THE INFLUENCE OF AUTOLOGOUS AND HETEROLOGOUS EXTRACT OF ANTIMICROBIAL PEPTIDES ON LEUKOCYTES ISOLATED DURING TITANIUM IMPLANT INSERTION IN RABBIT AND OVINE MODEL

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Abstract

This study evaluated the in vitro leukocyte response to titanium implants in the presence of autologous or heterologous antimicrobial peptides extracts. Antimicrobial peptides (AMPs) appeared to be a new approach both against microorganisms and for regulation of inflammatory and repair processes. To evaluate their potential usefulness in regenerative medicine, we prepared different extracts of neutrophil-derived AMPs from rabbit, ovine or porcine blood which contained AMPs of different compositions, mainly defensins, cathelicidins and fragments thereof. Then, we assessed in vitro the influence of different AMPs extracts on the neutrophils and monocyte-derived macrophages (MDM) activity. For this purpose, these cells were obtained from experimental animals, rabbits, or sheep submitted to insertion of a titanium implant into the tibial defect. The cultured cells stimulation was autologous or heterologous, dependently on the AMPs extract origin and the experimental animal species. The neutrophil activity was assessed on the basis of the enzymes release from azurophilic and secondary granules and the free radicals generation. The MDM functional assessment was based on the NO and superoxide generation and arginase activity. Additionally, morphological changes were evaluated in the cell cultures. Our results indicated that the origin of AMPs extract is crucial for its activity. The autologous extracts stimulated anti-inflammatory responses, whereas the heterologous extracts displayed pro-inflammatory effect on neutrophils and macrophages. These results might be considered during the introduction of new preparations in regenerative medicine.

Keywords: *titanium implants, neutrophil extract, implantation of biomaterial, neutrophils, monocyte-derived macrophages, natural antimicrobial peptides*

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Introduction

Titanium (Ti) is considered to be a relatively inert biomaterial which evokes minimal adverse effects. However, it has been described in the literature that the titanium particles released to circulation may be harmful under some circumstances. The adverse effect is noted especially during frictional wear of prostheses or screws used for the implant to bone attachment, the bone to bone fixation or the soft tissue fixation. Side effects in soft tissues with the presence of foreign body giant cells have been described in reaction to rough surface titanium alloy medical prostheses together with increased plasma concentration of Ti ions. The presence of Ti particles in the regional lymph nodes and in the tissue around the implant can lead to long-term metabolic, neoplastic and immunologic effects [1].

Despite these limitations, Ti is favourable because it is endowed with advantageous biomaterial characteristics, such as mechanical strength, corrosion resistance, chemical stability, and biocompatibility. For instance, due to the thin TiO_2 layer, the surface becomes highly resistant to corrosion. However, in the last decade, an increasing number of reports have been published about excessive inflammatory reactions and bone loss around implants, so-called peri-implantitis involved in implant side effects [2].

Within the white blood cells (WBC) population both neutrophils and blood monocytes migrate to the site of injury and participate in tissue repair [3]. These cells are essential for the regeneration, repair and remodelling in many tissues and can act as secondary pro-inflammatory factors capable of releasing cytokines and other stimuli. It should be emphasized that, apart from other effects, titanium ions form particles that induce inflammatory cells activation, especially monocytes and macrophages [2,4-7]. On the other hand, neutrophils are increasingly considered as a heterologous population with high regulatory potential. However, chronic inflammation and persistent neutrophils and macrophages activity are often involved in tissue destruction and fibrosis [3,8]. For these reasons, current studies are focus on interactions between components of the host defence and medical implants in order to improve the implanted biomaterials properties. Therefore, we decided to conduct the research that goes beyond basic biocompatibility studies and extends their scope.

Antimicrobial peptides are widely recognized for their multifunctional antimicrobial and immunomodulatory activities. These functions refer to modulation of pro- and anti-inflammatory responses, including macrophage differentiation and modulation of wound healing. AMPs can suppress pro-inflammatory responses, as in the case of human cathelicidin LL-37, thanks to limiting the release of pro-inflammatory mediators and the lipopolysaccharide (LPS) neutralization [9]. A similar effect was observed for porcine cathelicidin PR-39 [10]. PR-39 is a proline-arginine rich peptide from porcine neutrophils with a broad antibacterial effect. This peptide, first identified in the porcine small intestine, displays a specific antibacterial activity against multiple gram-negative bacteria. It inhibits the protein synthesis and exerts the membrane-disruptive effect on microbes.

