EFFICACY OF SERONEUTRALIZATION OF A SYSTEMIC TOXICO-INFECTION CAUSED BY *Staphylococcus pseudintermedius* ON MICE

(UTILIZAÇÃO DE MODELO BIOLÓGICO EXPERIMENTAL NA AÇÃO SORONEUTRALIZANTE DE TOXINFECÇÃO SISTÊMICA POR CEPA DE Staphylococcus pseudintermedius)

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SUMMARY

The aim of this study was to evaluate how effective the antitoxic hyperimmune serum is against intraperitoneal toxinfection caused by *Staphylococcus pseudintermedius* (*S. pseudintermedius*) on mice passively immunized with 0.5 mL of canine polyclonal serum with 512 anti-hemolysin Beta titer, which was considered the phenotypical marker of toxic virulence. Sixty-four mice (*Mus musculus*) were divided in two groups (I and II) with 32 mice each, one of which, the control group (II) received equal volume of physiologic saline solution by the same route. Both challenged groups received inoculum with 2,5 x 10^{10} C.F.U. diluted in broth with 16 HU (Hemolytic Units) of Beta toxin, corresponding to 1.5 DL_{50}. The survival index was evaluated 24 hours after the initial experiment, and reached 97% and 33% in group I and II, respectively. The evaluation of the antitoxic serotherapy efficacy, measured by Preventable Fraction, showed a protection index of 87%. In the passively immunized group, the presence of bacteria in the peritoneal fluid was observed during the whole experiment. The bacterial counts in the spleen of immunized animals showed decreasing tendency up to 84 hours after the challenge. At necropsy, small abscesses localized mainly on the liver surface, mesenteric lymph nodes, parietal peritoneum and diaphragm were observed in mice of group I, 108 hours after the challenge. The protection conferred by the polyclonal hyperimmune serum played an important role with respect to toxigenic virulence factors of this bacterium. In spite of the constant bacterial presence in the peritoneal cavity of passively immune animals, the neutralization of toxins allowed the survival of challenged animals showing their important role in the virulence of *S. pseudintermedius*, the main etiologic agent of canine pyoderma. Based on the results of this biological model, the therapeutic use of hyperimmune serum in immune-compromised patients as well as the preventive use of a toxoid vaccine can be a viable alternative to control these toxic infections in dogs.

KEY-WORDS: Biological model. *Mus musculus*. Serum neutralization. *Staphylococcus pseudintermedius*.

RESUMO

O objetivo deste estudo foi avaliar a proteção conferida por soro hiperimune policlonal contra a toxinfecção intraperitoneal por *Staphylococcus pseudintermedius* (*S. pseudintermedius*) utilizando como modelo biológico experimental camundongos passivamente imunizados pela via intraperitoneal com 0.5 mL de soro canino com título 512 de anti-hemolisina Beta a qual foi considerada como marcadora da expressão fenotípica de virulência tóxica. Para realização do experimento foram constituídos dois grupos de animais (I e II) compostos por 32 camundongos (*Mus musculus*) cada um, tendo o grupo controle (II) recebido igual volume de solução fisiológica pela mesma via. Os dois grupos foram desafiados com inóculo contendo 2,5 x 10^{10} U.F.C. diluídas em caldo BHI com 16 UH (Unidades Hemolíticas) de toxina Beta, correspondente a 1.5 DL_{50}. O índice de sobrevivência foi avaliado 24 horas após o início do experimento alcançando 97% no grupo I e 33% no grupo II. A avaliação da eficácia soroterápica antitóxica, medida pela Fração Evitável, alcançou índice de proteção de 87%. No grupo imunizado passivamente, observou-se a presença bacteriana nos lavados peritoneais durante todo o experimento. As contagens bacterianas no baço dos animais imunizados apresentaram tendências decrescentes até as 84 horas após o desafio. A partir de 108 horas após o desafio observou-se no grupo I a formação de pequenos abscessos localizados principalmente na superfície hepática, linfonodos

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mesentéricos, peritôneo parietal e diafragma. A proteção conferida pelo soro hiperimune policlonal desempenhou um papel importante em relação aos fatores de virulência toxigênicos do *S. pseudintermedius*. Apesar da constante presença bacteriana na cavidade peritoneal dos animais imunizados, a neutralização das toxinas possibilitou a sobrevivência dos animais desafiados demonstrando o importante papel das mesmas na virulência do *S. pseudintermedius***,** principal agente etiológico nas piodermites caninas. Face aos resultados de proteção obtidos neste modelo biológico, a utilização terapêutica de soro hiperimune em pacientes imunocomprometidos ou a utilização de um toxoide em infecções localizadas poderão se tornar uma alternativa viável no controle destas toxinfecções em cães.

