STUDY ON THE TOTAL PROTEINS AND MAIN PROTEIN FRACTIONS IN RABBITS EXPERIMENTALLY INFECTED WITH STAPHYLOCOCCUS AUREUS


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ABSTRACT
Infections with Staphylococcus aureus are a common cause which can induce severe skin infections in rabbits leading to important economic losses. The present study was conducted to evaluate the changes in the concentrations of total protein, albumin, globulin, fibrinogen, and albumin/globulin ratio during S. aureus infection using a model for experimental infection of rabbits. The experiment was carried out on 13 rabbits at the age of 3 months. Infection was induced by inoculation of 7 rabbits by 100 μL of bacterial suspension of a field S. aureus strain (density: 8x10⁸ cfu/mL) and 6 other rabbits were not treated (controls). Blood samples for principal protein analysis were collected before (0 h) and at 24, 48 and 72 h on day 7, 14, and 21 after infection. Total protein level showed little changes in the experimental group. The concentration of albumin decreased in the experimental group significantly (P<0.05) in the experimental group and slightly in the control group. Albumin/globulin ratio was lowered and significantly different on day 4 (P<0.01) and on day 7 (P<0.001). The concentration of fibrinogen was used as an acute phase protein with aim to confirm the incidence of infection. In parallel, rectal temperature and skin lesions (abscesses) were recorded. In all infected animals, formation of abscess due to the proliferation of the inoculated strain was observed within 48-96 h following the bacterial inoculation and these lesions have gradually extended leading to purulent exudates several days after and sometimes to secondary abscesses in surrounding muscles.

Key words: Rabbits, blood, protein fractions, infection, Staphylococcus aureus

INTRODUCTION
Proteins are present in all body fluids, but the blood plasma proteins are examined most frequently for diagnostic purposes (1, 2, 3, 4, 5). Over one hundred individual proteins have a physiological function (transport, humoral immunity, maintenance of oncotic pressure, enzymes, protease inhibitors, buffering) in the plasma. In fact, 22 of the blood proteins comprise approximately 98% of the protein content of plasma or serum, and the remaining 2% comprise the 1000s of low-abundance proteins that are likely to be useful as biomarkers (6).

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Principal plasma proteins are albumins (Alb) and prealbumin; α₁-globulins (α₁-antitrypsin, α₁-acid glycoprotein), α₂-globulins (haptoglobin, α₂-macroglobulin, ceruloplasmin); β-globulins (transferrin, low density lipoproteins, C₃) and γ-globulins (fibrinogen and immunoglobulins) (7, 8, 9). They are large, osmotically active proteins with an average molecular weight of 69 kDa. Globulins (Glb) have molecular weights ranging from 90 kDa (β₁-globulins) to 156 kDa (γ-globulins) (10).

Changes in serum protein concentrations result in a variety of clinical signs and systemic effects and are associated with a number of disease processes and synthesis of approximately 40 acute phase proteins (APPs), such as α₁-antitrypsin, α₁-acid glycoprotein,
haptoglobin, α2-macroglobulin, ceruloplasmin, transferrin, fibrinogen, C-reactive protein, serum amyloid A, tumour necrosis factors (11), and etc. The concentrations of these plasma proteins often increase (positive APPs) and more rarely decrease (negative APPs) during the acute phase response to inflammation or infection. They play important defensive roles (12, 9, 13, 14, 15), and their expression represents effector mechanisms of innate immunity (16, 17). These remarkable APPs have been extensively studied in humans and in laboratory animals (18, 11, 19).

The response to infection involves a large number of changes and leads to a wide-ranging APP response in acute inflammation. Within the literature there are few systematic data concerning APPs variations induced by bacteria in rabbits, but variations of the major plasma protein concentrations after inflammatory process are poorly investigated (20, 21, 19).

*S. aureus* is a versatile opportunistic pathogen that causes a wide spectrum of pathologies (22). This bacterium affects rabbits of different ages, infects dermal lesions and invades subcutaneous tissues (23), resulting in different pathologies, including subcutaneous abscesses, exudative and supportive dermatitis (24, 25); pododermatitis (26, 27); mastitis (26); infertility and death (28). Economic losses in industrial livestock husbandry attributed to staphylococcal infections are considerable at a worldwide.

