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Blood-derived products can moderate the activity of neutrophils isolated after biomaterial implantation in a sheep model

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Summary

Neutrophils play a pivotal role in both the inflammatory phase and subsequent repair processes, making their activity crucial after contact with biomaterials. Excessive neutrophil activation can lead to complications, including implant rejection, highlighting the importance of modulating their activity. This study investigates the long-term interaction of circulating ovine neutrophils with titanium (Ti) and silicon-doped layer (Si-DLC) coated implants. Additionally, the effects of platelet-rich plasma (PRP), platelet-poor plasma (PPP), and antimicrobial neutrophil crude extract (ANE) on neutrophil activity were assessed. Twelve female sheep were divided into Ti, Si-DLC implant and a control group. Neutrophils were stimulated with PRP, PPP, ovine ANE (oANE), or rabbit ANE (rANE), and their enzymatic activity and reactive oxygen and nitrogen species (RONS) generation were evaluated. Hematological parameters were also analyzed. Results showed no adverse effects at implant sites, and all hematological parameters remained within reference values. Ti and Si-DLC implants did not alter neutrophil activity. PRP significantly increased neutrophil activity and RONS production, while PPP decreased activity. oANE reduced enzymatic activity and RONS generation, indicating potential anti-inflammatory effects. rANE showed varied effects on neutrophil function. Both ANEs lacked antimicrobial activity against tested pathogens. This study demonstrates the hemocompatibility of Ti and Si-DLC implants and highlights the potential of blood-derived products in modulating the inflammatory response to implants.

Keywords: antimicrobial neutrophil crude extract, titanium implant, Si-DLC implant, platelet rich plasma, reactive oxygen and nitrogen species

Neutrophils play a significant role both in the inflammatory phase and in subsequent repair processes, and are strongly involved in both the initiation and amplification of the inflammatory response (14, 15). They can interact with other immune cells, mediating the transmission of pro- and anti-inflammatory signals. Therefore, their activity is particularly important after contact with biomaterials (27). Excessive activation of neutrophils may cause serious complications up to implant rejection, thus modulation of their activity

is of great importance for the success of biomaterial implantation (15, 38). The bone composition and remodeling patterns in adult sheep closely resemble those in humans. This model provides an opportunity to compare implant outcomes with ex vivo results, which is not ethical in human subjects (14). It has been assumed that circulating neutrophils during prolonged interaction with the implant may contribute to adverse effects (36).

Some blood-derived products, among others; platelet-rich plasma (PRP), platelet-poor plasma (PPP), and antimicrobial peptides (AMPs), play a significant role in enhancing the tissue repair process and may be considered as factors for the enhancement of healing and to reduce excessive undesired inflammatory response (10, 36). PRP can release some growth factors and pro-inflammatory cytokines to enhance healing in chronic disorders involved in disturbances in inflammatory response (10). PPP, in turn, is a by-product obtained during preparation of PRP, characterized by a significantly lower platelet count than in whole blood. The main components of PPP include fibrinogen, fibronectin, and thrombin. PPP functions in the haemostasis process, acts as a substrate for cell attachment, and promotes the cell division of fibroblasts and epithelial cells (22). Although PPP has a lower platelet concentration compared PRP, its application can support cell growth and survival. PPP is also capable of promoting functions related to wound healing and accelerating the migration and proliferation of fibroblasts (40).

Another promising blood-derived product is antimicrobial neutrophil crude extract (ANE), which contains a mixture of antimicrobial peptides (AMPs). AMPs are produced by various cell types, such as neutrophils, and are integral components of the innate immune system with antimicrobial and immunomodulatory properties such as immune cell differentiation, inflammatory responses, cytokine production, and chemotaxis (5). Previous work has confirmed the immunomodulatory and antimicrobial properties of ANE from various animal species (3, 12, 28), such as ovine ANE (28) and rabbit ANE (30, 31). Ovine-derived ANE (oANE) contains the cathelicidins, SMAP29, OaBac5mini, and OaBac7.5 (2), while rabbit ANE (rANE) is rich in the 15 kDa antimicrobial peptide, cathelin-like peptide, CAP-18 and defensins (26). It can be assumed that, depending on the origin, homologous (ovine) or heterologous (rabbit) ANE may have various effects on ovine neutrophils, potentially serving as an agent modulating the inflammatory response (37).

