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# Immunological parameters of macrophages infected by methicillin resistant/sensitive *Staphylococcus aureus*

## *Parâmetros imunológicos de macrófagos frente à infecção por Staphylococcus aureus metilicina sensível/resistente*

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### ABSTRACT

**Introduction:** Could changes in *Staphylococcus aureus* cellular walls, which are commonly associated with multidrug resistance phenotype, influence their immune evasion mechanisms? **Objective:** To evaluate the microbicide response and survival of alveolar macrophages after in vitro infection with methicillin-sensitive and methicillin-resistant *Staphylococcus aureus*. **Material and methods:** We used 20 adult, male, albino, Wistar rats. The alveolar macrophage samples were obtained after tracheostomy and bronchoalveolar lavage. The alveolar macrophages were cultured in the proportion of 1:1 cells/ml Roswell Park Memorial Institute (RPMI) medium/well and isolated by plate adhesion. For the assessment of immunological parameters, four systems were established: negative control, positive control, methicillin sensitive *S. aureus* (MSSA) and methicillin resistant *S. aureus* (MRSA). **Results:** When comparing MRSA and MSSA systems, there was no significant difference as to adhesion and phagocytosis rates, superoxide anion production and macrophage viability. By analyzing the kinetics of nitric oxide production, after 4 to 10 hours of cellular culture incubation, there was lower average production of this radical in the MRSA system when compared to MSSA. However, after 12 hours, no differences were detected between both systems. **Conclusion:** It is claimed that methicillin resistance may be a factor that influences the bacteria's ability to escape from macrophage microbicide response. Although the results of some immunological parameters were similar in the surveyed systems, the oscillations occurred during the production of nitric oxide may contribute significantly to the survival of *Staphylococcus aureus*.

**Key words:** macrophages; *Staphylococcus aureus*; methicillin.

### INTRODUCTION

The organism invasion by pathogens may cause deregulation of microbicide response in immune system cells<sup>(23)</sup>. This mechanism is triggered by several microorganisms, since it enables the development of the infection process and it also optimizes host survival<sup>(11)</sup>.

In order to spread infection, *Staphylococcus aureus* has developed several mechanisms to resist to the immunological system, which involve countless virulence factors that are coordinately expressed during the different stages of infection. This wide range of factors, either occurring on the surface or secreted in the external medium, may reduce phagocytic microbicide response, induce cellular death

and/or inhibit antibiotic action<sup>(8, 23)</sup>. Despite its scientific relevance, there are hardly any studies that evaluate the activation and survival of macrophages after infection by multi-resistant bacterial strains.

Since the 1960's, infections by methicillin resistant *S. aureus* (MRSA) have become a major public health problem worldwide, inasmuch as it is linked with higher mortality rates<sup>(5)</sup>. It is believed that MRSA strains have distinctive virulence mechanisms, which may be more exacerbated in comparison with MSSA strains<sup>(8)</sup>.

Several clinical studies based on morbidity and mortality rates show that MRSA is more virulent than MSSA. Nevertheless, laboratory studies that assess the presence and magnitude of pathogenic

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mechanisms and virulence factors of MSSA and MRSA strains have yielded conflicting results<sup>(12, 20)</sup>. Thus, the present investigation had the objective to evaluate immunological parameters of alveolar macrophages of rats after in vitro cellular infection with MSSA and MRSA strains.

## MATERIAL AND METHODS

The study protocol was approved by the Ethics Committee in Animal Experiments from Universidade Federal de Pernambuco – Centre of Biological Sciences (Protocol 239/09 – 24/11/2009).

### Animals

We applied 20 male, albino, Wistar rats (90-120 days) from the vivarium of UFPE-Nutrition Department. The animals were kept in propylene cages with galvanized wire lid, containing 3 animals per cage at most, with free access to water and food. The room temperature was controlled at  $22 \pm 1^\circ\text{C}$ , and the animals were exposed to 12:12h photoperiodic light-dark cycles.

