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A new protocol for the induction of chronic mastitis with intramammary infusion of lipopolysaccharide (LPS) in Balb/c mice

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Abstract: Mastitis is inflammation of the mammary tissue and is commonly observed in farm animals. The problem causes severe financial losses in the dairy industry in terms of veterinary costs, milk disposal, and treatment expenses. Bacteria are the main actors in the etiology and cause acute and chronic inflammatory changes in the mammary tissue. Acute inflammatory changes are easily recognized clinically, and treatment is initiated immediately, but subacute inflammation progresses insidiously and leads to chronic inflammation with irreversible fibrotic changes. Standardized experimental models for the induction of acute mastitis in laboratory animals are available. Usually, infusion of bacteria or some bacterial structural components into mammary tissue is easily applied for this purpose. However, there are few studies on the induction of chronic mastitis with fibrotic changes, and the applications are relatively complex. In this study, LPS was infused through the teat duct three times on days 0, 5, and 10 to induce chronic mastitis in mice. Tissues were sampled on days 1, 6, and 15 to evaluate histopathological changes. While severe neutrophil infiltrates, a component of acute inflammation, were observed on day 1, lymphocyte infiltrates increased on day 6, consistent with subacute inflammation. On day 15, lesions representing chronic mastitis, such as fibrosis and lymphocyte infiltration, were observed. A model similar to the lesions in chronic mastitis of dairy cattle was successfully and easily established by LPS infusion in mice.

Keywords: *Balb/c, Chronic mastitis model, Lipopolysaccharide, Mammary gland, Masson's Trichrome.*

Balb/c farelerinde meme içi lipopolisakkarid uygulaması sonucu yeni bir kronik mastitis modelin indüklenmesi

Özet: Mastitis, meme dokusunun iltihaplanmasıdır ve dünya genelinde çiftlik hayvanlarında yaygın olarak görülmektedir. Bu sorun süt endüstrisinde veteriner masrafları, sütün imhası ve tedavi giderleri açısından ciddi mali kayıplara neden olmaktadır. Bakteriler etiyolojinin ana aktörleridir ve meme dokusunda akut ve kronik inflamatuar değişikliklere neden olurlar. Akut enflamatuar değişiklikler klinik olarak kolayca tanınır ve tedavi hemen başlatılır, ancak subakut inflamasyon sinsice ilerler ve geri dönüşü olmayan fibrotik değişikliklerle kronik inflamasyona yol açar. Laboratuvar hayvanlarında akut mastitis indüksiyonu için standartlaştırılmış deneysel modeller mevcuttur ve genellikle bakterilerin veya bazı bakteriyel yapısal bileşenlerin meme dokusuna infüzyonu bu amaçla kolayca uygulanmaktadır. Bununla birlikte, fibrotik değişikliklerle birlikte kronik mastitis indüksiyonu üzerine az sayıda çalışma vardır ve uygulamalar nispeten karmaşıktır. Sunulan çalışmada, farelerde kronik mastitisi indüklemek için LPS 0, 5 ve 10. günlerde üç kez meme kanalından infüze edilmiştir. Histopatolojik değişiklikleri değerlendirmek için 1, 6 ve 15. günlerde dokulardan örnek alınmıştır. Akut inflamasyonun bir bileşeni olan şiddetli nötrofil infiltratları 1. günde gözlenirken, lenfosit infiltratları subakut inflamasyonla uyumlu olarak 6. günde artmıştır. 15. günde, fibrozis ve lenfosit infiltrasyonu gibi kronik mastitisi temsil eden lezyonlar gözlenmiştir. Süt sığırlarının kronik mastitisindeki lezyonlara benzer bir model, farelerde LPS infüzyonu ile başarılı ve kolay bir şekilde oluşturulmuştur.

Anahtar Kelimeler: *Balb/c, Kronik mastitis modeli, Lipopolisakkarit, Meme bezi, Masson Trikrom.*

