

## Procalcitonine, Nitric oxide and C-Reactive Protein concentrations in rats with experimentally-induced infectious and noninfectious inflammation

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**Summary:** The aim of this study is to determine the alterations in procalcitonine (PCT), nitric oxide (NO) and C-reactive protein (CRP) levels in infectious and noninfectious inflammation and to evaluate their potential in the differentiation of inflammation. Totally 70 female Wistar rats (Group 1: n=21; Group 2, n=21, Group 3: n=21; Day 0: n=7), weighing 250-300 grams, were used in this study. Before the applications, blood samples were collected in seven rats and the time is accepted as Day 0. Three groups were formed each containing 21 rats. *Staphylococcus aureus* were administered at a concentration of  $10^6$  CFU in the infection group (Group 1), subcutaneously. Turbentine oil at a dose of 0.5 mg/kg were injected subcutaneously to twenty-one rats in the noninfectious inflammation group (Group 2). In the control group, 21 rats were injected with saline subcutaneously. Blood samples were collected from randomly selected 7 rats in each group on the 1<sup>st</sup>, 4<sup>th</sup> and the 7<sup>th</sup> days. PCT, NO, and CRP analyzes were performed with commercial test kits in the ELISA reader. According to the obtained results, we determined that serum PCT, NO and CRP levels increased after administrations. The rises of CRP and PCT concentrations were not statistically significant but it was higher in infectious group levels than the other two groups. Based on these results, it can be said that NO shows significant increases during infectious inflammation and can be used for differential diagnostic purposes in clinics.

Key words: CRP, inflammation, NO, PCT, rat.

### DeneySEL Olarak İnfeksiyöz ve Noninfeksiyöz Yangı Oluşturulmuş Ratlarda Prokalsitonin, Nitrik Oksit ve C-Reaktif Protein Konsantrasyonları

**Özet:** Bu çalışmanın amacı, infeksiyöz ve noninfeksiyöz yangı durumunda prokalsitonin(PCT), Nitrik oksit (NO) ve C-reaktif protein (CRP) düzeylerini belirlemek ve yangının ayırımının yapılmasında kullanılabilirliğini değerlendirmektir. Bu çalışmada toplam olarak 250-300 gram ağırlığında 70 dişi Wistar rat (Grup 1: n=21, Grup 2: n=21, Grup 3: n=21 ve 0. Gün n=7) kullanıldı. Uygulamalardan önce 7 rattan kan örnekleri alınıp 0. gün olarak kabul edildi. Ayrıca her biri 21 rat içeren 3 grup oluşturuldu. İnfeksiyon grubunda (Grup 1) bulunan 21 rata *Staphylococcus aureus*  $10^6$  CFU derialtı olarak verildi. Noninfeksiyöz yangı grubunda bulunan 21 rata Turbentin yağı 0.5 mg/kg derialtı uygulandı. Kontrol grubundaki 21 rata derialtı fizyolojik tuzlu su verildi. Uygulamalardan sonra her gruptan rastgele seçilen yedişer hayvandan, 1., 4. ve 7. günlerde kan örnekleri toplandı. PCT, CRP ve NO düzeyleri ELISA cihazında ticari test kitleriyle çalışıldı. Elde edilen sonuçlara göre PCT, NO ve CRP düzeylerinin uygulamalardan sonra arttığını belirlendi. CRP ve PCT düzeylerindeki artış infeksiyöz grupta diğer iki gruba göre daha fazlaydı ancak istatistiki önem saptanamadı. Bu çalışmanın sonuçlarına dayanarak, NO'in infeksiyöz yangıda önemli düzeyde arttığı ve kliniklerde ayırıcı tanı amaçlı kullanılabileceği söylenebilir.

Anahtar sözcükler: CRP, PCT, rat, NO, yangı.

### Introduction

Early differentiation of bacterial infection and noninfectious inflammation is important due to selection of different treatment models. Sometimes, discrimination of infectious and noninfectious inflammations is difficult because of similar clinical and laboratory findings (17). The differentiation of inflammation and diagnosis of the disease is important for selecting the appropriate treatment and thus preventing complications in the long term (29).