### *Staphylococcus aureus* strains

We used methicillin resistant *Staphylococcus aureus* (The American Type Culture Collection [ATCC] 33591) and methicillin sensitive *S. aureus* (ATCC 29213) due to its distinctive resistance profile and its relevance concerning public health. The bacteria were maintained in Tryptic Soy Broth (TSB) with 20% glycerol at  $-20^\circ\text{C}$ . 24 hours prior to each experiment, strains were seeded in blood agar dishes (agar with 5% lamb blood) and incubated at  $37^\circ\text{C}$ . Some colonies were transferred to tubes containing sterile PBS in order to achieve approximately 0.15 nm turbidity by spectrophotometer at 570 nm. According to Lu and McEwan<sup>(12)</sup>, this absorbance corresponds to  $10^6$  UFC/ml of PBS.

### Bronchoalveolar lavage (BAL)

The animals were anesthetized with chloralose-urethane (0.5 and 12.5%, respectively) at the proportion of 8 ml/kg administered intraperitoneally. The BAL was collected with a saline injection (0.9%) through a plastic cannula inserted in the trachea. Several aliquots of 3 ml were injected and collected in 50 ml conical polypropylene tubes (Falcon, Sigma). We obtained approximately 30 ml of BAL per animal.

### Alveolar macrophage culture

The BAL was centrifuged at 1,500 RPM for 15 minutes. The precipitate was resuspended in RPMI 1640 (Gibco-Invitrogen Corporation<sup>®</sup>) with 3% fetal bovine serum (Gibco-Invitrogen Corporation<sup>®</sup>) and antibiotics (penicillin 100 U/ml and streptomycin 100 µg/ml). After dilution in Trypan blue (1:10), the

BAL cells were counted with the aid of a Neubauer camera. The concentration  $10^6$  cells/ml of RPMI was standardized. Subsequently, the cells were transferred to 35 mm diameter culture dishes (6 wells, Falcon<sup>®</sup>). After an hour at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$ , the supernatant with non-adherent cells was discarded and 1 ml of RPMI medium was added.

### Systems

Two systems were applied for the assessment of phagocytic index:

- MSSA, MA and 100 µl of bacterial inoculum of sensitive strain (ATCC 29213);
- MSSA, MA and 100 µl of bacterial inoculum of resistant strain (ATCC 33591).

Three systems were established for the adhesion index (AI) and superoxide anion kinetics: negative control (NC), containing exclusively alveolar macrophages, MSSA and MRSA, as described previously. The positive control (PC) was included for the assessment of nitric oxide kinetics and for the viability of alveolar macrophages, which contained MA and 10 µl of LPS (*Escherichia coli* serotype; 055:B5, Sigma<sup>®</sup>). The plates were incubated at  $37^\circ\text{C}$  in humid atmosphere and 5% of  $\text{CO}_2$ .

### Adhesion index

After incubation for an hour, 10 µl of culture supernatant was collected and added to Trypan blue at 1:10 dilution. The counting was performed with Neubauer camera. The AI was calculated through the formula described by Fuente *et al.*<sup>(2)</sup>:  $\text{AI} = 100 - \text{non-adherent cells/ml divided by the initial number of cells/ml} \times 100$ . The results were expressed in percentage of non-adherent cells.

### Phagocytic index (FI)

In this research, two systems (MSSA and MRSA) were established in test tubes. The contents were homogenized and uniformly distributed onto slides for optic microscopy. The slides were incubated at  $37^\circ\text{C}$  for an hour. Subsequently, they were washed for the removal of non-adherent cells, stained and assessed through optic microscopy by a skilled observer, who was unaware of the systems surveyed herein. The results were expressed in percentage of phagocytic cells after total counting of 100 cells.

### Kinetics of anion superoxide production ( $\text{O}_2^-$ )

$\text{O}_2^-$  was detected by its ability to reduce iron-cytochrome C chemically (30 mg/ml in Hank's Balanced Salt Solution [HBSS],  $2.4 \times 10^{-3}$  M, iron-cytochrome C of horse mitochondrion, type VI, SIGMA Chemical Company, St. Louis, MO). After adding phorbol miristate acetate/ PMA (Sigma<sup>®</sup>) to Hank's solution (HBSS, Gibco-Invitrogen Corporation<sup>®</sup>), 3 systems of discontinuous analysis (NC, MSSA and MRSA) were prepared at 2 µg/ml with assessment between the first and the second hour of incubation. The reading was performed

through immunosorbent assay linked with enzyme (ELISA- BIORAD, Model 680) with a 55 nm filter. The quantification of  $O_2^-$  was expressed in nM/ml.