Moreover, PR-39 improves wound healing by upregulating the surface expression of syndecan-1 and syndecan-4 on mesenchymal cells. In the mouse model of sepsis, this peptide showed a protective effect as it increased the nitric oxide (NO) production in the liver and limited the reactive oxygen species (ROS) generation [11]. Furthermore, AMPs influence the functions of neutrophils, i.e. the major innate immune effector cells of the early-phase response to injury. Neutrophils are both producers and receivers of AMPs. They are a source of both defensins and cathelicidins which are stored and released from granules during the neutrophil degranulation. AMPs can enhance the neutrophils influx both by a direct chemotactic function and indirectly by promoting the secretion of these neutrophil products [12].

It was estimated that, dependently on their properties, AMPs can induce the macrophage differentiation towards M1, M2 or the intermediate phenotype between pro-inflammatory M1 and anti-inflammatory M2 macrophages. PR-39 influences the porcine macrophages polarisation from the M2 to a M1 phenotype, and promotes the bactericidal functions of these phagocytes [11].

In our experiment, we evaluated the influence of various AMPs extracts on the activity of neutrophils and monocyte-derived macrophages (MDM) obtained from experimental animals, rabbits or sheep, having inserted the Ti implants into their bones.

Materials and Methods

The study was conducted on a rabbit and ovine model for biomaterial implantation. The response of neutrophils and MDM was evaluated *in vitro* after the titanium implant insertion. The neutrophil activity was assessed on the basis of the enzymes release, reactive oxygen species-ROS and reactive nitrogen intermediates-RNI generation. The MDM response was assessed based on the nitric oxide (NO) and superoxide generation, arginase activity and morphological changes of these cells. Before the experiment, the AMPs extracts of autologous or heterologous origin were prepared. Additionally, some components of the AMPs extracts (namely PR-39 and protegrin mixture) were separated via the gel filtration chromatography.

Titanium implants

Titanium Grade 2 discs were prepared for this experiment, having pre-treated the implants surface with a triple surface etching formula (TSE). The Tollens method was used to incorporate silver on the titanium surface so as to obtain the titanium implants modified with silver nanoparticles [13].

Rabbits and surgical procedure

Six healthy male New Zealand White (NZW) rabbits, aged between 7 and 9 months, with the body weight of approx. 4000 g were used for the experiment. The rabbits were housed and treated according to the laboratory animal treatment and care guidelines. The study protocol was approved by the Local Ethics Committee of the University of Life Sciences in Lublin and the experiment was performed in compliance with the animal protection regulations.

Prior to the surgical procedure, the rabbits were carefully examined. After premedication with xylazine (Sedazin; Biowet, Pulawy, Poland), 5 mg/kg and ketamine (VetaKetam; Vetagro, Lublin, Poland) 30 mg/kg intramuscularly, the animals received ketamine (0.35 mg/kg/min) intravenously. The surgical procedures were conducted under standard sterile conditions. After the hair removal, shaving, disinfection and draping, a straight 3-cm skin incision was made over the medial proximal tibia. Then, after the surgical exposure, the 4 mm defect was made using an electric surgical drill. The titanium implant was inserted into the tibial defect and fixed to the cortical bone. Then, the muscle tissue and skin were sutured. After the surgery, the rabbits were examined daily for clinical signs of complications or adverse reactions.

Preparation of autologous (rabbit) AMPs and heterologous (porcine) AMPs extract and its isolated components (PR-39, protegrin mixture), neutrophils isolation and stimulation

Porcine neutrophils for the AMPs extract were isolated from the blood collected at an abattoir. The red blood cells were lysed by the addition of 0.83% ammonium chloride to the blood sample and then centrifuged. The remaining pellet was washed twice with phosphate-buffered saline (PBS). The final cells were homogenized to release the neutrophil granules. The granules were collected (25 000 x g, 40 min, 4°C), suspended in the 10% acetic acid and stirred overnight at 4°C to extract the antimicrobial peptides. The solution containing the peptides was separated from the granules (25 000 x g, 20 min, 4°C) and the obtained extract was considered as the AMPs neutrophil extract. Then, the peptides were isolated according to their molecular mass via the gel filtration chromatography. The obtained products, namely the crude AMP extract, PR-39 and protegrins were used to stimulate the neutrophils or macrophages cultures. Rabbit AMPs were prepared similarly from rabbit blood.

