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Research Article

Kojic acid, a secondary metabolite from *Aspergillus* sp., acts as an inducer of macrophage activation

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Abstract

KA (kojic acid) is a secondary metabolite isolated from *Aspergillus* fungi that has demonstrated skin whitening, antioxidant and antitumour properties among others. However, limited information is available regarding its effects on macrophages, the major cell involved in cell defence. The aim of the present study was to analyse whether KA affects functional properties related to macrophage activation, such as phagocytosis and spreading ability over a substrate. Treatment of resident macrophages with 50 μg/ml KA for 1 h induced both morphological and physiological alterations in cells. Immunofluorescence microscopy revealed enhanced cell spreading and an increase in cell surface exposure, associated with a rearrangement of microtubules, actin filaments and intermediate filaments. KA also potentiated phagocytosis by macrophages, as demonstrated by the increase in phagocytic activity towards yeast, when compared to untreated cells. KA increased the production of ROS (reactive oxygen species), but not NO (nitric oxide) production. Three tests were used to assess cell viability; MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide], NR (neutral red) uptake and PI (propidium iodide) exclusion test, which showed that macrophages maintain their viability following KA treatment. Results indicate that KA can modulate macrophage activation through cytoskeleton rearrangement, increase cell surface exposure, enhance the phagocytic process and ROS production. The study demonstrates a new role for KA as a macrophage activator.

Keywords: cytoskeleton; kojic acid; macrophage activation; phagocytosis; secondary metabolite

1. Introduction

KA (kojic acid) is a secondary metabolite produced by some species of fungi from the genera *Aspergillus*, *Penicillium* and *Acetobacter*. This molecule inhibits tyrosinase activity (Chang, 2009) and is used as a food additive (Burdock et al., 2001; Blumenthal et al., 2004; Bentley et al., 2006), a skin-whitening agent for the treatment of melasma (Lim et al., 1999; Nohynek et al., 2004; Lin et al., 2007; Mi Ha et al., 2007), antioxidant, antitumour agent (Gomes et al., 2001; Burdock et al., 2001; Tamura et al., 2006; Moto et al., 2006) and radioprotective agent (Emami et al., 2007). Recently, *in vitro* antiproliferation and cytotoxic activities of KA derivatives have been reported (Fickova et al., 2008). Although KA has numerous biological functions, limited information is available regarding its effect on host immune cells. Enhanced phagocytosis, the generation of ROS (reactive oxygen species) and the concentration of calcium in neutrophils (Niwa and Akamatsu, 1991) in response to KA have been demonstrated, but the effects of KA on macrophages are unknown.

Macrophages are among the most important defence cells that specifically recognize and respond to foreign bodies, apoptotic cells and pathogens (Mosser and Edwards, 2008). Through the activation process, there is enhanced proliferation of resident macrophages, which undergo several morphological changes, such as an increase in spreading and adhesion abilities, phagocytosis activity, ROS generation, antigen presentation and cytokine production (Crume et al., 2007; Bilitewski, 2008). Most of these activities are regulated by cytoskeleton components (Amer and Swanson, 2002; Cruz et al., 2007; Morrow et al., 2007; Mosser and Edwards, 2008). The cytoskeleton is the main compound for microtubules and actin filaments, which work together for the well-synchronized progress of many functions (Salmon and Way, 1999). Microtubules are essential for motility, intracellular organization, transport and immune system regulation (Patel et al., 2009). Microtubule stability is closely involved in the transportation of cytokines and vesicles and is essential for both cellular and humoral immune responses. Stability is necessary for cell spreading and recognizing large particles in activated cells (Binker et al., 2007). Microtubules participate in the Fcγ-mediated internalization process, phagosome recycling and cell migration (Damiani and Colombo, 2003; Calle et al., 2006; Hehnly and Stamnes, 2007). Furthermore, actin filaments are also involved in features such as motility, intracellular transport and phagocytosis (Hehnly and Stamnes, 2007; Kustermans et al., 2008). Intermediate filaments are another component of the cytoskeleton and contribute to the maintenance of cell integrity in the presence of mechanical stress (Garg et al., 2006). Thus, well-synchronized assembly and

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; KA, kojic acid; LM, light microscopy; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NBT, nitroblue tetrazolium salt; NO, nitric oxide; NR, neutral red; PBS–BSA–Tw, PBS, pH 8.0, containing 1.0% BSA and 0.01% Tween 20; PI, propidium iodide; ROS, reactive oxygen species; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

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disassembly of cytoskeleton components are required for a number of cell functions (Damiani and Colombo, 2003).

As macrophages are important immune effector cells, and little is known regarding the effect of KA on innate immune function, the aim of the present study was to analyse whether KA affects functional properties related to macrophage activation, such as phagocytosis, ROS generation and spreading ability over a substrate.

