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Changes in the activity of ovine blood-derived macrophages stimulated with antimicrobial peptide extract (AMP) or platelet-rich plasma (PRP)

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Abstract

Introduction: Antimicrobial peptides (AMP) are a large group of innate immune effectors, which apart from antimicrobial activity show immunomodulative properties. Platelet-rich plasma (PRP) is a source of autologous growth factors and is used for stimulation of bone and soft tissue healing. The purpose of this study was to assess the influence of PRP and AMP extract on ovine monocyte-derived macrophage cultures**. Material and Methods:** The study was conducted on ovine macrophages (Mfs) previously stimulated with LPS or dexamethasone and then with preparations of PRP or AMP. Following activation of the Mfs their morphological and functional features were assessed. **Results:** The study revealed pro-inflammatory influence of both examined preparations on Mfs cultures on the basis of morphology, ROS generation and arginase activity. Both preparations enhanced the pro-inflammatory response of cultured Mfs. **Conclusion:** This activity may intensify the antimicrobial action of Mfs, however, in cases of excessive and prolonged inflammation the use of these preparations should be limited.

Keywords: monocyte-derived macrophages, antimicrobial peptides, platelet-rich plasma.

Introduction

Antimicrobial peptides (AMP), also known as host defence peptides, are important components of innate immunity. As has been stated, some of these peptides have immunoregulatory activity in addition to their potent antimicrobial action. AMP display highly diverse sequences and biological functions. In mammals, they are divided into two main groups: cathelicidins and defensins (19). Cathelicidins are the host defence peptides which show antimicrobial activity together with multiple functions relating to innate immunity and tissue repair (2, 13). Mammalian defensins are a large family of cysteine-rich antimicrobial peptides divided into three subfamilies: α-, ß-, and θ-defensins (19).

Some mixtures of natural preparations based on antimicrobial peptides have been evaluated as therapeutic means for fighting infections and strengthening host defence mechanisms (2). As noted previously, a rabbit AMP extract was evaluated which consisted of a 15 kDa antimicrobial peptide, cathelin-like fragments, CAP 18, and rabbit defensins NP-1, NP-2, NP-3a, NP-3b, NP-4, and NP-5, and may be regarded as a promising new therapeutic approach for regulation of the intensity of the inflammatory process with the potential for immunomodulation (24).

Platelet-rich plasma (PRP) has been used for several years during surgical procedures to enhance peri-implant soft tissue and bone healing and has been applied for regeneration of bone, cartilage, and ligaments. The main rationale for the use of PRP stems from the growth factors released from platelet α-granules, including platelet-derived growth factor (PDGF), transforming growth factor (TGF), vascular endothelial growth factor (VEGF), and insulin-like growth factor (IGF) (20). All these growth factors were detected previously in preparations of PRP from ovine blood together with antimicrobial peptide (PF4), which enhances their antimicrobial properties (23). These

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antimicrobial properties of platelet components would be best taken advantage of producing a combined neutrophil and platelet extract to maximise their activity (2).

Macrophages (Mfs) are the main effectors and regulators of innate inflammation. The key functions of these cells are phagocytosis, killing of invading pathogens, and release of inflammatory mediators; they also play a role in healing processes (9). Owing to Mf plasticity, there are possibilities to shift them from being pro-inflammatory to being regulatory suppressive cells (9). The study of the Mf response to stimulation with two different blood-derived agents, AMP extract and PRP could shed new light on the application of alternative substances in enhancement of tissue healing and infection fighting. Therefore, the aim of this study was to assess the influence of autologous ovine PRP and rabbit AMP extracts on ovine monocyte-derived Mf (MDM) populations, in respect of their pro-inflammatory or tissue repair properties.

Material and Methods

Preparation of the neutrophil crude extract of rabbit AMP. In the first step of the experiment neutrophils were purified from 2–3 mL portions of blood obtained from six healthy New Zealand White rabbits in accordance with animal protection regulations. After red blood cell lysis with 0.83% ammonium chloride solution followed by differential centrifugation, the final polymorphonuclear leukocyte cells were then homogenised to release the neutrophil granules. These granules were centrifuged at 25,000 × *g* for 40 min at 4°C, then acid extraction with 10% acetic acid was performed and insoluble material was sedimented (25,000 × *g*, 20 min, 4°C). The supernatant containing antimicrobial peptides was considered AMP extract and lyophilised. Portions of 40 μg/mL of this extract were dissolved in PBS, and then used for stimulation of macrophage cultures (25).

