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Original Contribution

EFFECT OF *STAPHYLOCOCCUS AUREUS* INFECTION ON THE SERUM AMYLOID A AND IRON LEVELS IN RABBITS

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ABSTRACT

The present study was conducted to examine the changes in serum amyloid A (SAA) and iron concentrations during *Staphylococcus aureus* (*S. aureus*) infection in rabbits. The experimental procedures were carried out with 12 male New Zealand white healthy rabbits, divided into two equal groups - experimental group (n=6, rabbits infected with *S.. aureus*) and control group (n=6, uninfected animals). Blood samples were collected at time 0 (before the infection), 24, 48 and 72 hours and also 7 and 14 days after the infection. The results in infected group showed a significant increase in the levels of SAA at the 24th hour ($p<0.001$), 48th hour ($p<0.05$), and 72th hour ($p<0.05$) post-infection with mean levels 72.13 ± 23.29 µg/mL, 37.57 ± 31.55 µg/mL and 18.03 ± 15.15 µg/mL respectively. The iron concentration decreased at the 24th hour and 14th day post-infection, reaching values of 178.8 ± 87.2 µg/dL ($p<0.01$) and 123.33 ± 17.8 µg/dL ($p<0.05$) respectively. In conclusion changes in SAA and Fe levels may be used as valuable biochemical indicators for the diagnosis and prognosis of staphylococcosis in rabbits.

Key words: *Staphylococcus aureus*, rabbits, serum amyloid A, iron, SAA

INTRODUCTION

Bacterial skin diseases in rabbits are very common infections and represent a suitable model for studying the relationship between the infection and changes in metabolism. *Staphylococcus aureus* is a Gram-positive bacterium that produces several toxins (leucocidin, exfoliatin, haemolysin, enterotoxins and TSS-toxin 1), causing severe skin damages and a variety of systemic responses in the organism including Acute Phase Response (APR) and changes in blood trace elements (1). APR is a part of the innate immunity appearing before the specific immune response raised (2). This initial body reaction against pathological processes (e.g. trauma, infection, surgery, neoplastic growth, etc.) includes different changes such as leukocytosis and activation of macrophages and monocytes to synthesize pro-inflammatory cytokines that transform the hepatic protein spectrum and trigger Acute Phase Proteins (APP) secretion (3-5). In addition, several other tissues are able to synthesize APPs e.g. the lungs, ovaries, uterus, digestive tract, testes, adipose tissue and mammary glands (4). The main purpose of these changes is to restore the homeostasis by isolating and destroying the harmful agent; to activate repair processes and to prevent tissues and organs from more severe damages (6). The APPs are a large group of plasma proteins that are released very quickly (within the first few hours) into the bloodstream, where each performs various and specific

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functions (6, 7). Some of them are constantly secreted and released in the blood, while other are secreted only during the APR (6). The type of APP expression considerably varies between animals (8). In rabbits, the APP spectrum includes C-reactive protein, haptoglobin, α1-macroglobulin and transferrin which are the most important positive APPs (9). Serum amyloid A (SAA) is also recognized as one of the most important positive APP in rabbits (9, 10). It is synthesized primarily by the liver, but extrahepatic secretion is also reported, e.g. smooth-muscle cells, intestinal epithelial cells and macrophages (3). Its function is not fully understood, but some activities have been described. Generally, SAA binds, transports and scavenges the cholesterol from the dying cells (4); inhibits the platelet aggregation (7) and detoxifies the endotoxins by binding to lipopolysaccharide (3). Also, SAA is able to bind to Gram-negative bacteria (11) and stimulate the migration of inflammatory cells to areas of altered tissues (12).

Iron is an essential trace element required for the normal course of various physiological processes in the living cells. As a part of hemoglobin, its main function is to transport oxygen from the lungs to the body tissues through the blood vessels. The iron is absolutely necessary for bacterial growth. In this connection, several defensive reactions in the host body during infections occur aiming to interfere iron utilization by pathogenic bacteria, which causes disorders of bacterial growth and replication. Some of these reactions include binding of hemoglobin to haptoglobin, which makes iron unavailable to microorganisms (13), storage of iron in hepatocytes (14), inhibition of intestinal absorption of iron (15), etc. However, if the iron concentration increases, oxidative stress may occur due to its well-pronounced redox activity. Therefore, iron balance is vital for the health of the body and its disturbance may lead to disease (16).

Given the above data, the present study aims to investigate the changes in concentrations of SAA and iron in rabbits, experimentally infected with *S. aureus* and to provide additional information for early diagnosis of rabbit skin staphylococcosis.