2. Materials and methods

2.1. Murine macrophages

Cells were obtained from peritoneal cavities of *Mus musculus Balb/c* mouse with DMEM (Dulbecco's modified Eagle's medium), pH 7.2, and incubated at 37°C in a humidified atmosphere containing 5% CO_2. After 1 h of incubation, non-adherent cells were washed away with PBS, pH 7.2, and macrophages were incubated overnight in DMEM medium supplemented with 10% heat-inactivated FBS (fetal bovine serum) at 37°C and in a 5% CO_2 in air atmosphere. All experiments were performed at least three times with treated and untreated cells. The experiments performed in this study were conducted in compliance with current Brazilian animal protection laws (CEPAE/ICB/UFPA - grant number BIO001-09).

2.2. Kojic acid

The secondary metabolite is highly soluble in water, ethanol and acetone. KA was obtained from either 0.5 g of mycelial pellets or 5 ml of a solution of *Aspergillus* spores in 400 ml of Czapek culture medium and 6% sterilized sucrose at 120°C for 15 min. The culture was maintained at 120 rev./min at a fixed temperature of 28°C. The liquid phase was filtered and lyophilized to obtain the product. Ethanol and water (80:20) were added, and consecutive extractions were performed to produce a product concentrate through the evaporation process. The final product was obtained through crystallization. Purity was evaluated by high-performance liquid chromatography and was higher than 95%. Dissolved in distilled water was 1.0 mg/ml and used as the standard solution for the assay.

2.3. Cell treatment

KA (stock solution of 1 mg/ml) was added to either tissue culture plates or flasks in different concentrations diluted in the culture medium (DMEM). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 in air for 1 h. The procedures were carried out after 1 h, 1 day or 2 days of treatment, depending on each assay. In all experiments, cells were treated for 1 h once a day. The culture medium without KA was added only to the control groups.

2.4. Cell viability assay

Cell viability was determined by three methods, as described below.

2.4.1. MTT assay

The MTT assay is based on the mitochondrial-dependent reduction of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] to formazan, following the procedure described by Fotakis and Timbrell (2006) with some modifications. Treatment was performed with 10–700 μg/ml of KA for 1 h. After washing and incubation (24 h), cells in 24-well tissue culture plates were incubated at 37°C in a humidified atmosphere containing 5% CO_2 in air for 2 to 3 h with 0.5 mg/ml of MTT dissolved in PBS. The cells were washed once with PBS, followed by the addition of DMSO and gentle shaking for 5 min so that complete dissolution was achieved.

Aliquots (200 μl) of the resulting solutions were transferred to 96-well plates and absorbance was recorded at 570 nm using the microplate spectrophotometer system (Bio-Rad Model 450 Microplate Reader). Results were analysed using the Biostat 4.0 program and were presented as the percentage of cells without treatment (control values). Cell survival was expressed as the percentage of the controls taken as 100%. Assay specificity was performed with non-viable cells treated with 10% formaldehyde in PBS.

2.4.2. NR (neutral red) uptake assay

The NR assay is based on the ability of viable cells to incorporate and bind the supravital dye NR in lysosomes, via active transport. NR is a weak cationic dye and penetrates cell membranes by non-ionic diffusion, subsequently binding intracellular to sites of the lysosome. Cell membrane injuries decrease the uptake and retention of NR. Treatments were performed with 10, 20 and 50 μg/ml of KA for 1 h. Cells were then maintained for 24 h at 37°C in a humidified atmosphere containing 5% CO_2 in air. Treated and control cells were incubated for 3 h with 10 mM of NR dissolved in DMEM. Cells were washed once with PBS, followed by the addition of elution medium (acetone/acetic acid – 50%/1%) and gentle shaking for 10 min, so that complete dissolution was achieved. After dissolution, aliquots (200 μl) of the resulting solutions were transferred to 96-well plates and absorbance was recorded at an OD (optical density) of 570 nm using the microplate spectrophotometer system. Assay specificity was performed with non-viable cells, treated with 10% formaldehyde in PBS.

2.4.3. PI (propidium iodide) assay

PI (Sigma) is a highly water-soluble fluorescent compound that is excluded by viable cells, but can penetrate non-viable cells, intercalating in double-stranded nucleic acids. Cells were treated with 50 μg/ml of KA for 1 h and then maintained for 48 h at 37°C in a humidified atmosphere containing 5% CO_2 in air. Treated and untreated macrophages were then incubated with 25 μg/ml PI for 5 min. An increase in PI–macrophage number indicated a decrease in cell viability. The number of PI fluorescent cells and non-fluorescent cells were determined by examining three coverslips for each treatment using a Zeiss Confocal LSM Pascal microscope (filter: 542–585 nm). At least 100 cells were counted,

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and results were expressed as the percentage of surviving cells when compared with controls.