Titanium (Ti) and its alloys are commonly used as materials for long-term applications such as orthopedic and dental implants (32, 35). However, they may corrode or gradually be released into surrounding tissues by mechanical processes such as friction, bending or scratching, which may lead to the release of titanium particles into the circulation and tissues. This may result in adverse effects such as disturbances in bone formation and the initiation of acute and chronic inflammation (9), which is associated with inappropriate activation and continuous recruitment of neutrophils and may result in failure of biomaterial implantation (7, 27). The introduction of a silicon-doped layer (Si-DLC) with high hemocompatibility onto the implant surface creates a solid diffusion barrier for metal ions, effectively separating the metallic material from body tissues (36, 41).

The aim of the study was to assess the long-term interaction of circulating ovine neutrophils isolated from sheep after Ti or Si-DLC coated implants insertion. It was assumed that blood-derived products can modulate neutrophil activity, therefore the second aim was to compare the response of cultured neutrophils to stimulation by some blood-derived products; PRP, PPP, and oANE or rANE.

Material and methods

Properties of Ti and Si-DLC implants. All implants were manufactured by Medgal, Białystok, Poland. The cylindrical Ti implants with a diameter of 4 mm and a length of 12 mm were made of titanium alloy, according to ISO 5832-3:2016, containing: Ti – 88.1%, Al – 6.75%, V – 4.5%, Fe – 0.3%, O – 0.2%, C – 0.08%, N – 0.05%, H – 0.015%. These implants were delivered in a sterile condition (26).

The second type of implant, Si-DLC, is made of implant steel coated with a carbon-silicon layer, according to ISO 5832-1:2016. The alloy composition is: Fe – 59.335%, Cr – 19%, Ni – 15%, Mo – 3%, Cu – 0.03%, Si – 1%, Mn – 2%, P – 0.025%, S – 0.01%, N – 0.1%. These implants had a cylindrical shape with a diameter of 4 mm and a length of 12 mm. This product was delivered in a non-sterile condition, ready for sterilization in a double paper-plastic sleeve intended for steam sterilization. The sterilization was carried out in an autoclave according to a validated sterilization method: exposure time 5 minutes, temperature 134°C, drying time 25 minutes (25).

Ovine models and surgical procedures. The study was conducted on the group of 12 female sheep line BCP, 12 months old, weighing 40-50 kg, and housed at the Bezek Experimental Farm of the University of Life Sciences in Lublin. The approval for animal management and surgical protocol were granted by the Local Ethical Committee at the University of Life Sciences in Lublin (No. 48/2021).

The animals were randomly divided into two groups; sheep with Ti implants (n = 4), and sheep (n = 4) with Si-DLC implants. The control group (n = 4) consisted of healthy sheep not subjected to the procedure and maintained under the same conditions as sheep in the experimental groups. After premedication with xylazine (Sedazin, 0.1 mg/kg) and butorphanol (Torbugesic Vet, 0.1 mg/kg) each sheep from the experimental groups had Ti or Si-DLC implants inserted into the tibia. Postoperative treatment included administration of Melovem (meloxicam 5%, 0.1 mg/kg) and Combi-Ject (200.000 IU/ml penicillin and 200 mg/ml streptomycin). Each sheep was monitored before and after surgery with clinical examination, including assessment

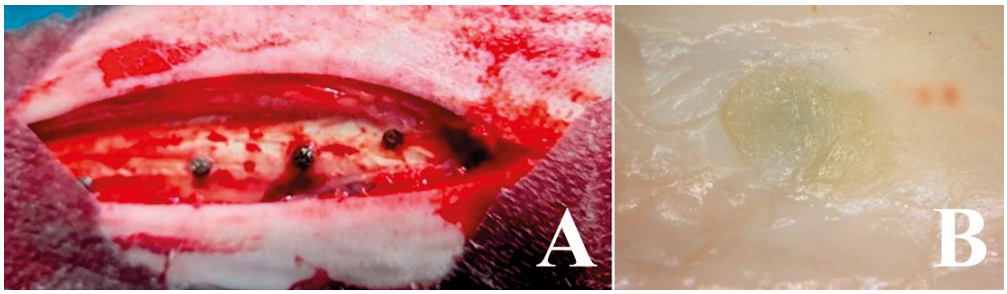

Fig. 1. A) Presentation of the implant inserted into the ovine tibia, B) Appearance of the implant after 10 months of implantation