In order to prepare the rabbit AMPs extract and cell culture, rabbit neutrophils were isolated from the blood collected from the ear vein. The blood was obtained 7 days before and 24 hrs after the surgery. The cell suspensions were supplemented as follows: the control group with PBS (marked as unstimulated), the other groups were stimulated with crude porcine antimicrobial extract (EXT), PR-39, protegrins or the crude rabbit antimicrobial extract (AMPs). Then, the cultures were incubated for 30 min and for 22 hrs at 37°C in the presence of 5% CO_2. Next, the neutrophils activity was assessed. The enzyme release from azurophilic granules was assayed based on the elastase or myeloperoxidase (MPO) release and compared to the maximal enzyme content obtained after the cells treatment with 0.5% Triton X-100. The elastase activity assay was based on the cleavage of azocasein as a substrate at 25°C for 10 min; thereafter, the absorbance value was assessed at 490 nm. The MPO release was measured as absorbance at 490 nm after cleavage of the substrate- o-phenylendiamine (Sigma-Aldrich, Poznan, Poland). The alkaline phosphatase (ALP) release, constituting a marker of specific granule response, was estimated after the 10 min incubation at 25°C with an equal volume of 4-nitrophenyl phosphate disodium salt hexahydrate, after which absorbance was measured at 405 nm. The nitric oxide level was determined by means of the Griess reaction [14]. Briefly, the equal volumes of the culture supernatant and Griess reagent (0.1% N-[1-naphtyl] ethylendiamine dihydrochloride 1% sulphanilamide and 2.5% H_3PO_4) were mixed and incubated at room temperature for 10 min and absorbance was measured. The obtained values were expressed as the nitrite concentration. The superoxide anion generation was measured by incubating neutrophils with a 0.1% nitroblue tetrazolium solution at room temperature for 10 min and reading absorbance at 545 nm. The superoxide generation was assessed using the extinction coefficient 21.1 nM [14].

Evaluation of the rabbit AMPs extract influence on the rabbit MDM

The influence of the rabbit AMPs extract on the rabbit MDM was assessed basing on morphological and functional changes. The blood for leukocytes isolation was obtained 7 days before and 24 hrs after the Ti implant insertion into the tibial defect. Mononuclear cells were isolated from the whole blood by gradient centrifugation over Histopaque-1077 and immediately cultured at a concentration of 1.0×10^6 cells/mL into 96-well flat-bottomed tissue culture plates at 37°C and 5% CO_2 for 72 hrs in Dulbecco's Modified Eagle's Medium (DMEM) with 10% bovine calf serum (BCS) to obtain MDM [14]. The cultures described as BCS did not undergo any additional stimulation. The other cultures were stimulated with 40 µg/mL of AMPs and marked as the rANE group. All these cultures were incubated for 3 days at 37°C and 5% CO_2, then the functional analysis was performed on the basis of the superoxide and NO generation and arginase activity of cultured MDM [15]. The morphology microscopic analysis was conducted using a reversed phase microscope (Olympus).

The study on the ovine model

The study was conducted on eight female sheep, BCP local breed, 4 months old, approximately 20 kg body weight, from the Bezek Experimental Farm, University of Life Sciences in Lublin. The sheep were clinically examined for possible infections and were found healthy with normal blood hematology and biochemistry parameters. The animal management and surgical protocol were approved by the local Ethics Committee number II in Lublin. The animals were anaesthetized with intramuscular xylazine (0.1 mg/kg) and butorphanol (0.1 mg/kg). For the local anesthesia, 2% Lignocaine was applied. The standard surgical approach for proximal tibia was used and a commercially available Ti plate was inserted. The postoperative care was Melovem (Meloxicam 5%, Dopharma Research B.V. 1.2 mL SC) as analgesic and Combi-ject (200 000 IU/mL Penicillin and 200 mg/mL Streptomycin) to prevent infections. The animals were monitored postoperatively for ten days to assess their breathing, heart rate, and body temperature. The postoperative wound and the skeletal system were examined to exclude motor disorders of the operated limb.