2.5. Phagocytosis assay and endocytic index

Phagocytosis was assessed by the interaction of macrophages and *Saccharomyces cerevisiae*. Cells were cultured as described above. After 24 h of growth, treated (50 μg/ml) and control cells were allowed to interact with *S. cerevisiae* at a ratio of 10:1 for 2 h in a medium without FBS. The microorganisms were then washed away, and the macrophages were rinsed with PBS, fixed with Bouin's fixative, stained with Giemsa and covered with Entellan® (Merck). For each slide, approximately 200 macrophages were examined using an Olympus BX41 microscope with a ×100 objective lens. The percentage of macrophages with ingested microorganisms, mean number of intracellular particles per macrophage and endocytic index were calculated as described by Araújo-Jorge and De Souza (1984).

2.6. ROS detection in KA-treated macrophages

ROS were detected cytochemically with NBT (nitroblue tetrazolium salt). NBT is a yellow dye that is converted to blue by a semi-quantitative reduction reaction when superoxide anion is present in cells.

Cells were cultured as described above. After 24 h of growth, cells were incubated with 50 μg/ml of KA and 0.5 mg/ml of NBT for 1 h. Cells were then washed with PBS and fixed with 4% freshly prepared formaldehyde, in a buffered solution, pH 7.2, for 30 min at room temperature. Macrophages incubated with *S. cerevisiae* and NBT were used as a positive control. For each slide, approximately 100 macrophages were examined and counted using an Olympus BX41 microscope with a ×100 objective lens. Results are presented as the percentage of macrophages that present formazan deposits.

2.7. Immunofluorescence microscopy of cytoskeletal structures

Cells were cultured on coated coverslips and fixed for 30 min in 3% freshly prepared formaldehyde in PBS. Control and treated cells (50 μg/ml) were then permeabilized with 0.1% Triton X-100 in PHEM buffer for 10 min, incubated with 50 mM NH_4Cl in PBS for 1 h and washed with PBS–BSA–Tw (PBS, pH 8.0, containing 1.0% BSA and 0.01% Tween 20). The cells were then incubated for 45 min with Alexa Fluor® 594 phalloidin (Molecular Probes Invitrogen) for actin filaments diluted 1:200 or incubated with polyclonal anti-tubulin antibody (Sigma) for microtubules diluted 1:100 or incubated for 1 h with polyclonal anti-vimentin antibody (Sigma) diluted 1:100. The cells were washed with PBS–BSA–Tw and incubated for 45 min with Alexa Fluor®-labelled goat anti-rabbit IgG (Molecular Probes Invitrogen) diluted 1:200 in PBS–BSA–Tw (except those labelled with phalloidin), and all were incubated with DAPI for nuclei detection. The coverslips were washed with PBS, covered with ProLong® Gold antifade reagent (Molecular Probes Invitrogen) and examined under a Zeiss Axiophot microscope.

2.8 LM (light microscopy)

Cells cultured on coverslips were treated with 50 μg/ml KA for 1 h. Control and treated cells were fixed with Bouin's fixative, stained with Giemsa and covered with Entellan® (Merck) as described above.

2.9. TEM (transmission electron microscopy)

Control and treated cells (50 μg/ml KA) were washed in PBS and fixed with 2.5% glutaraldehyde and 4% freshly prepared formaldehyde in a buffer solution containing 60 mM Pipes, 20 mM Hepes, 10 mM ethyleneglycol-bis-(B-aminoethylether)-*N*,*N*,*N′*-tetraacetic acid, 70 mM KCl and 5 mM $MgCl_2$, pH 7.2, for 1 h at room temperature. The cells were then washed in the same buffer and postfixed in a solution containing 1% osmium tetroxide, 0.8% ferrocyanide and 5 mM calcium chloride for 1 h. The cells were washed, dehydrated in graded acetone and embedded in Epon®. Thin sections were stained with uranyl acetate and lead citrate and examined in a Zeiss LEO 906E TEM.

2.10. SEM (scanning electron microscopy)

Control and treated cells (50 μg/ml of KA) were fixed with 4% formaldehyde and 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 1 h. The cells were washed and postfixed in 1% osmium tetroxide, dehydrated in graded ethanol, critical point dried (CO_2 in air), coated with gold and examined with a LEO 1450VP SEM.

2.11. Statistical analysis

All experiments were performed in triplicate. The means and S.D. of at least three experiments were determined. Statistical analyses of the differences between mean values in the experimental groups were performed using the Student's *t* test. All P-values <0.05 were considered statistically significant.