Tab. 1. Mean white blood cells (WBC), red blood cells (RBC), platelets (PLT) concentration in whole blood, platelet rich plasma (PRP) and platelet poor plasma (PPP)

Blood cells/yield	Whole Blood	PRP	PPP
WBC (× 10^9/l)	4.0 ± 2.00	20.11 ± 1.00*	0.1 ± 0.05*
RBC (× 10^{12}/l)	11.0 ± 0.5	1.5 ± 1.00*	0.01 ± 0.05*
PLT (× 10^9/l)	200.5 ± 30.00	1028.00 ± 420*	5.00 ± 1.50*
Yield (%)	–	512.72 ± 102.3	–

Explanation: * $p < 0.01$ in comparison with values obtained in whole blood

of respiration, pulse, and body temperature. Additionally, the surgical wound areas inspection was carried out, and the animals' mobility was evaluated to exclude locomotory disorders.

Obtaining blood-derived products. Preparation of platelet derived products: To obtain PRP and PPP, the Curasan-based system was employed, following the instructions provided by the manufacturer (36). Cells content in platelet-derived products was assessed using Abacus Junior Vet analyzer (Diatron, Budapest, Hungary) and shown in the Table 1.

Preparation of ANE: Neutrophils were isolated from fresh ovine blood collected in 3.8% sodium citrate (33). The initial concentration of the cell suspension was determined using an R1 Automated Cell Counter (Olympus, Warsaw, Poland). Isolated cells were homogenized using DIAX 900 (Heidolph, Schwabach, Germany; 12.5 rpm for 15 minutes) to release neutrophil granules, then centrifuged at 25,000 × g for 40 minutes at 4°C and stirred overnight in 10% acetic acid at 4°C. After determining the protein concentration, the extract was lyophilized and stored at –70°C. Rabbit ANE was obtained using the same procedure (31).

Evaluation of antimicrobial properties of oANE and rANE. The antimicrobial activity assay was performed according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) method. Antibacterial activity was tested on the following strains: *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213 and *Pseudomonas aeruginosa* ATCC 27853. Peptides were serially diluted in Miller Hinton (MHB) liquid medium (Biomaxima, Lublin, Poland) and each dilution (in triplicate) was introduced into volumes of 50 µl into wells on a 96-well titration plate. Finally, the activity of peptides was assessed at eight concentrations from 128 to 1 µg/ml. A bacterial suspension from a 24-hour culture on MHB medium was then introduced into each well, previously adjusted to a density of 5 × 10^5 CFU/ml. A suspension of each of the tested strains in MHB was used as a growth control. The plates were incubated at 37°C for 20 h. The results were read visually, and the minimum bactericidal concentration (MBC) was additionally determined: the suspension from the well indicating the MIC value was serially diluted and inoculated on solid MH medium in triplicate. The plates were incubated at 37°C for 20 h together with the positive control.

Neutrophils culture and stimulation. Neutrophils were isolated from whole blood according to the procedure described previously (33) and were suspended in phosphate-buffered saline (PBS). Then cell in concentration of 1 × 10^6 cells/ml were seeded into 24-well plates. Each of the groups (control and two experimental) was separately stimulated with individual stimulants: PRP (2.5 × 10^8 platelets/ml), and PPP (similar volume as PRP), oANE (20 µg/ml), and rANE (20 µg/ml). Each plate was incubated for 24 hours at 37°C and 5% CO_2 concentration. Subsequently, the enzymatic activity of neutrophils was assessed based on the release of MPO and ALP, as well as the generation of reactive oxygen and nitrogen species (RONS) (37).

Assessment of hematological parameters. Hematological parameters including total white blood cell count (WBC), lymphocytes (LYM), granulocytes (GRA), total erythrocyte count (RBC), and hematocrit (HCT) were analyzed using Abacus Junior Vet analyzer (Diatron, Budapest, Hungary).

Statistical analysis. The statistical analysis for this study utilized Statistica 13.3 software (StatSoft, Cracov, Poland). The experimental results were presented as mean ± standard error (SE). Student's t-test was applied to compare multiple groups, and statistical significance was considered at a p-value below 0.05 ($p < 0.05$).

