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THE INFLUENCE OF PORCINE CATHELICIDINS ON NEUTROPHILS ISOLATED FROM RABBITS IN THE COURSE OF BONE GRAFT IMPLANTATION

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Abstract: Antimicrobial peptides are important elements of host defence because of their direct antimicrobial activity and modulatory role in innate immune response. The purpose of the study was to determine whether porcine peptides PR-39, protegrins (PGs) and low molecular weight extract (LMWE) are able to influence the neutrophil response during bone graft implantation in rabbits. The study was conducted on 10 White New Zealand rabbits and neutrophil activity was assayed on the basis of elastase, myeloperoxidase, and alkaline phosphatase release as well as free radical generation. Our study showed that PR-39 and PGs inhibited enzyme release from neutrophils except for elastase, which is essential in the first phase of injury. Superoxide and nitric oxide generation under the influence of PR-39 and PGs were also decreased. Moreover, we found that unlike separated peptides PR-39 and PGs, LMWE acts proinflammatorily, intensifying the neutrophil secretory response and free radical generation. These results should be taken into account in treatment with natural antimicrobial peptides. The increased neutrophil responses in the first phase of inflammation during surgery may be useful in prevention of infection, but LMWE should not be used in conditions in which excessive neutrophil response is injurious.

Key Words: cathelicidin, neutrophil, bone, rabbit.

INTRODUCTION

The early phase of inflammation in the course of implantation of a biomaterial is of great interest to researchers, since it can provide useful information concerning the healing process, as well as the biocompatibility of the material used for the bone grafting. The role of the inflammatory phase, which lasts a week in rabbits, is to remove the debris resulting from trauma and provide appropriate signals for tissue repair and regeneration. During an acute inflammation, the emigration of leukocytes, mainly neutrophils, from the vascularisation to the site of injury is noted. Their major role is to release lysosomal enzymes and phagocytose microorganisms (Ramstedt *et al.,* 2010). Similarly, in the first phase of bone graft incorporation during the first 18 h, the arrival of chemotactic factors and an accumulation of neutrophils as inflammatory cells are observed. This process is connected with elastase release and generation of inflammatory factors such as collagenous and fibronectin peptides (Kaveh *et al.*, 2010).

The use of biomaterial together with tissue injury in the course of implantation induces an immune response which may be detrimental. For this reason, one key to successful implantation of a biomaterial is in-depth understanding of the host inflammatory and healing response in order to support the healing process (Franz *et al.,* 2011).

Despite some reports about the role of neutrophils in acute post-injury inflammation, the function of neutrophils stimulated by antimicrobial peptides in the course of bone graft implantation in rabbits has not been investigated in detail to date.

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Cationic antimicrobial peptides are present in domesticated animals and are divided into 2 major groups, i.e. cathelicidins and defensins. Cathelicidins are characterised by their highly conserved pre-pro-region, and their inactive proforms are stored in the neutrophil granules. Apart from their antimicrobial effects, these peptides are also known to stimulate a broad range of biological effects relevant to inflammation and immunity (Zanetti, 2004; Brown and Hancock, 2006). Among all species, pigs have the most diverse collection of cathelicidins, which comprises proline-phenylalanine-rich prophenin-1 (PF-1) and prophenin-2 (PF-2), proline-arginine-rich 39-amino-acid peptide (PR-39), and cysteine-rich protegrin-1 to 5 (PG 1 to PG 5) (Sang and Blecha, 2009).

PR-39 has unique bactericidal properties because it kills bacteria by a non-pore-forming mechanism that inhibits DNA and protein synthesis in gram-positive and gram-negative bacteria, potentiating neutrophil chemotaxis and phagocytosis, as well as the induction of angiogenesis (Shi *et al.,* 1996; Hoffmeyer *et al.,* 2000; Zanetti, 2005). Other effects of PR-39 include increased expression of syndecan, a proteoglycan involved in wound healing. This peptide also prevents neutrophil recruitment following ischemia and reperfusion (I/R) by downregulation of endothelial cell adhesion molecules. A study conducted by Hoffmeyer *et al.* (2000) revealed that administration of PR-39 significantly reduced myocardial infarction following ischemia and reperfusion by inhibition of neutrophil interaction with the endothelium, resulting in reduced PMN accumulation.

