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Antimikrobiyal Peptid Konjuge Edilmiş PGCL Cerrahi İpliklerin *In Vitro* Yara İyileşmesi Üzerine Etkisi

The Effect of Antimicrobial Peptide Conjugated PGCL Sutures on *In Vitro* Wound Healing

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***Özetçe*— Yara iyileşmesi kazalar veya travmalar sonrasında dokunun iyileşmesini sağlayan; kompleks, hayati ve fizyolojik bir süreçtir. Bu sürecin hızlandırılması ise, hastalık oranını ve tedavi süresini azaltmak için hayati bir öneme sahiptir. Cerrahi iplikler, yaraların kapanmasında kritik bir rol oynar. Bununla birlikte, vücutta implante edilen diğer tıbbi cihazlara benzer şekilde, cerrahi iplikler, yara bölgesindeki yabancı cisimlerdir ve bakteriyel bağlanma için bir nidus görevi görür. Bu nedenle, sütürlerin varlığı, bir yaranın enfeksiyonlara duyarlılığını önemli ölçüde arttırır ve cerrahi bir bölgede bir enfeksiyon riskini arttırır. Bu durumda, pek çok farklı organizma tarafından çeşitli patojenlere karşı birincil savunma hattı olarak üretilen antimikrobiyal peptitlerin (AMP), oluşabilecek bölgesel enfeksiyonlarla başa çıkmakla birlikte yara iyileştirmesinin desteklenmesi için kullanılabilecek uyumlu bir biyomalzeme olabileceği düşünülmektedir. Bu çalışmada, AMP konjuge edilmiş poli(glikokaprolakton) (PGCL) cerrahi ipliklerinin yara iyileşmesi üzerine etkisi, insan keratinosit hücre hatları kullanılarak hücre proliferasyon ve *in vitro* yara kapanması analizlerinin uygulanması ile araştırılıp antimikrobiyal etkinlikleri ise *Pseudomonas aeruginosa* ve MRSA (Metisilin Dirençli Stafilokok Aureus) üzerinde incelenmiştir.**

Anahtar Kelimeler —Antimikrobiyal Peptit, Yara İyileşmesi, Cerrahi Alan Enfeksiyonları, Cerrahi İplikler

***Abstract*—Wound healing is a complex, vital and physiological period for supplying skin repairing after an injury or a trauma. Acceleration of the wound healing process is crucial for decreasing the morbidity and the period of the hospitalization. Sutures play major role in the closure of wounds. However, similar to other medical devices implanted in the body, sutures are foreign bodies in the wound site and serve as a nidus for bacterial attachment. Therefore, the presence of sutures significantly increases the susceptibility of a wound to infections and increases the risk of an infection in a surgical site In this case, antimicrobial peptides (AMP) which are synthesized by several organisms as first line of defense against multiple types of pathogens might be the favorable biomaterial to overcome with the surgical site infections by supporting the tissue and accelerating wound healing process. In this study, the effect of AMP conjugated poly (glycolic acid-co-caprolactone) (PGCL) surgical sutures on wound healing process was investigated by *in vitro* scratch assays on human keratinocyte cell lines and the antimicrobial effect was investigated on *Pseudomonas Aeruginosa* and MRSA.**

Keywords — antimicrobial peptide wound healing, surgical site infections, surgical sutures

I. INTRODUCTION

Sutures are natural/synthetic biocompatible medical devices which are used to keep the tissues together and prevent bleeding or leakage of body fluids after a surgical incision or an injury, until the wound heals [3]. Sutures are foreign bodies on the wound and act as a nidus for bacteria similar to other medical devices that are implanted in the body. Since susceptibility of a wound to infections significantly increases in the presence of sutures, the risk of an infection on a surgical site increase. The main goal of the sutures is to prevent possible scar tissue formation after surgical operation and injury and accelerate the wound healing process while keeping the tissues together without causing any infection [4].

Surgical site infections (SSIs) are the commonly encountered life-threatening risk factor in clinics after the surgical operation. SSIs occur when the pathogenic organisms proliferate through the wound area and decelerate wound healing. Surgical sutures can become source for the formation of the SSIs. The presence of sutures on a wound site increases the risk of infection by about 10.000 times [5]. Moreover, the antibiotic exposure of bacteria is significantly reduced when they are colonized on the surface of the suture [6]. In this case, it is vital to develop an antimicrobial suture to overcome the surgical site infections.