Procalcitonine (PCT) is a polypeptide composed of 116 amino acids that are produced and secreted by thyroid C cells (22). When bacterial infections or septicemia occurs, PCT level increases in circulating blood. The origin of the PCT during infectious conditions are considered to be extrathyroidal such as lung, liver, pancreas, colon and other organs (17). PCT behaves like an acute phase protein similar to other positive reactants, such as CRP, the production of which is also increased by inflammatory stimuli, including infections (23). After

a bacterial stimulus in healthy volunteers, the PCT concentration rise within 4 hr, reaching peak levels after 6 hours, and maintaining a plateau between 8 and 24 hr (29). PCT levels do not increase or slight increase in localised inflammation or infection that no systemic finding (17).

C- reactive protein (CRP) is a marker of nonspecific inflammation and it was used for monitoring the treatment (4, 10). CRP, is reported as major acute phase protein in human and canine (4, 10, 15). CRP is a useful marker of inflammatory clinical situation (12). In the rat CRP has been studied as a marker of inflammation using experimental models (2,12,18). But some researchers investigated that CRP was less sensitive marker of inflammation in the rat than in the human (3, 6).

Nitric oxide (NO), is an endogenous mediator of numerous physiological processes. NO, produced from L-arginine catalysed by nitric oxide synthase (7). NO, is produced by macrophages and many other cell types in response to inflammation or infection (11). NO levels may be useful to determine inflammation and diseases (17). Some reporters demonstrated NO concentrations can help to use in discrimination of the inflammation (5, 13).

The object of present study was to determine the availability of use of serum PCT, CRP and NO levels in infectious and noninfectious inflammations induced in rats. Moreover it was aimed to determine the alterations in these parameters and evaluate their potential usage in differentiation of inflammation.

### Materials and Methods

**Animals:** This study was approved by The Institutional Animal Ethics Committee of Adnan Menderes University, Turkey (Date:01/03/2011, Number: 2011/18). Experiments were carried out in a semi-acclimatised room at 22°C, with 50-70% humidity and 12/12-hour light/darkcycle. A total of 70 female Wistar albino rats 3 month-old were used in this study. The mean weight of rats was 250-300g. Animals were acclimatised for at least 3 weeks before the experiment.

**Study Design:** Rats were randomly divided into three groups for applications.

Group 1: Infectious inflammation group (n=21) *Staph. aureus* was given by subcutaneous injection at  $10^6$  CFU/mL (19).

Group 2: Non-infectious inflammation group (n=21) 0.5 mg/kg turpentine oil were administered by subcutaneous injection (27).

Group3: The control group (n=21) received an equal volume of physiologic saline by subcutan way.

Day 0 (n=7): Before the applications, blood samples were collected in rats.

Blood samples were collected via hearth of randomly selected 7 rats from each group at 1, 4 and 7 days after *Staphylococcus aureus*, turpentine oil and physiologic saline injections. Blood samples were taken under the anaesthesia Serum were separated by centrifugation at 1700 g for 10 min and they were kept frozen -20°C until analysis.

**Biochemical analyses:** CRP levels were measured using competitive enzyme immunoassay (AssayPro, Assay Max Rat CRP, USA). This assay employs a quantitative sandwich enzyme immunoassay technique that measures rat CRP. The final absorbance of the samples in a microplate reader at 450 nm (Optic Ivyman System 2100C, Spain). The sample concentrations of CRP is determined by using the standart curve and multiply the value by the dilution factor. PCT was measured a with manuel rat spesific commercial ELISA test kits (CUSABIO, rat Procalcitonin ELISA Kit). This assay employs quantitative sandwich enzyme immunoassay. Determine the final absorbance of each well usuna a microplate reader set to 450 nm (Optic Ivyman System 2100C, Spain). The results is calculated the calibration curve using Professional 'Curve Expert 1.3'. NO levels were measured by test combination for determination of NO via nitrate (Nitric Oxide Colorimetric Assay, Roche, USA) on microtiter paltes (Thermo Scientific Multiscan GO, USA). NO, is detected in biological fluids via nitrite. The nitrate present in the sample is reduced to nitrite by reduced NADPH in the presence of the enzyme nitrate reductase. The nitrite formed reacts with sulfanilamde and N-(1-naphtyl) ethylendiamine dihydrochloride to give a red-violet diazo dye. The diazo dye is measured on the basis of its absorbance in the visible range at 550 nm. The result is calculated from the calibration curves using the standart solutions.

**Statistically Analyses:** SPSS 11.5 package was used for all computations. All data were checked for normal distribution with Shapiro-Wilk and homogeneity of variance with Levene's test. If the data were not normally distributed, logarithmic or square root transformation was performed in order to normalize the distribution. Two-way analysis of variance (ANOVA) was conducted to assess the effect of intervention and time on PCT, No and CRP. Post hoc multiple comparisons were performed using Tukey HSD (Honestly significant difference) test. P values <0.05 were considered to be significant. The results were presented as the means  $\pm$  SE.

