

Rat Model of Cystic Neutrophilic Granulomatous Mastitis by *Corynebacterium Kroppenstedtii*

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Background: Cystic neutrophilic granulomatous mastitis (CNGM) poses a significant threat to the physical and mental health of women due to its increasing incidence, complex clinical manifestations. Developing an appropriate animal model will help further study the pathogenesis of CNGM.

Methods: Seventy-two rats were randomly assigned to seven groups: group A (n=12, tissue suspension 0.2mL), group B (n=12, 1×10^8 CFU/mL *Corynebacterium kroppenstedtii* (CK) suspension 0.1mL), group C (n=12, 1×10^9 CFU/mL CK suspension 0.1mL), group D (n=12, tissue suspension 0.1mL + 1×10^8 CFU/mL CK suspension 0.1mL), group E (n=12, tissue suspension 0.1mL + 1×10^9 CFU/mL CK suspension 0.1mL), group F (n=6, phosphate buffer saline solution 0.1mL), and group G (n=6, physiological saline 0.1mL + Complete Freund's adjuvant suspension 0.1mL). Groups A to E constitute the experimental groups with 12 rats each, while groups F and G served as control groups with 6 rats each. Tissue suspension of patients with granulomatous mastitis and different concentrations of CK solution were injected into the fourth pair of mammary glands of rats. Tissue samples were harvested on the 3rd, 7th, and 14th days post-implantation. The breast tissue specimens were stained with HE stain and Gram stain to observe the histopathological characteristics and the presence of Gram-positive bacteria. Bacterial culture was performed to observe the presence of CK. The expression levels of C-reactive protein and interleukin-1 beta were detected.

Results: Rats in groups A, D, and E exhibited breast masses with erythema, with some showing ulceration, and granulomatous structures in pathological. Lipid vacuoles and Gram-positive rods observed in groups D and E. Pus cultures from groups D and E showed growth of CK. Histopathology revealed minimal inflammatory cell infiltration and no granulomatous formation in groups B and C. Group F showed no masses or inflammatory cell infiltration. Rats in group G presented with masses without ulceration, only chronic and acute inflammatory cell infiltration in pathological. Levels of C-reactive protein and interleukin-1 beta were significantly elevated in groups A and E at day 14.

Conclusion: Components of pathological tissues from granulomatous mastitis patient combined with CK suspension, can successfully induce CNGM in rat models.

Keywords: cystic neutrophilic granulomatous mastitis, *Corynebacterium kroppenstedtii*, rat model, granulomatous mastitis

Introduction

Cystic neutrophilic granulomatous mastitis (CNGM) is a benign inflammatory breast disease associated with bacterial infections. In 2002, Paviour et al¹ first identified its distinctive histologic features in 24 female patients with mastitis: a cystic granuloma consisting of a central lipid vacuole surrounded by neutrophils and an outer capsule of epithelioid histiocytes, accompanied by a mixed inflammatory infiltrate of Langhans-type giant cells, lymphocytes, and neutrophils. Some of the lipid vacuoles may contain sparse Gram-positive bacilli. In 2011, Renshaw et al² introduced the term “cystic neutrophilic granulomatous mastitis”, highlighting the characteristic histologic pattern of *Corynebacterium* infections in inflammatory breast disease, which includes “cystic vacuoles formed by neutrophilic encapsulation” and “Gram-positive bacteria in the cystic vacuoles”. In 2020, Wu et al³ proposed the diagnosis of CNGM based on histologic features, Gram-staining results, and microbiologic studies. Recent studies have shown that CNGM significantly differs from

