

THE INFLUENCE OF PORCINE PROPHENIN ON NEUTROPHILS ISOLATED FROM RABBIT BLOOD DURING IMPLANTATION OF CALCIUM SULPHATE GRAFT MATERIAL INTO BONE TISSUE

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ABSTRACT: Immune dysfunction induced by surgical trauma may comprise either an inappropriately exaggerated inflammatory response or a profound suppression of cell-mediated immunity. Neutrophils are the leading cells in the first response to trauma. It is known that they mediate initial resistance to bacterial infection. Activated neutrophils can degranulate and release some enzymes such as elastase and myeloperoxidase (MPO). The function of elastase is, among others, to kill bacterial, whereas MPO is a specific enzyme of primary granules of neutrophils and a marker of *in vivo* neutrophil activation. Previous reports estimated that some cathelicidins could act to increase or diminish an innate immune response in which neutrophils participate. The aim of this study was to evaluate prophenins (PF) isolated from porcine leukocytes in respect to neutrophil activity and survival during implantation of calcium sulphate bone grafts substitution in rabbits. Obtained results pointed out that neutrophils responded to PF depending upon concentration. Thirty min from implantation of calcium sulphate graft, we observed the greatest release of elastase (57.01±0.49% of maximal release) in cultures stimulated with 10 µg/mL of PF, at 0 µg/mL was 51.15±0.23%, while after 24 h of incubation the greatest response was at a concentration of 20 µg/mL. MPO release after 30 min from surgery decreased significantly at 10 µg/mL. In higher concentrations, the inhibition was less pronounced. Moreover, we estimated that PF causes cytotoxicity in the highest concentration as well as the apoptosis of neutrophils.

Key Words: Neutrophil, antimicrobial peptides, cathelicidin, prophenin, rabbit.

INTRODUCTION

Animal fracture healing models are essential to understanding the biological process of bone repair. Rabbits are the most popular animal models for this kind of lesions in humans. Unlike rodents, the rabbit's size allows multiple collections from the same bone for testing of biomechanical or histopathological properties. From these models it is possible to propose new techniques to manage and accelerate fracture consolidation in humans and animals (Matos *et al.*, 2008).

An injury-induced, neutrophil-mediated inflammatory response is involved in the regulation of chondrogenic and osteogenic processes, leading to bony tissue formation at the injury site. It plays a role in suppressing mesenchymal cell osteoblastic differentiation and increasing chondrogenic differentiation in the repair of injured growth plate cartilage (Chung *et al.*, 2006).

Apart from the role in tissue repair, neutrophils are the leading cells in the initial cellular response to pathogen. To ward off a microbial infection, neutrophils circulating in blood migrate by endothelium to the site of infection and combat pathogens using killing mechanisms. These

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<http://dx.doi.org/10.4995/wrs.2012.1149>

mechanisms consist of some antimicrobial enzymes such as elastase and myeloperoxidase (MPO) (Lentz *et al.*, 2007; Borregaard, 2010). Moreover, MPO is a specific enzyme of primary granules of neutrophils and a marker of *in vivo* neutrophil activation (Hans *et al.*, 1991). This enzyme takes part in oxidative stress by formation of hypochlorous acid from the H₂O₂-MPO system (Faurischou and Borregaard, 2003). However, while local neutrophil migration to the site of tissue damage is important for wound healing and protection against invading organisms, systemic activation can lead to sequestration in organs and can cause tissue damage from released enzymes and free radicals (Lentz *et al.*, 2007; Wessely-Szponder, 2008). Hence, diminution of the inflammatory response may provoke postoperative complications such as bacterial infections, whereas excessive inflammation can lead to tissue injury and multiple organ failure (Zhou *et al.*, 2007).

The new promising therapeutic approach for regulation of intensity of inflammatory process consists of cathelicidins with the potential for immunomodulation (McPhee and Hancock, 2005; Barlow *et al.*, 2006; Alalwani *et al.*, 2010). Prophenin (proline-phenylalanine-rich peptides, PF) belongs to cathelicidins, which are expressed in porcine neutrophils and exert properties such as antimicrobial activity (Zang *et al.*, 2000; Wessely-Szponder *et al.*, 2010). Previous reports estimated that some cathelicidins could act immunomodulatorily by increasing or diminishing immune response, including the influence on neutrophils. They can change neutrophil degranulation or regulate inflammatory response by promoting or decreasing apoptosis as the means of modulating inflammation (Aarbiou *et al.*, 2006). Recently, Chou *et al.* (2010) conducted experiments with the use of 2 antimicrobial peptides, magainin 2 and synthetic pexiganan acetate, to prevent implant infection in rabbit animal models. It is known that the prophenin used in earlier studies had antibacterial activity at concentrations from 20 µg/mL (Wessely-Szponder *et al.*, 2010), but its influence on inflammatory cells has not been evaluated to date.