Protegrins (PGs) exhibit a potent, broad-spectrum, microbicidal activity against various gram-positive and gram-negative bacteria, mycobacteria, fungi, and enveloped viruses (Zang *et al.,* 2000). These peptides are principal antibacterial factors in porcine wound fluids (Ceccarelli *et al.,* 2001). The small size together with effectiveness and low cytotoxicity to host cells makes PGs promising topical therapeutics (Zang *et al.,* 2000).

Apart from synthetic peptides, naturally obtained cathelicidins from neutrophils are considered for therapeutic usage (Anderson and Yu, 2003). Previously, the ovine neutrophil extract was assessed as the source of cathelicidins (Anderson and Yu, 2008). However, the crude extract obtained from porcine neutrophils has not been evaluated to date.

The aim of this study was to draw a comparison between a mixture of low molecular weight peptides (LMWE) and isolated main peptide components of this extract, namely PR-39 and PGs, to assess their influence on rabbit neutrophils in the course of implantation of biomaterial into the tibial defect.

MATERIAL AND METHODS

Isolation of porcine antimicrobial peptides from neutrophils

Antimicrobial peptides were isolated according to the method described previously (Wessely-Szponder *et al.,* 2010). The solution containing the peptides was separated from the granules (25000×g, 20 min, 4°C), lyophilised and stored at –20°C. To test the purity, the obtained fractions F1 and F2 were lyophilised, resuspended in a solvent (0.1% trifluoroacetic acid [TFA] in water v/v) and then loaded onto a C18 RP-HPLC column (250×4 mm, particle size 5 µm, LiChrospiner 100, Merck, Germany) with a flow rate of 0.7 mL/min. Peptides were eluted with gradient 0-60% acetonitrile in 0.1% TFA and monitored for absorbance at 225 nm.

The components present in the crude extraction were separated according to their sizes using gel filtration chromatography. The extract was passed through a Sephadex G-50 (Fine, Sigma-Aldrich) column, using a running buffer of 5% acetic acid at 0.5 mL/min. Absorbance of the eluate (every 5 mL) was monitored at 280 nm. Concentrations of PR-39 (in fraction 1-F1) and PGs (in fraction 2-F2) were assessed using the extinction coefficient (Kokryakov *et al.*, 1993). The fractions with molecular masses between 1 and 5 kDa were pooled from another portion of the crude extract to obtain LMWE.

MALDI-TOF analysis

Mass spectrometric studies were performed on the time-of-flight mass spectrometer with the MALDI ion source. The apparatus was built at the Molecular Physics Division, Institute of Physics, Maria Curie-Sklodowska University, Lublin, Poland.

The nitrogen laser (LN 300C, Laser Photonics) had a wavelength of 337 nm, an output length of 5 ns, maximum pulse energy specified as 250 µJ and a rectangular output shape of approximately 4×9 mm. The angle of

incidence of the laser beam focused on the plane of the sample was 30°. The greatest intensities for the samples investigated here were obtained using a slightly defocused laser beam. In this case, the calculated dimensions of the defocused laser beam spot were approximately 400×900 μm and the power density was ~14 $MW \times cm^{-2}$. The ions were accelerated by a voltage of 17 kV and detected by a 2 channel-plate (Hamamatsu) ion detector operated at a voltage of 2.3 kV. The data were acquired using a 500 MHz (1 G sample/s) digital oscilloscope (Hewlett Packard HP 54615B). In the presented investigations, the linear mode of the mass spectrometer was used. All the mass spectra were collected in the positive polarity of the ion source. To enhance the signal-to-noise ratio, each of them was averaged from 256 results obtained for consecutive laser shots (Karas *et al.,* 1987; Gruszecka *et al.,* 2008; Urban *et al.,* 2011).