granulomatous mastitis (GM) in clinical presentation, histopathological features, and the detection rate of *Corynebacterium kroppenstedtii* (CK). Yang et al⁴ found that patients with CNGM were more likely to present with larger breast masses, ulcers, and nipple discharge than those with GM. Additionally, the prognosis of mastitis patients with CK was worse than those without CK. Li et al⁵ identified CNGM as an inflammatory breast disease with a unique histologic pattern closely related to CK. The strong association between CNGM and CK has been repeatedly confirmed in other studies,^{2,6–8} establishing CNGM as an infectious disease with a histomorphologic pattern consistent with *Corynebacterium* infection.⁹ Therefore, CNGM is recognized as a distinct disease entity.³ In recent years, CNGM has gradually received attention due to its complex clinical manifestations, prolonged course, and high recurrence rate. Fewer than a thousand cases of CNGM have been reported, accounting for about 1% of all breast specimens.¹⁰ In addition to the possible role of bacterial infection in CNGM formation, milk stasis may be the basis for the pathogenesis of the disease.¹¹ Autoimmune reactions may play an important role in the development of this disease.^{12,13} The etiology and pathogenesis of CNGM remain unclear, therapeutic experience is limited, and there is no consensus on a standardized treatment, leading to poor efficacy and a tendency for recurrence.^{9,14–16} Developing an animal model of CNGM could help deepen our understanding of this disease and provide a valuable platform for subsequent drug treatment research.

Materials and Methods

Instruments and Materials

Corynebacterium kroppenstedtii standard strain (Beijing Beina Biological Company, LOT: 353961); Rat interleukin-1 beta (ELISA) kit (Beijing Solabao Technology Co., Ltd., LOT: SEKR-0002); Complete Freund's adjuvant (Sigma-Aldrich Co., Ltd., USA); Isoflurane (Shenzhen Reward Life Science and Technology Co., LOT: 20221004); Tiletamine hydrochloride and zolazepam hydrochloride for injection (Vickers France Ltd., LOT: BN972LA).

Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) (Zhuhai Meihua, China); Turbidity Comparator (DensiCHEK Plus, bioMerieux, France); AutoStainer XL(ST 5010) (Leica Camera AG, Germany); Microscope (ZEISS Axio Lab. A1) (Carl Zeiss AG, Germany); Automated Tissue Dehydrator (Leica TP1020) (Leica Camera AG, Germany); Paraffin Embedding Machine (Leica EG1140 h) (Leica Camera AG, Germany); Columbia blood agar plate (BNFUTURE, China).

Animals

Seventy-two Sprague-Dawley (SD) rats (300±20g) with a documented maternal history were selected and purchased from Beijing Huafukang Bio-technology Co. Ltd. (Animal License: SCXK (Beijing) 2019–0008). This study was reviewed and approved by the Animal Ethics Committee of the Institute of Chinese Materia Medica China Academy of Chinese Medical Sciences with the approval number: No. 2023D024, dated October 23, 2023. The guideline followed for the welfare of the laboratory animals is National Standard “GB/T 35892–2018 Laboratory animal-Guideline for ethical review of animal welfare”. All experimental animals were housed in an ABSL–2 level biosafety laboratory of the Institute of Chinese Materia Medica China Academy of Chinese Medical Sciences, and all the operations in this study were conducted strictly according to the standardized operating procedures required for biosafety laboratories.

Tissue Suspension

Lesion tissues from patients with GM who underwent surgery were selected and mixed with saline in a 1:3 ratio. The tissue obtained by surgical excision were of various sizes, without peritoneum, and the cut surface was solid, grayish in color and hard, with scattered foci of necrotic lesions in the form of rotting flesh, and in some cases abscesses. The mixture was crushed and ground by a tissue grinder. The supernatant of the crushed tissue homogenate was collected and mixed with Complete Freund's adjuvant (CFA) in a 1:1 ratio by volume at the temperature of 0–4 °C. The prepared suspension, the homogenate of diseased mammary tissue, was stored at 4 °C for future use. The clinical samples were reviewed and approved by the Medical Ethics Committee of Beijing Hospital of Traditional Chinese Medicine, Capital Medical University with the approval number: No. 2023BL02-120-02, dated December 05, 2023. And informed consent has been obtained from patients for the use of clinical samples. This study complies with the Declaration of Helsinki.