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AMPs are heterogenic molecules that are produced by every multicellular organism as a first line of defense for directly killing distinctive class of microorganisms such as; bacteria, virus, fungi and even cancer cells [4]. In recent studies, the use of AMP as a new antimicrobial agent for therapeutic purposes is increasing. The increase and rapid spread of pathogenic microorganisms, which are resistant to conventional drugs and which are becoming a major public health problem in the world and which are becoming a cause of increasing morbidity, are the reasons for the widespread use of AMP. In addition, resistance to AMP is very rare, as AMP acts directly on the cell membrane, which is vital for cells, compared to resistance to common antibiotics. For these reasons, natural and synthetic AMP are a potential alternative to antibiotics in the treatment of multidrug-resistant infections. In the present study, antimicrobial effect of the AMP conjugated poly (glycolic acid-co-caprolactone) (PGCL) surgical sutures on *P. aeruginosa* and MRSA (Methicillin-resistant *Staphylococcus aureus*) and wound healing process on HS2 keratinocyte cell line were investigated. For this purpose, two AMP sequence KRFRIRVRV(KRF), RWRWRWRW (RW) were synthesized and PGCL surgical sutures were conjugated by single and 1:1 combination of the AMP (KRF/RW). Cell proliferation assays and *in vitro* wound scratch assays were performed for the observation of their impact on wound healing. Additionally, antimicrobial effect of the AMP conjugated PGCL sutures were tested on *Pseudomonas aeruginosa* and MRSA as gram negative and gram positive model organisms, respectively.

II. MATERIAL & METHODS

A. Solid Phase Peptide Synthesis for AMP Sequences

Two different cationic AMP sequences as Arg-Trp-Arg-Trp-Arg-Trp-Arg-Trp (RWRWRWRW)-NH_2 and Lys-Arg-Phe-Ile-Arg-Val-Arg-Val (KRFRIRVRV-NH_2) were synthesized by manually with solid phase peptide synthesis by using Rink Amide AAPTec resins (substitution = 0.67 mol/g) [15-17]. Briefly, 400 mg resins were put into 8 ml dimethylformamide (DMF) solution for 30 minutes. The resins were put into the 20% piperidin-DMF solution to remove Fmoc-protecting groups for 20 minutes. The ninhydrin test was performed to control whether the Fmoc-protecting groups are removed. For the amino acid coupling F-moc protected amino acid was dissolved into DMF (equivalent of 2 according to the resin substitution). HBTU (equivalent of 2), hydroxibenzotriazol (HOBT) (equivalent of 2), N, N-diizoproplyethylamin (DIEA) (equivalent of 4) were added onto the mixture of resin and amino acid. The reaction took 3 hours. Ninhidryn test was performed after every coupling and deprotection process. When the all amino acids were coupled, the peptide was removed from the resin by using trifluoroasetic acid (TFA): triizopropylsylan (TPS): sterile water (H_2O) as a ratio of 95:2.5:2.5. After, TFA was evaporated under high vacuum, the cold diethyl ether was used for washing the peptides. The remained pellet were cooled and dried by freeze drying .

B. Conjugation of AMPs to the PGCL Suture Surfaces

In this study, sterile, absorbable, United States Pharmacopeia (USP) size 1, two braided multifilament PGCL sutures were used. All suture samples were generously donated by KATSAN A.S (İzmir, Turkey). The conjugation of AMPs through the PGCL suture surfaces was occured by the help of the carboxyl and hydroxyl functional groups of PGCL sutures in their structure. For this purpose, KRF, RW and KRF/RW (1:1) were used for conjugation of PGCL suture surfaces [18]. First, PGCL suture surfaces were treated by cold plasma for producing the carboxyl and hydroxyl functional groups on PGCL sutures. 2mM 1-ethyl- (dimethylaminopropyl) carbodiimit (EDC) and 5mM N-hydroxy-sulfosuccinimit (NHS) solution were mixed and put into 0.1 Molar MES solution. Then, the mixture was added onto the PGCL sutures and put into incubator for 40 minutes at 37 °C. After this step, the sutures were washed by PBS three times. 1mM AMP solution in PBS was added on PGCL sutures for 24h at 4 °C.