Previously, it was estimated that calcium sulphate (CS) is a biocompatible material that can be used safely and conveniently in a wide variety of bony defects. CS has been widely used as a bone graft substitute for over 100 yr and has never been associated with unusual inflammatory or foreign body reactions. However, it was recently noted that the use of purified, surgical-grade material evoked fast resorption and may lead to accelerated graft resorption and the accumulation of calcium rich fluid responsible for the inflammatory response (Robinson *et al.*, 1999; Lee *et al.*, 2002). However, the rabbit neutrophil response to CS has not previously been investigated.

The aim of this study was to analyse the effect of PF on the interaction between neutrophils and CS particles in the course of the implantation of bone graft material in rabbits.

MATERIALS AND METHODS

Isolation of prophenins

A PF mixture was isolated from neutrophils derived from fresh porcine blood collected with anticoagulant 3.8% citrate at an abattoir as described previously (Wessely-Szponder *et al.*, 2010). Briefly, a crude extraction of blood neutrophils was obtained after red blood cells were lysed by the addition of 0.83% ammonium chloride. The cells were then homogenised to release the neutrophil granules. These granules were collected (25 000 g, 40 min, 4°C), suspended in 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 g, 20 min, 4°C). Then, gel filtration chromatography with Sephadex G-50 (Fine, Sigma-Aldrich) column was carried out for separation of the molecules with an expected molecular mass between 8 and 9 kDa. The purity of the obtained fraction was tested by High Performance Liquid Chromatography. PF concentration

in the separated fraction was assessed using the extinction coefficient (Kokryakov *et al.*, 1993). These fractions were then pooled in 5 mL, lyophilised, and stored at -20°C .

Animals and study design

The study protocol was approved by the Local Ethics Committee. The aim of the surgical procedure was to implant calcium sulphate graft material into the tibial defect. The study was carried out on 6 healthy New Zealand White (NZW) rabbits, males with mean body weight \pm standard deviation of 4000 ± 100 g. A physical examination of each rabbit was performed before the surgical procedure. 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 to administer the anaesthesia. The animals received ketamine (0.35 mg/kg per min) intravenously at a continuous rate infusion diluted in 5% of glucose. Heart rate and temperature were monitored every 20 min during infusion. Mean surgical procedure duration was 30 min.

Blood samples were obtained 30 min (t1) and 24 h after bone graft material (t2) implantation. Blood was taken into tubes with 3.8% sodium citrate (anticoagulant) at a proportion of 1:10. Haematological tests were done using haematological analyser EXIGO VET.

A second clinical examination was conducted after 24 h for close observation of the surgical intervention site as well as the general condition and vital parameters of all animals.

Isolation of neutrophils from rabbit blood and cell culture

Rabbit neutrophils were isolated from peripheral blood. After red blood cells were lysed by the addition of 0.83% ammonium chloride at a ratio of 3:1, the remaining pellet was washed twice with phosphate-buffered saline (PBS-Biomed, Lublin, Poland). The final cell pellet was resuspended in 1 mL of Dulbecco's Modified Eagle's Medium (DMEM-Sigma). After isolation, the viability of polymorphonuclear cells (PMNs) was determined by trypan blue exclusion. After cell counting and differentiation (>85% of neutrophils on May-Grunwald-Giemsa-stained preparations), cell suspensions were adjusted to a final concentration of 2×10^6 cells/mL. Then, cell cultures were incubated for 30 min and for 24 h at 37°C and 5% CO_2 with PF in final concentrations of 10, 20, and 50 $\mu\text{g/mL}$ of culture. Control groups (0 $\mu\text{g/mL}$ of PF) were supplemented by PBS in equal volume.