3. Results

3.1. Effect of KA on phagocytosis

The endocytic index was analysed by Giemsa staining and given as the percentage of macrophages with ingested microorganisms. Cells were previously treated with 50 μg/ml of KA for 1 h and then infected with *S. cerevisiae*. After 24 h of interaction, internalized *S. cerevisiae* were counted. The statistical analysis revealed a significantly higher number of particles in the KA-treated cells in comparison with the untreated cells (Figure 1).

3.2. Effect of KA on microbicidal response

For ROS detection, treated macrophages were analysed with a semiquantitative cytochemical assay using NBT. Macrophages treated with 50 μg/ml for 1 h showed formazan deposits distributed in the entire cellular cytoplasm (Figure 2b), in comparison with the untreated cells (Figure 2a). The reaction was observed in

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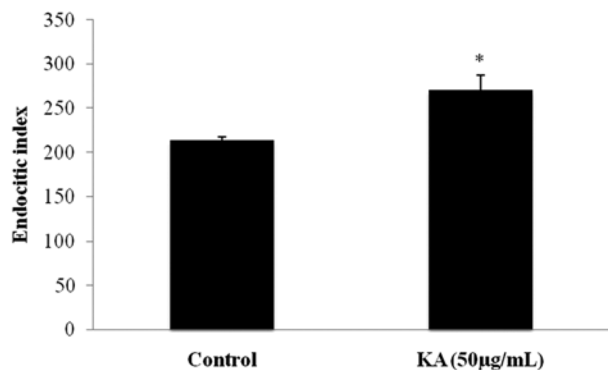

Figure 1 Endocytic index of murine peritoneal macrophages after treatment with 50 μg/ml of KA for 1 h once a day over 3 days
The endocytic index significantly increases with *S. cerevisiae*; $^{*}P<0.05$, when compared with control.

approximately 70% of the cells (Figure 2c). On the other hand, NO (nitric oxide) production was not observed in macrophages treated with 10, 20 and 50 μg/ml of KA (data not shown).

3.3. Effect of KA on macrophage morphology

Based on previous results, the concentration of 50 μg/ml of KA was chosen for morphological analysis. Control and treated cells were analysed by LM, TEM and SEM (Figure 3). LM and SEM demonstrated that KA was able to activate the treated cells, with greater spreading and more numerous cellular projections (Figures 3b, 3d) in comparison with the control cells (Figures 3a, 3c). TEM revealed typical activated cell morphology in KA-treated cells, with an increase in membrane projections, a higher number of vacuoles and endoplasmic reticulum (Figure 3f, arrows) in comparison with the untreated cells (Figure 3e). Furthermore, the mitochondria exhibited normal morphology (Figure 3f, inset).

3.4. Effect of KA on peritoneal cytoskeleton of macrophages

Cytoskeleton compounds of treated and untreated macrophages were analysed using fluorescence microscopy (Figure 4). Control cells exhibited a normal cell shape for actin filaments (Figures 4a–4c), microtubules (Figures 4g–4i) and vimentin (Figures 4m–4o), which is characteristic of resident macrophages. The cells treated with 50 μg/ml KA exhibited expressive alterations, particularly in the actin filaments, with filopodium establishment and a greater concentration of actin in these regions (Figures 4e, 4f – small arrows). Labelled microtubules exhibited enhanced polymerization, extending from the nucleus membrane to the cell membrane (Figures 4k, 4l – arrows). Vimentin filaments exhibited peripheral distribution (Figures 4q, 4r – thin arrows).

