PHAGOCYTOSIS IN \textit{Staphylococcus aureus} BACTERIAEMIA IN DOGS*

\textit{FAGOCITOZA KOD Staphylococcus aureus BAKTERIJEMLJE KOD PASA}

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\textit{Staphylococcus aureus} is the leading pathogenic cause of nosocomial infections, especially in bacteriaemia and sepsis. Phagocytosis is known to be an essential factor of innate immunity. The aim of this study was to evaluate phagocytic activity of leucocytes in experimental bacteriaemia.

Bacteriaemia was induced in six adult male mongrel dogs (experimental group) by intravenous injection of \textit{Staphylococcus aureus} isolate ($1.2 \times 10^9$ cells/ml). Phagocytic activity was evaluated before infection (0 h.), and on the 2nd, 6th, 24th, 48th hour, and on the 7th, 14th and 21st day after infection. Six control animals were tested in the same dynamics. Phagocytic activity was evaluated by using the Nitrotetrazolium blue reduction (NBT) test, and the immune fluorescence method (Samnaliev et al., 1995) for detection of phagocytic index and percentage of phagocyting leucocytes (FITC marked \textit{Staphylococcus aureus} were used).

Percentage of phagocyting leucocytes showed an increase in experimental animals, compared to control animals, on the 24th h.

Production of reactive oxygen species, evaluated by NBT, showed changes within experimental group as follows: an increase on the 24th h. compared to the 2nd h., 6th h., and the 14th day after infection; and an increase on 48th h., compared to the 2nd h. and 14th day.

Comparison of reactive oxygen species production between groups, revealed an increase in the experimental group, as compared to the control group, on the 24th h. and on the 48th h.

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In conclusion, cell elements of innate immunity are mostly activated on the first two days after inducing Staphylococcus aureus bacteremia in dogs.

Key words: Staphylococcus aureus, experimental bacteremia, phagocytosis, dog

Introduction / Uvod

Staphylococcus aureus is the leading pathogenic cause of nosocomial infections, especially in bacteremia and sepsis. Factors of innate resistance of the organism play a major role in pathogenesis of staphylococcal infection [5]. Phagocytosis is an essential factor of innate immunity [19]. Phagocytes play an important role in mechanism of immune response by means of cytokine production and antigen destruction by enzymes and cytotoxic components [2, 22]. Release of exotoxins by gram positive bacteria, most of which are superantigens i.d. stimulate T-cells, leads to differences in cell response in relatively low levels of tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1) and interleukin-6 (IL-6) and high levels of interleukin-8 (IL-8) [4, 24, 30]. Gram positive bacteria have immunogenic elements on their surface- lipoteichoic acid and peptidoglicans [24, 30]. Monocytes contacting these molecules start producing proinflammatory cytokines [25]. Macrophages, together with monocytes, play an important role in the defense against staphylococcal infection. Some proinflammatory mediators activate macrophages to produce intracellular oxide radicals [18]. Engulfment and destruction of microorganisms, a process known as phagocytosis, is based on the production of oxide radicals, other cytotoxic products or lysosomal components [23, 16].

The ability of phagocytes to kill bacteria is decisive, because such a defense is not specific and does not depend on initial counteraction between micro and macroorganism [7]. Gram negative microorganisms can be neutralized in intercellular space by complement and antibodies [26], while gram positive microorganisms are submitted to intracellular killing by neutrophils and macrophages [31]. During bacterial infections phagocytic activity has been reported either increased or decreased depending on the kind of infection, the used techniques and the parameters detected.

The aim of the conducted study, was to evaluate changes in phagocytic activity of neutrophils during staphylococcal infection, experimentally induced by intravenous injection (v. jugularis) of bacterial culture (1.2x10^9 cells/ml) after 24 hours' growth.
Material and methods / Materijal i metode rada

Experimental animals / Eksperimentalne životinje

We used 12 healthy, mature, mongrel dogs, 6-7 years of age, weighing 16 ± 2 kg. The dogs were placed in individual cages of 1.5 m² and 1.8 m height. Each dog was fed individually and had free access to water. During the experiment the animals were fed a standard maintenance diet for adult dogs „Canil”-21% (Sosil Guyomarc, H, Sao Paolo, Brasil), as recommended by the producer.