Sample preparation for the MALDI measurement was performed using α-cyano-4-hydroxycinnamic acid (CCA, 189.2 Da, Sigma Aldrich) as a matrix. The matrix solution was prepared by dissolving 0.01 g of CCA in 1 mL of acetonitrile and 0.1% TFA (1:1, v/v) mixture. The peptide lyophilisate portion was dissolved in 1 mL of a mixture: TFA (0.1%), acetonitrile (60%) and distilled water (49.9%). The peptide and the matrix solutions (a few microlitres) were applied directly onto the surface of the stainless steel sample holder and the solvents were evaporated in air at room temperature.

Animals and study design

The aim of the surgery was the implantation of biomaterial into the tibial defect. The study was carried out on 10 healthy New Zealand White (NZW) rabbits, males with mean body weight of 4000±100 g (standard deviation). Physical examination of each rabbit was performed before the surgical procedure. The study protocol was approved by the Local Ethics Committee. After induction of anaesthesia with xylazine (Sedazin; Biowet, Pulawy, Poland) 5 mg/kg and ketamine (VetaKetam; Vetagro, Lublin, Poland) 30 mg/kg intramuscularly, the marginal ear vein was catheterised with a 22-gauge sterile catheter for administration of anaesthetic mixture. The animals received ketamine infusion diluted in 5% of glucose (0.35 mg/kg per min) intravenously at a continuous rate.

Blood was taken into tubes with 3.8% sodium citrate as an anticoagulant at a proportion of 1:10. Blood samples were obtained at 3 time points: 48 h before the surgical procedure (T=–48 h), 30 min after the surgical procedure (T=30 min), and 24 h after the surgery (T=24 h). Haematological tests were done using a haematological analyser EXIGO VET.

Isolation and stimulation of rabbit neutrophils

Rabbit neutrophils were isolated from peripheral blood as described in Wessely-Szponder *et al.* (2012). After the red blood cells were lysed by the addition of 0.83% ammonium chloride at the ratio of 3:1 for 10 min, the remaining pellet was washed twice with phosphate-buffered saline (PBS-Biomed, Lublin, Poland) by double centrifugation (700×g, 10 min, 4°C). The final cell pellet was resuspended in 1 mL of PBS. After isolation, the viability of PMNs cells was assessed by trypan blue exclusion (>95% of live cells). After cell counting and differentiation (>85% of neutrophils on May-Grunwald-Giemsa-stained preparations) the cell suspensions were adjusted to a final concentration of 2×10^6 cells/mL.

The cell suspensions were supplemented as follows: the control group with PBS, the PR-39 group with PR-39 to a final concentration of 20 μg/mL, the PGs group with PGs (10 μg/mL), and the LMWE with LMWE (40 μg/mL). Then, the samples were incubated for 30 min at 37°C and 5% CO_2.

Rabbit neutrophils degranulation assay

Neutrophil degranulation was assessed by elastase, myeloperoxidase (MPO), and alkaline phosphatase (ALP) release. 100% of enzyme content was estimated by treating the cells with 0.5% Triton X-100 (Sigma). Elastase activity assay was performed by incubating samples with azocasein (Sigma) as a substrate at 25°C for 10 min; absorbance at 492 nm was assessed using ALAB-PLATE READER ELISA thereafter. MPO release was determined by measuring the absorbance at 492 nm after 10 min incubation of the sample with an equal volume of o-phenylendiamine (OPD-Sigma). ALP activity was estimated after 10 min incubation at 25°C with an equal volume of 4-nitrophenyl phosphate disodium salt hexahydrate (pNPP-Sigma); absorbance was then measured at 405 nm (Wessely-Szponder, 2008).