Preparation of PRP from sheep blood. Blood samples of 8.5 mL were collected from the jugular vein of Polish Lowland sheep into a monovette containing citrate phosphate, dextrose, and adenosine (CPDA) from a PRP kit (Curasan AG, Germany). A two-step procedure was applied, in which first the blood samples were centrifuged at 160 × *g* for 20 min at room temperature to separate the plasma containing the platelets. Then the plasma fraction was collected and centrifuged (400 × *g*, 15 min). The top layer was discarded and the remaining volume was platelet-rich plasma (23).

Monocyte isolation and generation of polarised monocyte-derived macrophages from sheep blood. Peripheral blood mononuclear cells were separated from ovine blood by gradient centrifugation with Lymphoprep (Nycomed, Norway) as the density gradient medium. The obtained cells were plated at a density of 1.0×10^6 cells/mL onto 96-well flat-bottom tissue culture plates (Nunc, USA) and cultured at 37°C in 5% CO_2 for 24 h in Dulbecco's modified Eagle's medium (DMEM) with 10% bovine calf serum (BCS) (Biomed, Poland). The adherent cells were cultured for an additional 48 h to allow monocytes to mature to functional macrophages (10). Then, polarisation of cultures began by changing media to one of three variants supplemented as follows: without stimulators, for generation of BCS Mfs as a control; with addition of 1 μg/mL LPS from *E. coli* serotype 055:B5 (Sigma-Aldrich, Poland), for generation of M1 (described as LPS); and with dexamethasone (DEX) at a concentration of 100 nM for generation of M2 (22, 25). After polarisation Mfs were additionally stimulated with one of two different stimulators: PRP or AMP. Addition of PRP generated cultures marked as PRP, LPS+PRP, or DEX+PRP, according to the previous stimulation. Addition of AMP (40 μg/mL of rabbit AMP extract reconstructed from lyophilisate) generated cultures marked as AMP, LPS+AMP, and DEX+AMP likewise corresponding to the previous stimulator used. On every subsequent day of culture the cells were subjected to microscopic analysis of their morphology using an inverted microscope. The functional analysis was conducted 24 h (time 1 – T1) and 48 h after stimulation (time 2 – T2).

***In vitro* functional analysis.** Nitric oxide (NO) concentration was assessed using the Griess method. Briefly, an equal volume of culture supernatant and Griess reagent (0.1% N-(1-naphthyl)ethylenediamine dihydrochloride 1% sulphanilamide and 2.5% H_3PO_4) were mixed and incubated at room temperature for 20 min and then absorbance was measured. The obtained values were expressed as a concentration of nitrite (the stable breakdown product of NO, which accumulates in the medium). Conversion of absorbance to micromoles (μM) was calculated from a $NaNO_2$ standard curve (25).

Superoxide production was measured using a method given previously (24). In brief, the media obtained from cultures of Mfs with different stimulators were incubated with 0.1% nitroblue tetrazolium (Sigma-Aldrich, Poland) solution at room temperature for 15 min and then absorbance was read. The amounts of the superoxide in nanomoles (nMs) were calculated using the extinction coefficient 21.1 nM.

Arginase activity was assessed by measuring the concentration of urea generated by arginase-dependent hydrolysis of L-arginine. Macrophages were lysed with 0.1% Triton X-100 and the cell lysates were incubated with 50 μL of 25 mM Tris-HCl and 10 μL of 10 mM $MnCl_2$. Then, after thermal activation of the enzyme (10 min at 55°C), L-Arginine (0.5M) was hydrolysed at 37°C for 120 min. Next, the reaction was stopped with 400 μL of $H_2SO_4/H_3PO_4/H_2O$ (1/3/7 vol/vol/vol). The urea concentration was measured after addition of 40 μL of α-isonitrosopropiophenone (Sigma-Aldrich, Poland) and heating at 100°C for 40 min. The activity of arginase was determined on the basis of urea

formation estimated by a comparison with a standard curve (7).

Statistical analysis. At least three independent experiments in four replications were performed. All data are expressed as the mean ± standard error (SE) for continuous variables. Significance was identified by one-way ANOVA using Statistica 13.1 (StatSoft, Poland) followed by Tukey's test. Differences were considered statistically significant when P was < 0.05.