In the adapting period, which lasted one week, the dogs were treated against parasites with Prazimec – D (Biovet Co, Peshtera, Bulgaria) (containing praziquantelum and abamectinum) at a dose of 1 tab./10 kg. Also, they were treated against ectoparasites with Tapilan (Dorvet, Israel).

Experimental design / Eksperimentalni postupak

Experimental infection was induced by intravenous injection (v.jugularis) of 5ml of bacterial culture of *Staphylococcus aureus* (1.2x10⁹ cells/ml) after 24 hours growth. A terrain strain was used. Identification of strain was done by "Sceptor - Becton Dickinson Diagnostic" system, in the department of “Hygiene, Microbiology, Infectious diseases and Epidemiology”, Faculty of Medicine, Thrakia University, Stara Zagora. Colonies had typical morphology of staphyloccoci (2-5 mm).

Parameters of innate immunity / Parametri urodjenog imuniteta

During the experiment the following parameters were detected:

Phagocytic activity of neutrophils-evaluated by their ability to produce oxide radicals using microscopic slide histochemic nitroblue tetrazolium chloride reduction (NBT) test.

Phagocytic index and percentage of phagocyting leucocytes: evaluated by the immune fluorescence method (Samnaliev et al., 1995-FITC marked *Staphylococcus aureus* were used).

Experimental animals were tested in the following dynamics: before infection (0h.), and on the 2⁰, 6⁰, 24⁰, 48⁰ hour and on the 7⁰, 14⁰ and 21⁰ day after that. Animals from the control group were tested in the same dynamics.

Statistical analysis / Statistička analiza

Results are presented as means±SE and were statistically processed by one-way ANOVA (StatMost, version 2.5), provided by DataMost corporation. Differences were considered statistically significant at the p<0.05 level.
Three parameters of innate immunity showed no statistically significant changes in animals of the control group (Fig. 1 and Fig. 2).

After inducing staphylococcal infection, comparison of phagocytic index between experimental and control group in dynamics, showed no statistically significant differences. The phagocytic index also did not change in infected animals within the period of experiment.

In the experimental group the percentage of phagocytizing neutrophils was significantly higher on the 2nd hour, 6th hour, 24th hour and 21st day compared to the 14th day as follows: 40.75±2.75>21.47±6.25, p<0.01; 36.45±3.46>21.47±6.25, p<0.05; 35.18±10.41>21.47±6.25, p<0.05; 31.64±6.1>21.47±6.25, p<0.05 (Fig. 1).

![Graph showing percentage of phagocytizing neutrophils in experimental and control groups.](image)

Figure 1. Percentage of phagocytizing neutrophils in dogs (n=6) with experimental S. aureus bacteriaemia, within 21 days lasting period ($\bar{x} \pm S\bar{x}$). Statistically significant changes are shown as follows: a1 – within experimental group (14 d./2h.); a2 – within experimental group (14 d./6h.); a3 – within experimental group (14 d./24h.); a4 – within experimental group (14 d./21 d.); c – in experimental group as compared to control group. ** - p<0.01; * - p<0.05

Slika 1. Procent fagocitirajućih neutrofila kod pasa (n=6) sa eksperimentalnom S. aureus bakterijemijom tokom perioda od 21 dana ($\bar{x} \pm S\bar{x}$). Statistički značajne promene su prikazane na sledeći način: a1 – unutar eksperimentalne grupe (14. d./2. sata); a2 – unutar eksperimentalne grupe (14. d./6. sata); a3 – unutar eksperimentalne grupe (14. d./24. sata); a4 – unutar eksperimentalne grupe (14. d./21. dana); c – u eksperimentalnoj grupi u poređenju sa kontrolnom grupom. ** - p<0.01; * - p<0.05
The comparison of the percentage of phagocyting neutrophils between two groups in the dynamics, revealed an increase in the experimental group compared to the control group on the 24th hour - 35.18±10.4>17±4.8, p<0.05. Trend was similar on the 7th day (31.1±10.41 in the experimental group and 14.98±9.71 in the controls, p<0.05) and on the 21st day (31.64±6.1 as compared to 19.98±6.75 in control animals, p<0.05) (Fig. 1).

When evaluating production of reactive oxygen species of neutrophils by the NBT-test in the dynamics in the experimental group we found a statistically significant increase on the 24th hour compared to the 2nd hour - 52.75±4.57>23±6.98, p<0.001 (Fig. 2). Similar changes occur on the 24th hour as compared to the 6th hour (35.5±13.9, p<0.05) and the 14th day (29.2±11.21, p<0.01). Values were also higher on the 48th hour (56.6±19.28) as compared to the 2nd hour (23±6.98, p<0.05), and the 14th day (29.2±11.21, p<0.05).