Bacterial Suspension

The standard strain of *CK* was inoculated onto a Columbia blood agar plate and incubated for 72 hours. A small amount of bacterial growth (2–3 mm single colony) was then picked from the plate and suspended in phosphate buffer saline (PBS). The bacterial suspensions were adjusted to concentrations of 1×10^8 CFU/mL and 1×10^9 CFU/mL using a turbidimeter.

Animal Grouping and Administration

Seventy-two rats were randomly divided into seven groups: group A (tissue suspension group, $n=12$), group B (low-concentration bacterial suspension group, $n=12$), group C (high-concentration bacterial suspension group, $n=12$), group D (tissue suspension + low-concentration bacterial suspension group, $n=12$), group E (tissue suspension + high-concentration bacterial suspension group, $n=12$), group F (PBS group, $n=6$), and group G (CFA group, $n=6$). Groups A-E served as the experimental groups, each containing 12 rats, while groups F and G were the control groups, each containing 6 rats. After the SD rats were anesthetized, the fur was removed using depilatory cream to expose the 4th pair of nipples. The pre-configured suspension and bacterial solution were injected into the mammary fat pads using a 1 mL syringe. All rats were injected bilaterally in the 4th pair of mammary glands. In group A, 0.2 mL of GM tissue suspension was injected into each mammary gland. In group B, 0.1 mL of 1×10^8 CFU/mL bacterial suspension was injected into each mammary gland, while in group C, 0.1 mL of 1×10^9 CFU/mL bacterial suspension was injected into each mammary gland. In group D, each mammary gland was injected with 0.2 mL of a 1:1 mixture of GM tissue suspension and 1×10^8 CFU/mL bacterial suspension. In group E, each mammary gland was injected with 0.2 mL of a 1:1 mixture of GM tissue suspension and 1×10^9 CFU/mL bacterial suspension. In group F, each mammary gland was injected with 0.1 mL of PBS solution, and in group G, each mammary gland was injected with 0.2 mL of saline mixed with CFA in a 1:1 ratio (Figure 1).

