

Influence of bacterial suspension storage in the inflammatory response in mice

Influência do armazenamento de suspensão bacteriana na resposta inflamatória em camundongos

Larissa Staurengo-Ferrari¹, Alexandre Yukio Saito², Jacinta Sanches Pelayo³, Sandra Regina Georgetti⁴, Marcela Maria Baracat⁵, Rubia Casagrande⁶, Waldiceu Aparecido Verri Junior⁷

Abstract

The preparation of bacterial suspension is an important procedure used in laboratories for inflammatory evaluation protocols and can be obtained by different methods that need or not need step storage. The aim of this work was investigate the influence of storage of *Staphylococcus aureus* suspension in bacterial viability and its influence in bacteria-induced inflammation *in vivo*. The bacterial suspension of *S. aureus* ATCC 6538 was prepared accordingly to 4th degree of McFarland's Scale by visual comparison. This suspension was used to determine by CFU counting the bacterial viability and for administration to the animals to induce septic arthritis and peritonitis. Twenty four hours of storage reduced the *S. aureus* CFU. As a consequence of reduced bacterial viability, was detected reduced mechanical hyperalgesia, edema and leukocyte recruitment in septic arthritis and leukocyte recruitment and cytokine production bacterial peritonitis. These results demonstrate that storage of bacterial suspension affected bacterial viability, which resulted in diminished inflammatory response *in vivo*, raising the importance of standard procedures for bacterial suspension preparation. A conceivable approach would be to determine the number of CFU at a specific McFarland's scale degree, which will allow the preparation and use a bacterial suspension in the same day for *in vivo* testing.

Keywords: McFarland's Scale. Neutrophil. Inflammation. Septic arthritis. *S. aureus*.

Resumo

O preparo de suspensão bacteriana é procedimento importante para avaliação da inflamação e pode ser obtida por diferentes métodos que precisam ou não do passo de armazenamento. O objetivo deste trabalho foi investigar a influência do armazenamento de suspensão de *Staphylococcus aureus* na viabilidade bacteriana e sua influência na inflamação *in vivo*. A suspensão bacteriana de *S. aureus* ATCC

¹Universidade Estadual de Londrina, Centro de Ciências Biológicas, Departamento de Ciências Patológicas. E-mail: larissaferrari@gmail.com

²Universidade Estadual de Londrina, Departamento de Ciências Biomédicas. E-mail: aysaito@yahoo.com.br

³Universidade Estadual de Londrina, Centro de Ciências Biológicas, Departamento de Microbiologia. E-mail: jspelayo@gmail.com

⁴Universidade Estadual de Londrina, Centro de Ciências da Saúde, Departamento de Ciências Farmacêuticas. E-mail: sangeorgetti@gmail.com

⁵Universidade Estadual de Londrina, Centro de Ciências da Saúde, Departamento de Ciências Farmacêuticas. E-mail: baracat@uel.br

⁶Universidade Estadual de Londrina, Centro de Ciências da Saúde, Departamento de Ciências Farmacêuticas. E-mail: rubiacasa@yahoo.com.br

⁷Universidade Estadual de Londrina, Centro de Ciências Biológicas, Departamento de Ciências Patológicas. Contato principal para correspondência. E-mail: waldiceujr@yahoo.com.br

6538 foi preparada visualmente seguindo o grau 4 da Escala de McFarland. A viabilidade bacteriana nessa solução foi determinada pela contagem de UFC, a qual foi utilizada para induzir artrite séptica e peritonite. O armazenamento por 24 h reduziu as UFC de *S. aureus*. Essa redução da viabilidade bacteriana resultou em diminuição da hiperalgesia mecânica, edema e recrutamento leucocitário na artrite séptica, e recrutamento leucocitário e produção de citocinas na peritonite bacteriana. Estes resultados demonstraram que o armazenamento de suspensão bacteriana afetou sua viabilidade, resultando em diminuição da resposta inflamatória *in vivo*, sugerindo a importância de padronizar procedimentos para o preparo de suspensão bacteriana. Uma abordagem concebível seria determinar o número de UFC em um específico grau da Escala de McFarland, o qual permitirá o preparo e o uso de uma suspensão bacteriana no mesmo dia para os testes *in vivo*.

Palavras-chave: Escala de McFarland. Neutrófilo. Inflamação. Artrite séptica. *S. aureus*.

Introduction

The estimated number of viable cells by determining the number of colony forming unities (CFU) in a bacterial suspension is of great importance in bacteriology, immunology, and inflammation/infection studies in general. There are different methodologies for estimating the number of CFU in a suspension of bacterial cells such as serial dilutions and plating, and using the McFarland's Scale with visual or nephelometric reading (BROCK et al., 1994; MCFARLAND, 1907).

The serial dilutions and plating to establish CFU (BROCK et al., 1994) is a lengthy process in which the suspension is prepared followed by plating a partial volume and counting the CFU after 24h, meanwhile the suspension is held at 8°C until use. The McFarland's Scale (which is basically a nephelometric scale) is a methodology for analysis of visual turbidity of a suspension and is used to estimate the number of bacteria in a given suspension. It consists essentially of a series of standard dilutions containing barium chloride precipitated in sulfuric acid, and the different turbidities indicate degrees of McFarland's scale. This process is useful to correlate the number of bacteria dispersed in isotonic solutions (saline or PBS) (MCFARLAND, 1907). Therefore, by comparing the turbidity of bacteria dispersed in saline or PBS with the standards of McFarland's Scale, it is possible to obtain a good estimation and

optimization of process to determine the number of viable bacteria within the same day of preparation of the bacterial suspension avoiding the storage of sample.