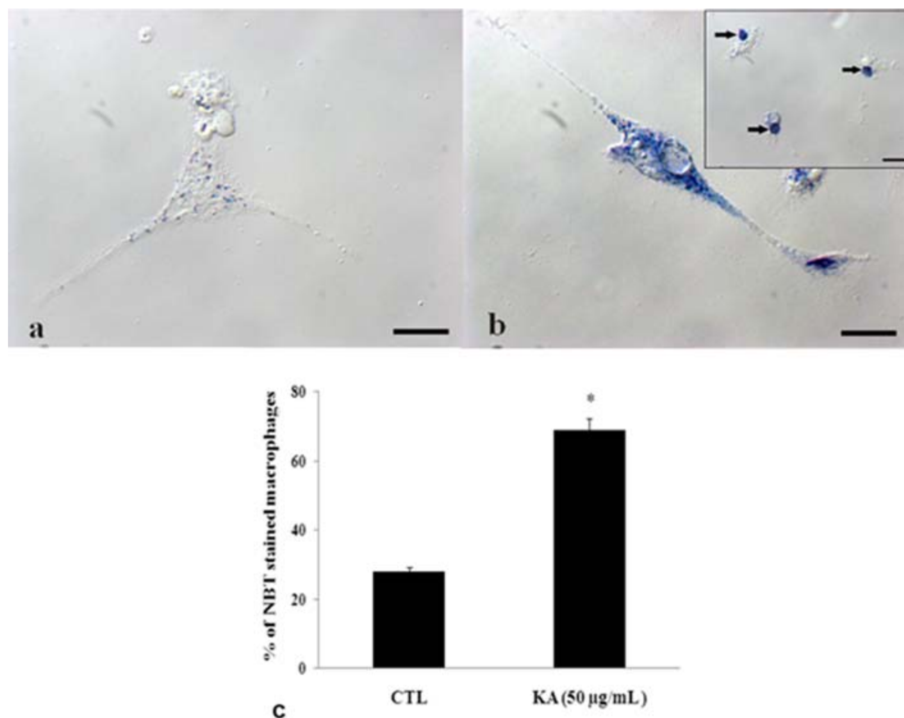

Figure 2 Detection of ROS production by NBT assay in macrophages treated with KA for 1 h
(**a**) Control cells. Non-treated macrophages; absence of formazan deposits. (**b**) Macrophages treated with 50 μg/ml KA; presence of formazan deposits distributed in entire cellular cytoplasm. Inset, macrophage infected with *S. cerevisiae* as positive control. Note the presence of reaction only at infection sites (arrows). (**c**) Percentage of macrophages that presented formazan deposits. CTL, control cells.

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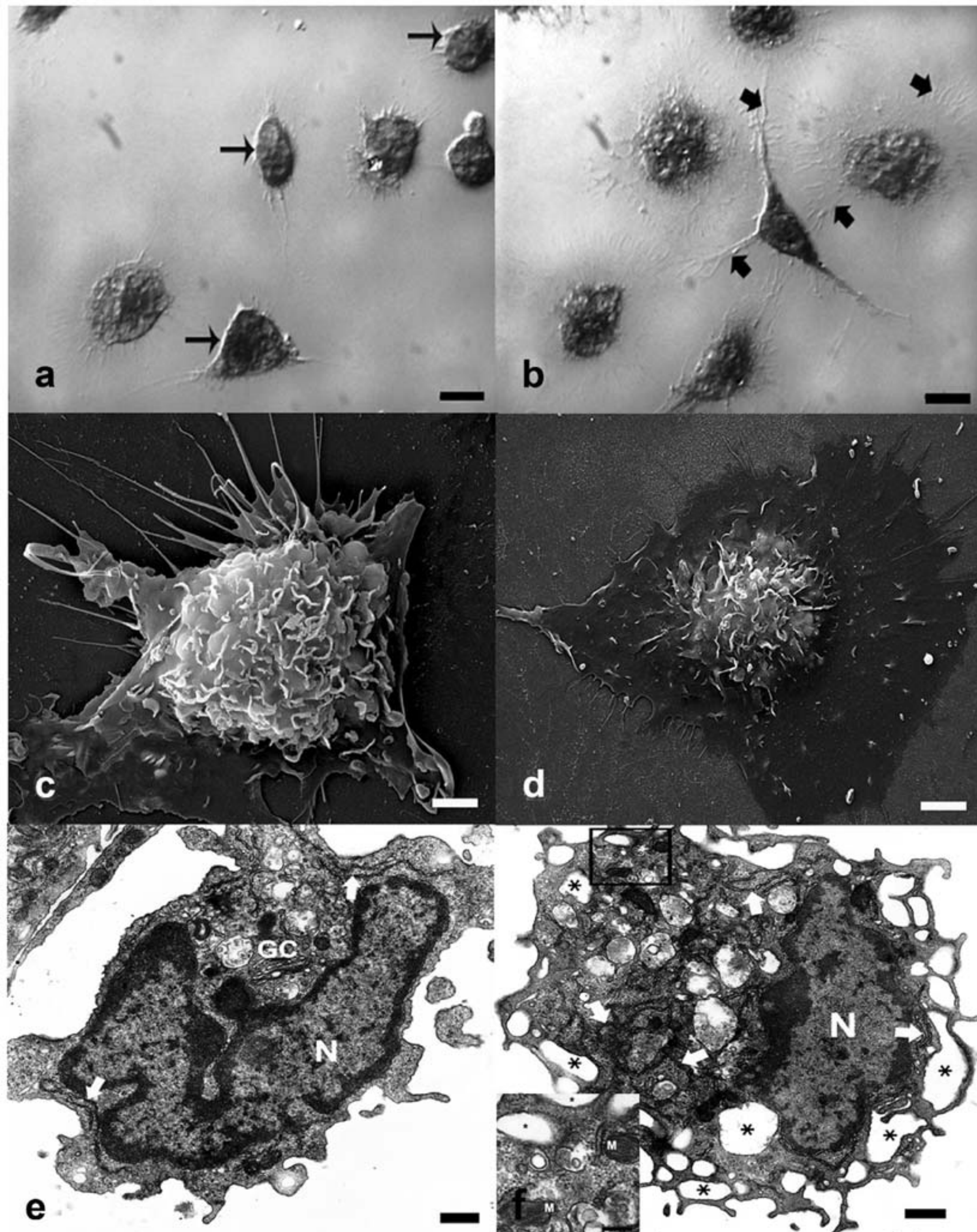

