INTRODUCTION

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Staphylococcus aureus (S. aureus) is an adaptive opportunistic pathogen, capable to persist and replicate under various conditions. This microbial species causes a wide range of diseases in both men and animals (1). In rabbits, problems of staphylococcosis arise when S. aureus bacteria infect small dermal lesions and invade subcutaneous tissue (2), provoking a number of lesions including pododermatitis, visceral abscesses and mastitis (3, 4, 5, 6). Occasionally, abscesses in internal organs, most commonly lungs, liver and uterus, are observed (3).

High virulence strains provoke significant economic losses. They are due to the epizootic spread of the infection throughout rabbitries and inherent chronic problems (7, 8). Increased plasma CK activity is ordinarily observed after muscular myopathies or damage, myocardial infarction and cerebral lesions. A parameter that is often used to estimate the degree of muscle injury after exercise is the serum creatine kinase activity (9, 10). In the available literature we did not find results for elevated CK after S. aureus infection.

According to GOLDBLAT (11) creatine kinase (CK, EC 2.7.3.2) is an enzyme expressed by various tissues and cell types, that consume ATP rapidly. CK catalyses the reversible conversion of creatine and consumes Mg adenosine triphosphate (Mg-ATP) to create phospho-creatine (PCr) and Mg adenosine diphosphate (Mg-ADP) (Figure 1). This enzyme reaction is reversible, which means that phosphate of creatine phosphate is readily exchangeable with the γ-phosphate of...
ATP through a reaction catalyzed by creatine kinase, leading to Mg-ATP formation from PCr and Mg-ADP (12). SCHLATTNER et al. (13) describes 3 cytosolic CK isoforms and 2 mitochondrial isoenzymes, the ubiquitous mtCK (present in non-muscle tissues) and the sarcomeric mtCK (present in sarcomeric muscle). Skeletal muscle expresses CK-MM (muscle type) (98%) and low levels of CK-MB (heart type) (1%). According to WALLIMANN et al. (14), mitochondrial mtCK and cytosolic CK are connected in a so-called PCr/Cr-shuttle or circuit. While mitochondrial creatine kinase is directly involved in the formation of phospho-creatine (PCr) from mitochondrial ATP, cytosolic CK regenerate ATP from ADP, using PCr shuttled to the cytosolic CK. The cytosolic enzymes are closely coupled to ATP-dependent processes, e.g. ATPases, like acto-myosin ATPase forming functional micro-compartment for muscle contraction, or ion pumps, like the calcium pump for muscle relaxation. The ATP regenerated by the cytosolic CK is used as an energy source by the ATPases in situ. Therefore PCr is not only an energy buffer but also a cellular transport form of energy between sub-cellular sites of energy production (mitochondria and glycolysis) and those of energy utilization (ATPases) (15).

Figure 1. Reaction catalyzed by the creatine kinase (CK)

According to LORENZO et al. (16) the rabbit muscle CK contain 17 histidine residues, whose only 5 are conserved in all guanidino-kinase sequences published to date. Four of the conserved histidines (H96, H105, H233 and H295) are not essential for activity, while the last one, H295 (17), is located in the active site of CK. Normal blood CK activities vary between 60 and 400 U/L in humans [1], and intervals of usual values are also established in various animals species (goats, sheep, cows, pigs, horses, dogs, cats, lamas, monkeys, rats, birds and fishes) (18). Elevated CK activity is an indication of damage to muscle. It is therefore indicative of injury, rhabdomyolysis, muscular dystrophy, myositis, malignant hyperthermia, and neuroleptic malignant syndrome. Isoenzyme determination has been used extensively as an indication for myocardial damage in heart attacks. The purpose of the current study is to determine plasma CK activity at different intervals after skin S. aureus infection. Rabbits were randomly allotted into 2 groups: 7 were infected (group 1) and 6 served as negative controls (group 2).