The present study was conducted to evaluate the changes in the concentrations of total serum protein (TSP) and major proteins, i.e. albumin (Alb) and globulins (Glb), also albumin/globulin (A/G) ratio and fibrinogen during experimentally induced *S. aureus* infection in rabbits.

**MATERIALS AND METHODS**

*Animals and study design.* The experimental procedure was approved by the Ethic Committee at the Faculty of Veterinary Medicine, Trakia University. Thirteen male, New Zealand White rabbits (3 months old), with average weight of 3.2 kg were used in this study. They were housed in grow-out batteries with access to water and commercial feed *ad libitum*. Rabbits were randomly divided into 2 groups: 7 were infected (group 1) and 6 served as negative controls (group 2). Animals from the first group were subcutaneously inoculated with 0.1 mL 8x10⁸ cfu/ml *S. aureus*. The experimental staphylococcosis was reproduced by a highly virulent field *S. aureus* strain, as described by Wills et al. (29). The not infected control rabbits were inoculated with saline. Blood samples were taken from the *v. auricularis* puncture into sterile tube with heparin as anticoagulant before injection, at the 6th, 24th, 48th, 72nd h and at the 7th, 14th and 21st days after inoculation. After centrifugation (1500g, 15 min, 4°C), plasmas were carefully harvested and stored at -20°C until analysis. Plasma samples were also stored at -20°C until assayed.

Samples from the abscesses of the sick rabbits and samples from the internal organs of one infected dead rabbit were cultivated on blood agar (BUL-BIO NCIPD, Sofia, Bulgaria) under aerobic conditions and temperature of 37°C for 24 h. Identification of the isolates was done using routine bacteriological practices according to Kloos and Bannerman (30).

**Analytical methods.** Swab samples of purulent exudates were collected from 6 rabbits from Group 1 at the time of fistulisation of the abscesses formed. Samples were inoculated on blood agar with 8-10% sheep blood (BUL-BOU base, National Institute of Infectious and parasitic diseases). Cultures were incubated aerobically for 24 h at 37°C. For dead animals and for animals slaughtered on day 21 post-infection, the identification of bacteria isolates was performed according the routine bacteriological techniques (15) from the internal organs (liver, spleen, kidney and heart), skin lesions and the site of bacteria injection.

The TSP concentrations were measured by the biuret method (31). Serum Alb concentrations were determined by the Bromcrezol green method with kit, supplied by “Human”, Gesellschaft fur Biochemica, Germany SU-ALBU INF 156001. Glb concentrations were determined by subtracting the Alb concentrations from the TSP concentration. The fibrinogen concentration was measured using method of Podmore, described by Todorov, (32).

The data for the microclimatic parameters of experimental animals are given in Table 1.
Table 1. Microclimatic parameters in the rabbit’s living area.

<table>
<thead>
<tr>
<th>Periods</th>
<th>Ambient temperature (°C)</th>
<th>Air humidity (%)</th>
<th>Air velocity (m/s)</th>
<th>NH₃ (ppm)</th>
<th>Light intensity (lx)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thermonutral period</td>
<td>20.50±2.5</td>
<td>65.07±4.59</td>
<td>0.2±0.05</td>
<td>traces</td>
<td>45.00 ±3.27</td>
</tr>
<tr>
<td>Reference values*</td>
<td>16–25</td>
<td>65–70</td>
<td>0.2</td>
<td>traces</td>
<td>50</td>
</tr>
</tbody>
</table>

*Reference values as per Anonymous Regulation 44/2006

The rearing conditions for the experimental rabbits were fully complied with the minimum requirements for humane treatment of experimental animals (Regulation 20/2012; Regulation 44/2006).

Statistical analysis. All data are expressed as mean ± standard deviation (SD). The statistical analysis of the data was performed using one way analysis of variance (ANOVA). The significance of differences of means between post infection and base line values and between values, observed in the experimental and control groups for the same time were evaluated by the one way test. Differences were considered as significant when p values were less than 0.05.