Results and discussion

During the whole experimental period no adverse effects at the implantation site were observed for up to 8 months, no motor disorders were observed in animals from the experimental groups. All hematological parameters remained within the reference values (Tab. 2). The absence of observed changes after implantation

Tab. 2. Mean hematological parameters (± SD) in control sheep (n = 4) and sheep after Ti implant (n = 5) and Si-DLC implant (n = 5)

Parameter	Control	Ti implant	Si-DLC implant
WBC (10^9/l)	3.6 ± 0.8	4.0 ± 1.26	3.8 ± 0.85
LYM (10^9/l)	2.5 ± 0.6	2.0 ± 1.0	3.0 ± 0.9
GRAN (10^9/l)	1.1 ± 0.9	2.32 ± 1.0	1.58 ± 0.8
RBC (10^{12}/l)	11.08 ± 1.5	10.0 ± 1.3	12.04 ± 1.1
HCT (%)	29.1 ± 0.95	30.5 ± 0.5	34.0 ± 1.1
HGB (g/dl)	15.6 ± 1.13	12.00 ± 1.4	11.65 ± 1.5

and the lack of systemic inflammation suggests that the studied implants (both Ti and those coated with Si-DLC) are hemocompatible.

The conducted research confirms that the Ti and Si-DLC implants used in the experiment did not disturb the response of circulating neutrophils, as assessed on the basis of their enzymatic activity and RONS generation, examined 8 months after implantation. In our experiment no significant differences in neutrophil enzymatic activity nor RONS generation were observed in experimental groups after comparison between the activity of neutrophils obtained from the control group (Fig. 2 and Fig. 3). This study extends the knowledge about the biocompatibility of biomaterials beyond the tests required according to the standard ISO 10993-6:2009 (1, 26). All materials intended for application in animals and humans as biomaterials undergo host responses. According to ISO 10993-4:2017 standards hemocompatibility tests evaluate the interactions between biomaterial and blood and/or blood components in five categories: thrombosis, coagulation, platelets, hematology, and immunology (1, 23).

It should be stressed that despite the long-standing use of metal implants, adverse reactions still occur, constituting a significant percentage of complications, potentially leading to implant rejection. These reactions may develop over an extended period. However, *in vivo* studies concerning neutrophils typically focus on the early inflammatory phase of healing. However, circulating neutrophils, acting as signaling cells, may play a crucial role in the late response to the implant (29). Avery et al. (4) demonstrated that the chemical composition of implants affects neutrophil activity.

The neutrophil response to stimulators used, namely PRP, PPP, oANE and rANE, was assessed based on enzymatic activity and RONS generation after 24 hours incubation at 37°C and 5% CO_2. Enzymatic activity was evaluated by measuring the release of MPO from azurophilic granules and ALP from secretory vesicles. RONS generation was assessed by measuring the generation of NO and $O_2^{\bullet-}$. The stimulation with PRP significantly increased the secretory activity and RONS production in all examined cultures of neutrophils isolated from animals from control and experimental groups compared to values obtained in unstimulated cell cultures from all studied groups. The statistically significant increase in response across all investigated parameters after PRP stimulation indicates a potential stimulating effect of factors present in PRP on neutrophils. Some studies on platelet-leukocyte interactions pointed out that platelets regulate inflammation, wound healing, and tissue repair acting via TLR-4 on