7 days before the experimental procedure the blood for hematological assays was collected from the jugular vein into tubes containing EDTA as an anticoagulant. A complete blood count was performed using an Abacus Junior Vet analyzer (Diatron, Budapest, Hungary) and hematological parameters in all animals were within the reference ranges. At the same time, blood aliquots of approximately 25 mL with 3.8% sodium citrate as anticoagulant were obtained for the AMP extract.

Evaluation of the ovine macrophages response to the AMPs extract

The blood for the MDM culture was obtained from each sheep before the Ti plate implantation into the proximal tibia and 5 months after the surgery. The AMPs extracts were prepared as previously described and stored. The mononuclear cells fraction (MNC) was isolated by gradient centrifugation over Histopaque-1077 to obtain the MDM, as in the case of the rabbit MNC. Then, the cultured MDM was stimulated with the ovine or porcine AMPs extract or left without additional stimulation as an unstimulated group. The MDM morphology and function was then assessed as described in the case of the rabbits.

Statistical analysis

Statistica 13.1 Software (Statsoft Poland) was used for statistical data analyses, with a two-tailed Student t test to compare two datasets and ANOVA for multiple comparisons, with the post hoc comparisons using Tukey's test. The values of $p < 0.05$ were considered as significant, the results were expressed as mean ± SD.

Results and Discussion

The rabbit neutrophil response to the autologous AMPs and the heterologous (porcine) AMPs extract and its isolated components (PR-39, protegrin mixture)

Having stimulated rabbit neutrophils with the autologous AMPs extract, their response diminished in comparison with the unstimulated cells (described as PBS). We observed the decreased MPO and ALP activity, as well as the diminished nitric oxide and superoxide generation in the measurements both before and after the implantation. On the contrary, the heterologous crude AMPs extract (described as EXT) had a pro-inflammatory effect on the neutrophils obtained before and after the implantation. We noted the increased elastase activity from 51 ± 0.8% before to 54.83 ± 0.98% after the implantation. On the other hand, we observed that some components of the porcine extract, namely PR-39 and protegrins, had a different influence on the neutrophil activity. PR-39 in most cases acted as a factor decreasing the neutrophil secretory activity both before and after the implantation, as compared to the unstimulated groups. In all the measurements, the activity of neutrophils treated with PR-39 was significantly lower ($p < 0.05$) than after the crude AMP extract stimulation. Similar results were noted after treating the neutrophils with PG (FIG. 1).

These findings confirmed the previous studies on the anti-inflammatory influence of PR-39 [11] and simultaneously indicated the AMPs potential to regulate the neutrophil inflammatory response. We also proved that the autologous AMP extract has an anti-inflammatory effect and could be a mean to decrease the excessive neutrophil response during surgical procedures.

The influence of the rabbit AMPs extract on morphology and function of the rabbit MDM obtained before and after the biomaterial implantation

We discovered that the rabbit macrophages treated with the autologous AMPs extract showed partially anti-inflammatory features with the decreased nitric oxide and superoxide generation and the unaltered arginase activity. The concentration of nitrite and superoxide was lower after stimulation with rANE, in comparison with the BCS groups in the two time points. After the implant insertion, the NO generation decreased from 3.63 ± 0.2 µM in the unstimulated groups (marked as BCS) to 3.24 ± 0.13 µM ($p < 0.05$) in the group treated with rANE. Similar results were obtained before the biomaterial implantation. The superoxide generation decreased from 5.6 ± 0.4 nM before to 5.4 ± 0.34 nM after the rANE addition in the group after the surgery (FIG. 2).