Introduction

Mastitis, the inflammation of udder tissue, is one of the most critical health issues of dairy cattle and causes serious economic losses. According to recent research, the average cost of an affected animal to the farm is about 444 USA dollars (Rollin et al., 2015). Infectious agents severely demolish mammary architecture and host inflammatory responses in acute and chronic mastitis (Ingman & Glynn, 2014; Zhao et al., 2015). Experimental induction of mastitis in dairy cattle may not be a good option for many reasons, including rearing conditions, contamination of teats with fecal bacteria after infusion of lipopolysaccharide (LPS), or live bacteria (Brouilette et al., 2023; Cheng & Han, 2020). Understanding the mechanism of mastitis is important not only for new treatment strategies but also for animal welfare (Cobirka et al., 2020). In this context, experimental animal models are strongly required to understand the mechanisms of tissue damage by various etiologic agents. So far, acute mastitis has been successfully induced by intramammary administration of various live bacteria such as *Escherichia coli*, *Staphylococcus aureus,* and *Candida krusei* or LPS in laboratory animals (Ingman et al., 2015). CD1 and Balb/c mice are very popular in studying mammary biology, function, and inflammation (Camperio et al., 2017). These models in CD1 and Balb/c breeds are primarily for the evaluation of early changes in the inflammatory process (Camperio et al., 2017). However, most infections in dairy cattle are subclinical, and damage to the udder tissue is often incurable when animals are suspected or diagnosed with mastitis (Cobirka et al., 2020; Lai et al., 2017). Ineffective treatments and prolonged inflammatory processes result in irreversible loss of parenchymal units and tissue fibrosis in mammary tissue (Kan et al., 2022). Fibrosis can be defined as the accumulation of interstitial cells and their unique extracellular matrix (ECM) proteins in tissues (Wynn, 2008; Wynn & Ramalingam, 2012). Mouse models utilized for the understanding of bovine chronic fibrosing mastitis are insufficient. In 1979, experimental chronic mastitis in mice was modeled by the intramammary inoculation of endotoxin 6 hours before *Staphylococcus aureus* administration (Anderson, 1979). Tuchscherr et al. (2005) have induced chronic mastitis in mice by the intramammary administration of particular strains of *Staphylococcus aureus* capable of producing polysaccharide wall components. However, these models have potential biological hazardous risks; *Staphylococcus aureus* has the capability to infect humans and other laboratory animals. If researchers aim to induce chronic fibrosing mastitis without the aforementioned risks, the submitted model herein can be a useful alternative. Establishing a fibrotic mastitis model in mice enables the investigation of specific genes, mediators, and immune cells enrolling in fibrosis formation. Development strategies of anti-fibrotic therapies can be easily possible. Therefore, we aimed to introduce a new model for generating mammary fibrosis in Balb/c mice.

Materials and Methods

The Animal Care and Use Committee of Bursa Uludag University, under the National Institute of Health Guide for the Care and Use of Laboratory Animals, approved all experimental procedures (Approval number: 2019-07/04).

Preparation of LPS: One mg of LPS from *Escherichia coli* O111:B4 (L4391 Sigma Aldrich, St. Louis, MO, United States) was dissolved in 5 ml of sterile phosphate-buffered saline (PBS) (P4417, Sigma Aldrich, St. Louis, MO, United States). Syringe-type filters (CLS431224, Sigma) were used to sterilize PBS (Barham et al., 2012).

Animals: Female Balb/c mice were obtained from the Experimental Animals Breeding and Research Center of Bursa Uludag University. They were housed at five mice per cage at temperatures of 20–22°C with 60-70% humidity in a controlled room set to a 12-h light/ dark cycle and had access to standard mice chow (Korkuteli, ANTALYA) and water ad libitum. Animals were mated at eight weeks of age, and lactating mice were used in experiments. After parturition, pups were allowed to suck their mothers for 8-10 days so that the inoculations could be given more easily. Pups were removed from dams 2-hour before intramammary injections and kept with nursing mice. Previous studies were based on determining the optimum dose of LPS to reduce the number of mice in preliminary experiments. The groups were first divided into 3 (control, PBS, LPS) according to the type of substance given. The number of animals in each group was nine, and a total of 27 animals were used. Three animals from each group were euthanized on day 1, three on day 6, three on day 6, and three on day 15, and the samples were examined.

Intramammary Infusion of LPS: All experimental procedures in mice were applied under anesthesia with sevoflurane (Sevorane liquid 250 mL, Abbvie, North Chicago IL, United States) using a portable anesthesia device (AMS Minor 612, Turkey). LPS solution (50 µl, 0.2 ug/µl) was infused directly into the right fourth mammary glands via the teat canal at days 0, 5, and 10. A 30-gauge blunt end needle (BD – Ultra Fine 0.5 mL insulin syringe, Becton, Dickinson, and Company, Franklin Lakes, NJ, United States) was utilized for all injections. An equal volume of sterile PBS solution was given into the right fourth mammary glands of mice in the vehicle group (Barham et al., 2012). Mice in the control group did not receive any infusion, three mice were euthanized at 1, 6, and 15 days for monitoring of healthy/or involuting mammary glands. To confirm the induction of mastitis following LPS injection, an animal was euthanized 24 hours after LPS infusion, tissue samples were taken and evaluated histopathologically.