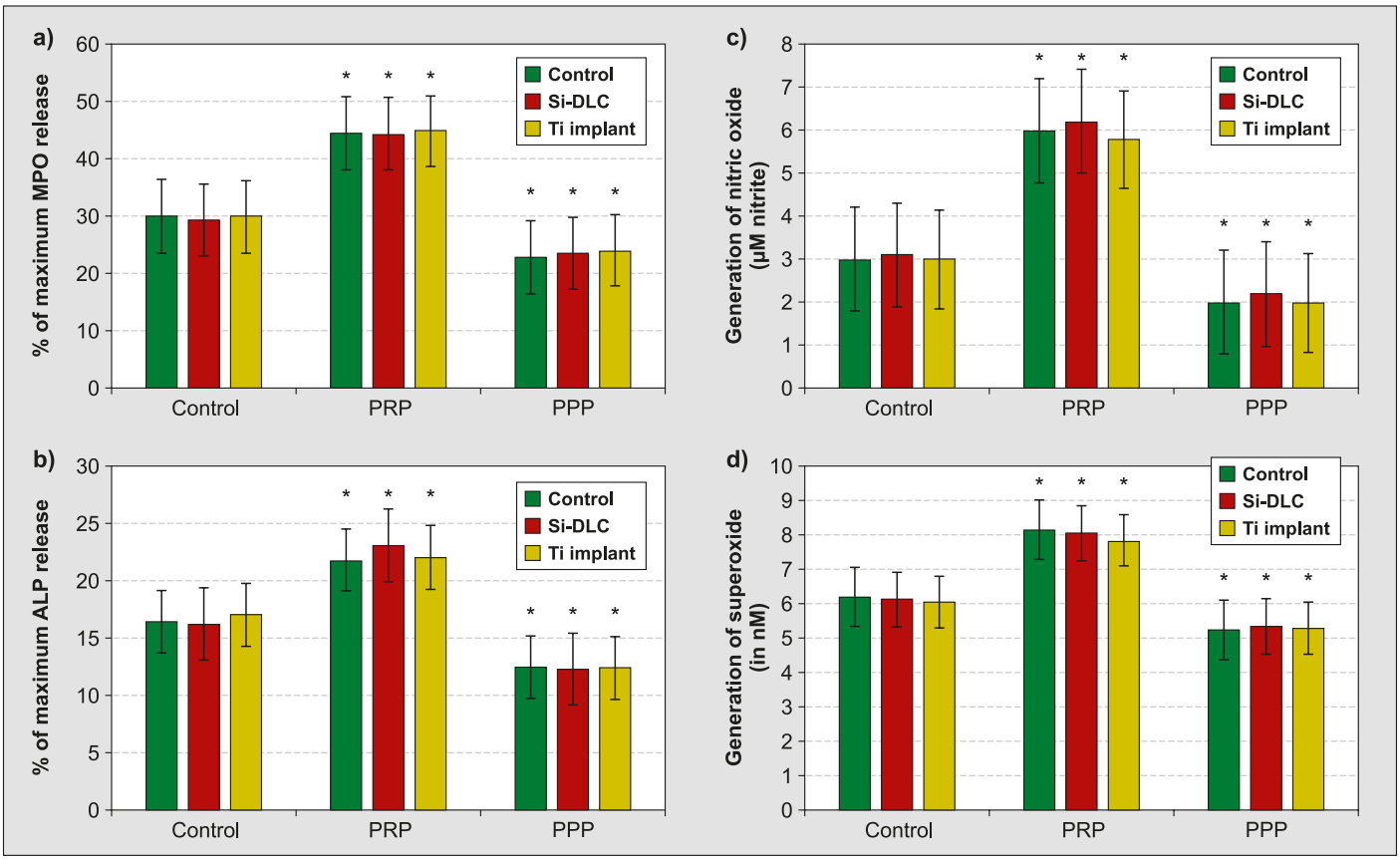

Fig. 2. Comparing the effects of stimulating with platelet-rich plasma (PRP) and platelet-poor plasma (PPP) on neutrophils isolated from the blood of sheep in experimental groups after the implantation of a titanium implant (Ti) and implantation of an implant with Si-DLC coating, as well as from the blood of sheep in the control group: a) % of maximum MPO release; b) % of maximum ALP release; c) generation of nitric oxide; d) generation of superoxide

Explanation: * – significant difference compared to the control group ($p < 0.05$)