Figure 3 Morphological alterations in murine peritoneal macrophages exposed to 50 μg/ml KA for 1 h
As seen under LM (**a**, **b**), SEM (**c**, **d**) and TEM (**e**, **f**); (**a**, **c** and **e**) control cells with typical morphology; (**b**, **d**) treated cells with cytoplasmic projections (arrows) and increased cytoplasm and spreading ability in comparison with untreated cells; (**f**) treated cells with a large number of vacuoles (*) and endoplasmic reticulum (white arrows), cytoplasmic projections and typical morphology of mitochondria (inset) and nuclei; M, mitochondria; N, nuclei; GC, Golgi complex. Bars (**a**, **b**) 10 μm; (**c**, **d**) 1 μm; (**e**, **f**) 0.5 μm, Inset: 1 μm.

3.5. Effect of KA on macrophage viability

Macrophages treated with different concentrations of KA were analysed by the MTT reduction (Figure 5a), PI (Figure 5b) and NR uptake (Figure 5c). The assays were performed after 1 h of treatment and maintained for 24 h in culture. No cytotoxic effect on the treated macrophages was observed. Cells treated with 50 μg/ml KA for 1 h demonstrated increased phagocytic capacities that occurred in a dose-dependent manner during incubation with NR, compared with the untreated cells. These results reinforce data showing that cells are stimulated during KA treatment.

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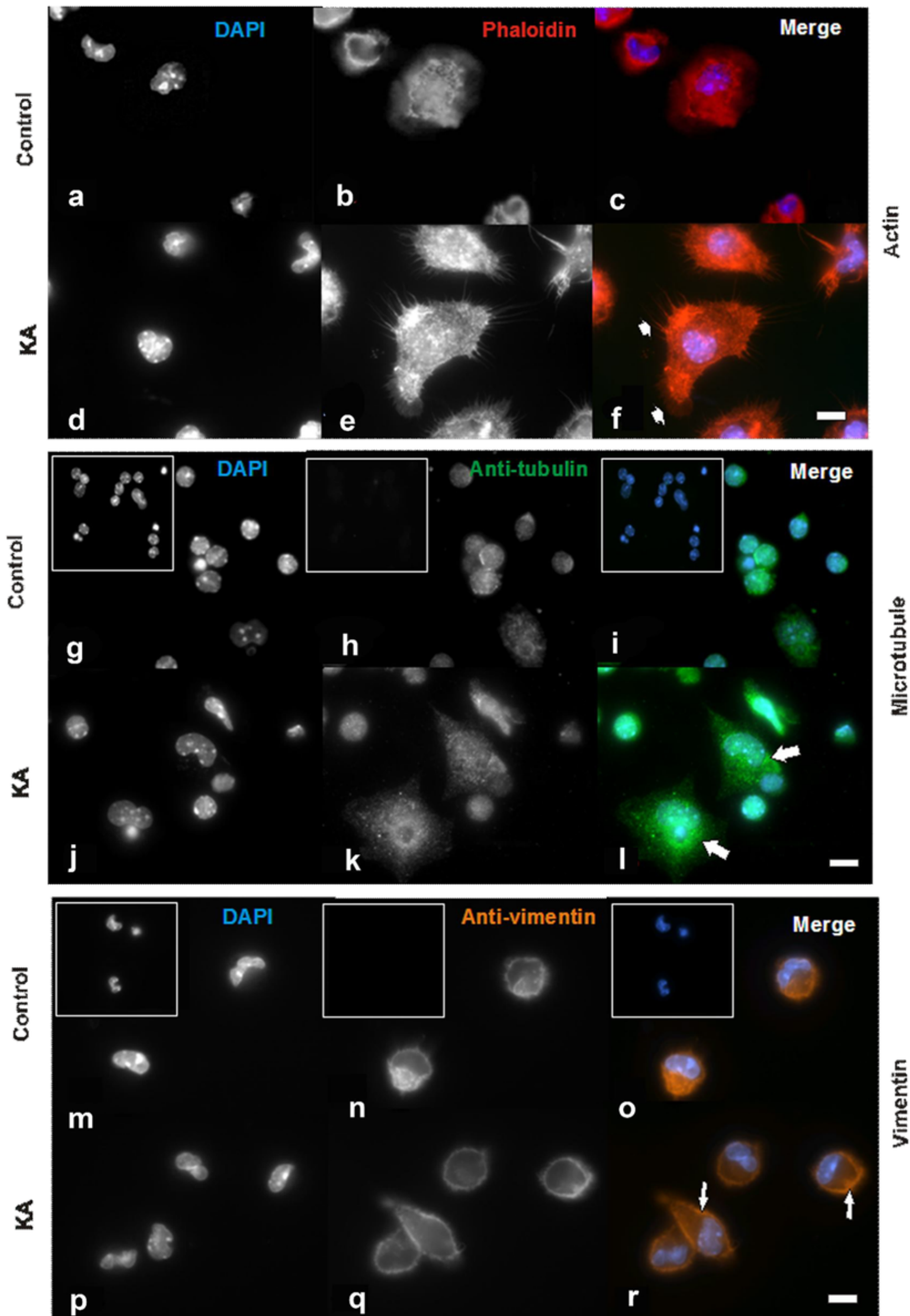