Elastase release assay

Elastase activity was measured with azocasein (Sigma) as a substrate after 10 min incubation at room temperature. The reaction was stopped by the addition of 10% TCA (trichloroacetic acid). 100% enzyme content was estimated by incubating cells in the presence of 0.5% CTAB (hexadecyltrimethylammonium bromide-Sigma) since CTAB results in complete cell lysis and the release of all granule enzymes. Absorbance was measured on ALAB-PLATE READER ELISA at 492 nm. All samples were assayed in duplicate (Wessely-Szponder, 2008).

MPO release assay

MPO release was measured spectrometrically after 10 min incubation at room temperature with an equal volume of o-phenyldiamine (OPD-Sigma). The MPO reaction was stopped by the addition of 2M H_2SO_4 . One hundred percent enzyme content was estimated by incubating cells in the presence of 0.5% CTAB. Absorbance was measured at 492 nm. All samples were assayed in duplicate (Wessely-Szponder, 2008).

Cell survival assay

Cell survival was determined by an MTT test (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide) based on a conversion of the tetrazolium salt MTT by mitochondrial dehydrogenase to a formazan colour product as measured at absorbance of 565 nm (Kakuta *et al.*, 2006). The values obtained were calculated as a percentage of 100% activity of cells without stimulation.

Apoptosis assay

Samples from neutrophil preparations containing 1×10^6 cells were stained with May-Gruenwald-Giemsa solution. Apoptosis morphology (chromatin condensation, cytoplasm vacuolisation) was evaluated by observation of slides with light microscopy at a magnification of $\times 1\,000$. In each preparation, 200 cells were counted and the percentage of apoptotic cells was determined as described by Kakuta *et al.* (2006).

Statistical analysis

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

RESULTS

Isolation of PFs

PFs were isolated from porcine neutrophil extract using a gel chromatography method (Figure 1). The peptide obtained was lyophilised and frozen at -20°C in portions of a known weight for further study. These portions were diluted in PBS to final concentrations of 10, 20, and 50 $\mu\text{g}/\text{mL}$ just before use.

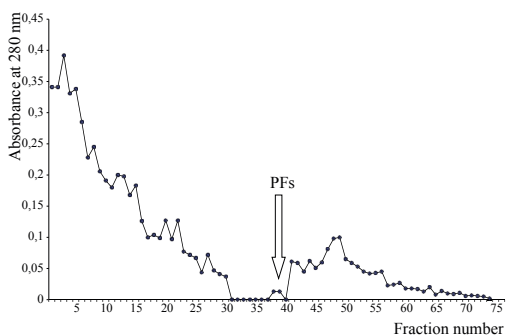


Figure 1: Gel filtration chromatogram for crude extraction applied on a Sephadex G-50 column in 5% acetic acid and monitored at 280 nm. Fractions were collected in tubes (0.5 mL/tube). The arrow indicates the fraction containing prophenins (PFs).

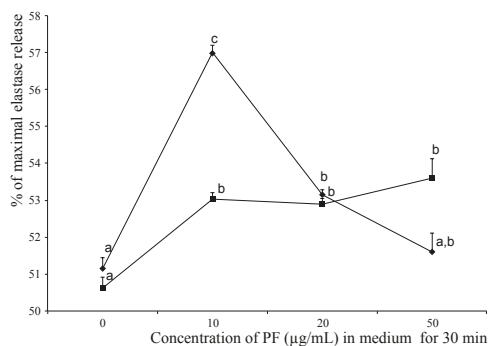


Figure 2: Dose-dependent elastase release by rabbit neutrophils isolated 30 min (t1- \blacklozenge) and 24 h (t2- \blacksquare) after surgery and incubated with different concentrations of PF for 30 min. Mean values marked with the same letter do not differ statistically; those marked with different letters differ statistically ($P < 0.05$).

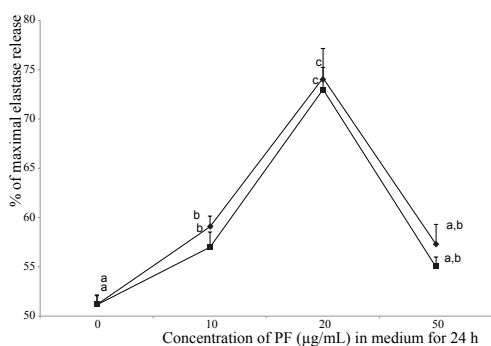


Figure 3: Elastase release by rabbit neutrophils isolated 30 min (t1-◆) and 24 h (t2-■) after surgery and incubated with different concentrations of prophenin for 24 h. Mean values marked with the same letter do not differ statistically; those marked with different letters differ statistically ($P<0.05$).