Figure 2. Production of reactive oxygen species of neutrophils, measured by NBT-test in dogs (n=6) with experimental S. aureus bacteriaemia, within 21 days lasting period (x±Sx). Statistically significant changes are shown as follows: a1 – within experimental group (24h./2h.); a2 – within experimental group (24h./6h.); a3 – within experimental group (24h./14d.); b1 – within experimental group (48h./2h.); b2 – within experimental group (48h./14d.); c – in experimental group as compared to control group; *** - p<0.001; ** - p<0.01; * - p<0.05
Comparing both groups in dynamics, we found higher reactive oxygen species production of neutrophils in the experimental group (Fig. 2). Values were higher as compared to the controls on the 24th hour (52.75±4.57>27.5±11, p<0.01), and on the 48th hour (56.6±19.28>30.25±9.14, p<0.05).

**Discussion / Diskusija**

Experimental infection in dogs, caused by intravenous application of a terrain strain of *Staphylococcus aureus*, is characterized by some changes in phagocytic activity of neutrophils. Cell elements of innate immunity are activated in the early stages of infection. This suggests rapid neutralizing of microorganisms.

Bacteremia and sepsis are often accompanied by the systemic inflammatory response syndrome (SIRS). In such cases macrophages produce a large number of pro-inflammatory mediators, which leads to the so called „inflammatory cytokine storm” [13]. Dysbalance between pro-inflammatory and anti-inflammatory mediators may lead to an exceedingly strong inflammatory response, followed by immunosupression, apoptosis and organ dysfunction. Neutrophils show changes in phagocytic activity [14] with high levels of neutrophil sequestration and reduction of their count [12, 28].

Cell elements of innate immunity, including neutrophils, have the ability to „recognize” bacterial cell components. Recognition can be direct or indirect by means of complement and antibodies bound to bacterial surface [31]. As a result opsonisation occurs, which makes easier lysis of bacteria by phagocytes [24]. In gram positive microorganisms these functions are done by lipoteichoic acid (LTA), peptidoglicans (PGNs), free and bound proteins (protein A, hemolysins and phenol soluble modulin) [15, 10].

LTA and PGNs cause release of NO, IL-1, IL-6 and TNF-α by monocytes and macrophages and activation of oxidative burst in vitro [32, 17].

In humans some changes in cell-mediated immunity during sepsis have been observed [1] - decrease in T-lymphocytes count, higher phagocytic activity of monocytes and macrophages and higher (over 50%) values of NBT-test, which is controversal to the above-mentioned hypothesis.

Phagocytosis is mostly activated in experimental staphylococcal infection in dogs within the 24th - 48th hour after infection [3]. This was also proved in the conditions of our experiment, where *Staphylococcus aureus* was induced intravenously.

After a subcutaneous injection of 5 ml bacterial suspension of *Staphylococcus aureus* 24 hours after of growth, Dimitrova et al., 2003, have observed a statistically significant increase of percentage of phagocytizing leucocytes up to 29.33 ± 0.42, as compared to initial levels 27.00 ± 052. Changes in phagocytic in-
dex are similar: 24 hours after inducing of infection values increase and are higher up to the end of the experimental period (1.85 ±0.05 on 8th day).

Although pro-inflammatory components and mediators stimulate phagocytosis (by increasing the number of engulfed bacteria and activating ROS production) and bactericidal activity of blood [6], in severe infections and intoxications phagocytosis may be suppressed [Krukowski & Smith, 1983]. It is possible that \textit{Staphylococcus aureus} uses the inflammatory response to reach certain structures of polymorphonuclear leucocytes, where it is protected from opsonisation and lysis [11].

### Conclusion / \textit{Zaključak}

Experimental \textit{Staphylococcus aureus} bacteriemia (in non lethal doses) leads to activation of cell elements of innate immunity mostly in the first two days after inducing the infection, characterized by an increase in percentage of phagocyting neutrophils and a higher production of oxide radicals, although it is known that \textit{Staphylococcus aureus} interacts with polymorphonuclear leucocytes, which prevents opsonisation and lysis.