Assessment of superoxide generation by neutrophils

Superoxide anion production was measured using the method described previously (Wessely-Szponder and Szponder, 2010). In brief, neutrophils were incubated with 0.1% nitroblue tetrazolium (NBT-Sigma) solution at room temperature for 10 min and then absorbance was read at 545 nm. All tests were done in duplicate.

Nitric oxide production by neutrophils

Nitric oxide (NO) level was determined by Griess reaction, as described previously (Wessely-Szponder and Szponder, 2010). All tests were done in duplicate. The obtained values were expressed as a concentration of nitrite, as the stable product of NO, which accumulates in the medium.

Statistical analysis

Statistical analysis was performed using the computer program STATISTICA 5.0 (StatSoft, Poland). The examined values were expressed as the mean±standard deviation and compared using an analysis of variance and Student's t-test and the differences were considered as significant at $P<0.05$.

RESULTS

Isolation of PR-39, PGs, and an extract of low molecular weight peptides

PR-39 and PGs were isolated from porcine neutrophil extract using a gel chromatography method (Figure 1). The peptides obtained were lyophilised and frozen at –20°C in portions of a known weight. These portions were diluted in PBS to the final concentrations of 20 and 10 µg/mL of PR-39 and PGs, respectively, just before use.

An extract of low molecular weight peptides was obtained from the crude extract after pooling fractions of molecular masses at between 1 and 5 kDa (Figure 2).

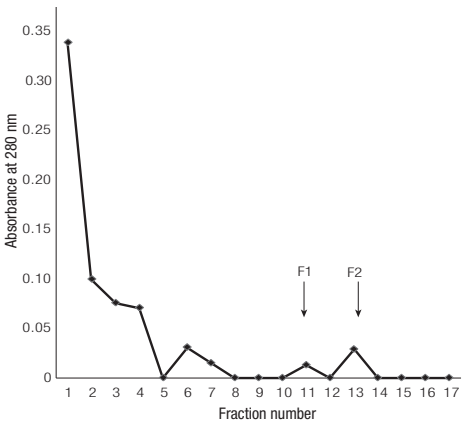

Figure 1: Gel filtration chromatograph for crude extract applied on a Sephadex G-50 column equilibrated with 5% acetic acid. Elution was performed with the same concentration of acetic acid, collecting fractions of 5.0 mL. Absorbance was monitored at 280 nm. Fractions containing PR-39 and protegrins were indicated with arrows as F1 and F2, respectively.

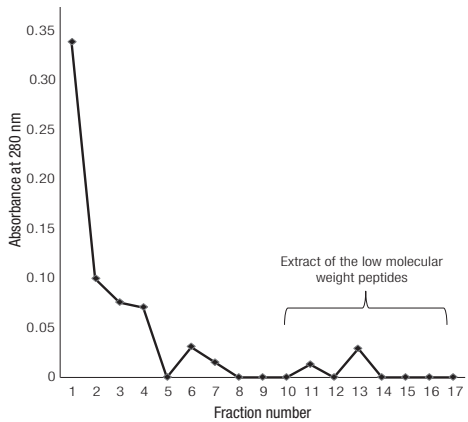

Figure 2: Gel filtration chromatogram for crude extract applied on a Sephadex G-50 column in 5% acetic acid and monitored at 280 nm. Low molecular weight extract fractions were pooled and marked in the figure.

MALDI TOF analysis

MALDI TOF analysis of fraction 1 revealed the presence of a peptide of a molecular mass of 4716 kDa corresponding to PR-39 (Figure 3). In fraction 2, we detected 3 small peaks of molecular masses of 1955.6; 2055.6 and 2154.5 Da, which correspond to protegrins PG 2, PG 3, and PG 1, respectively (Figure 4.) The analysis of LMWE demonstrated the peaks of molecular masses corresponding to both PR-39 and PGs, as well as other peaks that could reflect the peptides formed from proforms of cathelicidins during the extraction process (Figure 5).