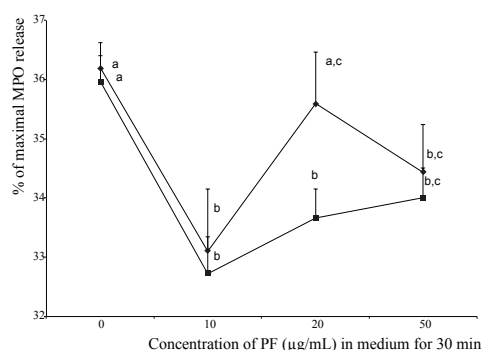


Figure 4: Myeloperoxidase (MPO) release by rabbit neutrophils isolated 30 min (t1-◆) and 24 h (t2-■) after surgery and incubated with different concentrations of prophenin for 30 min. Mean values marked with the same letter do not differ statistically; those marked with different letters differ statistically ($P<0.05$).

Neutrophil elastase release after 30 min of incubation

The measurement 30 min after surgery (t1) indicated an increase of elastase level in cultures stimulated for 30 min with all used concentrations of PF. The greatest elastase release was at 10 µg/mL (from 51.15±0.23% at 0 µg/mL to 57.01±0.49% at 10 µg/mL; $P<0.01$). Results obtained at measurement 24 h after surgery (t2) were less pronounced (Figure 2).

Neutrophil elastase release after 24 h of incubation

After incubation for 24h, all used concentrations of PF demonstrated an augmentation of elastase release. The greatest neutrophil response was noted at a concentration of 20 µg/mL in both groups of cultured neutrophils (obtained at t1 and t2). In the highest PF concentration, the elastase release decreased and reached values approximate to those of the control group (Figure 3).

Neutrophil MPO level after 30 min of incubation

In our study, the MPO release after 30 min from bone graft implantation decreased significantly at 10 µg/mL ($P<0.01$). In higher concentrations, MPO level also decreased, but to a lesser extent. Similar results were

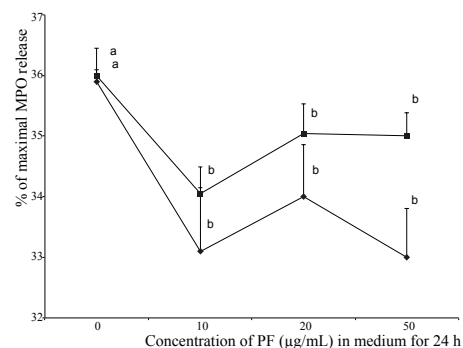


Figure 5: Myeloperoxidase (MPO) release by rabbit neutrophils isolated 30 min (t1-◆) and 24 h (t2-■) after surgery and incubated with different concentrations of prophenin for 24 h. Mean values marked with the same letter do not differ statistically; those marked with different letters differ statistically ($P<0.05$).

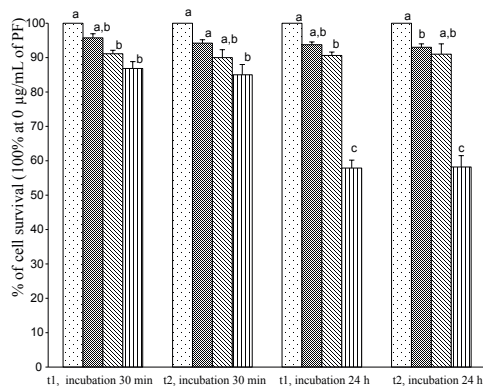


Figure 6: The effect of different concentrations of prophenin on MTT test reduction in neutrophils after 30 min and after 24 h of incubation expressed as a percentage of values obtained at each time point in unstimulated control group. Mean values marked with different letters differ statistically ($P < 0.05$). □ 0 µg/mL of PF, ▨ 10 µg/mL of PF, ▩ 20 µg/mL of PF, ▪ 50 µg/mL of PF.

