

Evaluation of nitric oxide level in prepartum heifer mastitis

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Summary: This study was carried out for detection of nitric oxide (NO) levels in healthy (Group 1, n:78) and infected (Group 2, n:63) quarters of pregnant heifers. The mean nitric oxide (NO) concentrations were calculated of about $15,58 \pm 2,21$ $\mu\text{M/ml}$ and $42,69 \pm 6,03$ $\mu\text{M/ml}$ for Group 1 and Group 2, respectively. A significant difference was found between two groups, statistically ($p < 0,001$). The mean NO concentrations were compared between quarters infected with *Staphylococcus aureus* (*S. aureus*) and coagulase negative staphylococcus (CNS) in Group 2 and measured to be; $38,50 \pm 8,47$ $\mu\text{M/ml}$ and $44,17 \pm 7,71$ $\mu\text{M/ml}$, respectively ($p > 0,05$). Additionally, lacteal secretions were classified to be thickened and watery according to viscosity. Intramammary infection rates (IMI) and NO concentrations were compared in low (watery) and high (thickened) viscosity samples. Risk of IMI was found higher in thickened samples than watery ($p < 0,001$). Every 1 unit ($\mu\text{M/ml}$) increase in the NO concentration boosted the risk of IMI 1,036-fold ($p < 0,001$).

Key words: Coagulase negative staphylococcus, heifer mastitis, nitric oxide, *Staphylococcus aureus*.

Prepartum dönemde mastitis şekillenen düvelerde nitrik oksit düzeylerinin değerlendirilmesi

Özet: Bu çalışma gebe düvelerin sağlıklı (Grup 1, n:78) ve enfekte (Grup 2, n:63) meme loblarında nitrik oksit düzeylerini belirlemek için planlandı. Grup 1 ve Grup 2 deki ortalama nitrik oksit düzeyleri sırasıyla $15,58 \pm 2,21$ $\mu\text{M/ml}$ ve $42,69 \pm 6,03$ $\mu\text{M/ml}$ olarak belirlendi ve iki grup arasında NO düzeyi bakımından istatistik olarak belirgin bir fark bulundu ($p < 0,001$). Grup 2'de *Staphylococcus aureus* ve koagülaz negatif stafilocoklar ile enfekte meme loblarındaki ortalama NO düzeyleri kıyaslandı ve sırasıyla; $38,50 \pm 8,47$ $\mu\text{M/ml}$ ve $44,17 \pm 7,71$ $\mu\text{M/ml}$ olarak ölçüldü ($p > 0,05$). Nitrik oksit düzeyine ek olarak lakteal sekresyonları viskozitesine göre yoğun ve sulu olarak sınıflandırıldı. Düşük (sulu) ve yüksek (yoğun) viskoziteli örneklerde meme içi enfeksiyon oranları ve NO konsantrasyonları kıyaslandı. Yoğun örneklerde meme içi enfeksiyon riski, sulu örneklerden daha yüksek bulundu ($p < 0,001$). Nitrik oksit konsantrasyonundaki bir birimlik artışın, enfeksiyon riskini 1,036 kat artırdığı belirlendi ($p < 0,001$).

Anahtar sözcükler: Koagülaz negatif stafilocoklar, düve mastitisi, nitrik oksit, *Staphylococcus aureus*.

Introduction

Heifers are the genetic future and future milking in all dairy herds. Mastitis is a disease that can influence future productivity (13). Although producers believe that the heifers are uninfected until the first calving, many studies have mentioned about etiology, prevalence, prevention and treatment (1,11,13,16). There are some diagnostic tests to estimate mammary gland epithelial cell damage in cows (9) but there isn't any test to estimate glandular damage in heifers other than bacteriologic examination. Staphylococci are the most isolated bacterial species in heifer mastitis (1,12). Both of *S. aureus* and CNS cause polymorphonuclear leukocyte (PMN) infiltration into cisternal and parenchymal mammary tissues (13). This phagocytes use an oxygen-dependent process to destroy bacteria (17). But, the mechanism that normally protects the tissue from infection can also cause extensive tissue damage. The mammary gland has some serial reactions under

inflammatory and immunological stimuli. Production of nitric oxide (NO) is one of these reactions. Nitric oxide can be endogenously synthesized from arginine by two constitutive (endothelial and neural) NO synthases (cNOS) and an inducible NOS (iNOS). iNOS is expressed by a variety of cells, especially monocytes, as a result of triggering with substances of microbial origin, such as endotoxins, i.e., lipopolysaccharides (LPS) and by cytokines, for example TNF- α . Cattle belong to the species whose monocytes react with a comparatively marked NO production in response to LPS. NO produced by macrophages in response to invading microbes acts as an antimicrobial agent, but it can also damage cells if produced excessively (2,4,14).