Staphylococcus aureus is a prominent gram-positive human pathogen, with an ability to produce systemic infections (BERENDS et al., 2010) and also is the major contributor to osteomyelitis, invasive endocarditis and septic arthritis (BANNAN, VISVANATHAN, ZABRIESKIE, 1999). Infection by *S. aureus* induces acute and chronic inflammatory response, causing tissue edema and the associated pain (WHALEY, BURT, 1996) and leading to pyogenic or suppurative inflammation (OKOLI et al., 2008). These bacteria are highly efficient phagocytosed by neutrophils culminating in an intense inflammatory response involving a complex cascade of cellular events to eradicate pathogens via oxidative and non-oxidative mechanisms (ANWAR et al., 2009).

As bacterial suspensions of *S. aureus* is used in different inflammatory models of infection, sepsis and septic arthritis (CROSARA-ALBERTO et al., 2002; GUO et al., 2009) or in different methodologies in laboratories, and there is evidence that temperature and time of storage can influence the viability of gram-positive bacteria in suspension such as *Clostridium sporogenes* (MAH; KANG; TANG, 2009), we investigated the effect of storage of bacterial suspension of *S. aureus* and the influence of this process in inflammatory models *in vivo*.

Methods

Animals

Male Swiss mice (25-30 g) from the Universidade Estadual de Londrina, Paraná, Brazil, were used in this study. Mice were housed in standard clear plastic cages with free access to food and water and a light/dark cycle of 12:12 h and kept at 21° C. All testing was performed between 9 a.m. and 5 p.m. in a temperature-controlled room. Animal care and handling procedures were approved by the Ethics Committee of the Universidade Estadual de Londrina, which follows the National Council of Science. All efforts were made to minimize the number of animals used and their suffering.

Staphylococcus aureus suspension preparation

S. aureus was obtained from ATCC (American Type Culture Collection, U.S.A.) number 6538. Twenty four hours before each experiment samples of bacteria were cultured in blood agar medium at 37° C. The bacterial suspension was centrifuged and the pellet was resuspended in sterile phosphate-buffered saline (PBS) to fit 4th degree of McFarland's Scale by visual comparison, for serial dilutions to confirm bacterial viability and administration to the animals to evaluate the effect of storage in inflammation. The number of colony forming units (CFU) of the bacterial suspension was determined through serial dilutions and plating on BHI agar dishes, and viable CFU determined after 24 h.

Induction of bacterial arthritis and neutrophil migration

The septic arthritis was induced by local injection in the femur-tibial joint of mice of *S. aureus* suspension. As control other animals received 10 µL of sterile saline. The mice were sacrificed at the 15th day after inoculation of bacteria and the synovial cavity of knee joint was washed twice with 5 µL saline contained 10 mM EDTA (ROCHA et al., 2008). The total number of infiltrating cells was determined in a Neubauer Chamber diluted in Turk's

solution and the differential counts were performed in slices stained by the Rosenfeld method.

Evaluation of articular hyperalgesia

The articular mechanical hyperalgesia (decreased nociceptive threshold withdrawn) of the femur-tibial joint was evaluated using a previous method with modification (PINTO et al., 2010; VERRI JUNIOR et al., 2008). In a quiet room, mice were placed in acrylic cages (12 x 10 x 17 cm high) with a wire grid floor 15-30 min before testing for environmental adaptation. Stimulations were performed only when animals were quiet, did not display exploratory movements or defecation, and were not resting on their paws. In these experiments, an electronic pressure-meter was used. It consists of a hand-held force transducer fitted with a polypropylene tip (IITC Inc., Life Science Instruments, Woodland Hills, CA, USA). For this model, a large tip (4.15 mm²) was adapted to the probe. An increasing perpendicular force was applied to the central area of the plantar surface of the hind paw to induce flexion of the femur-tibial joint followed by paw withdrawal. A tilted mirror below the grid provided a clear view of the hind paw. The electronic pressure-meter apparatus automatically recorded the intensity of the force applied when the paw was withdrawn. The test was repeated until three subsequently consistent measurements (i.e. the variation among these measurements was less than 1 g) were obtained. The flexion-elicited mechanical threshold was expressed in grams (g).

Evaluation of joint edema

The edema of femur-tibial joints was evaluated by measurement of the transverse diameters of femur-tibial joints using an analogical caliper (Mitutoyo Corp., Kanagawa Japan). Values of femur-tibial joint thickness are expressed as the difference between the diameter measure before (basal) and after induction of articular inflammation in millimeters (VALERIO et al., 2009).

Peritonitis Model and neutrophil migration

The peritonitis was induced through intraperitoneal injection of *S. aureus* suspension. The animals received bacterial suspension or saline into the peritoneal cavity in a volume of 500 μ L. The quantity of bacteria injected was 5×10^8 CFU/ml. Neutrophil migration was assessed 6h after i.p. injection of bacterial suspension. The peritoneal cavities of mice were then washed with 2 mL of saline containing 10 mM EDTA (VERRI JUNIOR et al., 2007). The exudates were collected by aspiration for determination of the total number of leukocytes in a Neubauer chamber diluted in Turk's solution. Differential cell counts were performed in slices stained by the Rosenfelt method (VERRI JUNIOR et al., 2007).

Determination of cytokine levels

The cytokine levels were detected in peritoneal exudates, 2h after i.p. injection of bacterial suspension. Peritoneal exudates were harvested by injecting 1 ml of PBS. IL-1 β and TNF- α levels were determined by ELISA according to the manufacture's instructions (eBioscience). The results are expressed as pg/mL.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 4.0 (La Jolla, 5 CA). Results are presented as means \pm SEM of 2 independent experiments. The "n" in the legends refers to the number of mice per group in each experiment. The differences between the experimental groups were compared by one-way ANOVA and individual comparisons were subsequently made by Tukey's post hoc test. The level of significance was set at $P < 0.05$.