Experimental design

Animals from the first group were subcutaneously inoculated with 0.1 mL 8x10^8 cfu/ml S. aureus. The experimental staphylococcosis was reproduced by a highly virulent field S. aureus strain, as described by WILLS et al. (19). The not infected control rabbits were inoculated with saline.

Blood samples

Blood samples were taken from the v. auricularis puncture into sterile tube with heparin as anticoagulant before injection, at the 6th, 24th, 48th, 75th hours and at the 7th, 14th and 21st days after inoculation. After centrifugation (1500g, 15 minutes, 4°C), plasmas were carefully harvested and stored at -20°C until analysis.

Clinical, biochemical and bacteriological analyses

The rectal body temperature was measured as well as the appearance and the size of the formed abscesses. Some parameters concerning the general condition of the animals, like behaviour, intake of water and food were also reported.

The CK activity was determined by a commercial kit from Hospitex Diagnostics...
(Italy), where creatine kinase catalysed conversion of creatine phosphate and ADP to form ATP. ATP connected with glucose and form glucose-6-phosphate and ADP. Glucose-6-phosphate dehydrogenase catalysed receiving of NADPH, measured at 340 nm.

Samples from the abscesses of the sick rabbits and samples from the internal organs of one died rabbit were cultivated on blood agar (BUL-BIO NCIPD, Sofia, Bulgaria) under aerobic conditions and temperature of 37°C for 24 hours. Identification of the isolates was done using routine bacteriological practices according to KLOOS and BANNERMAN (20). The fibrinogen concentration was measured using method of Podmore (21).

**Haematological investigations**

Individual blood samples of rabbits were collected from v. auricularis externa in Na₂EDTA tubes before (hour 0); on hours 6, 24, 48, 72 and days 7, 14 and 21 days after the challenge with *S. aureus*. White blood cell counts (G/l) were assayed on automated haematology analyzer BC 2800 Vet MINDRAY-China. Differential blood cell counts were determined on Romanovski-Giemsa stained blood smears.

**Statistical analysis**

The statistical analysis of the data was performed using one way analysis of variance (ANOVA). Differences were considered as significant when p value was less than 0.05.

**RESULTS**

The rabbits from the experimental group were lethargic, without taking of water and food, since within the first 24 hours after the injection. The body temperature started to increase and reached 42°C in one rabbit and fluctuated around 40°C up to 96 h after infection.

Within 48-96 hours post infection, abscesses appeared in all rabbits at the injection site. Fourteen days post inoculation an abscess of about 2-3 cm in diameter has appeared at the site of bacterial suspension application in one rabbit. The overlying skin was necrotic and a creamy greyish-yellow purulent discharge was observed (Figure 2). In one rabbit, on day 21, there was a spreading diffuse subcutaneous swelling (phlegmon) that affected the left lateral and the ventral thoracic region (Figure 3A). Dorsally, fistulae and purulent discharge were detected. After overlying skin removal, the purulent exudate appeared as a thick yellowish gray matter (Figure 3B). At the same time, disseminated abscesses were observed deeply in the thoracic and abdominal musculature (Figure 3B). The bacteriological examination of swab samples from abscesses has confirmed the presence of the inoculated *S. aureus* field strain and no bacteria was isolated from the internal organs. The mortality rate during the 21 day longs period was 28.6% (2/7) in the group of the inoculated rabbits.

![Figure 2](image-url)

*Figure 2.* An abscess of about 2-3 cm in diameter has appeared at the site of bacterial suspension application (black arrow) in one rabbit 14 days after the subcutaneous inoculation (100 μl) of a highly virulent field *S. aureus* strain (8x10⁸ cfu/ml). The overlying skin was necrotic and a creamy greyish-yellow purulent discharge (white arrow) was observed.
Figure 3. A. Spreading diffuse subcutaneous swelling (phlegmon) that affected the left lateral and the ventral thoracic region (white arrow) appeared at the site of bacterial suspension application (black arrow) in one rabbit 21 days after the subcutaneous inoculation (100 µl) of a highly virulent field S. aureus strain (8x10^8 cfu/ml). B. After overlying skin removal, the corresponding purulent exudate (black arrow) associated with abscesses in the thoracic musculature (white arrow).