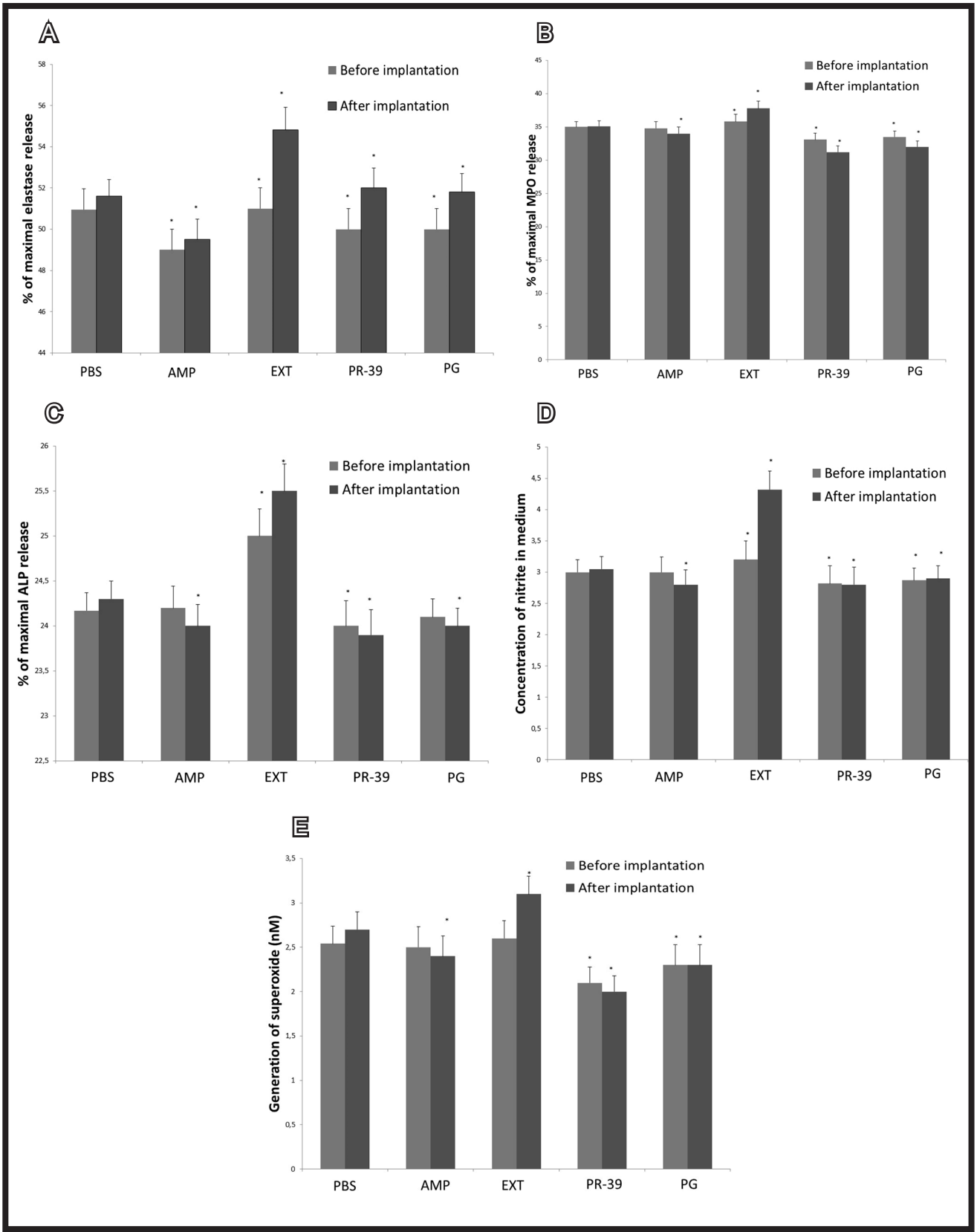

FIG. 1. The response of rabbit neutrophils to the autologous rabbit AMPs extract (AMP), the heterologous porcine extract (EXT) and isolated products of the porcine extract (PR-39, protegrin-PG) before and after the implantation. (A) The elastase release, (B) myeloperoxidase release, (C) alkaline phosphatase release, (D) nitric oxide generation, (E) generation of superoxide. All of the data expressed as the mean ± SD. *p < 0.05.