PALAVRAS-CHAVE: Modelo biológico. *Mus musculus*. Soro neutralização. *Staphylococcus pseudintermedius*. Toxoide.

INTRODUCTION

Staphylococcus bacteria are commonly found in the nasopharyngeal mucosa, skin and when the innate defense barriers are overwhelmed they may infect other parts of body, thus gaining access to the bloodstream and lymph nodes. *Staphylococcus pseudintermedius* has been frequently isolated from blood cultures of dogs with bacteremia and endocarditis due to discospondylitis and endocarditis. However, the most common bacteremic sources include abscesses, deep wounds and pyoderma (CALVERT & WALL, 2006). Skin infections represent a large percentage of cases in dogs. In a study by Scott & Paradis (1990) 25.3% of the studied dermatosis were infectious. *S. pseudintermedius* species is the main microorganism isolated from skin lesions in dogs (HAUSCHILD & WÓJCIK, 2007). In most cases, the underlying diseases such as allergies, ectoparasites, seborrhea and hypothyroidism lead to an imbalance of the skin micro-environment allowing secondary infection by virulent strains of *S. pseudintermedius* (SCOTT *et al.*, 2001). A number of toxins and enzymes are involved in its pathogenesis, among these catalase and hemolysins are highlighted because they inhibit phagocytosis by polymorphonuclear cells. The recruitment of neutrophils and their phagocytic action are critical steps to resolve bacterial infections (CAMPBELL, 1976). Mölne *et al*. (2000) showed the importance of these cells in bacterial elimination of *Staphylococcus aureus* (*S. aureus*) when studying skin infection on mice. Moreover, it has been proved that the beta hemolysin produced by *S. aureus* exerts a cytotoxic action on different types of eukaryotic cells, including leukocytes (WISEMAN, 1968). According to Carlotti (2003), the cytological examination of superficial pyoderma shows degenerated neutrophils (swollen, discolored, hypersegmented and pyknotic nuclei) free of bacterial colonies. It is assumed that the phagocytic activity of these cells on the infection initial focus does not occur with the expected efficiency and often allows bacteremia to install, thus making *S. pseudintermedius* the main infectious agent isolated from blood cultures, according to Calvert & Wall (2006). The hypothesis of a toxic action on these defense cells seems plausible. Given the frequency of this cytopathological observation among cases of canine pyoderma, this study aimed at investigating the role of neutralizing antitoxin in infections produced by toxigenic strains of *S. pseudintermedius* on mice (*Mus musculus*).

MATERIAL AND METHODS

Sixty-four adult female mice (*Mus musculus*) of the Rockefeller breed, from the Instituto de Tecnologia do Paraná (TECPAR), were used in the experiment. Also a healthy dog of the Beagle breed (*Canis familiaris*) was used for hyperimmunization with the toxoid *S. pseudintermedius*.

To obtain the polyclonal hyperimmune serum for the dog and mice, we chose the strain of *S. pseudintermedius* that is able to produce Beta hemolysin isolated from a dog with pyoderma, identified by colony morphology, expression of the double hemolysis on sheep blood agar, the catalase proof, linked coagulase, acetoin production and mannitol fermentation, according to Greene & Lämmler (1993). The strain was named strain 66.