### Results

All results of this study are shown in Table 1. According to the results of this study, serum PCT levels were not found any statistically significant when compared between groups and days. In spite of PCT

Table 1. Mean serum PCT, NO and CRP concentrations in saline, turbentine and *Staphylococcus aureus* groups on days of 0, 1, 4 and 7. Tablo 1. Fizyolojik tuzlu su, turbentin ve *Staphylococcus aureus* gruplarında 0, 1, 4 ve 7. günlerde ortalama serum PCT, NO ve CRP konsantrasyonları.

PARAMETER	GROUP	DAYS				Overall (mean±pooled SEM) (Group effect)	P values		
		0	1	4	7		Group	Time	Group* Time
PCT (ng/ml)	Saline	0.125±0.09	0.099±0.05	0.105±0.08	0.081±0.01	0.103±0.02	0.586	<0.05	0.617
	Turbentine		0.060±0.05	0.175±0.01	0.084±0.08	0.119±0.02			
	<i>Staph.aureus</i>		0.060±0.01	0.152±0.07	0.047±0.02	0.096±0.02			
	Overall (mean±pooled SEM) (Time effect)	0.125±0.02 <sup>cd</sup>	0.084±0.02 <sup>cd</sup>	0.144±0.02 <sup>c</sup>	0.071±0.02 <sup>d</sup>				
NO (µM/ml)	Saline	10.31±1.83	8.79±1.24 <sup>b</sup>	7.44±2.42 <sup>*ab</sup>	5.29±0.52 <sup>*#b</sup>	7.96±0.53 <sup>d</sup>	<0.001	<0.001	<0.001
	Turbentine		7.26±1.62 <sup>*b</sup>	8.71±1.15 <sup>a</sup>	6.76±0.61 <sup>*ab</sup>	8.26±0.53 <sup>d</sup>			
	<i>Staph.aureus</i>		19.53±6.24 <sup>*a</sup>	5.45±0.66 <sup>*#b</sup>	9.89±6.81 <sup>#a</sup>	11.30±0.53 <sup>c</sup>			
	Overall (mean±pooled SEM) (Time effect)	10.31±0.61 <sup>c</sup>	11.87±0.61 <sup>c</sup>	7.21±0.61 <sup>d</sup>	7.32±0.61 <sup>d</sup>				
CRP (µg/ml)	Saline	2.59±0.66	4.14±1.2	4.37±1.12	3.27±0.51	5.68±1.43	0.906	<0.05	0.914
	Turbentine		3.32±0.54	3.96±0.62	4.58±1.54	5.08±1.43			
	<i>Staph.aureus</i>		5.94±2.45	2.93±0.19	4.96±2.19	4.88±1.43			
	Overall (mean±pooled SEM) (Time effect)	2.59±0.66 <sup>d</sup>	5.29±1.43 <sup>cd</sup>	8.34±1.43 <sup>c</sup>	4.62±1.43 <sup>cd</sup>				

\* Statistical difference between output values (Day 0) and other sampling days in same group (same line) (P<0.001).

# Statistical difference between first day (Day 1) and other sampling days in same group (same line) (P<0.001).

a,b Statistical difference between groups with different letters in the same day (same column) (P<0.001).

c,d Statistical difference between overall values with different letters for group and/or time effect (P<0.001).

Data represents mean value of experiment ± SE. PCT: Procalcitonine; NO: Nitric oxide; CRP: C-reactive protein.

\* Aynı grup içinde çıkış günü (0. gün) ile diğer kan alım günleri arasındaki istatistiksel fark (Aynı satır)(p<0.01).

# Aynı grup içinde ilk gün(1. gün) ile diğer kan alım günleri arasındaki istatistiksel fark (p<0.001) (Aynı satır).

ab: Aynı gün içinde gruplar arası istatistiksel fark önemli (aynı sütun). (p<0.001)

cd: Farklı sütunlardaki tüm değerler arasında grup ve /veya zaman etkisi için istatistiksel fark (p<0.001)

Veriler ortalama değer±SE olarak verilmiştir. NO. PCT: Prokalsitonin Nitrik oksit CRP: C- reaktif protein

levels were raised 0.175 ng / ml in turbentine group and 0.152 ng / ml in *Staph. aureus* group in 4 day after application, the levels of increases were not found statistically significant.