MATERIAL AND METHODS

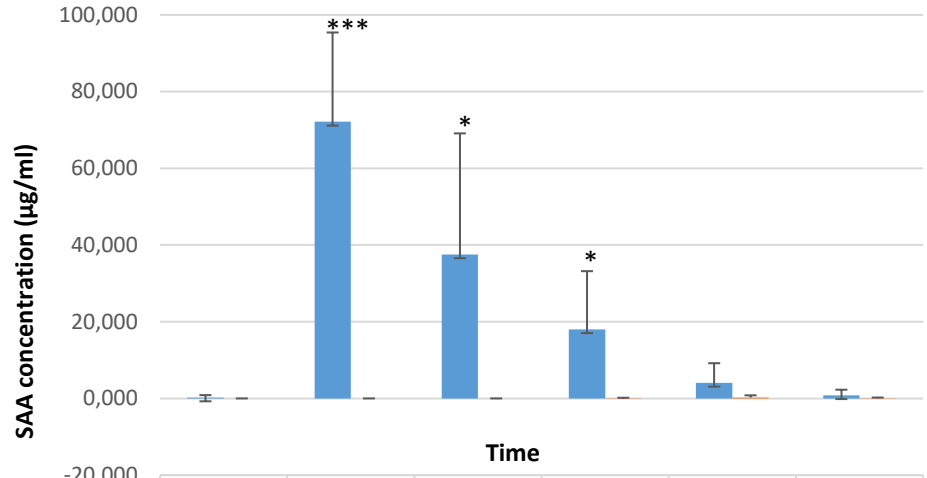
The experiment was approved by the Bulgarian Food Safety Agency (Approval protocol №158/2016). The experimental procedures were carried out with 12 male New Zealand white healthy rabbits, divided into two equal groups - experimental group (n=6) and control group (n=6). The rabbits from each group were reared separately into two premises and were placed in previously disinfected individual metal cages with a grill floor, and at a constant temperature of about 20-22°C. The animals were fed with granulated feed and have free access to water. It was provided a two-week acclimatization period before performing the infection in the experimental group. The experimentally induced staphylococcosis was reproduced by subcutaneous injection of the rabbits with 100 µL of a bacterial suspension of *S. aureus* field strain (density: $8x10^8$ cfu/mL). The rabbits of the control group were injected with saline solution. The inoculation site (flank) was pre-shaved and prepared for manipulation. Blood samples were collected from each rabbit by venipuncture of *v. auricularis caudalis* into sterile heparinized tubes at time 0, 24, 48, 72 hours and also 7 and 14 days post-infection. The blood samples were centrifuged (1500 rpm x 10 min); plasma was separated and frozen at -20 ºC until analyses. Thawed plasma was used for determination of SAA and iron. SAA (µg/mL) was determined using a multispecies enzyme linked immunosorbent assay. Briefly, samples were diluted 1:500 in 0.05 % Phosphate-buffered saline (PBS)-Tween and 100 µl of the sample was added along with standards and controls to a 96 well plate. All approaches were done in duplicate. The plates were incubated at 37ºC for 1 h and then washed four times using 300 µl of PBS-Tween per well. After tapping to remove excess wash solution, 100 µl of a ready to use horseradish peroxidase labelled monoclonal antibody was added to the micro wells and the plate incubated for a further 30 min at 37ºC. The wells were then washed with PBS-Tween before 100 µl tetramethylbenzidine(TMB)-substrate was added for 10 minutes. The reaction was stopped by addition of 100 µl of 0.1 M sulphuric acid and the plate was read at an absorbance of 450 nm by photometer. Samples with a concentration above the highest measurable concentration were further diluted and retested. Iron (Fe, µg/dL) concentration was determined with a biochemical analyser Olympus (Spain) at the Faculty of Veterinary Medicine at the Autonomous University of Catalonia, Barcelona, Spain. Statistical analysis was performed by means of ANOVA (Statistics for Windows, Stat Soft.) and the differences were considered significant at $p<0.05$.

RESULTS

SAA values varied within a wide range between the experimental and control groups. As shown in **Figure 1**, SAA concentrations in the experimental group increased significantly at the 24th hour ($p<0.001$), 48th hour ($p<0.05$), and 72th hour

(p<0.05) post-infection with mean levels 72.13 ± 23.29 µg/mL, 37.57 ± 31.55 µg/mL and 18.03 ± 15.15 µg/mL respectively. Also, SAA levels decreased at the 7th and 14th days, reaching the baseline. In experimental rabbits, Fe concentration at the 24th hour and 14th day post-infection decreased significantly compared to the control animals, reaching values of 99,28 ± 31,38 µg/dL (p<0.01) and 123,50 ± 43,88 µg/dL (p<0.05) respectively (**Figure 2**).