Histopathologic examination: Mammary tissue samples were fixed in 4% paraformaldehyde solution for 24 hours, cut into small pieces, and transferred to tissue cassettes, dehydrated in ascending series of ethanol, cleared

in xylene, embedded in paraffin, cut into 4-µm serial sections with a microtome, and sections were placed into Poly-L-Lysine coated slides. Sections were stained with Hematoxylin-Eosin (H&E), and inflammatory changes were evaluated under the light microscope (CX41, Olympus Corporation, Shinjuku City, Tokyo, Japan).

Masson's Trichrome Staining: All slides were stained with Masson's Trichrome using a commercially available kit (Bio-Optica, Milano, Italy) according to the manufacturer's instructions to evaluate fibrotic changes.

Results

Histopathological findings:

On day 1;

Three animals from each experimental group (nine animals in total) were euthanized for day 1 evaluations. No inflammatory changes were detected in the control and PBS-vehicle groups microscopically (Fig. 1 A-B).

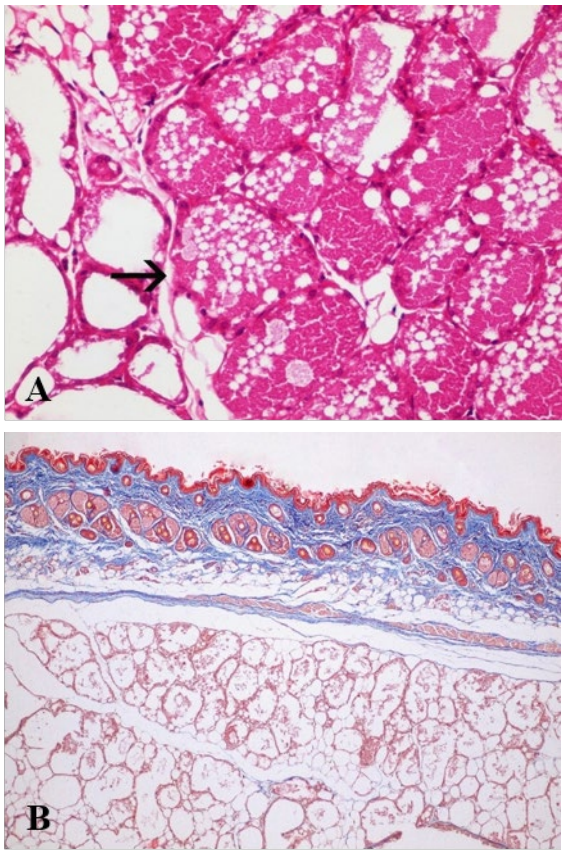

Figure 1. A) Healthy mice mammary tissue, no inflammatory changes were seen, and mammary tissue is active, alveolar lumens are filled with milk, H&E staining, control group x200 magnification, **black arrow:** mammary tubules. **B)** Healthy mice mammary tissue, no fibrous tissue around the alveoli, Masson's Trichrome staining, control group, x40 magnification.

Mammary tissue was active, and alveolar lumens were filled with milk in the control and PBS groups. Severe acute inflammatory changes represented by hyperemic blood vessels, neutrophil leucocyte infiltration within lumens of alveoli, and secretory tubules were observed along with degenerated-exfoliated secretory and tubular epithelium. Reactive mammary lymphadenopathy was evident in animals euthanized after the day of the first LPS injection. In addition to inflammatory changes, most alveoli continued synthesizing milk after a single LPS infusion (Fig. 2).

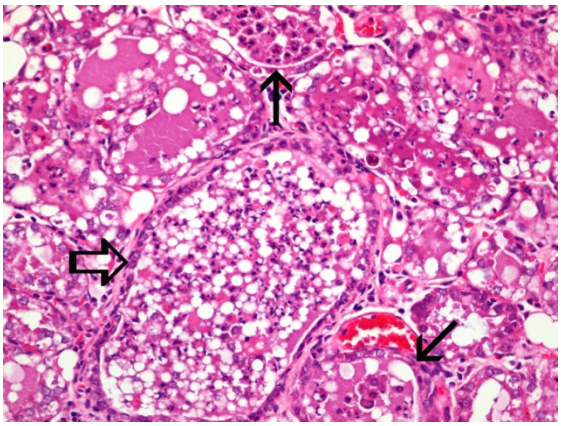

Figure 2. Acute mastitis on day 1, mice mammary tissue, severe neutrophils infiltration in tubules lumen **(transparent arrows)** and alveoli lumen **(black arrows)**, H&E staining, LPS group, x200 magnification.