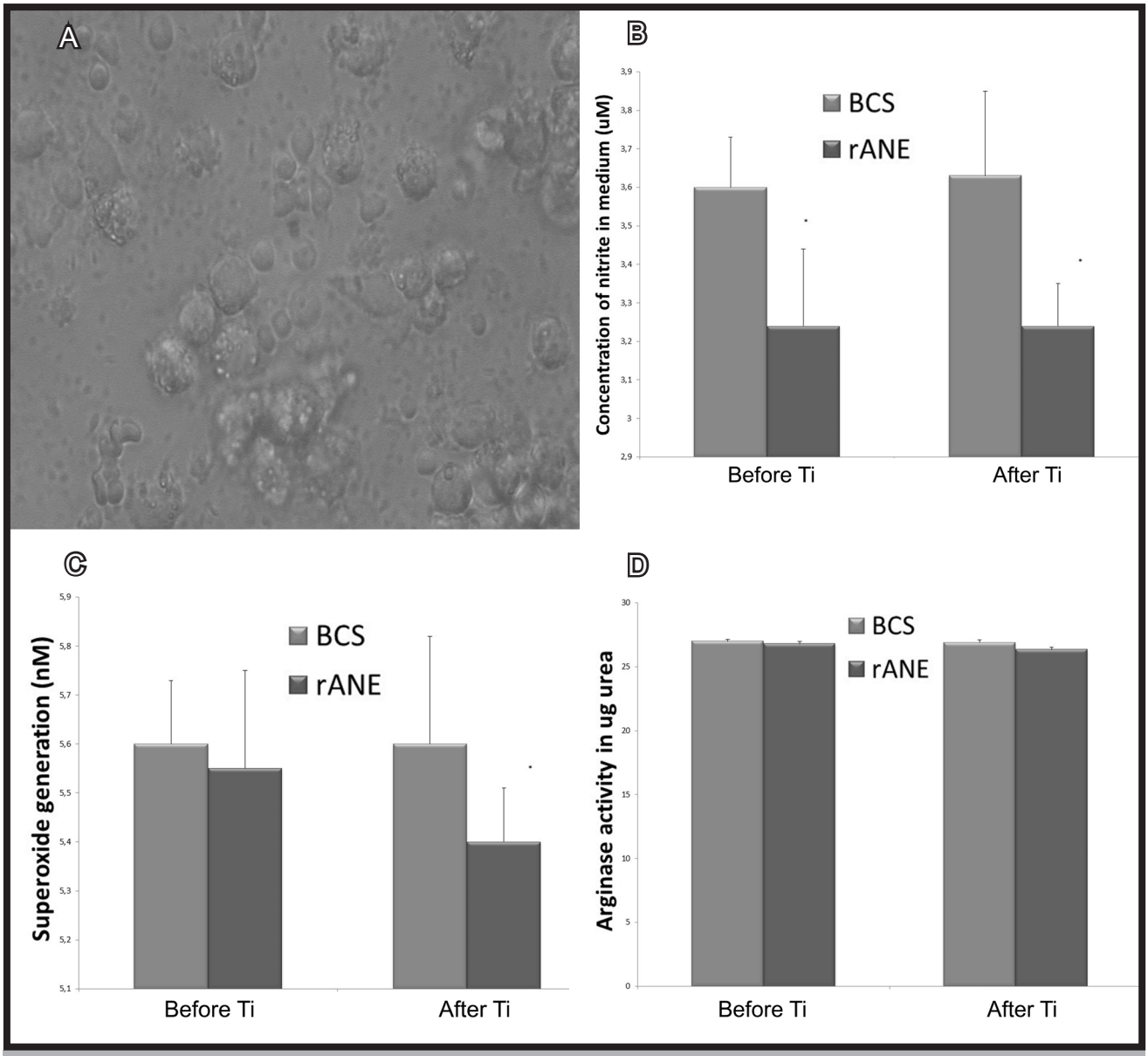

FIG. 2. The influence of the rabbit AMPs extract (rANE) on morphological and functional changes of MDM before and after the Ti implant insertion. (A) The MDM morphology after the rANE treatment , (B) nitric oxide generation, (C) superoxide generation, (D) arginase activity. All of the data expressed as the mean ± SD. *p < 0.05 compared with the cultures before the implantation.