The toxoid was obtained from the culture of the strain 66 in 400 mL of BHI (Brain Heart Infusion-Himedia) broth at 37˚C during 10 days in Erlenmeyer flask. Beta hemolysin was chosen as the marker of the phenotypic expression of toxic virulence. Subsequently, the culture was centrifuged at 6,000 g during15 minutes to obtain a clear supernatant, which was filtered on nitrocellulose membrane 0.45 µm (Millipore). Titration of hemolytic activity of hemolysin Beta was performed according to Adlam *et al*. (1977). The bacterial filtrate of strain 66 had 16 HU (hemolytic units) and protein dosage 6.3 $g.L^{-1}$ according to Lowry (1951). A 150-mL filtrate volume was frozen at -20˚C for further protein profile analysis by the SDS-PAGE technique. The filtrate, divided into 5-mL-aliquots, underwent lyophilization and was stored at 4˚C. To obtain the toxoid, the lyophilized was resuspended in 5 mL of 6% phenolated saline solution at 37˚C during 5 days for its inactivation (CARTER, 1969). Subsequently, to verify if Beta hemolysin was inactivated, sterile paper filter disks were embedded in 50 mL of the filtrate, deposited on Petri dishes that contained sheep blood agar and incubated at 37˚C in a moist chamber for 24 hours. Commercial Beta hemolysin (Beta toxin, Hardy Diagnostic, USA) was used as positive control.

The inoculum (bacterial cells and toxins) consisted of a culture obtained from strain 66 in Erlenmeyer flask contained 150 mL of BHI broth incubated at

37˚C during 72 hours in static culture containing 9.7 x 10^{10} U.F.C. mL^{-1}. Subsequently, 16 mL of the broth with the bacterial culture was used to resuspend the lyophilized filtrate obtained previously.

To determine the electrophoretic protein profile by the SDS-PAGE technique, the staphylococcal exoproteins from the filtrate were measured according to Lowry (1951). The precipitation was performed according to Adlam *et al*. (1977) and separated by electrophoresis on a polyacrylamide gel under denaturing conditions (SDS-PAGE) on 10% polyacrylamide and 5% concentrator gel in batch system according to Laemmli (1970). Proteins on the gel were stained with Coomassie blue.

To obtain the polyclonal hyperimmune serum, we chose a healthy dog with titer of 32 anti-beta hemolysin. Three subcutaneous immunizations each with 1 mL of toxoid in 21-day intervals were performed. Fifteen days after the third immunization, 25 mL of whole blood was collected aseptically, in two separate occasions, in order to obtain the serum volume necessary for immunization. The test for Beta hemolysin neutralization with hyperimmune serum was performed according to Colque-Navarro (1998) using defibrinated sheep blood as hemolysis indicator.

The modified biological model by Menzies & Kernodle (1996) was used for passive immunization and challenge in mice. Twenty-four hours before the challenge, 32 mice of group I received intraperitoneally 0.5 mL of serum from dogs hyperimmunized with strain 66 toxoid, while Group II (control) received via same route, an equal volume of sterile physiological solution. The inoculum for intraperitoneal challenge was 0.25 mL per mouse consisting of 2.5 x 10^{10} C.F.U. of *S. pseudintermedius* corresponding to approximately 1.5 DL_{50}.

Serum therapy effectiveness was evaluated by the avoidable antitoxic fraction using the following Abbott equation, cited by Cardella & Kolbe (1976), considering only the surviving animals from both groups, 24 hours after inoculation.
x 100

Peritoneal washes of four mice from each group was collected according to Abe *et al*. (1979) at time intervals of 6, 12, 36, 60, 84, 108, 156 and 204 hours after the challenge. Log 10 fold serial dilutions were prepared from 1 mL of the washes to proceed with bacterial counting. Each dilution was seeded in duplicate in 0.1 mL volume on the surface of Mannitol agar plates (Himedia) using Drigalski handle. The plates were then incubated at 37˚C during 48-72 hours. White colonies, not Mannitol fermenters, with features typical of the species were considered in the total counts of $UFCmL^{-1}$ as *S. pseudintermedius.*

Spleens were collected at the same time intervals and number as the peritoneal washes, washed twice in sterile saline solution and, immediately afterwards, crushed using a mortar and pestle in 4 mL of the same solution with sterilized sand. Serial dilutions were performed starting with 1 mL of homogenate, sowing and total counts of CFU/spleen, following the interpretations and parameters already mentioned.

The values were submitted to ANOVA and, when significant, submitted to Tukey test at 5%. Furthermore, the data were submitted to t test (software Statview). The mean counts in CFU of each harvest and their standard deviation were also calculated. This study was approved by the Ethics Committee on Animal Experiments (Comitê de Ética em Experimentação Animal, CEEA), Setor de Ciências Biológicas, Universidade Federal do Paraná under process number 23075.034859/2007-29, on October 9, 2007.