Results

Standardization of the number of S. aureus CFU in 4th degree of McFarland's scale suspension

S. aureus suspension was prepared from colonies incubated overnight at 37oC on blood agar plates.

At three different days, suspensions were prepared in accordance with the 4th degree of McFarland's scale. The suspensions were diluted sequentially for subsequent plating in triplicate with a fixed volume to determine the number of CFU in each suspension after 24 h incubation at 37oC. The number of bacteria was considered within the range of 30 to 300 CFU per plate, as previously described¹. The mean of three experiments performed in triplicate was 1.67×10^9 /mL (Table I).

Table I - Mean values of CFU in suspensions prepared accordingly to 4 degree of McFarland's scale at different days

McFarland's Scale	Means of Values (CFU $\times 10^9$ / ml)
Day 1	1.375
Day 2	2.050
Day 3	1.605
Mean of Means \pm SEM	1.67 \pm 0.198

Source: Authors

A suspension of *Staphylococcus aureus* ATCC (American Type Culture Collection, U.S.A.) number 6538 was cultured in Blood Agar at 37°C for 24 hours and prepared in PBS to fit the 4 degree of McFarland's Scale. Serial dilutions of the suspension were prepared and immediately plated. After 24 hours of incubation at 37°C it was determined the number of viable bacteria (colony forming unity – CFU). The number of bacteria was considered within the range of 30 to 300 CFU per plate, as previously described (BROCK et al., 1994). There was no significant difference between experiments.

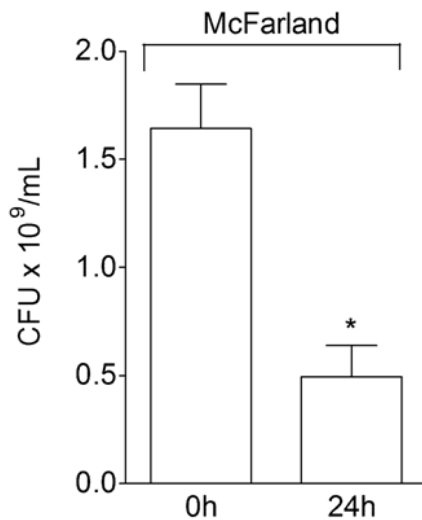
This value was considered the mean CFU in 4th degree of McFarland's scale and was used for

subsequent experiments. There was no significant difference between the three experiments.

Effect of storage in S. aureus suspension viability

To examine the effect of storage in the viability of bacteria, a bacterial suspension of *S. aureus* fitting 4th degree of McFarland Scale was plated for serial dilution at 0h (before storage) and 24h after storage at 80C, which is a usual temperature in laboratory routine. The number of viable bacteria was determined by plated serial dilution and counting the number of CFU 24h after plating. Total number of CFU per plate was considered valid if ranging between 30 and 300 CFU per plate¹ (Figure 1). It was detected that the storage for 24h significantly reduced the number of viable *S. aureus* (Figure 1).

Figure 1 - Storage of *S. aureus* suspension diminishes its viability.



Source: Authors

S. aureus ATCC (American Type Culture Collection, U.S.A.) number 6538 were cultured in blood agar medium at 37° C for 24 hours. A bacterial suspension was prepared accordingly to 4th degree of McFarland Scale. The number of colony forming units (CFU) was determined through serial log

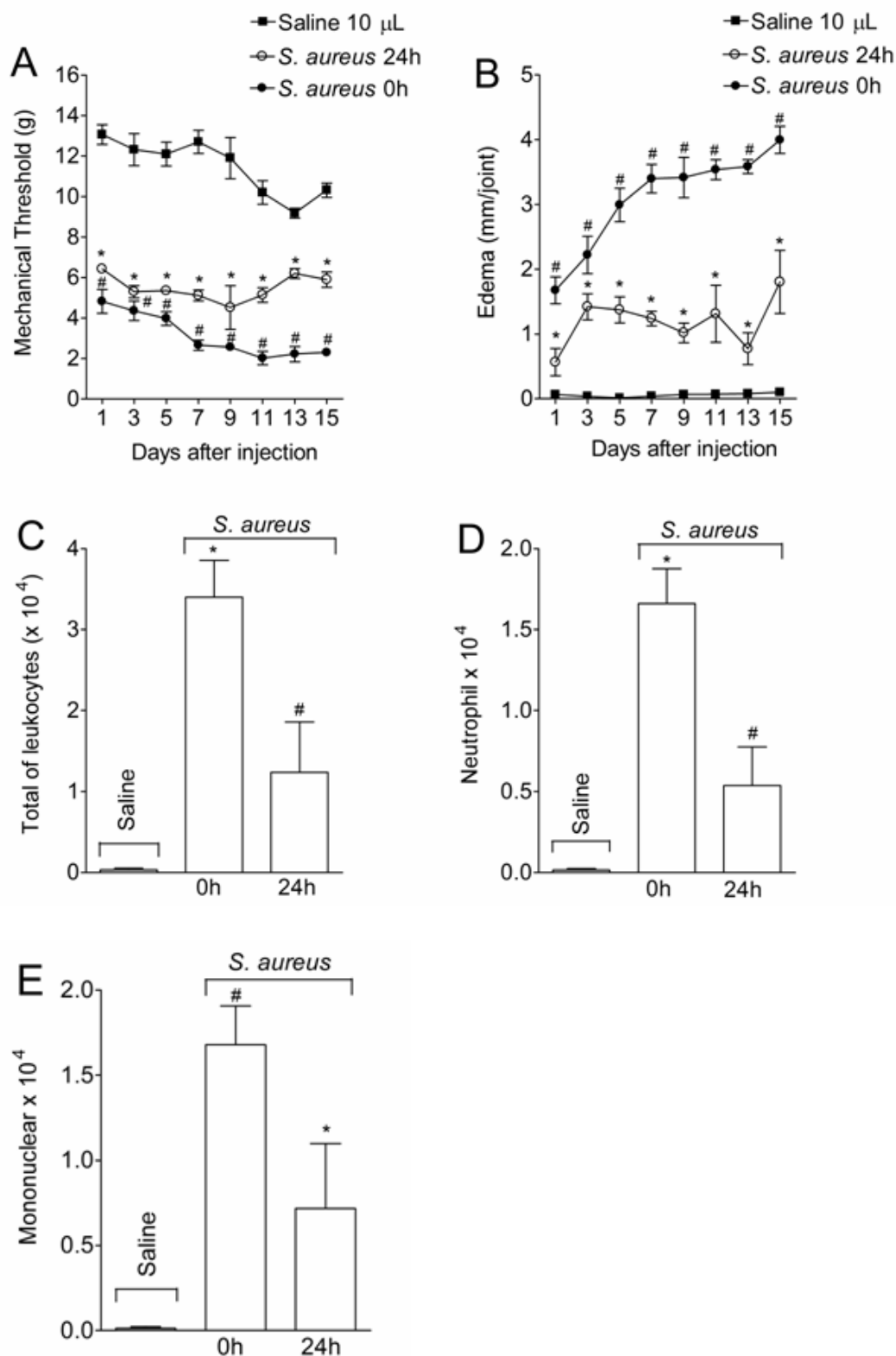
dilution and plating on BHI agar dishes using non-stored (0 h) and stored (24 h) bacteria suspension samples. The storage was performed at 80C for 24 h. n = 3, representative of two separated experiments. *P<0.05 compared to 0 h suspension. *t* test