Influence of studied peptides on elastase release by rabbit neutrophils

Neutrophils without stimulation released elastase at the level of 50.96±0.71% of maximal release. After stimulation with PR-39, the enzyme release was significantly inhibited at the first measurement ($P<0.05$). At t=30 min elastase activity slightly increased under the influence of PR-39 and PGs, but without statistical significance. At this time point we observed a significant increase of elastase release after the stimulation with LMWE. In the 3rd measurement (after 24 h from the surgery), all the values obtained were lesser in comparison with the 2nd measurement. In this measurement the elastase release under the influence of LMWE was significantly lower than 30 min after the surgery ($P<0.05$; Figure 6).

MPO release by rabbit neutrophils under the influence of studied peptides

In the 1st measurement before the surgical procedure, we noted a significant inhibition of MPO release in the group of neutrophils cultured with PR-39 and PGs in comparison with unstimulated cells ($P<0.05$). We observed the increase of MPO release in neutrophils stimulated with LMWE, but without statistical significance. The neutrophil response intensified 30 min after the surgery, whereas in the 3rd measurement the inhibition was lesser. We observed increased MPO release by neutrophils stimulated by LMWE, which was significant ($P<0.05$) in the 2nd measurement (Figure 7).

Influence of studied peptides on ALP activity in rabbit neutrophils

The neutrophils stimulated with LMWE revealed higher ALP activity than these stimulated with separated peptides; the greatest response was shown at the 2nd time point, 30 min after surgery (Figure 8).

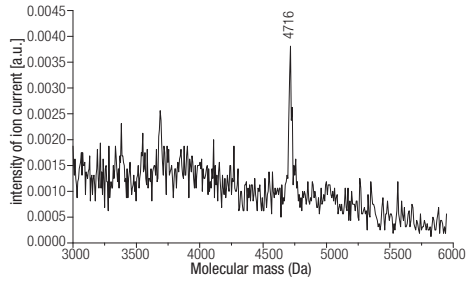

Figure 3: The MALDI TOF mass spectrum of PR-39 (4716 Da) current in fraction 1. The mass spectrum is an average of 256 results obtained for consecutive laser shots. α-cyano-4-hydroxycinnamic acid (CCA) was used as a matrix.

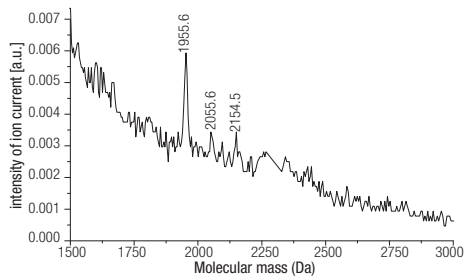

Figure 4: The MALDI TOF mass spectrum of protegrins: PG 1 (2154.5 Da), PG 2 (1955.6 Da), and PG 3 (2055.6 Da) obtained from the fraction 2. The accelerating voltage was 17 kV. The mass spectrum was averaged from 256 measurements.

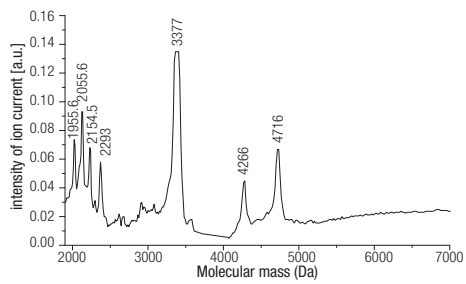

Figure 5: The mass spectrum of low molecular weight extract with the mass peaks corresponding to PR- 39 (4716 Da) and protegrins: PG1 (2154.5 Da), PG2 (1955.6 Da), and PG 3 (2055.6 Da). Moreover, signal peptide for protegrins (3377 Da) and cathelicidin fragments were seen.