Results

Changes in morphology of MDM after stimulation with different factors are shown in Fig. 1. Unstimulated MDMs (cultured for 72 h with DMEM enriched with 10% calf serum) were classified as BCS (Fig. 1A). The addition of LPS (Fig. 1B) generated dendric-like MDMs with large filopodia. Treatment of MDM cultures with AMP extract resulted in well-spread cells with multiple filopodia (Fig. 1C). In contrast, addition of dexamethasone induced an increase in rounded cells (Fig. 1D). After stimulation with PRP, MDMs adopted a dendric-like morphology with long filopodia (Fig. 1E), whereas, after previous stimulation with LPS, addition of PRP caused cells to be well spread and dendric-like with large filopodia (Fig. 1F).

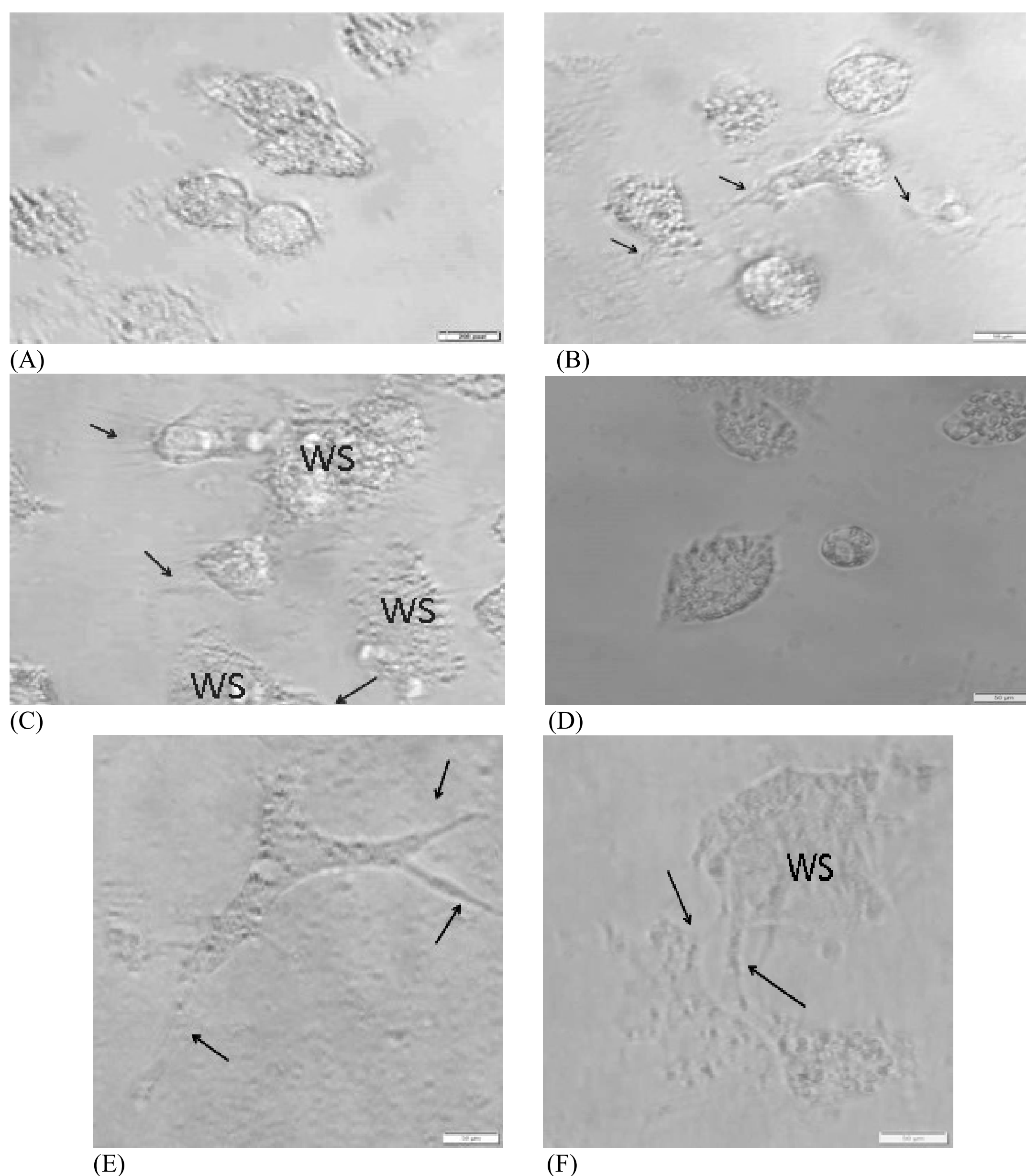

Fig. 1. Changes in morphology of MDM after stimulation with different factors. A – unstimulated MDMs (cultured for 72 h with DMEM enriched with 10% calf serum) classified as BCS. B – M1 Mfs after addition of LPS (black arrows indicate large filopodia). C – Mfs after addition of AMP extract, well-spread cells with multiple filopodia (black arrows). D – Mfs culture after addition of dexamethasone (DEX). E – stimulation of Mfs with PRP, cells with long filopodia (black arrows). F – Mfs culture after previous stimulation with LPS and addition of PRP, well-spread cells (WS) and dendric-like cells with large filopodia (black arrows). The morphology of Mfs was assessed by phase-contrast microscopy. Original magnification × 40 (CK-40, Olympus, Japan). Representative images are shown from n = 6 replicates