RESULTS AND DISCUSSION

Diseases caused by toxico-infectious bacterias are preventable by toxoid or anatoxins vaccines, remarkably the infections caused by the genus *Clostridium* and *Corynebacterium pyogenes* (CARDELLA & KOLBE, 1976; ALOUF, 1982). Most immunogens produced to fight infections caused by bacteria of the *Staphylococcus* genus uses bacterial cells or lysed (CHAMBERS & SEVERIN, 1984; GREENBERG *et al*., 1987; DeBOER *et al*., 1990; CURTIS *et al*., 2006). On the other hand, there are very few studies using only staphylococcal toxoids (ADLAM *et al*., 1977; MENZIES & KERNODLE, 1996). The biological model advocated by Menzies & Kernodle (1996) was used in this study in order to cause an intraperitoneal infection in mice challenged with viable bacterial cells and toxins preformed *in vitro*. Thus, it would be possible to evaluate the neutralizing potential of the hyperimmune serum in the immunized group and the role of toxins in the infections caused by *S. pseudintermedius* in the control group. If the toxins played a secondary and less important role in intraperitoneal infection, there should be in both groups a similar number of deaths from septicemic peritonitis. In the current study, the protein profile by SDS-PAGE electrophoresis of toxigenic broth used to inoculate mice had approximately 15 bands, thus confirming the presence of multiple exoproteins that could play a synergistic role in producing a septicemic toxico-infection. From the inactivation of the toxigenic broth, after three inoculations of a healthy dog, we obtained hyperimmune serum used in the passive immunity trial with mice. The antitoxic protection protected the challenged mice with 1.5 DL_{50} corresponding to 2.5 x 10^{10} CFU of *S. pseudintermedius*, and 16 HU of Beta hemolysin. Animal survival and toxico-infection control were kept throughout the experiment to prevent toxico-septicemic infection. The efficacy of the serum was proven by the Abbott equation, which considered only the animals that survived 24 hours after the beginning of the experiment, except for the 16 animals (8 from each group) that were euthanized during the first 12 hours for bacterial count procedures of the

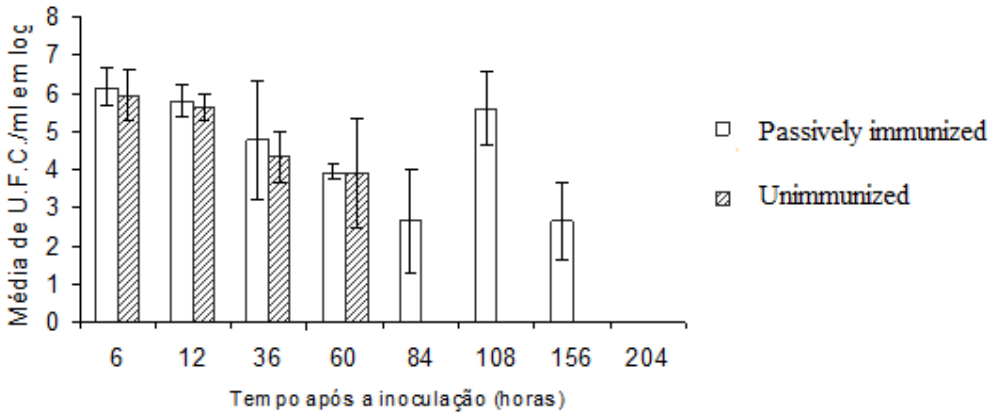

Figure 1 - Kinetics of systemic bacterial elimination (spleen) in mice challenged intraperitoneally with toxico-infectious inoculum of *S. intermedius*.