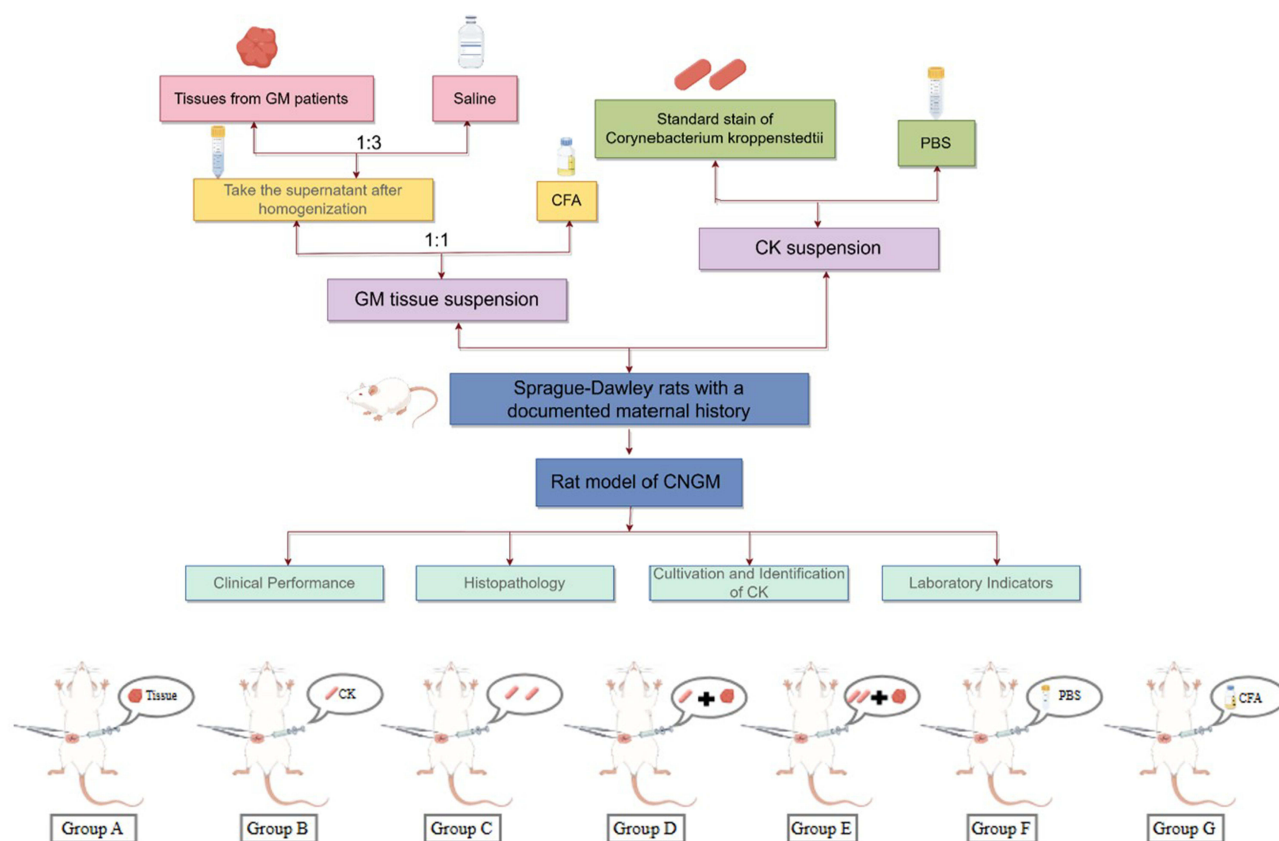


Figure 1 Flowchart of the experiment and group schematic diagram.

Abbreviations: GM, granulomatous mastitis; CFA, Complete Freund's adjuvant; PBS, phosphate buffer saline; CK, *Corynebacterium kroppenstedtii*. CNGM, cystic neutrophilic granulomatous mastitis.

Observation Indicators and Time

Sampling was conducted on the 3rd, 7th, and 14th day of modeling. In each of the A-E groups, 4 random samples were taken per group, and in each of the F and G groups, 2 random samples were taken per group.

Clinical Performance

The clinical performance of the rats was observed daily, with regular recording of the changes in each group. Observations included the skin color of breast, the size of any swelling, and the presence of rupture formation.

Histopathology

The fourth pair of mammary glands from each rat was excised. One of the mammary glands was fixed in formalin solution, then routinely embedded in paraffin wax and sectioned. Sections with a thickness of 4 μm were dewaxed, stained with Hematoxylin-eosin (HE), dehydrated, and sealed for observation. The morphology of the mammary tissues was examined under a light microscope for pathological diagnosis. Additionally, 6 μm thick sections were selected for Gram staining after dewaxing, then dehydrated and sealed to observe the presence of Gram-positive or Gram-negative bacteria under microscope.

Cultivation and Identification of CK

On the 14th day, pus specimens from the breasts in groups A, D, and E were collected using sterilized swabs. The pus specimens were inoculated on the blood plate and then cultured in an incubator at 35°C and 5%CO₂ for 72h. After the colony growth was stable, it was transferred to a new blood plate and incubated in an incubator for 48h. A single colony was selected and identified by Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS).

Laboratory Indicators

C-reactive protein(CRP) levels were detected after blood was drawn from the abdominal aorta. The level of interleukin-1 beta (IL-1 β) in one mammary gland tissue from each rat was determined using an ELISA kit.

Statistical Analysis

SPSS 25.0 statistical software was used for data analysis and processing. Measurement data conforming to a normal distribution were described as mean \pm standard deviation, while non-normally distributed data were described as median (interquartile range). Categorical data were described using a percentage composition ratio (%). Comparisons between groups were made using chi-square tests for normally distributed data, and nonparametric rank-sum tests for non-normally distributed data. Differences were considered statistically significant at $P < 0.05$.