As expected, Mfs under the proinflammatory stimulation with LPS showed M1 features in the form of increased NO (Fig. 2) and superoxide generation (Fig. 3) and decreased arginase activity in comparison with BCS Mfs (Fig. 4). Addition of DEX, in turn, evoked an anti-inflammatory response representative of M2 Mfs, presenting as a decrease in ROS generation at both T1 and T2 (Fig. 2 and Fig. 3). The ratio of urea generation to nitrite generation measured against that of BCS Mfs fell significantly ($P < 0.05$) in the LPS group and rose significantly in the DEX group (Fig. 5).

A significant increase in NO generation from 3.5 ± 0.4 $\mu M/10^6$ cells in BCS Mfs to 5.2 ± 0.29 $\mu M/10^6$ cells in the PRP group and to 5.64 ± 0.4 $\mu M/10^6$ cells in the LPS + PRP group was detected in the second measurement (Fig. 2). Stimulation with PRP caused generation of more superoxide, especially at T1, and in cultures previously stimulated with LPS this response was more pronounced (Fig. 3). Arginase activity was diminished in all groups stimulated with PRP (Fig. 4). The ratio of urea generation to nitrite generation was significantly ($P < 0.05$) lower than that in the BCS group and near the value obtained for M1 Mfs (Fig. 5).

Stimulation of Mf cultures with AMP significantly ($P < 0.05$) depressed superoxide production at T2 in all cultures (Fig. 3). On the other hand, stimulation of Mfs with AMP caused greater NO generation, especially at T2 (Fig. 2). Stimulation of Mf cultures with AMP alone inhibited arginase activity, however, previous supplementation with other stimulators weakened this response (Fig. 4). In all groups stimulated with AMP the ratio of urea generation to nitrite generation was significantly lower than that in the BCS group, with the highest value seen in the DEX + AMP group (Fig. 5).