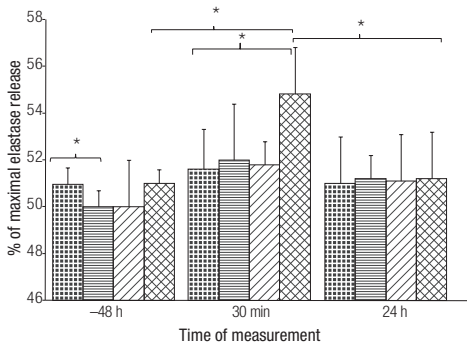

Figure 6: Elastase release by rabbit neutrophils isolated 48 h before surgery, 30 min after surgery, and 24 h after surgery and incubated for 30 min at 37°C and 5% CO_2 with PBS (without stimulation) ▦, PR-39 (20 µg/mL) ▤, protegrins (10 µg/mL) ▨, or low molecular weight extract (40 µg/mL) ▩. * $P<0.05$.

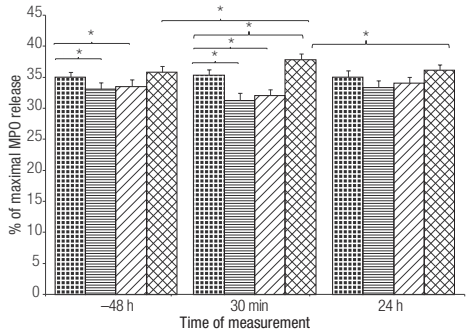

Figure 7: Myeloperoxidase release by rabbit neutrophils isolated 48 h before surgery, 30 min after surgery, and 24 h after surgery and incubated for 30 min at 37°C and 5% CO_2 with PBS (without stimulation) ▦, PR-39 (20 µg/mL) ▤, protegrins (10 µg/mL) ▨, or low molecular weight extract (40 µg/mL) ▩. * $P<0.05$.

Influence of studied peptides on superoxide anion generation by rabbit neutrophils

We observed that superoxide anion generation by neutrophils depended on the peptides used for stimulation. Under the influence of PR-39, we noted a significant inhibition of superoxide anion generation in the first 2 measurements, especially in the 2nd measurement, whereas the influence of PGs was less pronounced. In groups stimulated with LMWE, we observed the increase of superoxide anion production, which was significant in the 2nd measurement (Figure 9).

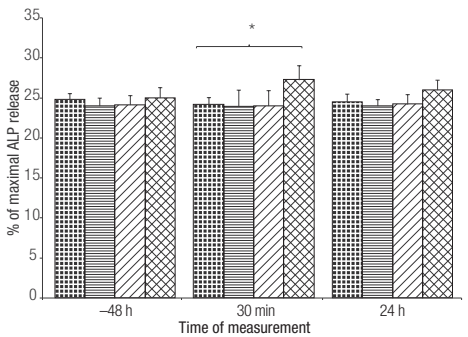

Figure 8: Alkaline phosphatase release by rabbit neutrophils isolated 48 h before surgery, 30 min after surgery, and 24 h after surgery and incubated for 30 min at 37°C and 5% CO_2 with PBS (without stimulation) ▦, PR-39 (20 µg/mL) ▤, protegrins (10 µg/mL) ▨, or low molecular weight extract (40 µg/mL) ▩. * $P<0.05$.

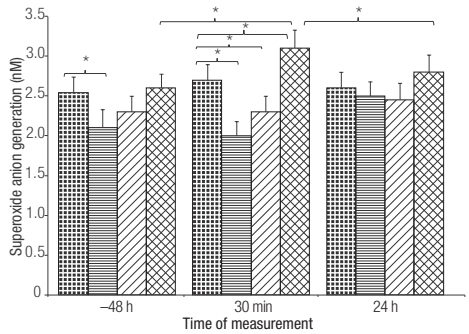

Figure 9: Superoxide anion generation by rabbit neutrophils isolated 48 h before surgery, 30 min after surgery, and 24 h after surgery and incubated for 30 min at 37°C and 5% CO_2 with PBS (without stimulation) ▦, PR-39 (20 µg/mL) ▤, protegrins (10 µg/mL) ▨, or low molecular weight extract (40 µg/mL) ▩. * $P<0.05$.