In the not infected control rabbits, the plasma CK activity fluctuated from 280,75±57,05 at 72h to 590,25±188,03 U/L at 48h according to time but not significantly. In the infected group, a marked increase in the plasma CK activity compared to controls (p < 0.05) was recorded on the 48 hour (Figure 4). In average, the enzyme activity was multiplied by a factor 2.75 compared to the mean value recorded in not infected rabbits at the same time and by a factor 4 compared to initial value in the same group. Thereafter the CK activity abruptly declined in all rabbits except for one in which a dramatically elevated value (6582 U/L) was obtained at 48h. This rabbit rapidly died after. On 72h , CK activities were closely related in the 2 groups.

Figure 4. Variations of the plasma CK activities in rabbits (n = 7) during a 21 days long period after S. aureus infection experimentally induced by a subcutaneous injection (100 µL) of a bacterial suspension (density: 8x10^8 cfu/mL). Control rabbits (n = 6) were not inoculated. Results are expressed as mean ± standard deviation. *: p < 0.05.

The dynamics of white blood cell counts in both groups was different (Table 1).

On hour 0 (before infection), total white blood cell counts in experimental and control groups non-obese rabbits were 8.91±2.07x10^9/l and 10.80±2.33x10^9/l, respectively. By the 7th post infection day, they attained 15.24±5.38x10^9/l in the experimental group (p<0.01) versus both baseline and control group and 9.18±4.37x10^9/l in non-infected controls (Table 1).
Table 1. Variations of the plasma white blood concentration in rabbits (n = 7) during a 21 days long period after S. aureus infection experimentally induced by a subcutaneous injection (100 μL) of a bacterial suspension (density: 8×10⁸ cfu/mL). Control rabbits (n = 6) were not inoculated. Results are expressed as mean ± standard deviation.

*p < 0.05; **p < 0.01; ***p < 0.001 between groups
a, b p<0.05; 0.01, 0.001 comparing with initial level in the experimental group

<table>
<thead>
<tr>
<th></th>
<th>Leu (G/L)</th>
<th>PsE (%)</th>
<th>Eo (%)</th>
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<tr>
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<tr>
<td>0 h</td>
<td>8.91 ± 2.07</td>
<td>35.00 ± 4.83</td>
<td>0.57 ± .54</td>
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<td>6 h</td>
<td>7.89 ± 1.43</td>
<td>34.43 ± 4.50</td>
<td>0.43 ± .55</td>
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<td>24 h</td>
<td>6.13 ± 3.32</td>
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<td>48 h</td>
<td>8.15 ± 3.42</td>
<td>40.67 ± 3.67</td>
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<td>72 h</td>
<td>7.65 ± 3.06</td>
<td>52.83 ± 5.71</td>
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<td>2.17 ± 0.75</td>
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<td>7 days</td>
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<td>14 days</td>
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<td>21 days</td>
<td>9.90 ± 3.25</td>
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<td>0 h</td>
<td>10.80 ± 2.33</td>
<td>30.25 ± 4.35</td>
<td>0.75 ± 0.96</td>
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<td>6 h</td>
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<td>48 h</td>
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<td>72 h</td>
<td>10.93 ± 3.80</td>
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<td>2.25 ± 0.50</td>
<td>72.50 ± 6.25</td>
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In the control rabbits, there were no significant changes in this parameter, while the infected animals exhibited a considerable increase (P<0.01) on post infection day 7, 2-3 days after the appearance of clinical signs. In the experimental group non-obese rabbits, pseudoeosinophil percentages increased statistically significantly since 35.00±4.83% to 52.83±5.71% (p<0.01) as early as the 72nd hour of infection. These counts persisted high (near the upper reference range limit) until the end of the trial period (post infection day 21). On day 7, pseudoeosinophil proportion was the highest – 57.60±14.01%, followed by gradual decline to 56.00±9.70% on day 14 and 54.20±6.83% on day 21. The levels of significance of differences versus controls after the 72nd hour were p<0.01 versus baseline levels and p>0.001 versus controls. At the same time, substantial variations in this cell population in the control group were not registered (Table 1).