Thus, these results indicate that storage of *S. aureus* at 80C for 24h reduces the number of viable bacteria in the suspension prepared accordingly to McFarland's Scale.

Effect of storing S. aureus suspension in septic arthritis development in mice

S. aureus suspension was prepared according to 4th degree of McFarland's scale, and 1 x 10⁷ CFU was injected intra-articularly (i.a., 10 µl) in the femur-tibial joint of mice at 0 h (before storage) and 24 h (after storage at 80C) (Figure 2). The i.a. injection of both suspensions (0 and 24 h) induced significant mechanical hyperalgesia (Figure 2A) and edema (Figure 2B) in mice. Nevertheless, the i.a. administration of 0 h *S. aureus* suspension induced significantly greater hyperalgesia (Figure 2A) and edema (Figure 2B) compared to 24 h *S. aureus* suspension. Concerning cellular recruitment, it was observed that i.a. administration of 0 h *S. aureus* suspension induced significant recruitment of total leukocytes (Figure 2C), neutrophils (Figure 2D) and mononuclear cells (Figure 2E) to the knee joint of mice compared to saline group. The cellular recruitment induced by 0 h *S. aureus* suspension was reduced by storage as observed with 24 h *S. aureus* suspension (Figure 2C, 2D and 2E).

Figure 2 - Storage of *S. aureus* suspension diminishes inflammatory response in the peritoneal cavity of mice.



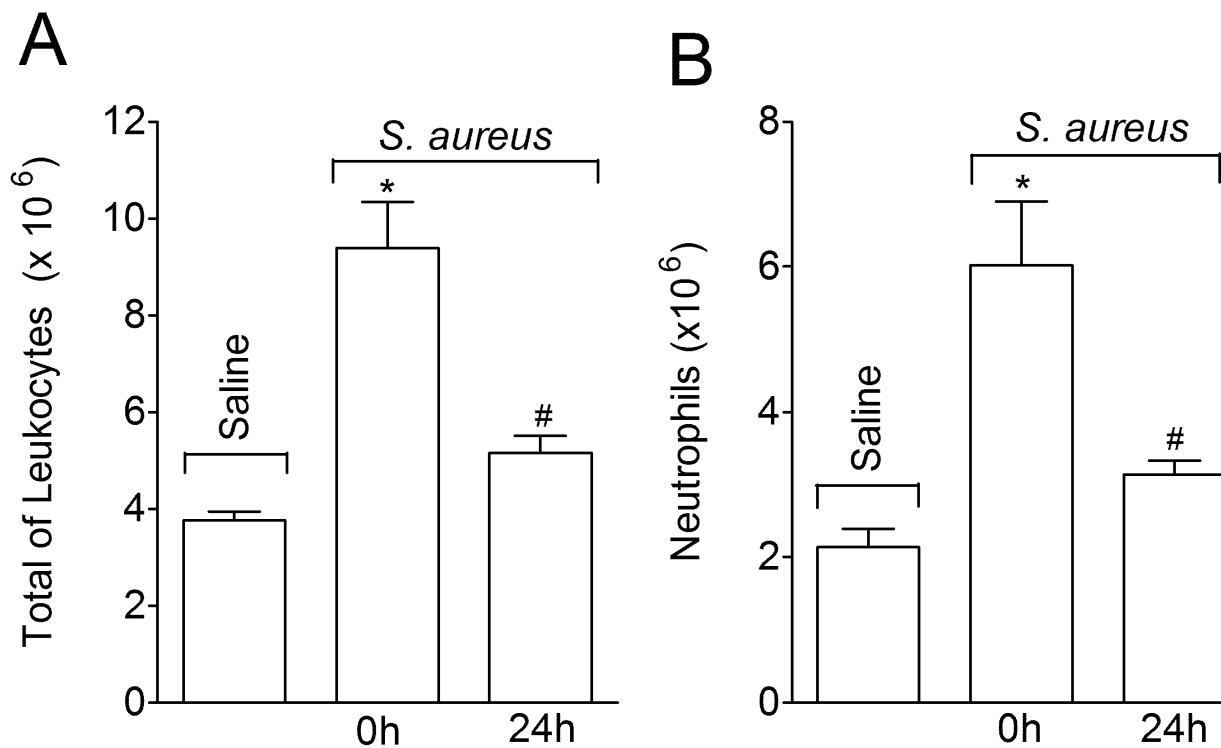
Source: Authors

The bacterial suspension of *S. aureus* (10^7 CFU) or saline (10 μ l) was injected in the femur-tibial joint of mice. The articular hyperalgesia and edema were evaluated over 15 days after bacteria injection with an electronic pressure meter (A) and caliper (B), respectively. Total leukocytes (C), mononuclear cells (D) and neutrophil (E) counts were determined using Newbauer chamber and Rosenfeld stained slices 15 days after *S. aureus* suspension injection. Data are means \pm SEM (n =5), representative of two separated experiments. * P < 0.05 compared to saline group, # P < 0.05 compared to saline group and stored bacterial suspension (24 h). One-way ANOVA followed by Tukey's t test