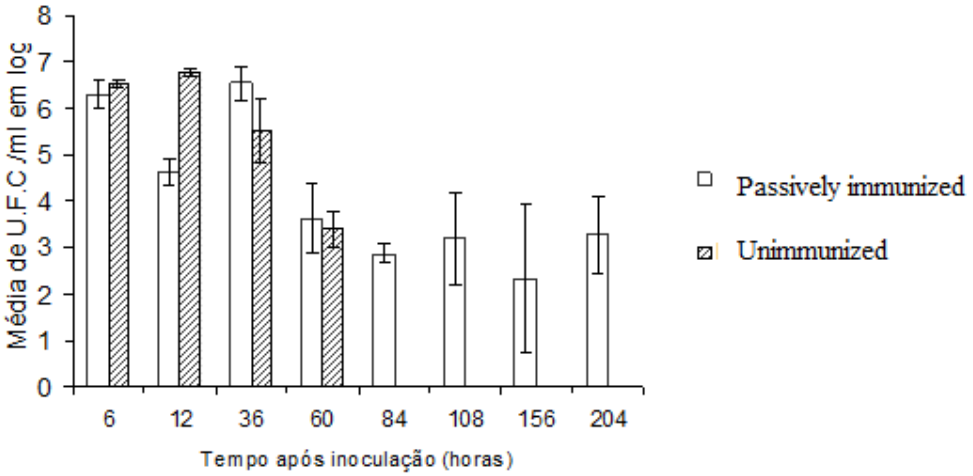

Figure 2 - Kinetics of bacterial elimination from the peritoneal washes in mice challenged intraperitoneally with toxico-infectious inoculum of *S. intermedius*. The asterisk shows significant difference between groups ($p<0.05$).

first 12 hours for bacterial count procedures of the peritoneal washes and spleen. From the 24 surviving animals of Group I (immunized), two died during the first 24 hours, yielding a survival percentage of 91.6%. On the other hand, there were 16 deaths in Group II (control) during the same period, yielding a survival percentage of 33.3%. Therefore, serum effectiveness was 87%. According to Tizard (1998) vaccine efficiency above 70% is considered satisfactory for protection. The preventable fraction provided by the vaccines varies according to laboratory and methodology used with different degrees of efficiency (GREENE & SCHULTZ, 2006). The clinical signs most commonly observed in Group II after inoculation

were anorexia, ruffled hair, abdominal breathing and prostration, typical of septicemic infection. On the other hand, Group I did not display the same behavior, except for anorexia in the first 12 hours, but returned to normal afterwards.

Bacteria was present in the peritoneal washes even after 204 hours and in the spleen of the group passively immunized for 156 hours, suggesting that the lack of mortality during this period was due to antitoxic protection (Figures 1 and 2). Significant differences in the peritoneal washes were seen only 12 hours after the challenge compared to control group (Figure 1).

It was observed that 108 hours after the challenge, all vaccinated animals had small abscesses in the abdominal cavity, located mainly in the liver, parietal peritoneum, diaphragm and mesenteric lymph nodes. Bacteriological tests and abscess culture confirmed the presence of *S. pseudintermedius* in these foci. The profuse polymorphonuclear cell infiltration seen in these foci suggests that the phagocytic cells were removing the microorganisms of the peritoneal cavity and enclosing them into the abscesses to prevent septicemia confirmed by complete bacterial elimination of the spleen. Similar study by Menzies & Kernodle (1996) also reports the presence of intraperitoneal abscesses in mice immunized with alpha toxin of *S. aureus*. According to Cox (2006), several virulence factors may contribute to the inhibition of phagocytic activity impairing effective host immune response.

It is known that pyoderma is among the most common staphylococcal infections in dogs. In addition, their cytological examinations show profuse colonization with *S. pseudintermedius,* the presence of degenerate neutrophils and extracellular cocci (IRKE, 2006). It is possible that in chronically infected animals, the antitoxic humoral immune response is not sufficiently effective due to inappropriate processing of toxins by these antigen carrier cells, which keeps the stubborn infections. Therefore, the use of antitoxic hyperimmune sera would be a viable alternative in immuno-compromised animals at risk of septicemia or contracting recurrent pyoderma.

Moreover, the preventive use of the immunogen containing the active form of various toxins, at high protein concentrations, would cause an efficient modulation of antibody responses against future infections caused by *S. pseudintermedius*, allowing adequate stimulation with subsequent production of neutralizing antibodies.

CONCLUSION

The therapeutic use of either hyperimmune serum or toxoid produced by *S. pseudintermedius* to prevent and control infections caused by this microorganism may be promising in view of the antitoxic serum therapy efficacy obtained in this study using mice as experimental biological model. The lack of research concerning the role played by exotoxins expressed *in vivo* by *S. pseudintermedius* justifies further investigations of the pathogenesis caused by this bacterium in order to enhance the effectiveness of commercially available immunogens.

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