On day 6;

Three animals from each experimental group (nine animals in total) were euthanized for day 6 evaluations. There were no inflammatory changes, and most alveolar lumens were filled with milk in the mammary tissues of the control and PBS groups. In some areas, signs of involution were characterized by the narrowing of alveoli and duct lumens and the absence of milk synthesis. Involuted mammary tissues were embedded in the increased amount of mammary adipose tissue. Acute inflammatory reaction altered mild to moderate subacute inflammatory response characterized by mononuclear cell infiltrations around alveoli and tubules; neutrophils were also observed in lumens of some secretory units (Fig. 3-A). Lymphoid follicular hyperplasia was seen in mammary lymph nodes. Mild to moderate ECM accumulation around the alveoli and ducts was initiated (Fig. 3-B).

On day 15;

Three animals from each experimental group (nine animals in total) were euthanized for day 15 evaluations. No signs of inflammation and whole mammary alveoli were involuted in control and PBS groups. Milk synthesis was not observed in any secretory unit; alveoli and ducts were isolated as islands in adipose tissue. In the LPS treatment group, mammary alveoli and ducts were heavily surrounded by mononuclear cells, including lymphocytes, macrophages, and plasma cells. Severe perialveolar and periductal fibrosis were observed in chronically inflamed mammary tissue. Mammary lymph nodes were greatly enlarged due to severe, diffuse follicular hyperplasia (Fig. 4-A). Masson's trichrome staining visualized the existence and accumulation of fibrotic tissue in mammary tissues (Fig. 4-B).

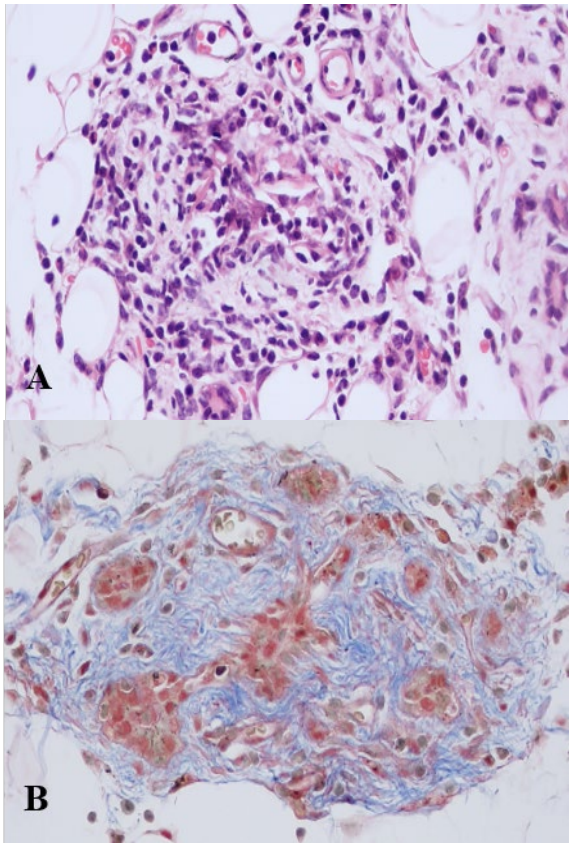

Figure 3. Mice mammary tissue on day 6 **A)** Around the tubules and alveoli lumens characterized by mononuclear and polymorphonuclear cell infiltration and increased fibrosis, H&E staining, LPS group, x200 magnification **B)** Increase connective tissue around the tubule and alveoli, Masson's Trichrome staining, LPS group, x200 magnification.