C. Cell Proliferation Analysis of AMP conjugated PGCL sutures

The cytotoxicity test of AMP conjugated sutures and bare PGCL sutures as control were performed according to ISO 10993-5 standards. The sutures and control group were incubated in serum free Dulbecco's Modified Eagle Medium (DMEM) cell culture medium for 24 hours (h) at %5 CO_2 37°C. After 24 hours, the extracts were taken from the media for cell proliferation analysis. Keratinocyte cells (HS2) were seeded on 24 well plates and incubated in DMEM medium supplemented by 10% Fetal Bovine Serum (FBS), 1% Penicillin-Streptomycin for 24 hours. The extract of sutures was added on the cells. MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) assay was applied for evaluating the cell proliferation of sutures. Briefly, the MTT solution was added on the each well and incubated for 2 hours at %5 CO_2 37 °C. After 2 hours, the MTT solution was removed from the cells and 500 µl Dimethyl Sulfoxide (DMSO) was put on the cells. The cell numbers were obtained by absorbance reading at 570 nm by using plate reader.

D. In Vitro Scratch Wound Assay of AMP Conjugated PGCL Sutures

In vitro wound scratch assay was evaluated by using HS2 human keratinocyte cell lines for evaluation of impact of the AMP conjugated PGCL sutures on the wound closure. Firstly, 10 cm long sutures were pre-incubated in 1ml serum free DMEM cell culture media after centrifuging at 120 rpm for 24 hours by using. After, $5x10^4$ cells were seeded on 24 well plate and incubated in the media for 24h. Then, 200 µl pipet tip was used to make scratch to mimic wound. Each scratch was controlled by inverted microscope (Olympus CKX41, Tokyo, Japan) for assuring the all the scratches were in similar size in all samples. Then, the DMEM media was removed from the each well and the cells were washed 1x PBS for removing any debris from the scratch. Finally, 500 µl extracted media from each AMP and control group were put onto the cells and incubated at %5 CO_2 37 °C. The scratch areas and cell

migration activity were observed by inverted microscope (Olympus CKX41, Tokyo, Japan) at 0h, 24h, 48h, and 72h

E. Colony Counting Assay of AMP conjugated PGCL sutures

In present study, *P. Aeruginosa* was used as gram negative bacteria and MRSA as gram positive model organism for antimicrobial tests of AMP conjugated PGCL sutures. 1 mL of frozen stocks of both strains were added in 9 mL of trypticase soy broth (TSB) medium and incubated in a shaker incubator at 200 revolutions per minute (rpm) and at 37°C for 24 h. Then, cultures were plated on trypticase soy agar (TSA) plates. Single colonies were picked from TSA plate and inoculated in TSB medium and incubated overnight in a shaker incubator at 200 rpm and at 37 °C prior to use in experiments. The bacterial concentration was adjusted to the desired number by measuring optical density using a spectrophotometer (PG Instruments Limited, Leicestershire, UK). After, the suspension cultures were diluted by using PBS for reaching the desired 10^8CFU/ml concentration. After serial dilutions of the suspension culture, 10 cm long sutures were suspended in 1 ml microbial suspensions in 10^5CFU/ml concentration and incubated for 6 hours at 37°C in a stationary incubator. Bacterial culture was diluted to 10^5 CFU/mL concentration using 1X sterile phosphate buffered saline (PBS) solution. 100 µL of 10^3 CFU/mL *P. aeruginosa* and MRSA were plated on TSA plates and incubated overnight at 37 °C. The following day, surviving colonies were counted. Results were expressed as log surviving colonies.

III. RESULTS

Since the cell number in AMP conjugated peptide groups didn't significantly change compared to the PGCL group, AMP conjugation doesn't cause any cytotoxic effect. Nevertheless, RW conjugated PGCL suture groups has the highest cell number compared to other groups.

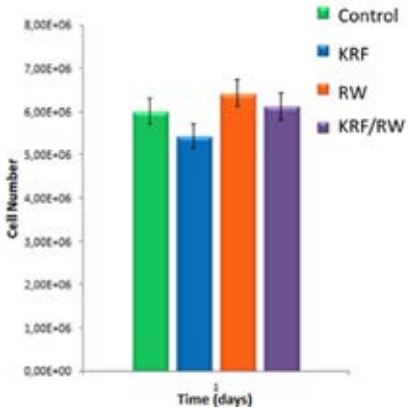

Figure 1. The cell number after cytotoxicity assay in A) KRF, B) RW, C)KRF-RW D) PGCL control groups

The effect of AMP conjugated PGCL suture on wound healing was evaluated by *in vitro* scratch assay by using human keratinocyte cell lines. As it's shown in Figure 2, all AMP conjugated PGCL suture groups accelerated the wound closure process compared to the PGCL control group after 72 hours. $(RW)_4$ AMP conjugated PGCL groups provided the fastest healing process. Additionally, KRFRIRVRV AMP conjugated PGCL group has supplied faster wound healing process then the PGCL control group.