Results

Clinical Performance

On the day of implantation, the 4th pair of breasts was observed without any signs of redness and ulceration in each group. By the 3rd and 7th days post-implantation, redness and swelling of breast was noted in groups A, D, E, and G. Although there was no statistically significant difference in the size of the swelling among the groups, the swelling was significantly larger compared to groups B, C, and F. On the 14th day post-implantation, breast mass with redness and swelling persisted in groups A, D, and E, with some rats exhibiting breast ulceration. There was no statistically significant difference in the size of the swelling among groups A, D, and E; however, the swelling was statistically larger compared to groups B, C, F, and G (Figure 2 and Table 1).

Histopathology

The results of HE staining of the histopathological sections of the mammary glands of SD rats showed that on day 3, inflammatory cell infiltration was observed in all groups except group F. Increased inflammatory cell infiltration was seen around the mammary ducts and lobules of rats in groups A, D, and E, accompanied by abscess formation. In contrast, only a small amount of inflammatory cell infiltration was observed in the mammary glands of rats in groups B, C, and G. By day 7, the inflammatory reaction had subsided in groups B and C, with no significant inflammatory cell infiltration

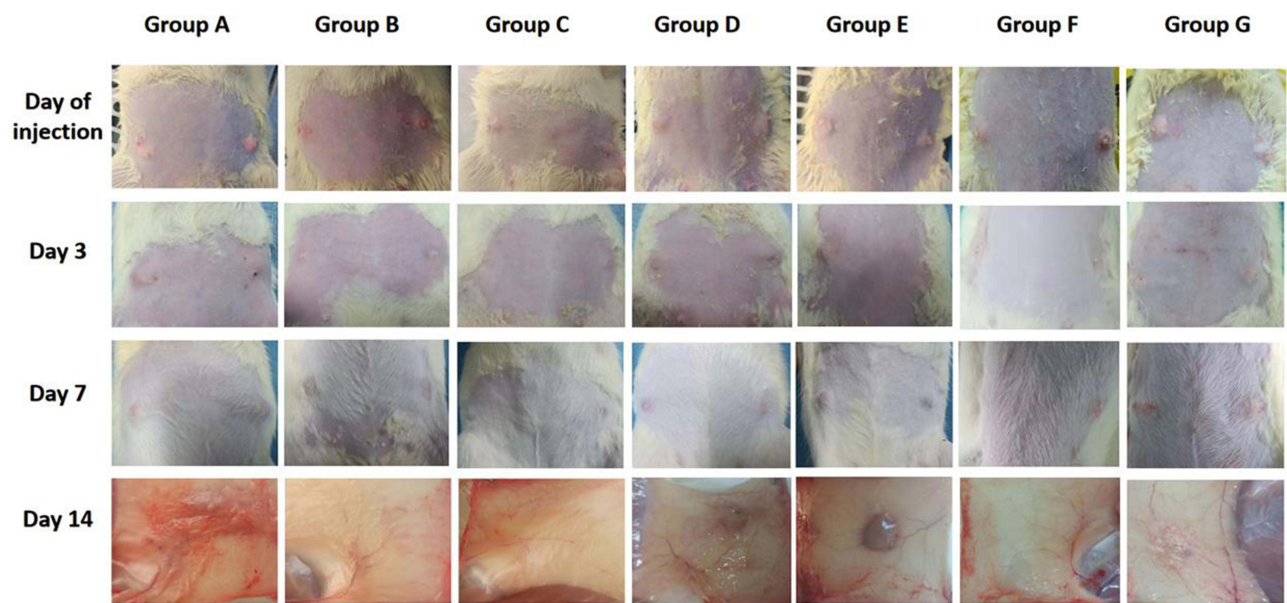


Figure 2 Breast changes in the 4th pair of mammary glands of rats in each group. The groups are as follows: Group (A) tissue suspension group; Group (B) low-concentration bacterial suspension group; Group (C) high-concentration bacterial suspension group; Group (D) tissue suspension + low-concentration bacterial suspension group; Group (E) tissue suspension + high-concentration bacterial suspension group; Group (F) PBS group; Group (G) CFA group.