Effect of bacterial suspension storage in mice peritonitis

S. aureus suspension was prepared according to 4th degree of McFarland's scale, and 5×10^8 CFU/ml were administrated via intraperitoneal route (i.p.) immediately (0h) or after being stored for 24 hours at 8°C (Figure 3). Saline was used (500 μ l/cavity) as negative control. There was a significant increase of total leukocytes (Figure 3A) and total neutrophils (Figure 3B) in the peritoneal cavity of mice that received 0 h *S. aureus* suspension compared to control group. On the other hand, the total leukocytes (Figure 3A) and total neutrophils (Figure 3B) were significantly reduced in 24 h *S. aureus* suspension compared to 0h *S. aureus* suspension. Furthermore, there was no statistical difference between saline group and 24h *S. aureus* suspension group (Figure 3A and 3B).

Figure 3 - Storage of *S. aureus* suspension diminishes inflammatory response in the peritoneal cavity of mice.



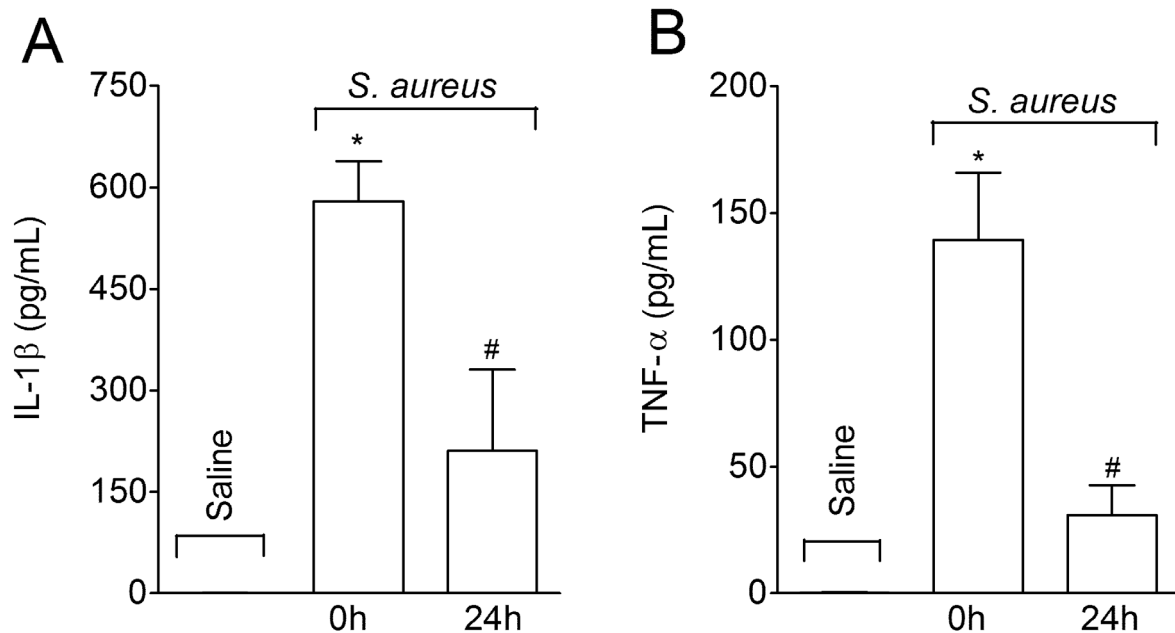
Source: Authors

Mice received an intraperitoneal injection of 5×10^8 CFU of *S. aureus*/ml suspension accordingly to 4th degree of McFarland scale or saline (0 h, 500 μ l). The same suspension was stored at 8°C for 24 h and administered in another group of mice at the same dose. The total number of leukocytes (A) and neutrophils (B) into the peritoneal cavity were quantified 6 h after stimulus injection. n = 7, representative of two separated experiments. *P < 0.05 compared to saline and #P < 0.05 compared to suspension 0 hour. One-way ANOVA followed by Tukey's test.

Effect of storing bacterial suspension peritonitis-induced cytokine production in mice

To examine whether the reduced leukocyte recruitment to the site infection observed in mice that received bacterial suspension stored for 24h at 8°C was due to reduced production of chemotactic mediators, we measured the levels of chemotactic cytokines in peritoneal cavity. Accordingly, as shown in Figure 4, the peritoneal exudates concentrations of IL-1 β (Figure 4A) and TNF- α (Figure 4B) in mice that received 24 h *S. aureus* suspension were significantly lowered compared with mice that received bacterial suspension immediately prepared (0h). Therefore, these data suggest that the reduction in leukocyte recruitment observed is due to reduced production of IL-1 β and TNF- α at the site of infection.

Figure 4 - Storage of *S. aureus* suspension reduces proinflammatory cytokine production in the peritoneal cavity of mice.