Apart from their antimicrobial activity, AMPs are regulatory molecules that limit inflammation. Therefore, they promote immune homeostasis. The AMPs anti-inflammatory function was previously confirmed by several studies on animal models. It was demonstrated that the deficiency of these peptides resulted in the overexpressed inflammatory responses; i.e. cathelicidin- deficient mice exhibited a more severe inflammatory phenotype, as compared with wild-type mice. Similarly, the reduced expression of β-defensin in human enterocytes has been noted in the Crohn's disease. Moreover, the critical role of defensins in maintaining the integrity of intestinal mucosa and immune homeostasis is well established. The exogenous application of AMPs, such as human cathelicidin LL-37, CATH-2, BMAP-28 or HBD2, and synthetic peptides (e.g. IDR-1 and IDR-1002) has proved to control inflammation in various animal models of infection and sepsis. Similarly, the LL-37-derived peptide controlled the disease process in a mouse model of inflammatory arthritis and the IDR-1002 effectively suppressed the airway inflammation *in vivo* [12].

The influence of the ovine and porcine AMPs extract on morphology and function of the rabbit MDM obtained before and after the biomaterial implantation

In the experiment on the MDM stimulation with the autologous (ovine) and heterologous (porcine) AMPs neutrophil extracts, we found out that the MDM response was different and related to the extract origin. The autologous extract causes the NO generation decrease, particularly after the implantation, from 3.03 ± 0.23 μM (unstimulated) to 2.65 ± 0.22 μM (stimulated with oANE). Moreover, the superoxide generation was also lower in comparison to the cultures described as BCS, whereas the arginase activity remained unchanged. It was confirmed that the local arginase activity is required for wound healing [16]. Thus, this effect could be considered as beneficial in the repair process. Conversely, the heterologous extract stimulation showed a significant ($p < 0.05$) increase in the free radical generation. We detected that the nitrite concentration in the cultures after implantation reached 4.48 ± 0.22 μM in comparison with 3.03 ± 0.23 μM in the unstimulated group. Additionally, the arginase activity was higher before and after the treatment (FIG. 3).