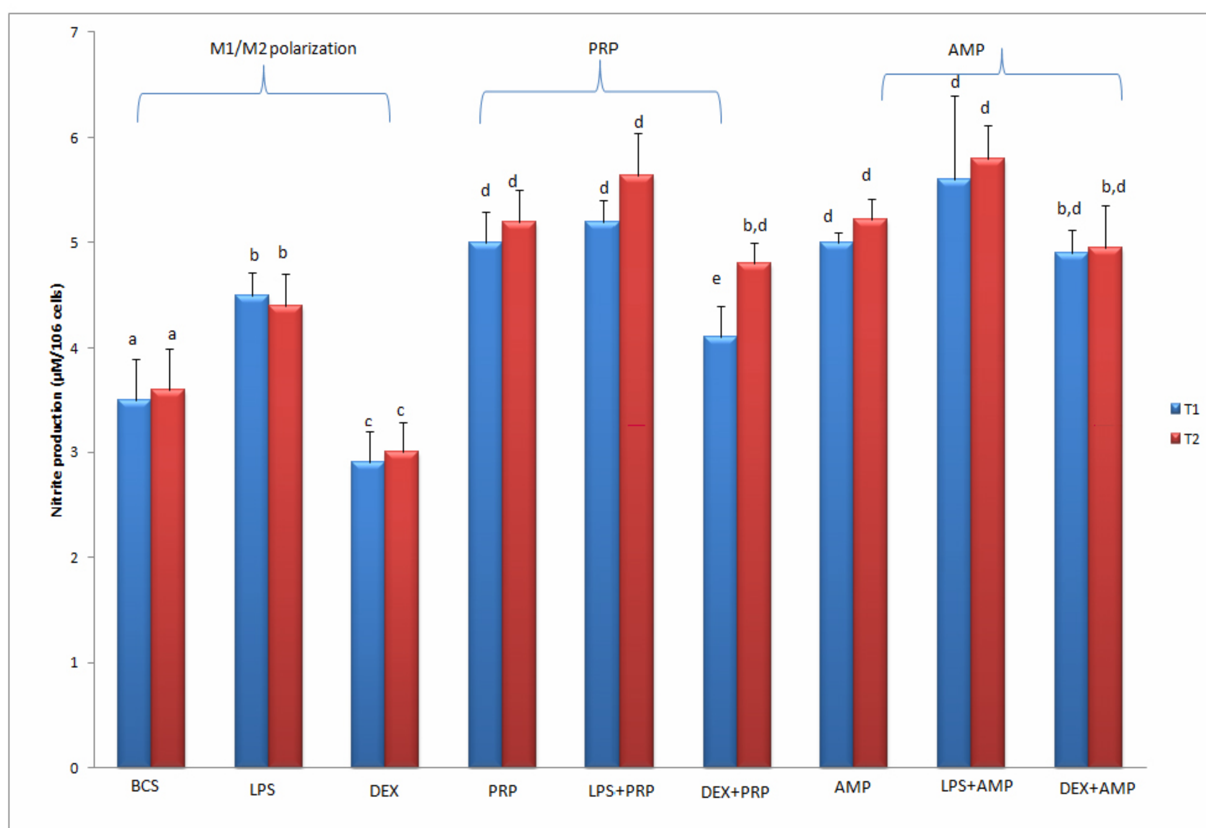

Fig. 2. Nitric oxide generation by Mfs after 24 h and 48 h stimulation as follows: BCS – cultures stimulated only with DMEM with 10% BCS, LPS – cultures stimulated with 1 µg/mL LPS, DEX – cultures stimulated with 100 nM of dexamethasone, PRP – cultures stimulated with PRP, LPS + PRP – cultures stimulated with PRP after previous incubation with LPS, DEX + PRP – cultures stimulated with PRP after previous incubation with dexamethasone, AMP – cultures stimulated with AMP extract, LPS + AMP – cultures stimulated with AMP extract after previous incubation with LPS, DEX + AMP – cultures stimulated with AMP extract after previous incubation with dexamethasone. Values are means ±SE obtained from each separate experiment conducted on three independent cultures in four replications. Mean values marked with different letters differ statistically ($P < 0.05$)