observed in groups B, C and F. However, in groups A, D, E, and G, the inflammation in the mammary glands further progressed, with a large amount of inflammatory cell infiltration noted, particularly in groups A, D and E, where abscess formation was also observed. By day 14, many inflammatory cells such as lymphocytes, mononuclear macrophages, neutrophils, and a few plasma cells were seen around the lobules and ducts of the mammary glands in rats from groups A, D, and E, with granulomatous structures formation in all these groups. More granulomatous structures were observed in groups D and E compared to group A, with lipid vacuoles surrounded by neutrophils also present in groups D and E. Gram-stained revealed the presence of bacilli within the vacuoles in groups D and E, and microscopic observation showed that the microorganisms were short rod-shaped and disordered. In group G, only a very small number of inflammatory cells were observed, and no significant inflammatory cell infiltration was seen in groups B, C, and F (Figures 3 and 4).

Cultivation and Identification of CK

The pus specimens were inoculated on the blood plate and then cultured in an incubator at 35°C and 5%CO₂ for 72h. Small and scattered grayish-white colonies can be observed on the plate. The pus samples were then transferred to a new blood plate and incubated in an incubator for 24h, very small scattered grayish-white colonies with a diameter <1mm were observed. After incubation for 48h, small grayish-white colonies were found, which were slightly larger than those at 24h, and the diameter was still <1mm. The peak of the mass spectrum of the isolates is shown in Figure 5.

Table I Breast Mass Size in Each Group [M (P25, P75)], (cm²)

| Time | Group A | Group B | Group C | Group D | Group E | Group F | Group G |
|--------|--------------------------------|----------------|----------------|--------------------------------|--------------------------------|----------------|-------------------------------|
| Day 3 | 0.880(0.8,1.2) ^{bcf} | 0.400(0.3,0.5) | 0.320(0.3,0.4) | 1.160(0.8,1.2) ^{bcf} | 0.920(0.7,1.1) ^{bcf} | 0.320(0.3,0.4) | 1.440(1.1,1.6) ^{bcf} |
| Day 7 | 1.050(0.7,1.4) ^{bcf} | 0.090(0.1,0.1) | 0.105(0.1,0.2) | 0.710(0.3,2.4) ^{bcf} | 0.600(0.4,0.9) ^{bcf} | 0.110(0.1,0.1) | 1.050(0.9,1.2) ^{bcf} |
| Day 14 | 2.800(2.5,5.3) ^{bcfg} | 0.085(0.1,0.1) | 0.105(0.1,0.2) | 0.975(0.6,2.0) ^{bcfg} | 0.530(0.5,0.6) ^{bcfg} | 0.120(0.1,0.2) | 0.150(0.1,0.2) |

Notes: ^b indicates a statistically significant difference compared with group B ($P < 0.05$). ^c indicates a statistically significant difference compared with group C, ($P < 0.05$). ^f indicates a statistically significant difference compared with group F, ($P < 0.05$). ^g indicates a statistically significant difference compared with group G, ($P < 0.05$).