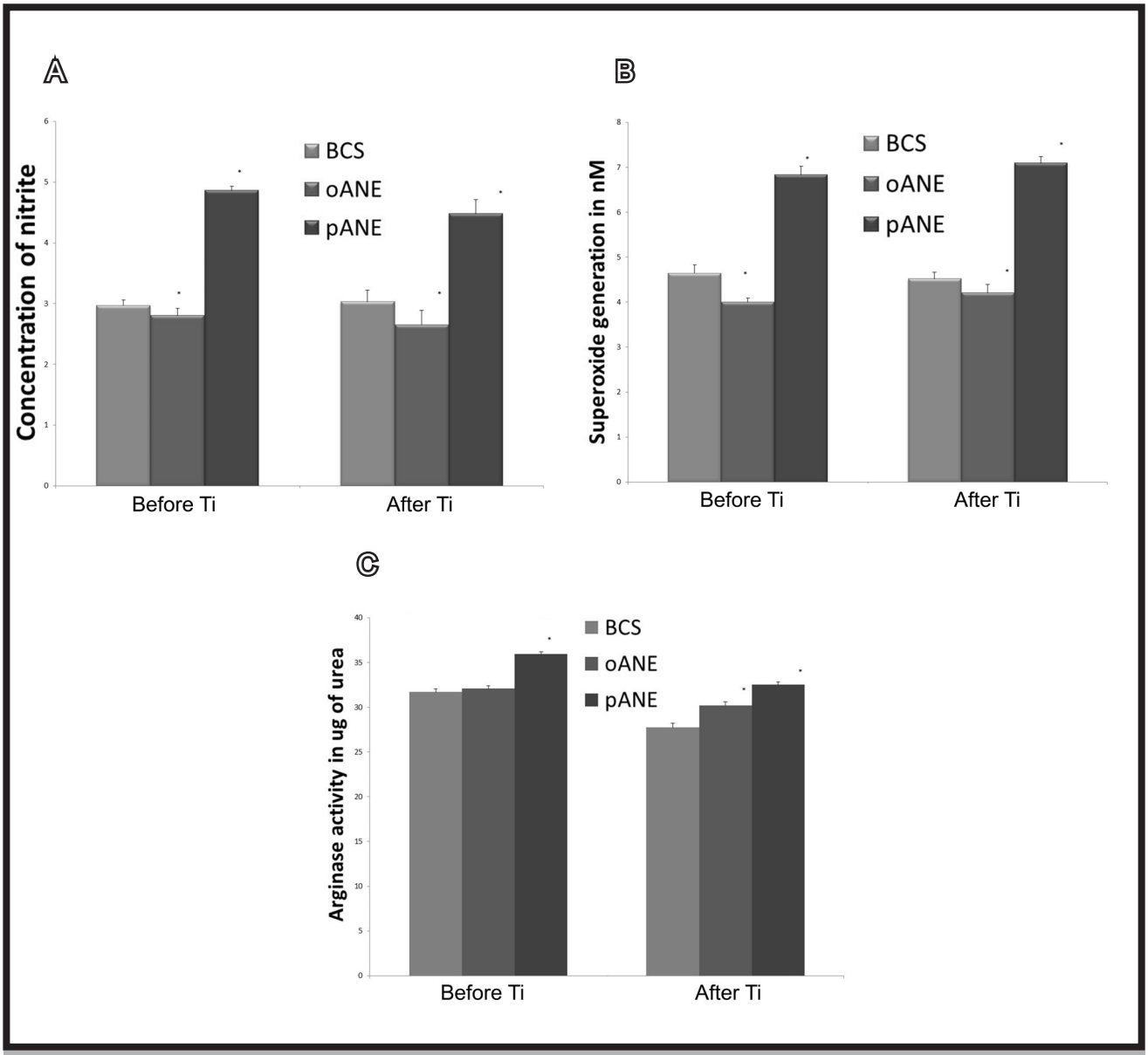

FIG. 3. The influence of the autologous (oANE) and heterologous (pANE) extract on the MDM function before and after the Ti implant insertion in the ovine model (A) nitric oxide generation, (B) superoxide generation, (C) arginase activity. The data expressed as the mean ± SD. *p < 0.05.

With regard to these results, we studied the influence of autologous and heterologous AMPs extracts on some components of the white blood cells system (WBC). Our study revealed that the response depended on the extract origin, the animal species, the cell type and the animal status (before or after the implantation). The study of neutrophils revealed that the autologous AMPs decreased their activity in respect of the enzymes release and the free radicals generation. Contrary to this, the heterologous AMPs extract increased the secretory activity of these cells.

After the implant insertion, MDMs are among the first cells at the implant site and they are considered as key regulators of both the initiation and the resolution of inflammation [17]. Therefore, in our experiment, we also evaluated the MDM response to the AMPs extracts. The MDM after stimulation with the autologous extract showed the decreased ROS and NO generation with the intact arginase activity in comparison with the cultures stimulated only with BCS. After the heterologus AMPs extract stimulation, in turn, these cells generated higher amounts of superoxide and NO and the higher arginase activity. These results indicated a mixed subpopulation of both pro- and anti-inflammatory features in which the unchanged arginase activity ensures an undisturbed healing process. A similar intermediate state was described in response to the synthetic IDR-1018 peptide when macrophages developed a unique capability to maintain particular proinflammatory activities while producing anti-inflammatory and regulatory mediators [9,18].

It should be emphasized that differences between the cellular response before and after the implantation resulted both from the biomaterial itself and the overall host reaction to the implantation procedure. The implant surface characteristics also influenced the host immune response. Different modifications of the implant surface, e.g. the pectin coatings, were described previously [19]. The authors evaluated the *in vitro* interactions of the implant and human bone marrow stromal cells regarding f the cell adhesion and proliferation. In our experiment, we used commercially available Ti discs with a silver coating and the response of circulating neutrophils and MDM was studied before and after the implantation.

Conclusions

The inflammatory response may be modulated using blood-derived products which not only display antimicrobial activity, but also regulate the inflammation processes, dependently on the organism needs. Therefore, different AMPs extracts can be used to enhance or suppress the inflammatory response. This may be applicable in surgical procedures where antimicrobial peptides will act both as an antimicrobial agent and a factor inhibiting the excessive reaction to the biomaterial implantation. The obtained results could be used to develop novel autologous blood-derived products for bone healing.

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