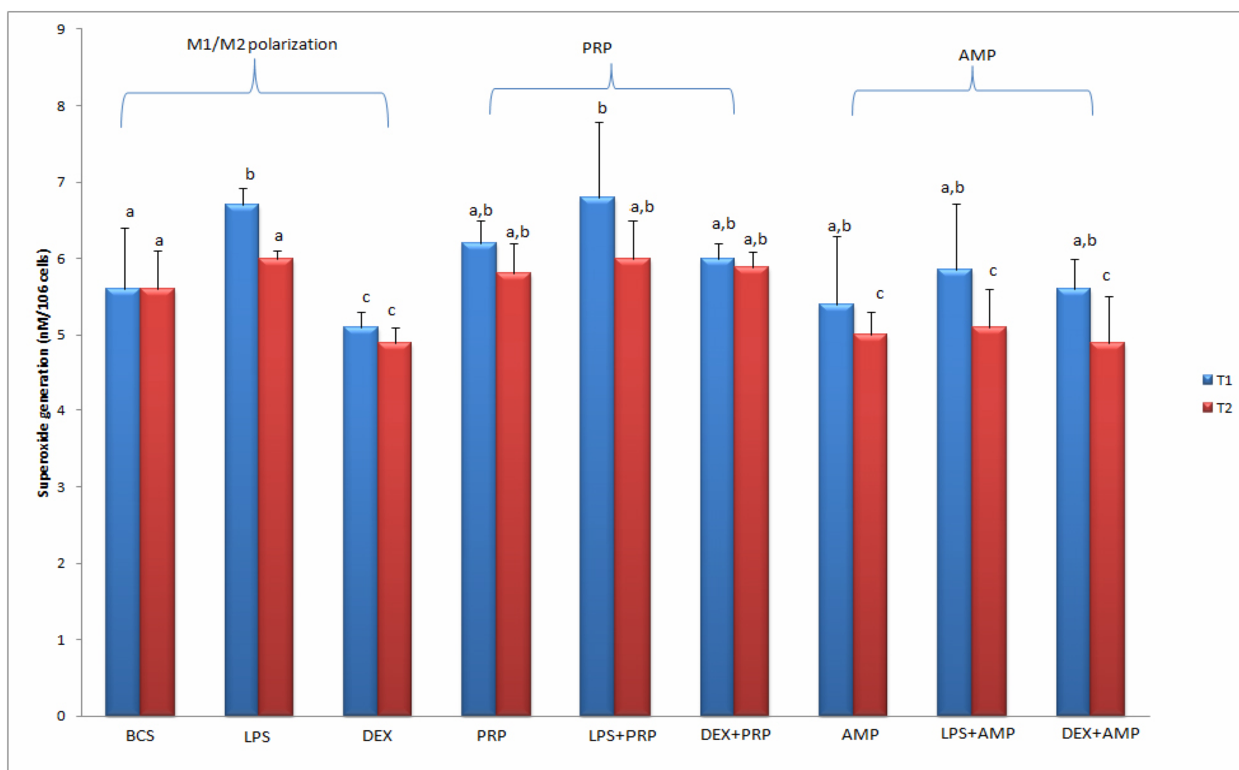

Fig. 3. Superoxide generation by Mfs after 24 h and 48 h stimulation. For abbreviations see footnote to Fig. 2. Values are means ±SE obtained from each separate experiment conducted on three independent cultures in four replications. Mean values marked with different letters differ statistically ($P < 0.05$)

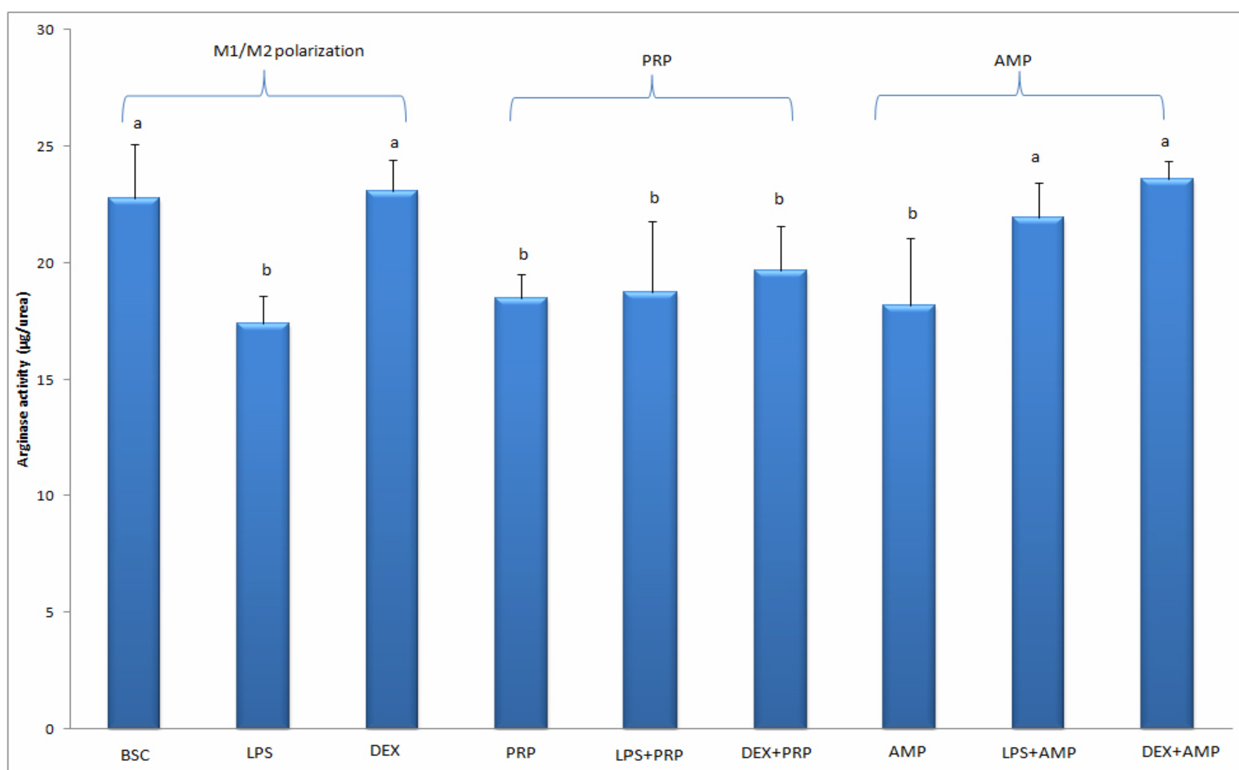

Fig. 4. The arginase activity of differentiated Mfs cultures. For abbreviations see footnote to Fig. 2. Values are means ±SE obtained from each separate experiment conducted on three independent cultures in four replications. Mean values marked with different letters differ statistically ($P < 0.05$)