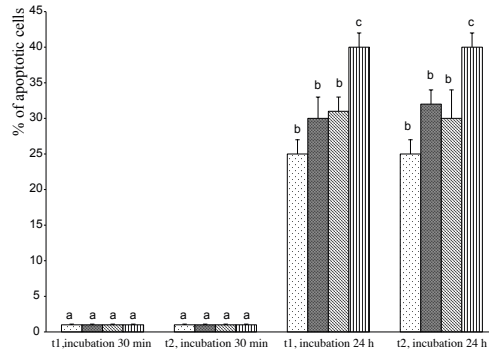


Figure 7: Percentage of apoptotic neutrophils isolated from rabbits 30 min and 24 h after surgery and incubated for 30 min and for 24 h with different concentrations of prophenin. Mean values marked with different letters differ statistically ($P < 0.05$). □ 0 µg/mL of PF, ▨ 10 µg/mL of PF, ▩ 20 µg/mL of PF, ▪ 50 µg/mL of PF.

seen at t2 but with greater inhibition of enzyme release in all concentrations than in the culture of neutrophils obtained at t1 (Figure 4).

Neutrophil MPO level after 24 h of incubation

We observed that neutrophils obtained after 30 min from implantation and incubated 24 h with PF released the lowest MPO ($P < 0.01$) at the concentration of PF 10 µg/mL, whereas at other concentrations the inhibition of MPO release was smaller. In neutrophils isolated after 24 h from implantation, the inhibitory effect was lesser (Figure 5).

Cell survival assay

The mitochondrial metabolic activity of rabbit neutrophils decreased under the influence of increasing concentrations of PF. This effect was observed in both measurements at t1 and t2, and was more pronounced in cultures of neutrophils incubated for 24 h than in cultures incubated for 30 min. The greatest inhibition of mitochondrial metabolic activity was seen in neutrophils obtained at t1 and t2 and stimulated with 50 µg/mL of PF for 24 h (Figure 6).

Apoptosis assay

The percentage of apoptotic cells after the first 30 min of incubation in both measurements was around 1%, independently on the concentration of PF used. However, the number of apoptotic cells increased with the incubation time. In cultures incubated for 24 h without stimulation, we observed 25±2% of apoptotic cells, whereas 40±2% of apoptotic neutrophils were seen in cultures stimulated with the highest concentration of PF (Figure 7).

DISCUSSION

The number of published animal studies about the effects of natural and synthetic antimicrobial peptides remains relatively few. Stallman *et al.* (2004) described the local use of synthetic antimicrobial peptides for prevention of *osteomyelitis* on rabbit models in pre-clinical studies of bone infection caused by resistant strains of *Staphylococcus aureus* and compared their efficacy with current antibiotic agents. Previously, the influence of 2 bovine-derived peptides, Bac2A and indolicidin, have been tested on human neutrophils. This study revealed that neither of these peptides is cytotoxic to human neutrophils in the concentrations used. Moreover, indolicidin displays major antiendotoxic properties, whereas Bac2A is a more potent chemotactic agent than indolicidin (Bowdish *et al.*, 2005). The PF used in our study for the stimulation of rabbit neutrophils were obtained according to the previously described method (Wessely-Szponder *et al.*, 2010). The usefulness of these antimicrobial peptides isolated from porcine neutrophils as a therapeutic that modulates inflammatory response during and after bone graft surgical procedure has not yet been evaluated.

The search for a satisfactory substitute for autogenous bone graft has been essential in orthopaedics for many years (Lee *et al.*, 2002). Despite the fact that CS has been recognised as a biocompatible material that can be used safely and conveniently in some bone defects, it was noted that the use of purified, surgical-grade material evoked fast resorption and may lead to accelerated graft resorption and an accumulation of calcium rich fluid responsible for the inflammatory response (Robinson *et al.*, 1999; Lee *et al.*, 2002). In addition, there are some reports that the injection of calcium-based cement can have a potential for adverse reactions, such as pulmonary embolism. Moreover, CS demonstrated local cytotoxic properties (Nystrom *et al.*, 2002). On the other hand, Orsini *et al.* (2004) reported that CS does not interfere with the healing process. In our study, we did not observe any alterations of neutrophil activity or adverse effects of CS implants on rabbits 24 h after surgical implantation in comparison with measurements after 30 min.

