	No reproduction	No reproduction	Exitus	24 x 10^6 CFU

Table II: Inflammation Scores according to Histopathological Evaluation System

Group 1A	**Day 7**	**Day 21**	**Group 1B**	**Day 7**	**Day 21**	**Group 2A**	**Day 7**	**Day 21**	**Group 2B**	**Day 7**	**Day 21**
1A-1	3	2	1B-1	3	2	2A-1	3	1	2B-1	3	1
1A-2	3	2	1B-2	3	1	2A-2	3	2	2B-2	3	2
1A-3	3	Exitus	1B-3	3	2	2A-3	3	2	2B-3	3	3
1A-4	3	2	1B-4	3	2	2A-4	3	1	2B-4	3	2
1A-5	3	Exitus	1B-5	3	Exitus	2A-5	3	2	2B-5	3	1
1A-6	3	2	1B-6	3	1	2A-6	3	1	2B-6	3	3
1A-7	3	2	1B-7	3	1	2A-7	3	2	2B-7	3	1
1A-8	3	3	1B-8	3	2	2A-8	3	2	2B-8	3	3
1A-9	3	3	1B-9	3	1	2A-9	3	3	2B-9	3	2
1A-10	3	1	1B-10	3	1	2A-10	3	Exitus	2B-10	3	2

RESULTS

A total of four rats, two from Group 1A, one from Group 1B, and one from Group 2A, died as a result of anesthesia complications and were excluded from the study. For this reason, the study was continued with eight rats in Group 1A, nine rats in Group 1B, nine rats in Group 2A, and ten rats in Group 2B.

In the statistical analysis using Fisher's exact test, the groups were compared as "positive growth" or "negative growth." There was no statistically significant difference between the groups inoculated with biofilm-forming S. aureus. There was a statistically significant difference between the group inoculated with slime factor (–) bacteria and without implant removal (Group 1A) and the group inoculated with slime factor (+) bacteria and without implant removal (Group 2A) (p=0.029). There was no statistically significant difference between the groups in which the implants were removed.

"Bacterial count" was evaluated with Mann–Whitney U Test. There was no statistically significant difference between the groups inoculated with biofilm-forming S. aureus strains. Statistical analysis showed that there was no significant difference between the biofilm-forming groups. There was a statistically significant difference between the group inoculated with slime factor (–) bacteria and without implant removal (Group 1A) and the group inoculated with slime factor (+) bacteria and without implant removal (Group 2A) (p=0.010). There was a statistically significant difference between the group inoculated with slime factor (–) bacteria and whose implants were removed (Group 1B) and the group inoculated with slime factor (+) bacteria and whose implants were removed (Group 2B) (p=0.024).

DISCUSSION

In parallel with advances in technology and the increasing number of patients with spinal problems, surgical interventions involving the spine have increased in the last two decades (50). In Türkiye, there has been an increase in the speed of intervention to emergency patients in the last 15 years (44). This enabled intervention in complicated cases able to reach the hospital in time. Thus, the increasing number of implant-dependent spine surgeries resulted in an increasing number of complications along with a larger patient population being followed.

In a meta-analysis on the risk of infection after surgical procedures on the spine, post-procedural infection rates were reported to vary between 0.7% and 16%. This wide range was attributed to different risks associated with different surgical interventions on the spine (37).

In the literature, the main microorganisms that cause infection after implant surgery are gram-positive ones, mainly S. aureus, S. epidermidis, and less frequently Propioniobacterium acnes (10,31,32,34,36,40,41,43).

Literature evidence shows that vancomycin is the most effectively used antibiotic in the treatment of methicillin-resistant S. aureus (MRSA) -associated surgical site infections (2,8,17). In the present study, antibiotherapy was initiated after growth was detected in the swab culture obtained from all subjects in the first week. All subjects were administered a single dose of 7 mg/kg per day for an equal amount of time, and the doses recommended in the literature were not exceeded (2,8). In addition, no growth was detected in some of the subjects in the wound washing fluid taken on the 21st day of the experiment, suggesting that the dose administered was appropriate. Before starting the study, vancomycin minimum inhibition concentration (MIC) values were determined for both bacteria in the antibiotic susceptibility test performed in Kahramanmaraş Sütçü İmam University Medical Microbiology Laboratory. For vancomycin, the MIC value of both bacteria was ≤1.

In this study, the initial aim was to develop infection in as many subjects as possible. Therefore, all subjects were inoculated with 0.1 ml 10^6 CFU bacteria. Swab culture samples taken in the first week of the study showed that infection developed in all subjects. The spinal infection model in rats was first described by Ofluoglu, in 2007 (33). In the present study, bacteria were inoculated above the amount suggested in the literature to detect infection. However, on the 21st day of the study, no growth in the washing fluid samples was observed of some subjects. Lack of growth at the end of the experiment suggests that the antibiotic had cured some of the subjects (18,24,28,30,35).