RESULTS

The TSP concentrations changed weakly in the experimental group (Figure 1), and had a tendency to be higher comparing initial levels and in absolute values started since 58.5±6.2 g/L to 60.8±6.9 g/L at day 21 after inoculation of the bacterial suspension. In the control group total protein concentration was relatively constant and varied between 59.7±5.0 g/L to 61.0±6.8 g/L.

Serum Alb concentrations in the experimental rabbits started since 32.2±0.3 g/L before experiment (Figure 2), gradually decreased according to time: on day 4 the mean value was 27.8±1.9 g/L and on day 7 it was 25.4±3.2 g/L. In the control rabbits serum Alb concentrations varied between 33.23±3.2 and 34.4±4.0 g/L (Figure 3). The variations of the Glb concentrations according to time were quite different in the control and infected rabbits (Figure 3). Whereas they dramatically increased in the experimental group till day 7 (from 26.3±4.0 g/L before experiment to 29.0±2.5 g/L at 72th h, and to 35.4±2.6 g/L on day 7), they remained roughly constant in control animals. Moreover there was statistically significant differences in Glb concentrations (P<0.05) between control and infected rabbits. Consequently, the A/G ratio (Figure 4), which started since 1.27±0.11 before experiment went to 0.96±0.04 at 72th h, continued to decrease on day 7. These values were significantly different at 72th h (P<0.01) and on day 7 (P<0.001). In the control group this ratio varied between 1.23±0.06 to 1.31±0.07. The maximum of the changes between groups occurred in the A/G ratio (Figure 4). The decrease in A/G ration between 72th h and day 7 was due from one hand to the decreasing of the Alb and to another hand to the increasing of Glb. Also there was a significant difference in the experimental group as compared the pre-challenge levels up days 4-7 (72th h - 168th h) of the experimental period (Figure 4).
Figure 2. Variations of serum Alb concentrations (g/L) according to time in rabbits subcutaneously infected by 100 μL of bacterial suspension of a field S. aureus strain (Density: 8x10^8 cfu/mL).

Figure 3. Variations of serum Glb concentrations (g/L) according to time in rabbits subcutaneously infected by 100 μL of bacterial suspension of a field S. aureus strain (Density: 8x10^8 cfu/mL).
* (p < 0.05) indicates a significant difference between the 2 groups compared
* superscripts indicates significant difference to initial values for a given group for a given time (*: p < 0.05).

Figure 4. Variations of the A/G ratio according to time in rabbits subcutaneously infected by 100 μL of bacterial suspension of a field S. aureus strain (Density: 8x10^8 cfu/mL).
* (p < 0.05), ** (p < 0.01), *** (p < 0.001) indicate a significant difference between the 2 groups compared.
Different superscripts indicate significant differences to initial values for a given group for a given time (*: p < 0.05, **: p < 0.01 and ***: p < 0.001, respectively).
The results of the present study further elucidate the behaviour of major blood proteins in response to infection in rabbits. The concentrations of TSP were weakly affected by the subcutaneously *S. aureus* administration. In inoculated rabbits, while hypo-albuminemia compared to control values was evidenced since the 7th day, blood Glb concentrations and essentially the A/G ratio markedly varied within the experimental period. The A/G ratio was significantly lowered on days 4-7 probably because of the dramatic decreasing in albumin concentration and increasing of the concentrations of some APPs from α2-globulins fraction like haptoglobin, ceruloplasmin at a lesser extent and γ-globulins concentrations.

The concentration of the fibrinogen at the beginning of the experimental group was 1,21±1,04 g/L (Figure 5). The tendency of increasing was observed since hour 6 after infection in the same group and at the hours 24, the difference between these two values (2,19±1,35 g/L) was significant (P<0,05). The fibrinogen values at experimental group at day 7 reached 4,17±3,81 g/L, and between day 14 to day 21 decreased to 3,15±2,57 g/L, but again were significantly higher (P<0,05) comparing the initial levels. In the control rabbits the values varied between 1,18±1,02 g/L to 2,31±1,82 g/L, and these values were not significant compared with initial levels. The significant differences between groups were established at the period since day 3 to day 21 after infection.