In infected non-obese rabbits, significant changes in neutrophil counts (p<0.001) occurred 72 hours after the challenge when they decreased from 63.00±5.32% to 45.00±5.76%. These changes consisted in reduction of lymphocytes to the lower limit of the reference range. The decline was maintained at a significantly lower level vs baseline (p<0.001) until the end of the 21-day experimental period (Table 1).

With respect to eosinophil and monocyte percentages, they did not change statistically significantly in control and experimental groups.

The concentration of fibrinogen at the beginning of the experiment in the infected group was 1.21±1.04 g/L (Figure 2). The increasing was established already at hour 6 after infection and at the 24th hour the concentration reached 2.19±1.35 g/L. At this time point the difference of fibrinogen concentration was significant (P<0.05) compared initial level in the experimental group. The mean value of fibrinogen measured at day 7 after infection was 4.17±3.81 g/L (P<0.001) and on days 14 and 21 were established decreasing of fibrinogen concentration. The concentration at day 21 was 3.15±2.57 g/L and the difference was...
significantly different compared the initial level (P<0.05).
In the control group the level of fibrinogen varied between 1,18±1,02 g/L and 2,31±1,82 g/L and there was not significant difference in the group. Significant difference between groups were established in the period between 48th hour and day 21 after infection.

**DISCUSSION**

In all rabbits infected with the virulent S. aureus field strain, abscesses occurred at the injection site within 48-96 hours and have greatly developed in some cases leading to the phlegmon formation and to secondary abscesses into the thoracic and abdominal musculature. The time course of abscess formation observed in the present study was similar to the time interval observed by WILLS et al. (19, 22). Moreover, the inoculated S. aureus strain was successfully re-isolated from all the swab samples from abscesses, proving the successful reproduction of the experimental infection.

It is often assumed that the CK activity is somehow related to the magnitude of muscle injury (12). The intriguing results from this experimental study concerning the plasma CK activity of infected rabbits revealed that this enzyme activity has effectively markedly increased but that the observed changes were transient despite the induced long-term inflammation. The deep-seated infection of the subcutaneous tissue that resulted in the progressive destruction of fascia and musculature, as well as the proteolytic enzymes of accumulated leukocytes led to muscle tissue lysis at the site of injury. The breakdown necrotic products may facilitate the disruption of muscle cell membranes and result in release of creatine kinase in plasma and afterwards, in autointoxication that caused the death of one rabbit at the 72nd hour post infection. Probably, staphylococcal exotoxins damage all skeletal muscle and heart muscle membranes, leading to marked increase in the plasma CK activity (13, 23). Among the bacterial exotoxins, those of the streptococcal origin (responsible for the rash of scarlet fever) probably play a critical part in the pathogenesis of the toxic shock syndrome. They act as super-antigens in vitro, interacting simultaneously with the major histocompatibility complex class II antigens on antigen-presenting cells and specific Vβ-regions of T-lymphocyte receptors in the absence of classic antigen processing. The consequence of such interaction is the concomitant synthesis of various cytokines (TNFα, IL1β, IL6, IL2, interferon γ, TNFβ) (3) which greatly amplify the inflammatory reaction. The general course of CK activity modulation is comparable with the earlier reported change in the plasma from stress susceptibility in pigs, or systematically blood sampling (9, 11). BACCOU and BRESCOT (23) revealed that repeated blood sampling induced in rabbits a reaction with a similar pattern, but a different intensity, expressed by an elevated plasma CK activity. In the same way, variations of the CK activities in the plasma of controls in the present study would be induced by the emotional stress due to handling and probably by the additional stress of the venipuncture. YERROUM et al. (24) reported that CK values were higher 564 U/L in rats held behind the front legs compared to control group (272 U/L) (p < 0.01). The physiological significance of the observed changes in plasma CK activity during acute inflammation could be associated with increased production of some positive acute phase proteins such as haptoglobin, fibrinogen, ceruloplasmin during prolonged inflammation, as shown in weaning rabbits with E. coli infection (25), and C-reactive protein in rabbits treated for 26 days twice weekly with turpentine (26).