### Kinetics of nitric oxide production (NO)

The NO production was determined through the concentration of supernatant nitric in culture cells<sup>(10)</sup>. 100  $\mu$ l of supernatant cells was collected every two hours totaling 24 hours of incubation. 50  $\mu$ l of Griess reagent (1.5% of sulfanilamide in 5% of  $H_3PO_4$ , 0.1% in  $H_2O$  of naphthyl ethylenediamine dihydrochloride) was added to the cell culture supernatants for nitric quantification. After 15 minutes at rest and at room temperature, the reading was performed by Elisa (BIORAD, Model 680) with a 550 nm filter. The nitric concentration was calculated through mean values of  $NaNO_2$  standard curve and the collected data were expressed in  $\mu$ M of nitrite/ml.

### Viability of alveolar macrophages

The cellular viability was assessed by mitochondrial reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyl tetrazolium bromide (MTT) to formazan, as described by Mosmann<sup>(15)</sup>. After a 24-hour incubation period, the cell cultures were washed with PBS (1 $\times$ ) at room temperature. Subsequently, they were incubated with 550  $\mu$ l of PBS and 55  $\mu$ l of MTT solution for two hours under light protection. After this period, 200  $\mu$ l of PBS and 200  $\mu$ l of dimethylsulfoxide (DMSO) were added and, afterwards, the cellular monolayer was scraped. The quantification of formazan solution was performed with ELISA reader (BIORAD, Model 680) with a 570 nm filter. The results were expressed in percentage of viable cells, applying NC results as reference.

### Statistical analysis

The analysis of variance (ANOVA) was applied for the comparison of multiple means. When ANOVA results revealed significant difference, Tukey test was performed in order to identify inconsistency in results. In the analysis of only two systems, which were used in the assessment of superoxide kinetics and FI, test *t Student* was employed for parametric data and Mann-Whitney test for non-parametric data. The results were expressed by mean  $\pm$  standard error. The significance was defined by  $p < 0.05$ . The statistical program employed in the analysis was Sigma Stat<sup>®</sup>.

## RESULTS

### Adhesion index

The analysis of non-adherent cells revealed no difference among the systems: NC ( $87.5 \pm 3.0$  %), MSSA ( $90.3 \pm 2.6$  %) and MRSA ( $93.3 \pm 3.1$  %),  $p > 0.05$  (Figure 1).

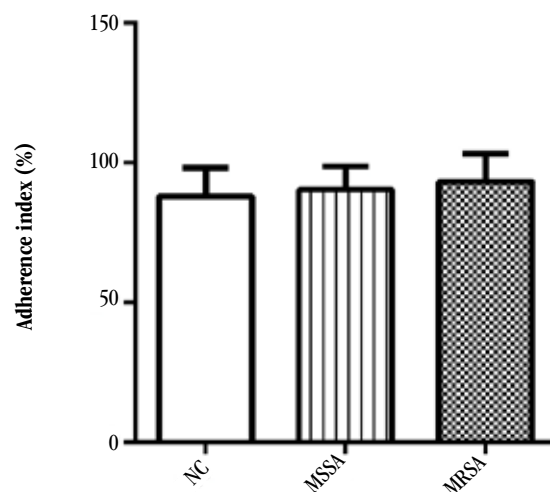


FIGURE 1 – AI of alveolar macrophages from adult Wistar rats for the following systems: NC, MSSA and MRSA. Values expressed as mean  $\pm$  standard error ( $n = 20$ )

AI: adhesion index; NC: negative control; MSSA: methicillin sensitive *S. aureus*; MRSA: methicillin resistant *S. aureus*.

### Phagocytic index

As to PI, there was no difference between macrophages infected in vitro with MRSA ( $12.1 \pm 3.0$  %) and MSSA ( $10.4 \pm 3.2$  %),  $p > 0.05$  (Figure 2).