Figure 4 Cytoskeleton compounds detected by fluorescence in murine peritoneal macrophages exposed to 50 μg/ml KA for 1 h
(**a–f**) Fluorescence labelling of actin filaments with phalloidin and DAPI in untreated cells (**a–c**), KA-treated macrophages (**d–f**) with enhanced filopodium establishment (small arrows); (**g–l**) fluorescence labelling of microtubules with polyclonal anti-tubulin antibody and DAPI in untreated cells (**g–i**), KA-treated macrophages (**j–l**) with microtubule polymerization extending from the nucleus membrane to the cell membrane (arrows); (**m–r**) immunofluorescence labelling of intermediate filaments (vimentin) with polyclonal anti-vimentin antibody and DAPI in untreated cells (**m–o**), KA-treated macrophages (**p–r**) with greater distribution of vimentin on the macrophage surface (thin arrows); Insets: negative control of Alexa594-labelled goat anti-rabbit IgG; Bars: 10 μm.

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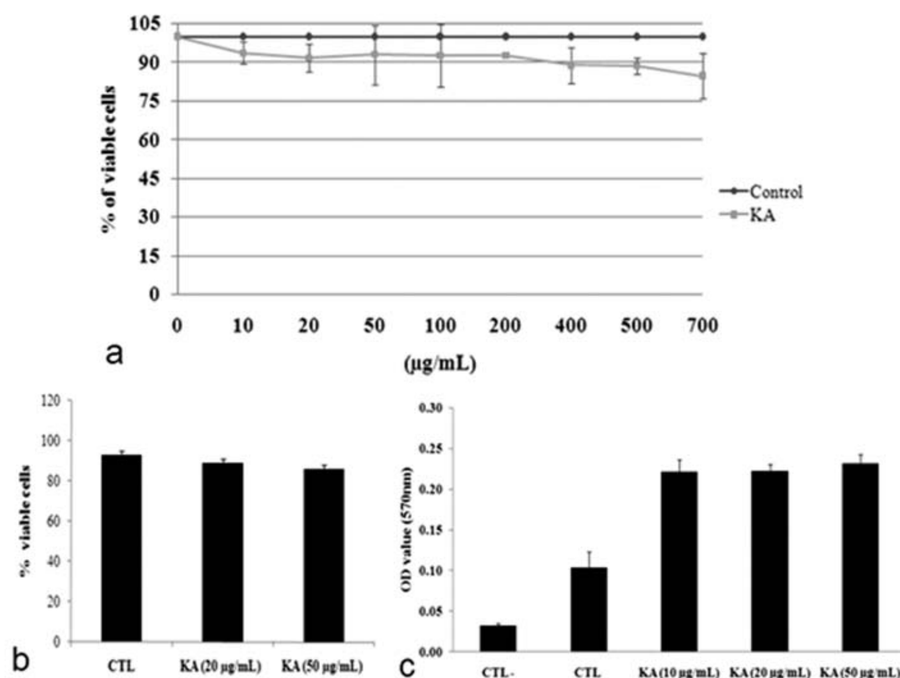

Figure 5 Viability of macrophages treated with KA, as measured by MTT reduction assay, PI assay and NR uptake after 1 h of treatment
(**a**) The MTT assay. The viability of the untreated control was taken as 100%, and the percentage viability was calculated for different concentrations of KA. No differences were found at 10–700 μg/ml KA when compared with the control. (**b**) PI assay. The viability of treated and untreated macrophages was recorded by microscopic analysis. PI-stained macrophages and non-stained cells were counted. Results were provided as the percentage of viable cells. (**c**) NR uptake assay. The viability of treated and untreated cells is shown as absorbance, recorded at an optical density (OD) 570 nm. CTL, control group, untreated macrophages; CTL – non-viable macrophages treated with 10% formaldehyde.