Immune system activation occurring under the effect of proinflammatory stimuli is characterised with changes in specific markers of inflammation. A classic example is the change in total leukocyte and in particular, neutrophil counts (27).

In our studies, a similar trend in the time course of white blood cell counts was also observed – leukocytosis with pseudoeosinophilia (in rabbits, pseudoeosinophils correspond to neutrophil granulocytes in other mammalian species). A statistically significant increase in total leukocyte counts was determined only by the 7th post infection day. The increase persisted until the end of the experimental period, but differed considerably versus hour 0 only on the 7th day.

The pseudoeosinophil percentages after staphylococcal infection increased statistically significantly after the 72nd hour and were maintained higher versus baseline values until the 21st day. At the same time, lymphocyte percentages in this group were reduced from post infection hour 72 until the end of the study.

The obtained results about white blood cell changes in rabbits with experimental staphylococcal infection are comparable to
those of two American research teams, who studied rabbits as a model toxic shock induced by purified \textit{Staphylococcus aureus} toxin (28) and the pathological response of rabbits to purified staphylococcal toxin challenge (5). In both investigations, there were no significant changes in red blood cell picture except for a slight reduction of haemoglobin content and gradual decrease in platelet counts. Simultaneously, the authors established a sharp elevation of total leukocytes until the 48th post treatment hour followed by increase in both total white blood cell counts and pseudoeosinophils (from 30% to 70-80%) on the account of lower proportion of lymphocytes.

Elevation of fibrinogen, haptoglobin and ceruloplasmin as acute phase proteins was recorded in our previous experiment with \textit{E.coli} in weaning rabbits (10) and also in chickens after \textit{E.tenella} and \textit{E.coli} invasions (29).

The significant elevation of fibrinogen in the present study may be attributed to the involvement of fibrinogen in homeostasis, providing a substrate for fibrin formation and tissue repair. Whereas the antioxidant markers remained stable in untreated control rabbits (n=6), significant decreases in paraoxonase1, ferric reducing antioxidant power (FRAP) and thiol concentrations compared to the initial and control values were recorded on day 1, 2 and 3, respectively in infected rabbits (n=7) and have persisted until the 7th day for the enzyme activity, the 14th day for the FRAP value and the 21-st day for the thiol concentrations (30).

In conclusion we assumed that disseminated abscesses were observed deeply in the thoracic and abdominal musculature in all rabbits at the period 48-96h after inoculation and 2 rabbits from the experimental group were died before the end of the experiment. By the 7th post infection day, total white blood cell counts in experimental group attained apparently twice more than the period before infection (p<0.01). On day 7, 2-3 days after the appearance of clinical signs, pseudoeosinophil proportion was the highest. The fibrinogen concentration in the experimental rabbits elevated significantly at 24 hour after invasion and extended till day 21.

Plasma concentration of CK activity could be used as a significant biomarker for early detection of severe skin infection affecting also the underlying musculature in rabbits simultaneously with the appearance of the first clinical signs. The amplitude and the duration in the elevation of the enzyme activity in rabbits is currently associated with muscles, brain and hearth diseases, and would be also indicative of the tissue damage provoked by the \textit{S. aureus} exotoxins.

**REFERENCES**

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