Effect of antimicrobial peptides on NO generation by rabbit neutrophils

We obtained a decrease of the generation of NO in groups supplemented with PR-39 and PGs in all measurements but without significance. However, stimulation by LMWE resulted in an increase of the generation of NO at all time points. The maximal neutrophil response was noted in the second measurement and was significantly different from both other measurements ($P<0.05$; Figure 10).

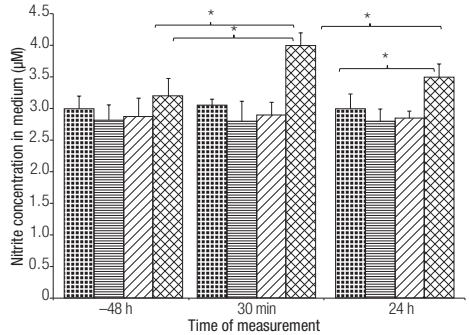

Figure 10: Nitric oxide generation by rabbit neutrophils isolated 48 h before surgery, 30 min after surgery, and 24 h after surgery and incubated for 30 min at 37°C and 5% CO_2 with PBS (without stimulation) ▦, PR-39 (20 μg/mL) ▤, protegrins (10 μg/mL) ▨, or low molecular weight extract (40 μg/mL) ▩. * $P<0.05$.

DISCUSSION

As estimated previously, besides a direct killing activity, cathelicidins are claimed to have an immunomodulating effect. Bovine cathelicidins altered the host defence responses by their impact on human neutrophils (Bowdish *et al.,* 2005). PR-39 in turn influenced the migration of neutrophils in a dose-dependent manner, by intensifying migration in response to concentrations from 0.01 to 2 μM, gradually inhibiting it in concentrations from 4 to 10 μM (Hang *et al.,* 1997). This peptide is also involved in the prevention of a neutrophil associated tissue injury. PR-39 preserved a myocardial function through the inhibition of the tumour necrosis factor α-induced degradation of the NFκB inhibitor IκBα, which in turn inhibited NFκB-dependent expression of leukocyte adhesion molecules, and thus decreased the accumulation of neutrophils and reduced the size of myocardial infarction. In a murine model of myocardial I/R injury, the PR-39 treatment inhibited leukocyte recruitment into the inflamed mesentery (Li, 2009). In another study, PR-39 significantly inhibited PMN vascular adherence as well as infiltration and blocked endothelial NADPH oxidase (Ikeda *et al.,* 2001). In the study of Bao *et al.* (2001), myocardial MPO activity was inhibited by PR-39 in the amount of 250 μM. However, the myocardial MPO activity in this study was assessed on the basis of neutrophil infiltration into the tissues. The study of the influence of PR-39 on elastase and ALP release has not been conducted to date. Our report showed that PR-39 inhibited elastase release, but only before the surgery. Moreover, it decreased MPO and ALP release by neutrophils in all measurements. The obtained results indicated that PR-39 inhibited secretory activity of neutrophils except for elastase release, which is essential in the first phase of injury (Kaveh *et al.*, 2010).

In the study conducted by She *et al.* (1996), it was estimated that PR-39 inhibited both cell-free and whole-cell superoxide anion production by neutrophils. At concentrations above 5 μM, the peptide completely inhibited generation of superoxide anion. This report revealed that PR-39 binds to $p47^{phox}$, a cytosolic component of NADPH oxidase, and that specific binding decreases superoxide generation (She *et al.,* 1996). Korthuis *et al.* (1999) showed that PR-39 pre-treatment inhibited oxidant production in rat neutrophils, preventing I/R injury in organs. In the study of Ikeda *et al.* (2001), PR-39 significantly reduced superoxide anion release from suspensions of rat neutrophils at concentrations of 4 and 10 μg by 22 and 46%, respectively.