3. Discussion

Macrophages are major components of the innate immune response and play diverse functions related to location and activation state. Activated macrophages exhibit increased adhesion to and spreading over substrates, altered phagocytic activity and increased ROS generation (Bilitewski et al., 2008). Studies have demonstrated that different drugs and bioproducts induce macrophage activation (Pereira et al., 2005; Lopes et al., 2006; Morrow et al., 2007; Maity et al., 2009; Tiwari and Kakkar, 2009).

The present study tested whether KA, which is a secondary metabolite produced by fungi, induces macrophage activation. Results demonstrate that KA induces both morphological and physiological alterations in resident macrophages, as shown by enhanced cell spreading and changes in the cytoskeleton pattern (confirmed by electron microscopy and immunofluorescence). Moreover, KA induced the reorganization of microtubules and actin filaments in the macrophages. Previous studies have shown that stable microtubules contribute to actin remodelling, and the extension of filopodium and actin filaments can lead to microtubule buckling and modulate microtubule turnover during cell spreading and phagocytosis (Gupton et al., 2002). In the present study, up-regulation of vimentin-type intermediate filaments was detected on the surface of macrophages treated with KA. This characteristic has also been reported in monocytes infected with *Mycobacterium tuberculosis*; the expression of vimentin on the monocyte surfaces was stimulated by TNF-α and must be related to natural killer cell-mediated lysis and through the activation of the oxidative mechanism (Mor-Vaknin et al., 2002; DePianto and Coulombe, 2004; Garg et al., 2006). In the present study, fluorescence microscopy also revealed actin filaments arranged in parallel in many filopodium structures in KA-treated cells labelled with phalloidin. Microbial products can stabilize actin filaments in monocytes/macrophages and increase their adhesion (Williams and Redley, 2000). Intermediate filaments seem to be associated with microtubules in order to ensure structural support for organelles, such as mitochondria (Correia et al., 1999; Tang et al., 2008) and also seem to be linked to actin filaments by fimbrin collocated in the filopodium. This result, therefore, suggests that KA is able to induce cytoskeleton rearrangement, associated with filopodium establishment, as previously demonstrated for the RAW 264.7 macrophage line, when treated with an exopolysaccharide, obtained from the mushroom, *Lentinus edodes* (Lee et al., 2008). Further studies are needed to identify the mechanism that induces this cytoskeleton reorganization.

Another characteristic feature seen in KA-treated macrophages was the greater phagocytic activity towards yeast,

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in comparison with untreated cells. The same results were observed using NR uptake, which showed a dose-dependent increase in phagocytic ability. KA has been reported to potentiate neutrophil phagocytosis (Niwa and Akamatsu, 1991). Previous studies have demonstrated that polysaccharides obtained from mushrooms, commonly used in Asian cultures, have a well-known immunomodulatory effect and enhance the host immune system by activating macrophages. This activation was associated with phagocytosis mechanism, which is important for immune reaction initiation and antigen presentation (Chen et al., 2010).

Phagocytosis is a mechanism of innate immune response for the removal of invading pathogens and clearance of apoptotic cells (Underhill and Ozinsky, 2002; Stuart and Ezekowitz, 2005). This mechanism can be potentialized by cytokines such as TNF-α and INF-γ, as well as by microbial products (LPS) and some drugs/bioproducts (Aderem and Underhill, 1999; Cho, 2008). KA seems to induce the phagocytosis of microorganisms by macrophages through cytoskeletal rearrangement and greater spreading over the substrate. This process was associated with ROS production, as detected by the NBT cytochemical reaction. Approximately 70% of KA-treated macrophages presented formazan deposits. Intrinsic ROS generation was also observed on neutrophils treated with KA (Niwa and Akamatsu, 1991). Other studies have shown the activation of macrophages after treating with polysaccharides from mushrooms. This activation is associated with higher cytokine rates, ROS/NO levels and phagocytosis mechanism (Lee et al., 2008; Kuo et al., 2008; Martins et al., 2008; Lee et al., 2009).

In conclusion, KA induced macrophage activation, cytoskeleton rearrangement, an increase in phagocytosis and in ROS production, with no cytotoxic effects on mammalian cells. Thus, this study provides further evidence that KA could be used to induce macrophage activation for the combat of pathogens.

Author contribution

Ana Paula Rodrigues was responsible for the cell culture and optical microscopy assays. Antônio Sergio Carvalho was in charge of the bioproduct obtention. Alberdan Santos was responsible for the fungal cultures and bioproduct obtention. Claudio Alves was responsible for the biotechnology assays. José Luiz do Nascimento was in charge of the biochemistry assays. Edilene Silva was responsible for electron microscopy assays.

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