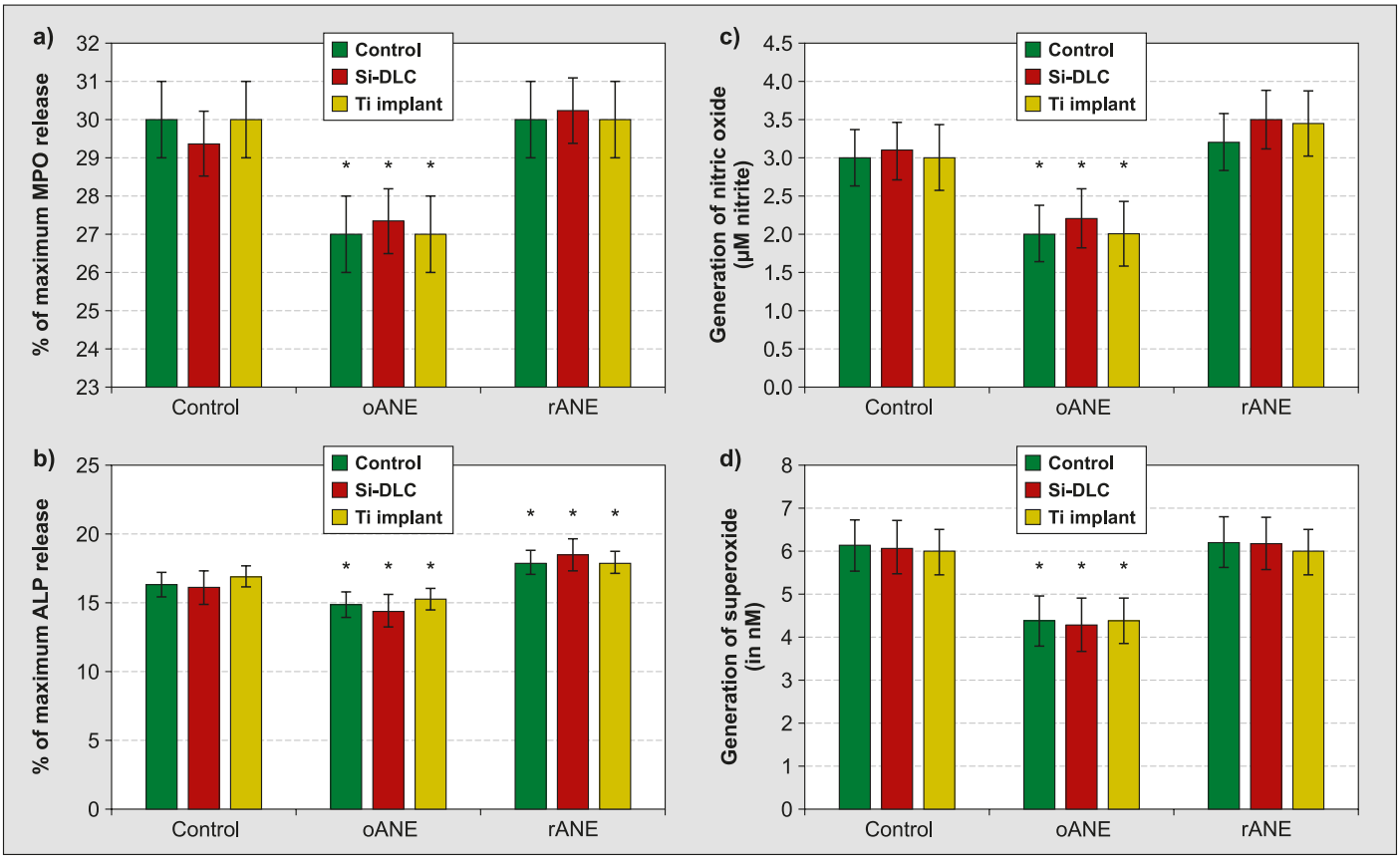

Fig. 3. The effects of stimulating with ovine (oANE) and rabbit (rANE) neutrophil extracts on neutrophils isolated from the blood of sheep in experimental groups after the implantation of a titanium implant (Ti) and implantation of an implant with Si-DLC coating, as well as from the blood of sheep in the control group: a) % of maximum MPO release; b) % of maximum ALP release; c) generation of nitric oxide; d) generation of superoxide

Explanation: * – significant difference compared to the control group ($p < 0.05$)

leukocyte by modulating oxidative burst with ROS generation and release MPO (10).

Conversely, the application of PPP significantly decreased release of MPO, ALP, as well as generation of NO, and $O_2^{\bullet-}$ in all studied groups compared to unstimulated culture (Fig. 2). The decrease in neutrophil activity following PPP stimulation may suggest that factors present in PPP could exert anti-inflammatory effect. Some biologically active components of PPP could be used in regenerative medicine, especially in treatment of some musculoskeletal disorders involved in excessive inflammation (18).

As estimated ANE prepared from ovine blood significantly ($p < 0.05$) reduced the release of MPO and ALP from neutrophils. A similar response was observed in RONS generation, with a significant decrease in the generation of NO and O_2^- upon stimulation with oANE. This effect could be considered in the context of anti-inflammatory activity.