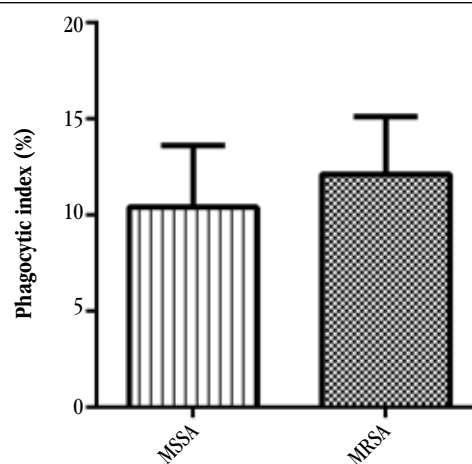
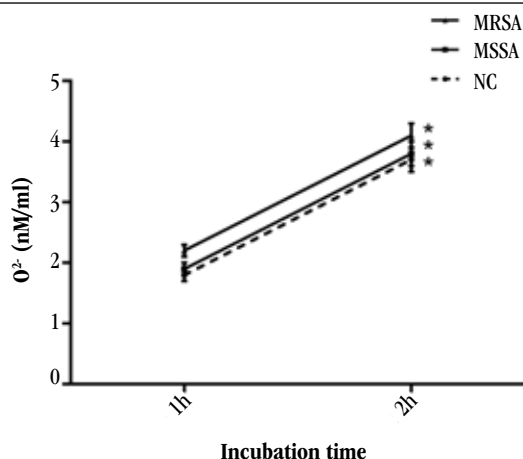


FIGURE 2 – PI of alveolar macrophages from adult Wistar rats for MSSA and MRSA systems. Values expressed as mean  $\pm$  standard error ( $n = 20$ )

PI: phagocytosis index; MSSA: methicillin sensitive *S. aureus*; MRSA: methicillin resistant *S. aureus*.

### Kinetics of $O_2^-$ production

The kinetics of  $O_2^-$  production in the supernatant of macrophage cultures was higher in all systems in the second hour of incubation: NC (1h:  $1.8 \pm 0.1$ /2h:  $3.7 \pm 0.2$  nM/ml,  $p < 0.001$ ); MSSA (1h:  $1.9 \pm 0.1$ /2h:  $3.8 \pm 0.2$  nM/ml,  $p < 0.001$ ); MRSA (1h:  $2.2 \pm 0.1$ /2h:  $4.1 \pm 0.2$  nM/ml,  $p = 0.009$ ). However, regarding comparison within systems, there was no difference in  $O_2^-$  production when analyzed in the first two hours of incubation,  $p > 0.05$  (Figure 3).



**FIGURE 3** – Kinetics of  $O_2^-$  production by alveolar macrophages from adult Wistar rats for NC, MSSA and MRSA systems after one and two-hour incubation periods. Values expressed as mean  $\pm$  standard error ( $n = 20$ )

\*:  $p < 0.05$  between 1h and 2h for NC, MSSA and MRSA.

NC: negative control; MSSA: methicillin sensitive *S. aureus*; MRSA: methicillin resistant *S. aureus*.

### Kinetics of NO production

The kinetics of NO revealed an increase in the production of NO when compared with other incubation periods ( $p < 0.001$ ). Similar results were detected in PC, MSSA and MRSA, with  $p < 0.001$ . The maximum liberation peak of this free radical occurred after a 22h incubation period in PC (CP-22h:  $7.55 \pm 0.295 \mu\text{M/ml}$ ), 4h in the MSSA system (MSSA-4h:  $11.778 \pm 0.413 \mu\text{M/ml}$ ) and 8h in the MRSA system (MRSA-8h:  $11.663 \pm 0.16 \mu\text{M/ml}$ ).