Figure 1: SAA content in control and infected groups of rabbits

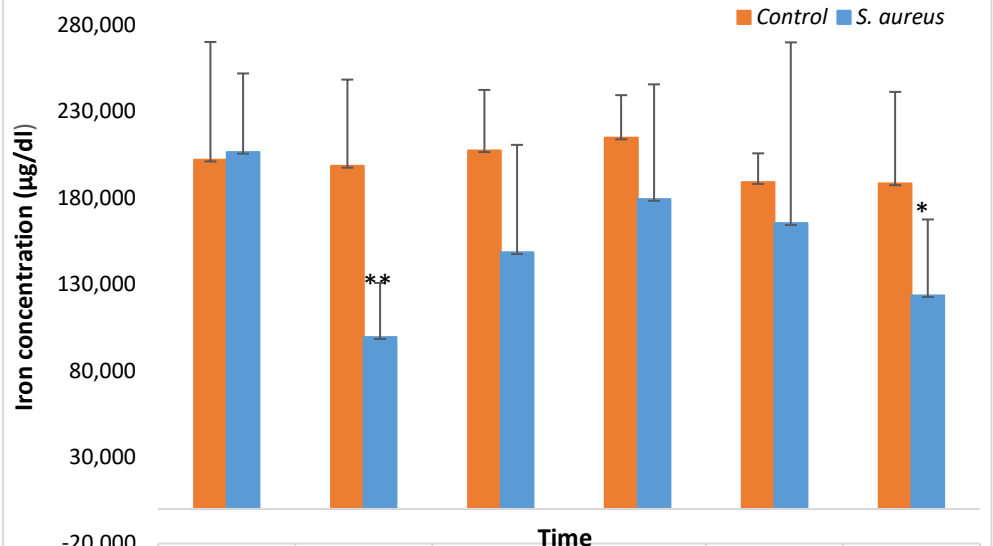


	0 hour	24 hours	48 hours	72 hours	7 days	14 days
Experimental group	0,250	72,133	37,567	18,033	4,083	0,833
Control group	0,000	0,000	0,000	0,050	0,233	0,067

Statistically significant difference between control and experimental groups:
*p<0.05; **p<0.01; ***p<0.001

Figure 1. Changes in serum amyloid A concentration in rabbits experimentally infected with *S. aureus*
Significance between groups *p<0.05; **p<0.01; ***p<0.001

Figure 2. Iron content in control and experimental groups of rabbits



	0h	24h	48h	72h	7d	14d
Control	201,900	198,333	207,333	214,667	188,933	188,167
S. aureus	206,400	99,283	148,383	179,150	165,183	123,500

Statistically significant difference between control and experimental groups:
*p<0.05; **p<0.01; ***p<0.001

Figure 2. Changes in iron concentration in rabbits experimentally infected with *S. aureus*
Significance between groups *p<0.05; **p<0.01

DISCUSSION

Data on changes in the blood levels of some APPs and trace elements during *S. aureus* infection in rabbits are still limited. The results of this study show that SAA increases extremely significant after infection with *S. aureus*. Cray (17) also reported an increase in the SAA concentration in rabbits with induced aseptic inflammation by applying turpentine oil. Similarly, the SAA levels increase in rabbits injected with lipopolysaccharides, casein and multiple inoculation of silver nitrate (17). According to Cray et al (18), there are no significant differences in SAA in rabbits infected with *Encephalitozoon cuniculi*. This might indicate that SAA does not actively participate in the APR in rabbits with encephalitozoonosis. The same authors found that only some of the animals demonstrate elevated SAA levels. Hadžimusić et all (19) established that after canine orchiectomy there is a rapid increase (within the first 24 hours after surgery) in the concentration of SAA and C-reactive protein. Similarly, results of a study in dogs with pyometra revealed a significant increase in SAA and CRP concentrations (20). According to Hari-Dass et al (21), SAA plays a major role during infections with numerous bacterial pathogens. It acts as an opsonin, rapidly binding with high affinity many Gram-negative bacteria, such as *Escherichia coli, Pseudomonas aeruginosa, Salmonella typhimurium, Shigella flexneri, Klebsiella pneumoniae* and *Vibrio cholerae* (21).

Information on changes in Fe blood levels during *S. aureus* infection in rabbits is completely lacking in the available references. The results of this study show that iron significantly decreases which is most pronounced at the 24th hour and 14th day after the infection. 22 revealed that Fe concentration in rabbits infected with *Trypanosoma brucei brucei* declined at the 18th day after the inoculation. Similarly, Baker et al (23) found a decrease in Fe levels in mice injected with endotoxins of *E. coli* or *Brucella abortus*, which is in general agreement with our findings. In this regard, William et al (24) reported that serum Fe in laboratory animals generally decreases after inoculation with lipopolysaccharide endotoxin, bacterial exotoxin, infectious microorganisms, or inflammatory agents. Such a reduction is recognized as a crucial protective mechanism aimed at preventing the use of Fe by pathogenic bacteria and therefore inhibiting bacterial replication. In contrast, an abnormal Fe availability in some clinical conditions may be the cause of fatal septicemia due to the fact that phagocytic cells are congested by the rapidly increasing number of multiplying microorganisms having free Fe access (25). In healthy people, the antibacterial capacity of the blood could not be maintained unless the level of iron is extremely low (25).

CONCLUSION

The results of this study show that SAA is a reliable marker for determination of *S. aureus* infection in rabbits. The changes in SAA and Fe levels may be used as valuable biochemical indicators for the diagnosis and prognosis of staphylococcosis in rabbits but more additional investigations are needed to demonstrate their full potential as diagnostic markers of the infection.

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