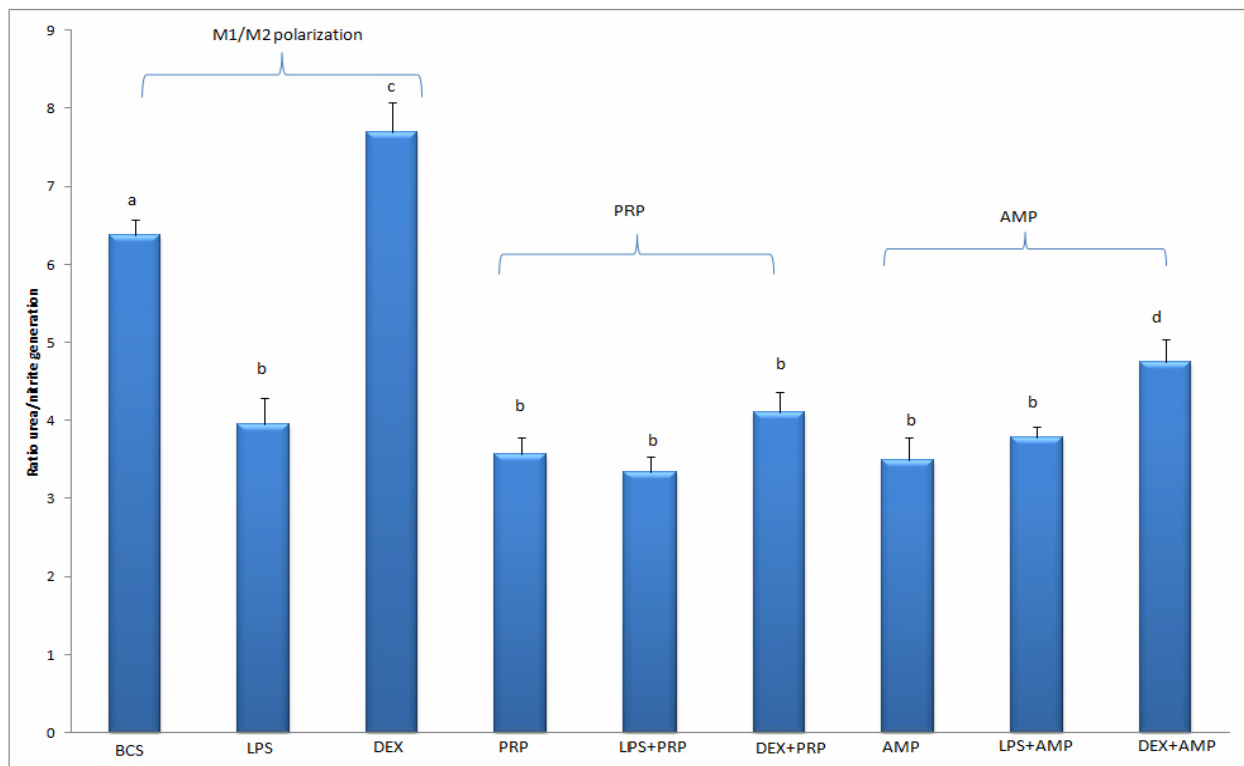

Fig. 5. The ratio of urea generation to nitrogen generation of differentiated Mfs cultures. For abbreviations see footnote to Fig. 2. Values are means ±SE obtained from each separate experiment conducted on three independent cultures in four replications. Mean values marked with different letters differ statistically ($P < 0.05$)

Discussion

In the first step of this study, we demonstrated that MDM morphology and function after polarisation differentiated to pro-inflammatory or repair phenotype according to the stimulator used. During tissue injury or infection the first-responder Mf usually exhibits a pro-inflammatory phenotype and secretes pro-inflammatory cytokines, superoxide, and NO, for activation of various mechanisms, which contribute to the elimination of invading organisms (15). However, the previous study by Campbell *et al.* (5) revealed that Mf phenotype is temporally regulated during wound healing such that M1 are present at early stages and M2 predominate during later stages of inflammation, with increased arginase activity as one of the markers. Local Mf polarisation has a great impact on healing, however, an excessive inflammatory response is a major problem in pathological inflammation (18).