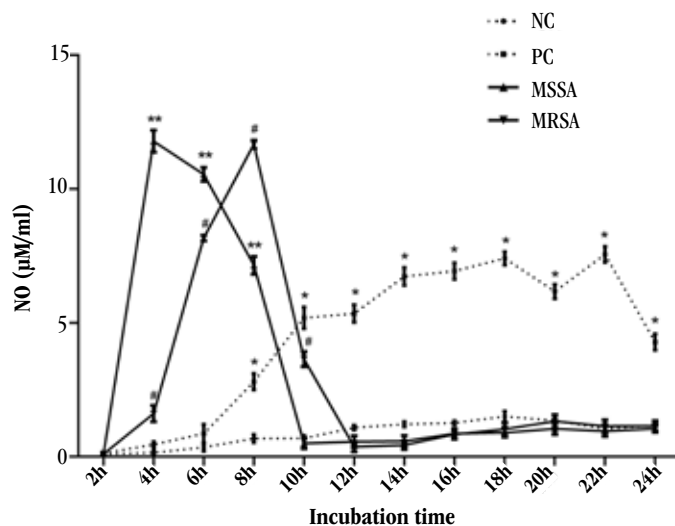
When comparing NC and PC, there was a higher production of nitrogen reactive species in PC after an 8h incubation period, remaining steady until the end of the analysis ( $p < 0.001$ ). In the MSSA and MRSA systems, there were differences in the production of NO in the following periods: 4h, 6h, 8h and 10h. Nevertheless, after 12h the nitrite concentration within the systems was similar to NC. During the kinetic period, the mean nitrite production was lower in MRSA in contrast with MSSA,  $p < 0.05$  (Figure 4).

### Viability of AM

There was a reduction in the percentage of viable cells infected with MSSA and MRSA strains in comparison with PC (LPS:  $69.2 \pm 0.8\%$ ; MSSA:  $18.5 \pm 0.22\%$ ,  $p < 0.001$ ; MRSA:  $20.7 \pm 0.2\%$ ,  $p < 0.001$ ) (Figure 5).

## DISCUSSION

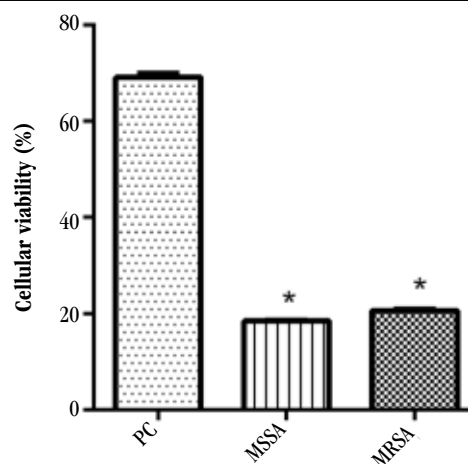
In the present investigation, the coinubation of alveolar macrophages with *Staphylococcus aureus* strains did not induce any change in the adhesion index of these cells. This finding may be justified by the capacity of *Staphylococcus aureus* to evade immune system recognition by means of polysaccharide capsules or biofilm



**FIGURE 4** – Kinetics of NO production by alveolar macrophages from adult Wistar rats for NC, PC, MSSA and MRSA systems. Values expressed as mean  $\pm$  standard error ( $n = 20$ )

\*:  $p < 0.05$  between NC and PC; \*\*:  $p < 0.05$  between NC and MSSA; #:  $p < 0.05$  between NC and MRSA.

NO: nitric oxide; NC: negative control; PC: positive control; MSSA: methicillin sensitive *S. aureus*; MRSA: methicillin resistant *S. aureus*.



**FIGURE 5** – Viability of alveolar macrophages from adult Wistar rats for PC, MSSA and MRSA systems. Values expressed as mean  $\pm$  standard error ( $n = 20$ )

\*:  $p < 0.05$  among PC, MSSA and MRSA.

and the fact that they produce or secrete specific molecules that block phagocyte receptors<sup>(3, 4)</sup>. Some studies suggest that both MSSA and MRSA present the same magnitude in terms of binding to human epithelial cells<sup>(4)</sup> and feline corneocytes<sup>(26)</sup>.

Furthermore, there was no difference as to phagocytic index when comparing MSSA and MRSA strains. Similar results were reported by Salgado *et al.*<sup>(21)</sup>, who compared phagocytic susceptibility to MSSA and MRSA. Mekontso-Dessap *et al.*<sup>(14)</sup> also observed no difference neither in the phagocytic function of Wistar rat neutrophils nor in the survival rate of bacteria inside the cell when compared with methicillin sensitive and resistant strains.