Bone fracture as well as bone graft implantation incites an inflammatory response which peaks 24 h following the injury and is completed by the first week (Mountziaris and Mikos, 2008, Velard *et al.*, 2009). Neutrophils are the most numerous inflammatory cells in the initial response associated with the up-regulated expression of various cytokines and growth factors in bony repair (Chung *et al.*, 2006). In the first phase of bone graft incorporation, during the first 18 h a chemotactic factor arrival and an accumulation of neutrophils as inflammatory cells are observed. This process is connected with elastase release producing chemotactic factors such as collagenous and fibronectin peptides (Kaveh *et al.*, 2010). Immediately following injury, neutrophils migrate toward the implant site and this recruitment is triggered by chemoattractants. Reaching the implantation site, neutrophils encounter the biomaterial surface and the subsequent engagement of integrins on the neutrophil surface triggers a phagocytic response and degranulation (Franz *et al.*, 2011). The study recently conducted by Velard *et al.* (2009) on the widely-used bone graft substitute - hydroxyapatite (HA) revealed that released HA particles induce an inflammatory response in the form of early recruitment and activation of neutrophils. HA particles cause the release of proinflammatory mediators as well as an increase in matrix metalloproteinase 9 activity. Moreover, the report of Eferic and Deierkauf (1987) pointed out that HA crystals induced enzyme release from human neutrophils. No studies on the influence of CS on neutrophils, or studies of the effect of PF on neutrophil degranulation, have been conducted to date.

We noted the increase of elastase release under the influence of PF. The greatest response after 30 min of incubation was at 10 µg of PF, whereas the highest elastase release after 24 h of

incubation was under the influence of 20 µg/mL of PF. MPO release was inhibited at all used concentrations of PF, especially at 10 µg/mL after 30 min and 24 h of incubation.

Elastase and MPO are stored in azurophilic granules of naïve neutrophils (Papayannopoulos *et al.*, 2010). However, as estimated by Damiano *et al.* (1988), only a fraction of azurophilic granules are elastase positive, whereas the others are elastase negative. It was suggested that neutrophils may react to the various stimuli by a graded series of responses in which azurophil granule constituents could be released either rapidly or slowly depending upon the type of stimulus. Thus, although elastase and MPO are both stored in azurophil granules, heterogeneity of granule contents can result in a different enzyme share (Eckle *et al.*, 1990). In our study, a significant ($P<0.01$) decrease in MTT reduction was observed after 24 h of incubation with PF at the highest concentration (50 µg/mL) in measurements 30 min and 24 h after surgical intervention. Previous studies revealed that cathelicidins are cytotoxic against eukaryotic cells at high concentrations (Aarbiou *et al.*, 2006). Our previous research estimated that there were no cytotoxic effects on studied cell lines in concentrations up to 20 µg/mL (Wessely-Szponder *et al.*, 2010).

Our study revealed that the proportion of apoptotic cells after 30 min of incubation was about 1% in all studied groups. However, in both measurements, at t1 and t2 the number of apoptotic cells increased after 24 h of incubation and reached 25±2% in cultures without stimulation and 40±2% at the highest concentration of PF.

In normal conditions neutrophils are programmed to die by apoptosis, in contrast to ‘accidental’ cell death, i.e. necrosis, which maintains the plasma membrane integrity and avoids releasing noxious granule contents that cause tissue injury. Thus, apoptosis of neutrophils is a way of regulating inflammation among others by the restriction of the release of proinflammatory neutrophil products. Therefore, apoptosis should be considered not only as a programme of cell death, but also as a highly organised, non-inflammatory means of cell clearance. (Savill and Haslett, 1995). However, despite the importance of this problem, previous studies have provided limited insight into the mechanism involved in cell death induction by antimicrobial peptides (Aarbiou *et al.*, 2006).

CONCLUSION

Our study revealed that PF in concentrations up to the level of an antibacterial effect inhibits MPO generation, thereby preventing oxidative destruction, whereas the level of elastase is increased significantly in concentrations of 10 and 20 µg/mL. The elastase release in the first phase of bone repair is related to the participation of this enzyme in bone graft incorporation. Marked cytotoxicity of neutrophils under the influence of PF as well as apoptosis is seen only in the highest concentration above antimicrobial level. Porcine PF can act immunomodulatorily, as seen in its influence on rabbit neutrophil degranulation, survival, and apoptosis. These peptides could be considered for use as a means of preventing infection and of stimulating effector cells for innate immunity during and after a bone graft surgical procedure.

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