The aim of this study was to detect nitric oxide concentrations in infected and healthy quarters of pregnant heifers and evaluate the relationship between IMI and NO increase in low (watery) and high (thickened) viscosity samples.

Materials and Methods

Study design and sampling: In the presented study, 141 glandular secretion samples from 57 Holstein-Friesian pregnant (6-8 months) heifers belonging to 3 dairy farms were used in Ankara region in Turkey. The heifers were chosen if they had no signs of clinical mastitis, had four quarters free of teat abnormalities, and hadn't received antibiotic or anti-inflammatory treatment during the previous 30 days. The pregnant heifers were detected by using rectal palpation and insemination data. Glandular secretion samples were collected from pregnant heifers according to recommended procedures for milk samples (8). Teat ends were cleaned by 70% alcohol-soaked gauze and nearly 3 ml udder secretions were collected using a gentle milking into 10 ml sterile plastic tubes, and numbered to identify the quarters. After that, iodine solution was applied to teat-ends. Samples were transported to the laboratory at +4°C.

Classification of samples and bacteriologic analysis: The samples were classified according to Hallberg and et al. (7). Low and high viscosity samples were evaluated by same person. Infected and healthy samples were cultured using conventional bacteriological methods (5,8). Samples were thawed and homogenized at room temperature, cultured on blood and Mac Conkey agar. They incubated at 37 °C for 24-48 hours. After the incubation period, colony morphology was evaluated as described (15). Gram staining was performed to detect Gram positive cocci. The catalase and coagulase tests were applied to Gram positive bacteria to identify *CNS* and *S. aureus*. Healthy (Group 1, n=78) and subclinically infected quarters (Group 2, n=63) with *Staphylococcus aureus* (*S. aureus*) and *Coagulase-Negative Staphylococcus* (*CNS*) were divided into 2 groups according to bacteriologic results.

Biochemical analysis: Nitric oxide is very rapidly deactivated by oxidation to nitrite and nitrate by physically dissolved oxygen and water in biological fluids. Owing to nitric oxide level was evaluated by measuring its more stable metabolites, nitrite and nitrate in samples. For NO assay, equal volume of samples and potassium phosphate buffer, pH 7.5, were placed in ultra-filter (10,000 MWCO Sartorius, Viva Science Cat. No: 13239-E, Germany) and centrifuged at 20°C for 45 minute (5000xg). The ultra-filtrate were used in the test. The nitrate presents in the sample was reduced to nitrite by reduced nicotinamide adenine dinucleotide phosphate (NADPH) in the presence of the enzyme nitrate reductase as already described (6). Briefly, 500 µl samples were mixed with 50 µl of co-enzyme solution (1.5 mg NADPH and 0,03 mg FAD in 1 ml of potassium phosphate buffer, pH 7,5) and 20 µl of nitrate reductase solution (4 U nitrate reductase in 0,6 ml of redist water) and incubated at room temperature for 30 minutes. Following that, 150 µl of incubated solution were mixed with equal volume

of Greiss reagent (75 µl of sulfanilamide reagent and 75 µl of N-(1-naphtyl)-ethylene diamine dihydrochloride reagent) in a microplate. The reaction allowed to proceed for 5 minutes and the resulted nitrite was reacted with sulfanilamide and N-(1-naphtyl)-ethylene diamine dihydrochloride to give a red-violed diazo dye. Total nitrite was evaluated by reading the optical density of each sample at 550 nm (6). The nitric oxide concentration was determined by comparison with a potassium nitrate standart curve (0 to 80 µM).

Statistical analysis: The mean levels of NO in both groups were calculated using T-test. Average NO concentrations depend on bacterial species in Group 2 and average NO concentrations in thickened and watery samples were calculated by T-test. The correlation between groups and viscosity of samples and their effects on NO concentration were calculated by variance analysis. The correlation among IMI, viscosity and NO were measured by logistic regression analysis. A difference was considered statistically significant when $p < 0,001$ and non-significant when $p > 0,05$ (PASW statistics version 18,0, SPSS Inc.).

Results

In the presented study, the mean NO concentrations were $15, 58 \pm 2,21$ µM/ml and $42,69 \pm 6,03$ µM/ml for group 1 and group 2, respectively. A significant difference was found between two groups, statistically ($p < 0,001$) (Table 1).