The phagocytosis of microorganisms activate oxidase dependant on adenine dinucleotide phosphatase (NADPH), which induces the production of high levels of superoxide, a process commonly denominated respiratory burst. Studies on the release of this anion describe that there is increasing production of  $O_2^-$  during the first 6h after antigenic stimulus with phorbol myristate acetate<sup>(17)</sup>. Herein, the generation of  $O_2^-$  in supernatant of alveolar macrophage culture increased in the first to the second hour of the incubation period in the control systems as well as in MSSA and MRSA strain systems. According to Mandell<sup>(13)</sup>, regardless of the resistance antimicrobial profile, *S. aureus* seems to neutralize oxygen intermediates formed after its internalization through the release of catalase and staphyloxanthin enzymes.

As to NO production, there was an increase after stimulation with LPS in all analyzed periods. Ferreira-Silva *et al.*<sup>(24)</sup> yielded similar results evaluating the production of NO in Wistar rat macrophages after contact with LPS, which corroborates our investigation. According to Hwang *et al.*<sup>(9)</sup>, LPS is regarded as an efficient macrophage stimulator, insofar as it induces the production of NO, hence being a positive control for the activation of these cells.

Pumerantz *et al.*<sup>(16)</sup> claimed that NO produced by alveolar macrophages play a major microbicide role against *S. aureus*. According to Sasaki *et al.*<sup>(22)</sup>, the expression of induced nitric oxide synthase is initiated 3h after *S. aureus* infection. During the kinetics, a maximum peak of NO release was observed after 4h in the MSSA system and 8h in the MRSA system. These results evinced that the resistant strain activates a more delayed microbicide response of macrophages in comparison with sensitive strains. Thus, this may enable the multiplication of resistant bacteria inside the phagocytes, inasmuch as they do not present antimicrobial mechanisms until the macrophagic activation occurs.

After NO production peaks, a reduction in the concentration of nitrite was detected in the supernatant of MSSA and MRSA systems, and after a 12 hour incubation period, it was similar to basal levels of NC. According to Richardson *et al.*<sup>(18)</sup>, *S. aureus* may evade multiple components of the innate immune response, including the microbicide action of NO. These researchers proved that *S. aureus* is able to adapt metabolically to nitrosative stress, inasmuch as it expresses inducible dehydrogenase (NO-L-lactato). Its production allows *S. aureus* to maintain homeostasis during nitrosative redox stress.

The kinetics of NO production in MSSA and MRSA strains showed that there was oscillation during 4h, 6h, 8h and 10h of incubation. The mean concentration of nitrite during this period was lower in MRSA in comparison with MSSA. This result implies that macrophages may have been activated with lower intensity during the infection by MRSA or that this bacterium may have distinctive mechanisms of evasion to microbicide response. Vaudaux e Waldvogel<sup>(25)</sup> analyzed PI cytolysis of polymorphonuclear leucocytes (PMN) through an in vitro assay. After 30 and 60 minutes of incubation, MRSA strains showed a better survival to phagocytic action of PMN in comparison with MSSA strains.

According to Cutler<sup>(1)</sup>, methicillin resistant staphylococcus present thicker cellular walls and different lipidic content in contrast with those sensitive to this antibiotic. Accordingly, it is possible to claim that the morphology of resistant strains enables a better protection against destruction by lysosomal enzymes or by the oxidative metabolism of activated phagocytes. In 2011, Ferreira *et al.*<sup>(6)</sup> affirmed that MRSA is a major pathogen in nosocomial infections and it is associated with high rates of mortality opposed to infections by MSSA.

As to the viability rate of Wistar rat alveolar macrophages, there was a reduction after a 24h incubation period with MSSA and MRSA in comparison with PC. This finding may explain the low levels of nitrite in culture supernatant. According to Deleo *et al.*<sup>(3)</sup>, there are two possible interactions between bacterium-phagocyte. The bacterial phagocytosis triggers the production of oxygen and nitrogen reactive species as well as lysosome degranulation. This process occurs along with apoptosis induction, hence combating the infection. Alternatively, some bacterial pathogens may cause lysis of phagocytes or induce apoptosis, enabling the bacteria to remain in the medium or the development of the disease.