We demonstrated that PR-39 in the amount of 20 μg/mL prevents excessive neutrophil activity by the restriction of enzyme release and free radical generation, but without inhibition of elastase release, as the enzyme essential for the first phase of bone repair and for the proteolytic activation of cathelicidins (Cole *et al.,* 2001).

Pig-derived PG1, when used as a therapeutic topically administered on wounds experimentally infected with *Pseudomonas aeruginosa* in pigs, significantly prevented colonisation and reduced the *in vivo* bacterial concentration (Ceccarelli *et al.,* 2001). In topical administration of PGs in a hamster model of oral *mucositis,* the accelerated recovery was noted (Levy, 2000). Moreover, PG 1 was the first of the mammalian cathelicidin peptides that had undergone clinical trials. Because there is a lack of reports on immunomodulatory effects of natural PGs, the synthetic analogue was taken into account. IB-367 is a structural analogue of PG 1 and acts anti-inflammatorily to ameliorate

oral *mucositis* in hamsters (Loury *et al.,* 1999). No studies of the influence of PGs on neutrophil degranulation have been conducted to date. Our work demonstrated that PGs inhibited elastase release before the surgery, but not after, whereas the MPO release was decreased in all measurements. Superoxide anion generation was also decreased, as well as NO.

Previously, preparations based on naturally obtained extracts of peptides were introduced to therapy. The surfactant components containing, among others, prophenin, are used for treatment of respiratory distress syndrome in premature infants. Curosurf as a modified natural surfactant isolated from minced porcine lungs consisted of phospholipids, prophenin, PF-18 and surfactant proteins SP-B and SP-C (Wang *et al.,* 2004). This cathelicidin-containing mixture was also used in treatment of experimentally induced bacterial pneumonia in neonate rabbits (Herting *et al.,* 1994).

Moreover, ovine antimicrobial peptides were evaluated as possible therapeutics, particularly for the treatment of respiratory diseases and wound infections and as bio-preservatives (Anderson *et al.,* 2004). Anderson and Yu (2003) showed that a crude extract obtained from ovine neutrophils had properties expanding beyond the properties displayed by proline/arginine rich peptides. This may be due to the presence of other ovine antimicrobial peptides which can act synergistically in the studied extract. Unprocessed cathelicidins (propeptides) may increase the antimicrobial potency of other peptides and exert host defence activities. Some of bovine cathelicidin propeptides inhibit *in vitro* activity of cathepsin L and are chemoattractant for monocytes at 10^{9}M, but the molecular mechanism of this activity has not been investigated in depth (Zanetti, 2005). These findings indicate that in certain cases it would be preferable to use the peptides in the crude extract form, instead of purifying them to be used individually.

In our study only isolated, non synthesised peptides were used for comparison between the effect of LMWE and separated peptides on rabbit neutrophils. As pigs have the most diverse range of cathelicidins, we decided to restrict our findings on crude extract only to peptides with low molecular mass between 1 and 5 kDA. In our work, contrary to the effect of PR-39 and PGs, we observed the greater elastase, MPO, and ALP release at all time points under the influence of LMWE. Moreover, we estimated an increased generation of free radicals in response to LMWE stimulation of neutrophils. It revealed that unlike the separated peptides PR-39 and PGs, the LMWE extract acts proinflammatorily, intensifying the neutrophil secretory response as well as free radical generation.

These findings should be taken into account in planning treatment including natural antimicrobial peptides. These responses were accelerated in the first phase of inflammatory response to injury during the implantation of biomaterial into the bone tissue, and may be useful in supporting antimicrobial activity of neutrophils, although in conditions in which excessive neutrophil response is harmful, the use of LMWE should be restricted.

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