NO levels 10.31 ± 1.83 µM/ml in day 0. This levels were raised to 19.53 ± 6.24 µM/ml in first blood sampling day in *Staphylococcus aureus* group. This level were significant (p<0,001) when evaluated between groups and days.

CRP levels increased in all groups after the applications and the high CRP levels were remained all sampling days. CRP levels raised at first day of the trial (approximately two fold of before administration day) in *Staph. aureus* group. But it was not found any statistical significance in CRP levels between groups and days.

Although no effects were seen among treatments, time- dependent changes were seen in PCT and CRP levels. It was observed that time and group has significant effects on NO concentrations. Moreover, there was a significant interaction between treatments and time. In entire experimental period, NO levels in *Staphylococcus*

*aureus* group were higher when compared to other treatments. A significant decrease starting from day 4 seen in this group (Table 1).

## Discussion and Conclusion

Identifying infectious and noninfectious inflammation is important because of the treatment procedure differs. Antibiotic treatment is urgent for bacterial infections while immunosuppressive treatment is needed to noninfectious inflammations such as autoimmune disease. Wrong drug choice may cause to deteriorate the patients condition and sometimes the results may be fatal (17). The objective of present study were to evaluate availability of use of serum PCT, CRP and NO levels in infectious and noninfectious inflammations induced in rats. This study showed that CRP, PCT and NO concentrations were higher in infectious group than the noninfectious inflammation group. However the significant difference was only in NO levels whereas CRP and PCT concentrations were not changed significantly.

PCT has stimulated great interest as a potentially more specific marker for bacterial infection. PCT is produced ubiquitously in response to endotoxin or mediators released in response to bacterial infections that is, interleukin (IL-1 $\beta$ ), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and interleukin-6 (IL-6) and strongly correlates with extent and severity of bacterial infections (28). PCT may serve as a useful marker for the detection of systemic bacterial infection in patients with systemic autoimmune disease (9). In this study, PCT levels were showed that nonsignificant increased in infectious and noninfectious group at 4<sup>th</sup> day after administration. The results of this study indicate that PCT levels were not altered from the cause of inflammation. It is related to PCT levels only rased significantly during systemic bacterial or fungal infections (7). In healthy subjects, PCT levels are lower than 0.10 ng/ml (7). Many mild increases in PCT concentrations were missed. The results of this study may be related to use of mild inflammation models.

It is reported that CRP is not a major acute phase protein in rats and basal levels of rat CRP is higher than human (24, 25). However, some studies indicated that rat CRP is used for the marker of inflammation (2,12,18). In this study it was determined that CRP concentrations were raised after administrations in three group but this increase was not statistically significant. Connolly et al. (6) reported that plasma CRP levels raised to %138 at 17 days post injection in a rat adjuvant arthritis model (6). Bürger et al.(3) described an increase at 4 and 15 day of post injection (3). The results of the present study show that CRP levels increased in two fold in *Staphylococcus aureus* group in first day after administration. We didn't find any significant difference among other treatment groups. Similarly, Myer et al. (21) using subcutaneously injected turbentine, determined increased CRP levels in two fold at 24 h post administration (21). Giffen et al.(14) was also determined a slight increase in CRP concentrations (14). This may reflect the difference in models of inflammation used, the route of administration and the method of detection system.

NO, plays an important role in many physiologic functions such as neurotransmission, regulation of immun system and vascular system. In septic shock, endotoxines and cytokines can stimulate NOS in various cells such as macrophages, endothelial cells, vascular smooth muscle cells, leading to NO production (1, 5, 17, 26, 27). NO plays a complex and incompletely understood role in infectious disease and similarly has complex effects on immun system and metabolism. Ahren et al.(1) reported that NO production increased in patients with acute infectious gastroenteritis and acute infectious disease such as acute pneumonitis, urinary tract infection and gastroenteritis (26). NO concentration increases as disease severity increases (27). The increase in NO concentrations is a general finding with acute infectious

diseases and may rather be associated with certain pathogens or sites of infection. Charmandari et al. (5) described an significant increase in plasma NO concentrations in infectious or noninfectious diarrhea (17). Similarly, in this study, NO concentrations in infectious group showed a statistically significant increase in first day after injection.

According to present study, it can be stated that availability of NO concentrations for discrimination of infectious and noninfectious inflammation is higher than PCT and CRP concentrations. Similar to this, NO levels can be a useful tool for diferential diagnostic purposes in clinics.

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