In order to escape cytoplasmic confinement, *S. aureus* induces phagocyte death by triggering pyroptosis or by releasing leukocidins<sup>(11)</sup>. The main characteristic of pyroptosis is the rupture of the plasmatic membrane with the release of pro-inflammatory intracellular content via caspase-1 (cytoplasmatic protease)<sup>(7)</sup>. The action mechanism of leukocidins causes leukocyte destruction due to the formation of pores in the cellular membrane. Kubica *et al.*<sup>(11)</sup> confirmed that after phagocytosis by macrophages, *S. aureus*, regardless of its antimicrobial resistance profile, survives intracellularly in vacuoles for 3-4 days prior to escaping into the cytoplasm and ultimate cellular lysis. Besides remaining several days inside macrophages, the bacterium was able to escape intracellular confinement and to proliferate in the host organism.

Different bacterial strains may express a higher or lower number of virulence factors such as adhesins and toxins or differ in their ability to produce biofilm and resist to phagocytosis. According to Rossney *et al.*<sup>(19)</sup>, the expression regulation of virulence factors plays a pivotal role in the pathogenicity of *S. aureus*. According to Gordon e Lowy<sup>(7)</sup>, MRSA strains may contain genetic factors that maximize virulence, which allow these bacteria to cause particular clinical syndromes. Further studies with molecular biology techniques could elucidate the differences in macrophage immune response to methicillin resistant and sensitive strains.

## CONCLUSIONS

There are some evidences that methicillin resistance constitutes a factor that influences the ability to evade microbicide response in alveolar macrophages from adult Wistar rats. Although the adhesion capacity, PI,  $O_2^-$  production and viability of infected phagocytes were not altered, there were oscillations in the kinetics of ON production, which may contribute significantly to its pathogenicity and bacterial survival.

## RESUMO

**Introdução:** Modificações nas paredes celulares das cepas de *Staphylococcus aureus* relacionadas com o fenótipo de multirresistência poderiam influenciar seus mecanismos de evasão frente à resposta imune? **Objetivo:** Avaliar a resposta microbicida e a sobrevivência de macrófagos alveolares após infecção in vitro com *Staphylococcus aureus* metilicina sensível/resistente. **Material e métodos:** Utilizaram-se 20 ratos adultos, machos, albinos e da linhagem Wistar. Os macrófagos alveolares foram obtidos após procedimento cirúrgico de traqueostomia, por meios da coleta do lavado broncoalveolar. Os macrófagos alveolares foram cultivados na proporção de 1:1 células/ml de Roswell Park Memorial Institute (RPMI)/poço e isolados pela capacidade de adesão à placa. Para avaliação de parâmetros imunológicos, foram estabelecidos quatro sistemas: controle negativo, controle positivo, *S. aureus* sensível a metilicina (MSSA) e *S. aureus* resistente à metilicina (MRSA). **Resultados:** Ao comparar os sistemas de MSSA e MRSA, não foi observada diferença no índice de aderência, na taxa de fagocitose, na produção do ânion superóxido e na viabilidade dos macrófagos. Ao analisar a cinética de óxido nítrico, houve menor produção média desse radical para o MRSA quando comparado com o MSSA, no período de 4 a 10 horas de incubação das culturas celulares. Entretanto, após 12 horas, não foi detectada divergências entre esses sistemas. **Conclusão:** Sugere-se que a resistência à metilicina poderá ser um fator que influenciará a capacidade de evasão da bactéria à resposta microbicida dos macrófagos. Apesar dos resultados de alguns parâmetros imunológicos terem sido similares entre os sistemas analisados, as oscilações ocorridas durante a produção do óxido nítrico poderão contribuir de forma importante para a sobrevivência da bactéria *Staphylococcus aureus*.

**Unitermos:** macrófagos; *Staphylococcus aureus*; metilicina.

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