Table 1: The mean concentrations of NO in groups.
Tablo 1: Gruplarda ortalama NO konsantrasyonları.

Group	n	NO µM/ml	SE	p-Value
Group 1 (Healthy)	78	15,58	2,21	0,0001
Group 2 (Infected)	63	42,69	6,03	

SE—Standard Error; NO—Nitric Oxide

In the second group, 24 quarters infected with *S. aureus* and 39 quarters infected with *CNS* were used. The mean NO concentration was $38,50 \pm 8,47$ µM/ml and $44,17 \pm 7,71$ µM/ml for *S. aureus* and *CNS* infections, respectively. Although the mean NO concentration in quarters infected with *CNS* was higher than *S. aureus*, a significant difference was not found statistically ($p > 0,05$) (Table 2).

Table 2: The mean concentrations of NO depend on bacterial species in Group 2.
Tablo 2: Grup 2 de bakteri türüne göre ortalama NO konsantrasyonları.

Group 2	n	NO µM/ml	SE	p-Value
<i>S. aureus</i>	24	38,50	8,47	0,804
<i>CNS</i>	39	44,17	7,71	

SE—Standard Error; NO—Nitric Oxide; CNS—Coagulase Negative Staphylococcus; *S. aureus*—*Staphylococcus aureus*

The relationship between intramammary infections (IMI) and NO increase were evaluated in low (watery) and high (thickened) viscosity. The mean nitric oxide concentration in thickened samples was measured higher than watery samples and a significant difference was found statistically ($p < 0,001$) (Table 3). Additionally, it was detected that thickened secretions boosted risk of IMI 6,77-fold and every 1 unit ($\mu\text{M}/\text{ml}$) increase in the NO concentration boosted the risk of IMI 1,036-fold ($p < 0,001$) (Table 4).

Table 3: The comparison of mean NO concentrations in thickened and watery secretions.

Tablo 3: Yoğun ve sulanmış sekresyonlarda ortalama NO konsantrasyonları.

Group	Viscosity	NO $\mu\text{M}/\text{ml}$	SE	p-Value
Group 1	Thickened	15,95	4,13	0,75
	Watery	14,73	6,33	
Group 2	Thickened	74,87	6,33	0,0001
	Watery	24,23	4,93	

SE—Standard Error; NO—Nitric Oxide

Table 4: The correlation among IMI, thickened secretions and NO concentrations.

Tablo 4: Meme içi enfeksiyonlar, yoğun sekresyonlar ve NO konsantrasyonları arasındaki ilişki.

Variable	P-Value	Odd Ratio	95% confidence interval	
			Lower	Upper
Thickened secretions	0,0001	6,77	2,95	15,52
NO		1,036	1,01	1,04

Discussion and Conclusion

A relationship between nitric oxide production and mammary gland damage was referred by some authors. They reported intramammary or in-vitro production of NO in quarters and cell cultures challenged by staphylococcal enterotoxins, cytokines, *Escherichia coli* lipopolysaccharides (2,3,4,10). High somatic cell count and NO release were stated during induced mastitis in cows (3). These findings were also confirmed under in-vitro conditions. The bovine epithelial cells and mononuclear phagocytes were found responsible for NO release under inflammatory conditions (4). Although the current study was performed on non-lactating heifers, similar results were detected. Nitric oxide production was found higher in infected quarters than healthy. On the other hand, the previous studies were reported for acute or chronic cases. Although this study was performed for only subclinical cases, the mean NO levels were measured much higher than previous results. The glandular secretions derived from heifers were more concentrated than milk. This difference might be effective on results.

Hallberg et al. (7) reported that precalving mammary quarters with high viscosity were usually uninfected. Conversely, risk of IMI was higher in thickened secretions. This result might be occurring due to bacteriologic method. Different from previous report, all samples were frozen, than thawed. The bacteriologic procedure might be providing to *Staphylococcus spp.* more detectable in high viscosity samples.

In conclusion, high NO concentration was detected during mastitis in heifers. This result shows that heifer's mammary glands are very sensitive to subclinical intramammary infections (IMI) and glandular tissue gives significant reactions which can be detected by NO increase. Additionally, CNS can be more harmful to glandular tissue than *S. aureus* during the first gestation. On the other hand risk of IMI is higher in thickened samples. The quarters have thickened secretion may be susceptible to heifer mastitis in the next gestation period and lactation. According to these findings, NO may be useful for early diagnosis of heifer mastitis but the results must be supported by further studies in the future.

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