Source: Authors

Mice received an intraperitoneal injection of 5×10^8 CFU/mL of *S. aureus* suspension accordingly to 4th degree of McFarland scale or saline (0 h, 500 μ l). The same suspension was stored at 80C for 24 h and administered in another group of mice at the same dose. IL-1 β and TNF- α concentrations (pg/mL) were quantified in the peritoneal exudate (A and B, respectively) 2h after injection by ELISA. n = 5, representative of two separated experiments. *P < 0.05 compared to saline and #P < 0.05 compared to suspension 0 hour. One-way ANOVA followed by Tukey's test

Discussion

An important question in bacteriology and in studies involving bacteria is determining how many living bacteria are in a sample, and its effect upon *in vivo* investigations. Herein, it was demonstrated that storage of bacterial suspension for 24 h to determine the number of bacteria by serial plating reduces the number of viable *Staphylococcus aureus* in the sample, resulting in reduced bacterial load *in vivo*. As a consequence, there is reduced inflammation (e.g. pain, edema, leukocyte recruitment and cytokine production). An interesting and reliable approach to determine bacterial counts in a sample is the use of McFarland's scale to estimate the number of CFU and avoiding the reduction of *S. aureus* viability that affects bacterial count-related *in vivo* inflammation.

It is a common/standard procedure in laboratory routine to determine the number of viable bacteria by preparing a bacterial suspension, which follows two separated procedures. In one procedure, this suspension undergoes serial dilutions followed by plating and determinations of the total number of colony-forming unities (CFU) (MADRID; FELICE; VALENTINUZZI., 1999; RICHARDS et al., 1978). The number of CFU must be in the range of 30-300¹. In the second procedure, the bacterial suspension is stored at approximately 80C for 24 h until the result of bacterial CFU counting is determined in the first procedure. Then, the bacterial suspension can be

used.

An interesting alternative to the CFU counting method is the use of McFarland's scale in which the number of bacteria is estimated by the turbidity of the suspension (MCFARLAND, 1907). This procedure can be performed using equipments to determine the optical density or by visual comparison with a standard scale. In the present study, we used the visual comparison with a standard scale to ensure the use of a method with maximal simplicity and low cost avoiding the need of equipment. The number of viable bacteria in the suspension was confirmed by CFU counting using random suspension (data not shown) and 4th degree of McFarland's scale. It was observed a reduction in the number of viable *S. aureus* CFU by plating the suspension for 24 h of storage compared to 0 h (before storage). Therefore, storage at 80C reduces *S. aureus* viability either using a random suspension (data not shown) or 4th degree of McFarland's scale suspension, indicating that the CFU counting method with 24 h storage will result in reduced *S. aureus* load. Indeed, temperature and time of storage also affects other bacteria such as *Vibrio spp* (SOUZU, 1980) and *Clostridium sporogenes* (MAH, KANG, TANG, 2009). This fact can be attributed to damage of the cytoplasmic membrane as well as to outer membrane of bacteria (MATCHES, LISTON, DANEALU, 1971).

S. aureus was chosen because it is a bacterium with clinical relevance. There is participation of *S. aureus* in septic arthritis (TARKOWSKI et al., 2001; TARKOWSKI et al., 2002) and sepsis originated in the peritoneum (BANNAN, VISVANATHAN, ZABRIESKIE, 1999). *S. aureus* infection induces inflammatory signs of pain, edema and recruitment of leukocytes. Therefore, in the septic arthritis model, it was investigated whether there is reduced inflammation by administrating of stored (24 h) compared to non-stored (0 h) *S. aureus* suspension using 4th degree of McFarland's Scale. The non-stored (0 h) *S. aureus* suspension induced significantly greater intensity of joint mechanical hyperalgesia, edema, and total leukocyte, mononuclear cells and

neutrophils recruitment to the knee joint of mice compared to stored (24 h) *S. aureus* suspension.

In the peritonitis model, it was also possible to determine that there is reduced inflammation by administrating of stored (24 h) compared to non-stored (0 h) *S. aureus* suspension using 4th degree of McFarland's Scale. The non-stored *S. aureus* suspension induced significantly greater recruitment of total leukocytes and neutrophils in the peritonitis model compared to stored *S. aureus* suspension. Thus, the storage of *S. aureus* suspension affected the inflammation in different sites of infection, and also a variety of inflammatory parameters.

The decrease in leukocyte recruitment observed was accompanied by reduced production of cytokines IL-1 β and TNF- α . These inflammatory cytokines are crucial for host immune response against bacterial infection, such *S. aureus*, by inducing the expression of cell adhesion molecules and chemokines and other proinflammatory cytokines that also facilitate the recruitment of leukocytes to the infection site to eliminate the pathogen (MEDZHITOV; JANEWAY JUNIOR, 2000).

The inflammatory phenomena are at some extend interconnected. For instance, neutrophils are important leukocytes recruited in acute inflammation that contribute to the development of mechanical hyperalgesia by further producing nociceptive mediators such as prostaglandin E₂ (CUNHA et al., 2008; GUERRERO et al., 2008; TING et al., 2008; VERRI JUNIOR et al., 2009). In this sense, reduced bacterial load will result in reduced activation of resident cells (e.g. macrophages) that will produce reduced amounts of chemotatic mediators (e.g. TNF α , IL-1 β) resulting in reduced neutrophil recruitment, edema and pain (VERRI JUNIOR et al., 2006; VERRI JUNIOR et al., 2007; VERRI JUNIOR et al., 2009).

Recruited neutrophils are also important for bacterial clearance by phagocytosis and production of microbicidal oxygen and nitrogen species. Thus, reduced bacterial load will result in reduced

neutrophil recruitment and activation (ANWAR et al., 2009).

This study raises implications concerning the interpretation of data. In sepsis, there is a failure (reduced) of neutrophil recruitment to the inflammatory foci because neutrophils are activated in the circulation by bacteria and bacterial products. Bacterial products such as lipopolysaccharide (LPS) down-regulate the expression of chemokine receptors (e.g. CXCR2) via GRK, therefore, there is no gradient towards inflammatory foci (ALVES-FILHO et al., 2010) It is possible to induce peritonitis, sepsis or septic arthritis by administrating a bacteria suspension. However, if the bacterial load is excessive, it is possible to induce septic shock instead of peritonitis since the resident cells and recruited cells will not be able to control infection in the inflammatory foci because the bacterial load is supra-maximal (ALVES-FILHO et al., 2010). In this sense, using a bacterial dose based on a literature study that used CFU counting plus storage, but using McFarland's scale will result in a significantly greater inflammation that can eventually lead to loose of local control of infection and septic shock. Other implications are that pharmacological treatments might be ineffective if the bacterial load is supra-maximal, or the window between negative (PBS or saline) and positive controls (bacteria) is too small to allow detection of reduction by a testing treatment.