In contrast, rANE did not affect the release of MPO, but showed a significant increase in ALP release ($p < 0.05$). The neutrophil response to RONS generation after stimulation of with rANE was insignificant (Fig. 3). Based on the obtained results, it can be seen that the ANE prepared from fresh blood of sheep and rabbits differs in its effect on enzyme release and the RONS generation by neutrophils *in vitro*. This differential response may be due to different neutrophil activation pathways and the mechanism requires further research (8). Ovine ANE significantly reduced the enzyme release, suggesting that it may modulate the response of neutrophils, potentially reducing their activity. Additionally, a significant decrease in the release of NO and O_2^- after stimulation with oANE indicates the possibility of inhibiting RONS generation. Therefore, anti-inflammatory and antioxidant activity of this product can be considered in clinical applications. The significant reduction in the release of MPO and ALP suggests that the ovine extract may have an inhibitory effect on oxidative stress. According to Avery et al., increased release of MPO from activated neutrophils is strongly involved in host/implants interactions (4). The homologous oANE exerts an inhibitory effect on the secretory activity of neutrophils and RONS generation, as noted in previous findings on other types of implants (36, 37, 39).

On the other hand, rANE did not affect MPO secretion, but showed a significant increase in ALP release. The different effects of rANEs suggest that they may affect other aspects of neutrophil function, such as ALP. However, the response of neutrophils to RONS secretion after rabbit ANE was statistically insignificant,

suggesting that rANE may not affect RONS production to the same extent as oANE. The antimicrobial neutrophil extracts used to stimulate neutrophils depending on the origin of the extract and the species of experimental animals were autologous ovine extract and heterologous rabbit extract (37).

Antimicrobial assays revealed that both oANE and rANE at concentrations up to 32 µg/ml, did not show antimicrobial activity against the tested pathogens. In the case of rANE, the MIC and MBC values were above the tested concentrations, except for *E. coli*, where a value of 128 µg/ml was found. In the case of oANE, the activity seems to be higher, but the MIC and MBC value was still above 32 µg/ml. However, the synergistic effect of using AMPs and antibiotics has been repeatedly confirmed (17, 21, 34) which could be used in therapy to reduce the doses of antibiotics used, and thus reduce their potential side effects. Despite high inhibitory concentrations, the obtained results seem to be promising and research should be continued to determine the synergism of the tested AMPs with selected antimicrobials, especially in the case of therapeutically difficult pathogens such as *P. aeruginosa* (Tab. 3).

Tab. 3. Results of antimicrobial activity of oANE and rANE

Antimicrobial Peptides Extract	*E. coli* MIC&MBC	*S. aureus* MIC&MBC	*P. aeruginosa* MIC&MBC
oANE	64 µg/ml	64 µg/ml	128 µg/ml
rANE	128 µg/ml	> 128 µg/ml	> 128 µg/ml

It is now well established that AMPs have multiple effects on various cell types throughout the body, including a stimulatory effect on immune cells. However, the concentration of AMP in body fluids and tissues is generally much lower (ranging from ng per ml to µg per ml), than the level of antimicrobial activity. This potential antimicrobial activity is inhibited by the presence of physiological concentrations of salt, serum proteins and/or lipoproteins and glycosaminoglycans (12). Considering the inhibitory effect of physiological conditions, most cathelicidins probably do not have direct bactericidal activities as their primary function *in vivo*. LL-37 stimulates the production of reactive oxygen species (ROS) in neutrophils, most likely in a NADPH-dependent manner. On the other hand, porcine cathelicidin PR-39 inhibits NADPH oxidase activity, which impairs the oxidative bacterial killing of neutrophils. The PR-39 mediated NADPH inhibition could be a negative feedback loop to inhibit superoxide formation and prevent tissue damage (13). The immunomodulatory effects of AMPs below their antimicrobial concentration enable the potential therapeutic application of AMPs below their killing effects, which lowers their toxicity threshold (7, 11).

The limitation of the study is the assessment of only the circulating neutrophils; further examination is required to assess the resident neutrophils. Moreover, a complete examination of the neutrophil response will require accurate determination of neutrophil subtypes.

Owing to their central role in the immune response to implants, particularly during the inflammation stage, neutrophils represent potential targets for therapeutic strategies aiming to address biomaterial-related dysfunctions during tissue regeneration, by modulating the host response (6).

Our study revealed that both implant types used in the experiment are hemocompatible. Additionally, some blood-derived products have the potential to regulate the inflammatory response to implants. Specifically, PRP acts as a pro-inflammatory and oxidative factor, increasing enzymatic activity and the generation of RONS. In contrast, PPP and homologous oANE exhibit anti-inflammatory properties. The pro-inflammatory activity of PRP could be beneficial in cases of chronic inflammation by enhancing the immune response. On the other hand, the anti-inflammatory properties of PPP and oANE could prevent excessive neutrophil activity, which might otherwise lead to adverse effects.

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