In our experiment, after stimulation with LPS Mfs adopted a fully differentiated ameboid morphology characteristic of M1. This feature of Mfs was previously described by Ploeger *et al.* (17). On the other hand, the rounded cells previously noted by Giles *et al.* (11) after treatment with glucocorticoids were also observed in our study after stimulation of Mfs with DEX (18). The addition of AMP changed Mf morphology; they transformed into well-spread cells with multiple filopodia, as described previously (24, 25). After contact with PRP, Mfs changed towards the pro-inflammatory response both in cultures after previous stimulation with LPS and in those without this stimulation.

Addition of LPS produced more abundant superoxide in Mf culture, as observed previously, and this response was involved in polarisation of Mfs towards M1. Contrastingly, supplementation with DEX resulted in M2 Mfs with augmented arginase activity and decreased free radical generation. This response noted by us was previously also described by other authors in the case of M2c Mfs (6, 12, 14).

In the further part of the study we demonstrated a significant decrease in superoxide generation in cultures stimulated after 48 h with AMP. Previously, it was seen that rabbit AMP extract diminished superoxide level in culture of rabbit MDMs (10), and a similar effect was also mentioned by Agier *et al.* (1). The stimulation of Mfs with PRP resulted in more superoxide generation, with the highest response evident after priming with LPS. Some authors mentioned a pro-inflammatory response of Mfs after stimulation with PRP (8), whereas others suggested Mf differentiation towards a repair profile (3).

Our experiment revealed more powerful generation of NO in cultures stimulated with PRP alone or in combination with LPS (LPS + PRP) or DEX (DEX + PRP). Similarly, all Mf cultures treated with AMP alone or with other stimulators showed uplifted production of NO in comparison with unstimulated BCS Mfs. This response indicates a pro-inflammatory

phenotype of Mfs, as described previously by Mantovani *et al*. (12).

Arginase activity significantly ($P < 0.05$) decreased after addition of LPS, PRP, LPS+PRP or AMP to MF cultures, whereas previous stimulation with DEX caused a slight increase in arginase activity. Additionally, the urea to nitrogen ratio came down in all groups with the exception of the cultures stimulated with DEX alone where increased arginase activity was detected. The balance between inducible nitric oxide synthase (iNOS) and arginase activity, which both compete for the common product L-arginine, is tightly regulated during repair, and decreased arginase activity is observed in human non-healing wounds, indicating its potential importance for healing. Moreover, an absence of iNOS delays healing, whereas iNOS upregulation correlates with faster healing (4).

According to Scull *et al*. (26), the interaction of human Mfs with autologous platelets results in scavenger-receptor-mediated platelet uptake and enhancement of LPS-induced cytokine secretion. Conversely, the research of Arrepol *et al.* (3) revealed that human autologous PRP programmed Mfs toward a repair profile. Our study did not confirm this effect from what was seen of ovine platelet-Mf interactions. Our results showed pro-inflammatory response to autologous PRP with a slight rise in arginase (as a marker) in comparison with LPS-stimulated M1. The previous work of Czakai *et al.* (8) indicated that human PRP intensified the antimicrobial activity against pathogens generated by human monocyte-derived Mfs as well as the pro-inflammatory Mf response to PRP alone.

During a local inflammatory process, activated platelets intensified the Mf pro-inflammatory response. However, Scull *et al.* (21) revealed that platelets can be redirected to exert anti-inflammatory effects on Mfs after dexamethasone-loading, suggesting this as a promising therapeutic approach to treating unresolved inflammation. In our experiment previous addition of DEX to Mfs cultures weakened the pro-inflammatory response of Mfs to PRP.

We demonstrated that the blood-derived preparations heterologous AMP extract and autologous PRP both shift Mfs towards a pro-inflammatory rather than a repair phenotype. If manipulated, pro-inflammatory responses must however be tightly controlled to prevent extensive damage of the host tissues. Therefore, because of the crucial role of Mfs in the regulation of inflammatory process, in cases of excessive inflammation the application of these preparations should be restricted.

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Animal Rights Statement: The experiments on animals were conducted in accordance with local Ethical Committee laws and regulations as regards care and use of laboratory animals.

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