Conclusion

The present results suggest that standardization of bacterial counts is an important step in bacteriology and related areas, and that storing or not the sample has implications such as the intensity of inflammation obtained *in vivo*, and the consequent interpretations of data. Accordingly, the use of the MacFarland scale is an effective approach to optimize and also to reliably estimate the number of bacteria in a sample. Importantly, in the United States Pharmacopoeia, bacteria are used after plate counting and incubation at 80C (UNITED..., 2011).

Therefore, the present data raises the fact that using MacFarland scale would be more appropriate than CFU counting together with 24h incubation procedure contributing to direct knowledge-based improvement in other fields.

Acknowledgements

We thank the excellent technical assistance of Pedro S. Dionísio Filho, Jesus A. Vargas, Claci Sandra, Miriam S. N. Hohmann, Renata M Martinez, Sandra S. Mizokami, and Talita Perdigão Domiciano (received a technician fellowship from SETI/Fundação Araucária and Parana State Government). This work was supported by Brazilian grants from Departamento de Ciência e Tecnologia da Secretaria de Ciência, Tecnologia e Insumos Estratégicos (Decit/SCTIE), Ministério da Saúde (MS) (Decit/SCTIE/MS) by means of Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and support of Fundação Araucária, and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). RC and MMB received a senior research fellowship from SETI/Fundação Araucária and Paraná State Government, and WAVJ received a senior research fellowship from CNPq.

References

ALVES-FILHO, J. C.; SÔNEGO, F.; SOUTO, F. O.; FREITAS, A.; VERRI JUNIOR, W. A.; AUXILIADORA-MARTINS, M.; BASILE-FILHO, A.; MCKENZIE, A. N.; XU, D.; CUNHA, F. Q.; LIEW, F. Y. Interleukin-33 attenuates sepsis by enhancing neutrophil influx to the site of infection. *Nature Medicine*, New York, v. 16, p. 708-12, 2010.

ANWAR, S.; PRINCE, L. R.; FOSTER, S. J.; WHYTE, M. K. B.; SABROE, I. The rise and rise of *Staphylococcus aureus*: laughing in the face of granulocytes. *Clinical and Experimental Immunology*, Oxford, v. 152, p. 216-224, 2009.

BANNAN, J.; VISVANATHAN, K.; ZABRIESKIE, J. B. Structure and function of streptococcal and staphylococcal superantigens in septic shock. *Infectious Disease Clinics of North America*, Philadelphia, v. 13, p. 387-396, 1999.

BERENDS, E. T. M.; HORSWILL, A. L. R.; HASTE, N. M.; MONESTIER, M.; NIZET, V.; VON KOCKRITZ-BLICKWEDE, M. Nuclease expression by *Staphylococcus aureus* facilitates escape from neutrophil extracellular traps. *Journal of Innate Immunity*, Basel, v. 2, p. 576-586, 2010.

BROCK, T. D.; MADIGAN, M. T.; MARTINKO, J. M.; PARKER, J. *Biology of microorganisms*. New Jersey: Prentice Hall, 1994. p. 321-360.

CROSARA-ALBERTO, D. P.; DARINI, A. L.; INOUEU, R. Y.; SILVA, J. S.; FERREIRA, S. H.; CUNHA, F. Q. Involvement of NO in the failure of neutrophil migration in sepsis induced by *Staphylococcus aureus*. *British Journal of Pharmacology*, London, v.136, p.645-658, 2002.

CUNHA, T. M.; VERRI JUNIOR, W. A.; SCHIVO, I. R.; NAPIMOGA, M. H.; PARADA, C. A.; POOLE, S.; TEIXEIRA, M. M.; FERREIRA, S. H., CUNHA, F. Q. Crucial role of neutrophils in the development of mechanical inflammatory hypernociception. *Journal of Leukocyte Biology*, New York, v. 83, p. 824-832, 2008.

GUERRERO, A. T.; VERRI JUNIOR, W. A.; CUNHA, T. M.; SILVA, T. A.; SCHIVO, I. R.; DAL-SECCO, D.; CANETTI, C.; ROCHA, F. A.; PARADA, C. A.; CUNHA, F. Q.; FERREIRA, S. H. Hypernociception elicited by tibio-tarsal joint flexion in mice: a novel experimental arthritis model for pharmacological screening. *Journal of Leukocyte Biology*, New York, v. 83, p. 122-130, 2008.

GUO, Y.; LI, J.; HAGSTRÖM, E.; NY, T. Protective effects of plasmin(ogen) in a mouse model of *Staphylococcus aureus*-induced arthritis. *Arthritis and Rheumatism*, Atlanta, v. 58, p. 764-772, 2009.

MADRID, R. E.; FELICE, C. J.; VALENTINUZZI, M. E. Automatic on-line analyser of microbial growth using simultaneous measurements of impedance and turbidity. *Cellular Engineering*, London, v. 37, p. 789-793, 1999.

MAH, J. H.; KANG, D. H.; TANG, J. Comparison of viability and heat resistance of *Clostridium sporogenes* stored at different temperatures. *Journal of Food Science*, Champaign, v. 74, p. 23-27, 2009.

MATCHES, J. R.; LISTON, J.; DANEALU, L. P. Survival of *Vibrio parahaemolyticus* in fishhomogenate during storage at low temperatures. *Applied Microbiology*, Oxford, v. 21, p. 951-952, 1971.