**Figure 5.** Variations of the plasma fibrinogen concentration in rabbits during a 21 days long period after *S. aureus* infection experimentally induced by a subcutaneous injection (100 μL) of a bacterial suspension (Density: 8x10⁸ cfu/mL)

* (p < 0.05), ** (p < 0.01), *** (p < 0.001) indicate a significant difference between the 2 groups compared. Different superscripts indicate significant differences to initial values for a given group for a given time (a: p < 0.05, b: p < 0.01 and c: p < 0.001, respectively).

The rabbits from the experimental group were lethargic, without taking of water and food, since within the first 24 h 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.

**Figure 6.** Severe lesions of subcutaneous tissue-purulent inflammations (phlegmons) affecting vast areas (white arrow) near the site of the bacterial *S. aureus* suspension injection (black arrow) (Density: 8x10⁸ cfu/mL, injection volume: 100 μL) in rabbits 21 days later.
Within 48-96h 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 6). In one rabbit, on day 21, there was a spreading diffuse subcutaneous swelling (phlegmon) that affected the left lateral and the ventral thoracic region. Dorsally, fistulae and purulent discharge were detected. After overlying skin removal, the purulent exudates appeared as a thick yellowish grey matter. At the same time, disseminated abscesses were observed deeply in the thoracic and abdominal musculature. The bacteriological examination of swab samples from abscesses has confirmed the presence of the inoculated *Staphylococcus aureus* field strain and no bacteria was isolated from the internal organs.

**DISCUSSION**

Archetti et al. (35) reported reference ranges for TSP between 32 and 61 g/L, for growing rabbits which is coincided with our results. Stezenic (cited by 16) reported for higher results in adult rabbits (43±0.5 g/L) for the serum Alb concentrations than measured in the present study in control rabbits. Our observed results for decreasing of serum Alb concentrations in infected rabbits from the 4th day to the 7th day was in agreement with the study of Orhue et al. (19). They reported a significant diminution of Alb 14 days after *Trypanosoma brucei* - infection in rabbits probably due to impaired liver synthesis and/or losses via the gut, kidney or both. Moreover, Alb could be considered as a negative APPs in mammalian and bird species (7, 36, 18, 37, 38, 39).

The TSP concentrations remained fairly constant 6 days after the induction of an inflammation process according to Schreiber et al. (18). In the present study, any significant variation of this parameter compared to controls was not recorded in *S. aureus* infected animals. Alb is the most appropriate for decreasing because of its metabolic adaptation: the half-life of the Alb is very long (18-19 days), its total body pool is the largest among the plasma proteins and it has no specific indispensable function. On the other hand, the amino acids necessary for positive APPs synthesis may be also supplied throughout the ubiquity dependent catabolism of muscle proteins (18).

The easy determination of the serum Glb concentrations may contribute to the detection of an inflammatory process because Glb are positive APPs (37, 9, 13, 14, 11, 41, 38). This parameter significantly increases in the infected rabbits at the 7th day. According to Burtis (7), increased immunoglobulin concentrations are seen in both acute and chronic infections, but the measurement of the specific immunoglobulin class concentration does not supply any positive diagnostic gain.

Elevation of fibrinogen, haptoglobin and ceruloplasmin as an acute phase proteins was recorded in our previous experiment with *E.coli* in weaning rabbits and also in chickens after *E.tenella* and *E.coli* invasions (42, 43).

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 21st day for the thiol concentrations (44).

**CONCLUSIONS**

The observed variations of blood Alb and Glb concentrations showed that Alb related as negative APPs in rabbits in response to an experimental bacterial subcutaneous infection and Glb were positive APPs. The A/G ratio could serve as a reliable and relatively precocious marker for the early detection of *S. aureus* infections in rabbits simultaneously with the appearance of clinical signs. Fibrinogen concentration was significantly higher in the experimental group that showed it as a positive acute phase protein.

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Trakia Journal of Sciences, Vol. 15, № 1, 2017


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