### References / \textit{Literatura}

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FAGOCITOZA KOD Staphylococcus aureus BAKTERIJEMIJE KOD PASA

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Staphylococcus aureus je vodeći patogeni uzročnik nozokomijalnih infekcija, a posebno kod bakterijemije i sepse. Zna se da je fagocitoza bitan faktor urođenog imu-
niteta. Cilj ovih istraživanja bio je da se obavi procena fagocitne aktivnosti leukocita kod ek-
sperimentalne bakterijemije.

Bakterijemija je izazvana kod šest odrašlih pasa mešanaca, mužjaka, (ek-
sperimentalna grupa) putem intravenske injekcije izolata Staphylococcus aureus (1.2x10⁹
čelija/ml). Fagocitna aktivnost je procenjena pre infekcije (0 sat), 2, 6, 24, 48. sata, 7, 14., 21. dana posle infekcije. Šest kontrolnih životinja je ispitano istom dinamikom. Fagocitna

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aktivnost je procenjena koristeći test nitrotetrazolium plave redukcije (NBT test), a metoda
imune fluorescencije (Samnaille i sar, 1995) korišćen je za detekciju fagocitnog indeksa i
procenata leukocita koji fagocituju (korišćeni su Staphylococcus aureus obeleženi FITC-
om).

Procent fagocitirajućih leukocita pokazao je povećanje 24. sata kod eksperiment-
entalnih životinja u poredjenju sa kontrolnim životinjama.

Proizvodnja reaktivnih vrsta kiseonika procenjena korišćenjem NBT testa po-
kazala je sledeće promene unutar eksperimentalne grupe: povećanje 24. sata u poredjenju
sa rezultatima 2. i 6. sata i 14. dana posle inficiranja; kao i povećanje 48. sata u poredjenju
sa rezultatima 2. sata i 14. dana. Poredenje proizvodnje reaktivnih vrsta kiseonika izmedu
grupa pokazalo je povećanje u eksperimentalnoj grupi u poredenju sa kontrolnom grupom
24. sata i 48. sata posle inficiranja.

Može da se zaključi da se čelijski elementi urođenog imuniteta aktiviraju uglavnom
 tokom prve dve dana nakon izazivanja bakterijemija sa Staphylococcus aureus u
pasa.

Ključne reči: Staphylococcus aureus, eksperimentalna bakterijemija, fagocitoza, pas

**РУССКИЙ**

**ФАГОЦИТОЗ У STAPHYLOCCUS AUREUS БАКТЕРИЕМИИ У СОБАК**

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*Staphylococcus aureus* ведущий патогенный возбудитель нозокомиаль-
ных инфекций, а отдельно у бактериемии и сепсиса. Известно, что фагоцитоз
существенный фактор врождённого иммунитета. Цель этих исследований была
совершить оценку фагоцитарной активности лейкоцитов у экспериментальной бак-
териемии.

Бактериемия вызвана у шести взрослых собак емисов, самцов, (эк-
спериментальная группа) путём внутрикожной инъекции изолята *Staphylococcus au-
reus* (1,2х10⁹ клеток/мл). Фагоцитарная активность оценена до инфекции (0 час), 2,
6, 24 и 48 часов и 7,14 и 21 день после инфекции. Шесть контрольных животных
испытали нами тот же опыт. Фагоцитарная активность оценена, пользу-
зуя нитротетразолий синей редукции (NBT тест), а метод иммунной флуоресцен-
ции (Самналев и сот., 1995) использован для детекции фагоцитарного индекса и
процента лейкоцитов, фагоцитирующие (пользуя *Staphylococcus aureus* ФИТЦ-
ом).

Процент фагоцитирующих лейкоцитов показал увеличение 24 часа у эк-
спериментальных животных в сравнении с контрольными животными.

Производство реактивных видов кислорода оценено пользованием
NBT теста показало следующие изменения внутри экспериментальной группы:
увеличение 24 часа в сравнении с результатами 2 и 6 часов после инфици-
рования; и увеличение 48 часов в сравнении с результатами 2 часа и 14
дней. Сравнение производства реактивных видов кислорода среди групп показало
увеличение в экспериментальной группе в сравнении с контрольной группой 24
часа 48 часов после инфицирования.
Можно сделать вывод, что клеточные элементы врождённого иммунитета активируются главным образом в течение первых двух дней после вызвания бактериемии с *Staphylococcus aureus* у собак.

Ключевые слова: *Staphylococcus aureus*, экспериментальная бактериемия, фагоцитоз, собака