MCFARLAND, J. The nephelometer: a instrument for estimating the number of bacteria in suspensions used for calculatin of the opsonic index and vaccines. *J. A. Med. Assoc*, New York, v.14, p.1176-1178, 1907.

MEDZHITOV, R.; JANEWAY JUNIOR, C. The toll receptor family and microbial recognition. *Trends in*

Microbiology, Cambridge, v. 8, p. 452-456, 2000.

OKOLI, C. O.; AKAH, P. A.; ONUOHA, N. J.; OKOYE, T. C.; NWOYE, A.; NWORU, C. S. *Acanthus montanus*: an experimental evaluation of the antimicrobial, anti-inflammatory and immunological properties of a traditional remedy for furuncles. *BMC Complementary and Alternative Medicine*, London, v. 8, p. 1-11, 2008.

PINTO L. G.; CUNHA, T. M.; VIEIRA, S. M.; LEMOS H. P.; VERRI JUNIOR, W. A.; CUNHA, F. Q.; FERREIRA, S. H. IL-17 mediates articular hypernociception in antigen-induced arthritis in mice. *Pain*, Amsterdam, v. 148, p. 247-256, 2010.

RICHARDS, J. C. S.; JASONS, A. C.; HOBBS, G.; GIBSON, D. M.; CHRISTIE, R. H. Electronic measurement of bacterial growth. *Journal of physics E: Scientific instruments*, London, v. 11, p. 561-569, 1978.

ROCHA, F. A.; LEITE, A. K.; POMPEU, M. M. M.; CUNHA, T. M.; VERRI JUNIOR, W. A.; SOARES, F. M.; CASTRO, R. R.; CUNHA, F. Q. Protective effect of an extract from *Ascaris suum* in Experimental arthritis models. *Infection and immunity*, Washington, v. 76, p. 2736-2745, 2008.

SOUZU, H. Studies on the damage to *Escherichia coli* cell membrane caused by different rates of freeze-thawing. *Biochim. Biophys. Acta (BBA)*, Amsterdam, v. 603, p. 13-26, 1980.

TARKOWSKI, A.; BOKAREWA, M.; COLLINS, L. V.; GJERTSSON, I.; HULTGREN, O. H.; JIN, T.; JONSSON, I. M.; JOSEFSSON, E.; SAKINIENE, E.; VERDRENGH, M. Current status of pathogenetic mechanisms in staphylococcal arthritis. *FEMS Microbiology Letters*, Amsterdam, v. 217, p. 125-132, 2002.

TARKOWSKI, A.; COLLINS, L. V.; GJERTSSON, I.; HULTGREN, O. H.; JONSSON, I. M.; SAKINIENE, E.; VERDRENGH, M. Model systems: modeling human staphylococcal arthritis and sepsis in the mouse. *Trends in Microbiology*, Cambridge, v. 9, p. 321-326, 2001.

TING, E.; GUERRERO, A. T.; CUNHA, T. M.; VERRI JUNIOR, W. A.; TAYLOR, S. M.; WOODRUFF, T. M.; CUNHA, F. Q.; FERREIRA, S. H. Role of complement C5a in mechanical inflammatory hypernociception: potential use of C5a receptor antagonists to control inflammatory pain. *British Journal of Pharmacology*, London, v. 153, p. 1043-1053, 2008.

UNITED States Pharmacopeia. 34. ed. Rockville: United States Pharmacopeial Convention, 2011. p. 48-56.

VALERIO, D. A.; GEORGETTI, S. R.; MAGRO, D. A.; CASAGRANDE, R.; CUNHA, T. M.; VICENTINI, F. T. M. C.; VIEIRA, S. M.; FONSECA, M. J. V.; FERREIRA,

S. H.; CUNHA, F. Q.; VERRI JUNIOR, W. A. Quercetin reduces inflammatory pain: inhibition of oxidative stress and cytokine production. *Journal of Natural Products*, Cincinnati, v. 72, p. 1975-1979, 2009.

VERRI JUNIOR, W. A.; CUNHA, T. M.; FERREIRA, S. H.; WEI, X.; LEUNG, B. P.; FRASER, A.; MCINNES, I. B.; LIEW, F. Y.; CUNHA, F. Q. IL-15 mediates antigen-induced neutrophil migration by triggering IL-18 production. *European Journal of Immunology*, Weinheim, v. 37, n. 12, p. 3373-3380, 2007.

VERRI JUNIOR, W. A.; CUNHA, T. M.; MAGRO, D. A.; GUERRERO, A. T.; VIEIRA, S. M.; CARREGARO, V.; SOUZA, G. R.; HENRIQUES, M. G.; FERREIRA, S. H.; CUNHA, F. Q. Targeting endothelin ETA and ETB receptors inhibits antigen-induced neutrophil migration and mechanical hypernociception in mice. *Naunyn-Schmiedeberg's Archives of Pharmacology*, Berlin, v. 379, p. 271-279, 2009.

VERRI JUNIOR, W. A.; CUNHA, T. M.; PARADA, C. A.; POOLE, S.; CUNHA, F. Q.; FERREIRA, S. H. Hypernociceptive role of cytokines and chemokines: targets for analgesic drug development? *Pharmacology & therapeutics*, Oxford, v. 112, p. 116-138, 2006.

VERRI JUNIOR, W. A.; GUERRERO, A. T.; FUKADA, S. Y.; VALERIO, D. A.; CUNHA, T. M.; XU, D.; LIEW, F. Y.; CUNHA, F. Q. IL-33 mediates antigen-induced cutaneous and articular hypernociception in mice. *Proceedings of the National Academy of Sciences of the United States of America*, Washington, v. 105, p. 2723-2728, 2008.

WHALEY, K.; BURT, A. D. *Muir's textbook of pathology*. London: Hodder Arnold Publication, 1996. p. 112-165.

Recebido em: 04 de abril de 2012
Aceito em: 26 de março de 2013