Rats were used in the present study because of low cost, ease of reproduction, and ease of surgical procedures. In the literature, animals such as rabbits, sheep, and pigs are usually preferred to create spinal infection models due to their large size, ease of radiological examination, and ease of surgery (9-11,13,22,25,27,29,38,39,45,47,48,50,52).

Many different treatment and prophylaxis methods have been used in the treatment of implant-related surgical site infections. In another study, it was found that the use of royal jelly in the treatment of implant-related surgical site infection reduced the severity of infection (16). There are also studies showing that the use of silver-coated screws reduces infection (19,42). A study using polymethylmethacrylate (PMMA) rod with added vancomycin showed a significant improvement compared to vancomycin alone (32). In a similar study, treatment with rifampicin in combination with vancomycin yielded significant results compared to the control group (20). In a study using bacteriophages, there was a significant difference in treatment compared to the control group, but not all subjects could be treated (51). Briefly, studies in the literature show that combined therapies in addition to vancomycin are effective but do not completely cure the subjects.

In these studies, however, treatment was initiated only on the basis of infection development in the control group without proving the development of infection after bacterial inoculation in test subjects. In contrast, swab cultures were taken from all subjects on the 7th day and microbiological evidence of infection was demonstrated in the present study. Antibiotherapy was started after microbiological evidence.

Although some studies in the literature have compared implant removal, wound irrigation, and antibiotherapy in the treatment of implant-related surgical site infection, the question of exactly when implant removal is required could not be answered (12,23). In some studies, rather than implant removal, criteria were determined according to the extent of infection and appropriate patients were followed-up with a closed drainage system (46). Some authors suggest that the treatment will be successful without removing the instrument, at least until fusion develops (26). However, there is no mention of the possibility of sepsis due to prolonged infection or precautions against complications due to prolonged immobilization (26). In addition, some studies argue that removal of the instrument is inevitable in terms of infection control (1).

Weinstein conducted a follow-up study with 22 patients; the instrument was left in place in 19 patients and removed in the other patients (49). The authors recommended waiting up to two years for fusion development (49). However, in the present study, it was observed that treatment was not possible in subjects infected with biofilm-forming bacteria and whose implants were not removed. In addition, none of the studies investigated the effect of the biofilm-forming ability of the bacteria on treatment decisions. In this respect, this study is unique in the literature.

CONCLUSION

In the present study, the effect of biofilm formation by S. aureus on the treatment of implant-related surgical site infections was investigated. Microbiological findings of the study revealed the following:

1. When treating a subject infected with a slime (–) bacterium, there is no difference between removing and retaining the implant.
2. In the presence of slime (+) bacteria, subjects cannot be treated when the implant is retained.
3. Although there was no significant difference between Groups 2A and 2B, the number of subjects who could be treated was higher in the group whose implants were removed (Group 2B).
4. When the implant is removed, treatment success increases regardless of whether the bacteria are slime positive or negative.

According to the results obtained in the present study, it can be suggested that when a patient with implant-related surgical site infection is encountered, if the patient's clinical condition is suitable for sampling, a sample should be taken in the first place. It is necessary to examine microbiologically whether the bacteria isolated in the sample have the ability to form biofilm. For this purpose, there are different methods that can be performed by the microbiology unit (Example: Congo red agar). Biofilm characteristics can be learned in a short time by staining. If the bacterium causing the infection has the ability to form a biofilm, removal of the implant may be recommended. If the bacterium causing the infection does not have the ability to form a biofilm and the patient's clinical condition is considered suitable for antibiotherapy, continuing the treatment without removing the implant may be considered.

S. aureus was used in the present study because it is the most common bacterium detected in implant-related surgical site infections. From this point of view, it would be appropriate to generalize the results of this study only for infections caused by S. aureus. Other bacteria that may be causative agents of implant-related surgical site infections were not used in the present study; thus further studies using different bacteria species that are causative agents of implant-related surgical site infections can support our results.

AUTHORSHIP CONTRIBUTION

Study conception and design: HT, ECK, KDS
Data collection: HT, ECK
Analysis and interpretation of results: IES, HT, MA, BK
Draft manuscript preparation: HT, ECK, KDS
Critical revision of the article: HT, ECK
Other (study supervision, fundings, materials, etc...): HT, KDS, IES, RE, BK, ZY
All authors (HT, ECK, KDS, IES, BK, MA, RE, ZY) reviewed the results and approved the final version of the manuscript.

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