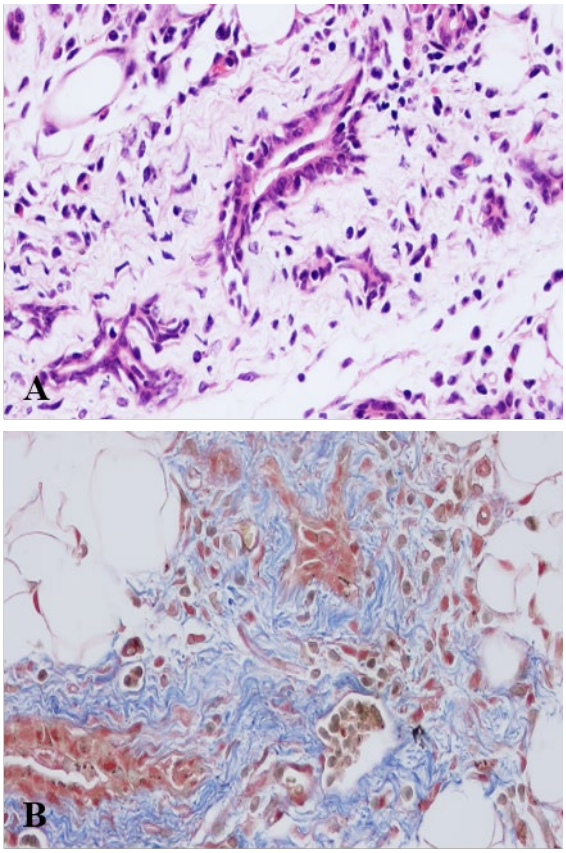

Figure 4. Mice mammary tissue on day 15; **A)** Severe perialveolar and periductal fibrosis and mononuclear infiltration in the perialveolar and periductal area in LPS group, H&E staining, x200 magnification, **B)** Severe fibrosis around the tubule and alveoli in LPS group Masson's Trichrome staining, LPS group, x200 magnification

Discussion

In this study, we have established a new model for fibrotic changes in mammary tissue after LPS infusion in Balb/c mice. Mastitis resulting in fibrosis is a common problem in dairy cattle, but its mechanism is poorly understood, and studies questioning pathogenesis are extremely limited. Mouse mastitis models are of utmost importance in understanding bovine udder health and disease when the costs originating from including cattle in experiments and their rearing conditions are considered. Alternative and inexpensive mouse models are applicable in almost all standard laboratory animal breeding facilities. In this context, several mouse mastitis models have been established using various inflammatory stimuli. The administration of live bacteria or bacterial cell wall fragments such as LPS via intramammary injection is a useful and well-established experimental approach in the induction of mastitis in laboratory animals. Most of these mouse models mimic acute inflammatory changes in mammary tissue. Even though acute mastitis is essential in cattle, most cases are subclinical insidious infections accompanied by tissue fibrosis resulting in early culling of affected animals. New, simple, and safe experimental models are needed since the data on the induction of chronic fibrosing mastitis in laboratory animals is very limited. Working with live bacteria may cause biological hazards to researchers and laboratory animals in the same facility. Additionally, culture, storage, and administration of bacteria in mice are difficult parts of experimental models. Different mouse mastitis models can be good alternatives for avoiding such disadvantages.

The number of experimental studies for initiating chronic mastitis in mice is limited. Tuchscherr et al. (2005) have reported that the administration of Staphylococcus aureus strains capable of producing capsular polysaccharides via the intramammary route elicited chronic mastitis in mice. In their model, severe tissue damage, polymorphonuclear, and mononuclear cell infiltrates in the mammary tissues of mice on the 4th, 8th, and 12th days following the administration of bacterial strains have been demonstrated. However, they did not mention changes compatible with increased connective tissue accumulation (fibrosis) in the mammary sample. In our study, major histopathological inflammatory changes were found to be similar to those of Tuchscherr et al. Hence, we successfully demonstrated the induction of fibrosis together with severe inflammatory changes in mammary tissue. Moreover, similar histopathological findings were noticed in tissue samples from slaughtered cattle selected due to chronic fibrotic mastitis (Özguden-Akkoc et al., 2023).

A recent study reported that single-dose subcutaneous injection of *S. aureus* suspension induced mammary tissue fibrosis in SPF Balb/c mice (Bi et al., 2020). However, such an administration route may cause injection site reactions in the dermis and subcutaneous tissues, leading to dermatitis, cellulitis, and abscesses. Further, this experimental manipulation increases the risk of lowering the bacterial burden at the injection site. Biohazard and zoonotic potential for humans should be kept in mind. In the present study, the infusion of LPS solution directly into teat canals allowed it to be diffused through the duct system and alveoli. Thus, an inflammatory reaction selectively involved the mammary tissue without affecting the adjacent tissues.

Conflict of Interest

The authors stated that they did not have any real, potential, or perceived conflict of interest.

Ethical Approval

This study was approved by the Bursa Uludag University Animal Experiments Local Ethics Committee (2019/07-04 Number Ethics Committee Decision). In addition, the authors declared that Research and Publication Ethical rules were followed.

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Similarity Rate

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Author Contributions

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Writing the Article: OY, AA
Critical Review: OY, AA

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