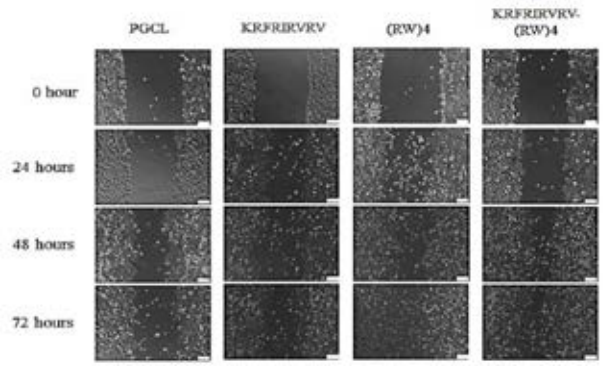

Figure 2. In vitro scratch assay results for 0 hour,24 hours, 48 hours and 72 hours with respect to; A) KRFRIRVRV, B) KRFRIRVRV- (RW)4, C) PGCL control group, D) (RW)4 by using inverted microscope . Scale bar represents 20µm)

Antimicrobial effect of AMP conjugated sutures was tested by using *P. aeruginosa* and MRSA. As shown in Figure 3, all AMP conjugated sutures show antibacterial effect on *P. aeruginosa* and MRSA. In KRF, RW and KRF/RW (1:1) group about 1-log, reduction of MRSA was observed while reduction of *P. aeruginosa* was less than 1-log in KRF KRF/RW group ($p < 0.001$).

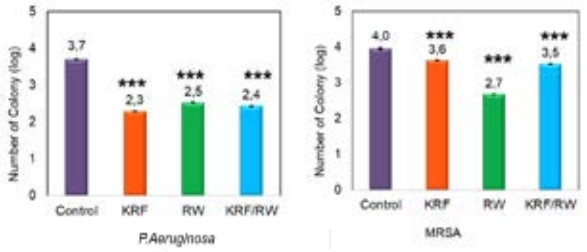

Figure 3. Colony counting assay results of planktonic forms of *P. Aeruginosa* and MRSA in KRF, RW,KRF/RW,Control groups

IV. DISCUSSION

In the present study, we produced 3 different types of AMP conjugated PGCL sutures and investigated their effect on wound closure and also antimicrobial effect on *P. aeruginosa* and MRSA. AMP conjugated PGCL surgical sutures have showed promising results *in vitro* for acceleration of wound healing process with the help of the increase cell migration according to control group at the end of the 72 hours. Furthermore, antimicrobial test indicates that KRF and RW conjugated PGCL sutures leads significant death of *P. Aeruginosa* and MRSA according to the control group, respectively. The positively charged KRF peptide conjugated to the PLGA surface targets negatively charged bacteria by electrostatic interaction and damage to the bacterial membrane by causing bacterial death. RW repeated chains were effective against both gram-positive and gram-negative bacteria, leading to significant death on both bacteria. The

positively charged Arginine interacts with the bacterial membrane by electrostatic attraction, while the non-polar Trp interacts with the lipid bilayers through hydrophobic interactions. These two activities on a single peptide help the peptide to interact with the bacterial membrane. This results in membrane instability and pore formation leading to bacterial cell death. Therefore, AMPs might be the favorable biomaterial to obtain faster wound healing process and also it might be the appropriate material to get antimicrobial efficiency against SSIs.

V. CONCLUSION

Our results have demonstrated that three group of the AMP conjugated PGCL sutures are not toxic for the keratinocyte cell lines and accelerated the wound closure materially according to the PGCL control group at the end of the 72 hours. However, the antimicrobial test results are promising. In conclusion, KRFRIRVRV, (RW)4 and KRFRIRVRV- (RW)4 AMP conjugated PGCL sutures have showed promising results on on *P. aeruginosa* and MRSA, specifically. AMP conjugated sutures could be considered as a possible alternative for preventing of suture-related surgical site infections.

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