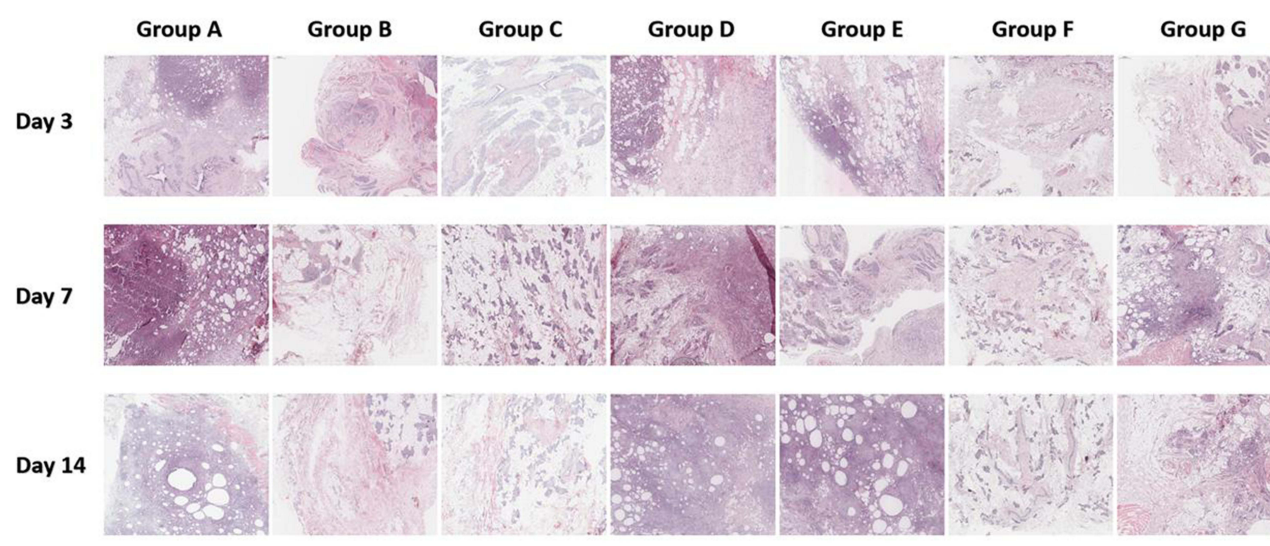


Figure 3 HE staining of the groups on day 3, 7 and 14 (10X). In groups (A,D and E), the border of the lobular tissue of the breast was unclear, and a large number of neutrophils and other inflammatory cells were seen infiltrating, and some of them were seen to have dilated breast ducts and abscess formation. Granulomatous structures were seen in all three groups at 14 days. A small amount of inflammatory cell infiltration such as lymphocytes was seen in groups (B,C and G). The infiltration of inflammatory cells in group (G) resolved at 14 days, and no granulomatous changes in the lobules of the mammary glands were seen in groups (B,C and G). No significant inflammatory cell infiltration was seen in group (F).

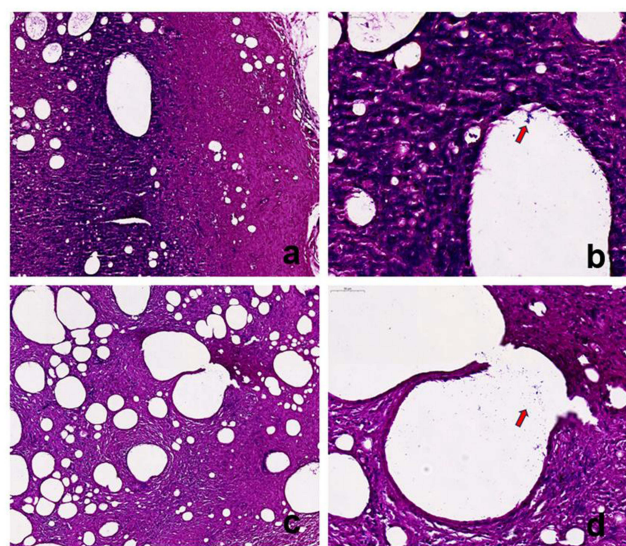


Figure 4 Gram staining of groups D and E on day 14: (a): group D, 20X; (b): group D, 63X; (c): group E, 20X; (d): group E, 63X. Short rod-shaped and disordered Gram-positive bacilli are visible at the locations indicated by the red arrows.

Laboratory Indicators

There was no significant difference in CRP levels between the groups on days 3 and 7. However, on day 14, CRP levels in groups A and E were higher than other groups, with statistically significant differences compared to groups F and G. IL-1 β levels were higher in groups D, E, and G on day 3 compared to the other groups, with statistically significant differences compared to groups A, B, C, and F. There were no statistically significant differences among groups D, E, and G themselves. On day 7, IL-1 β levels were higher in groups A, D, and E compared to the other groups, with statistically significant differences compared to groups B, C, F, and G. On day 14, IL-1 β levels were higher in group A than in the other groups, and the difference was statistically significant. Additionally, IL-1 β levels in group C were higher than in groups F and G, with a statistically significant difference. IL-1 β levels in groups D and E were higher than in groups B,

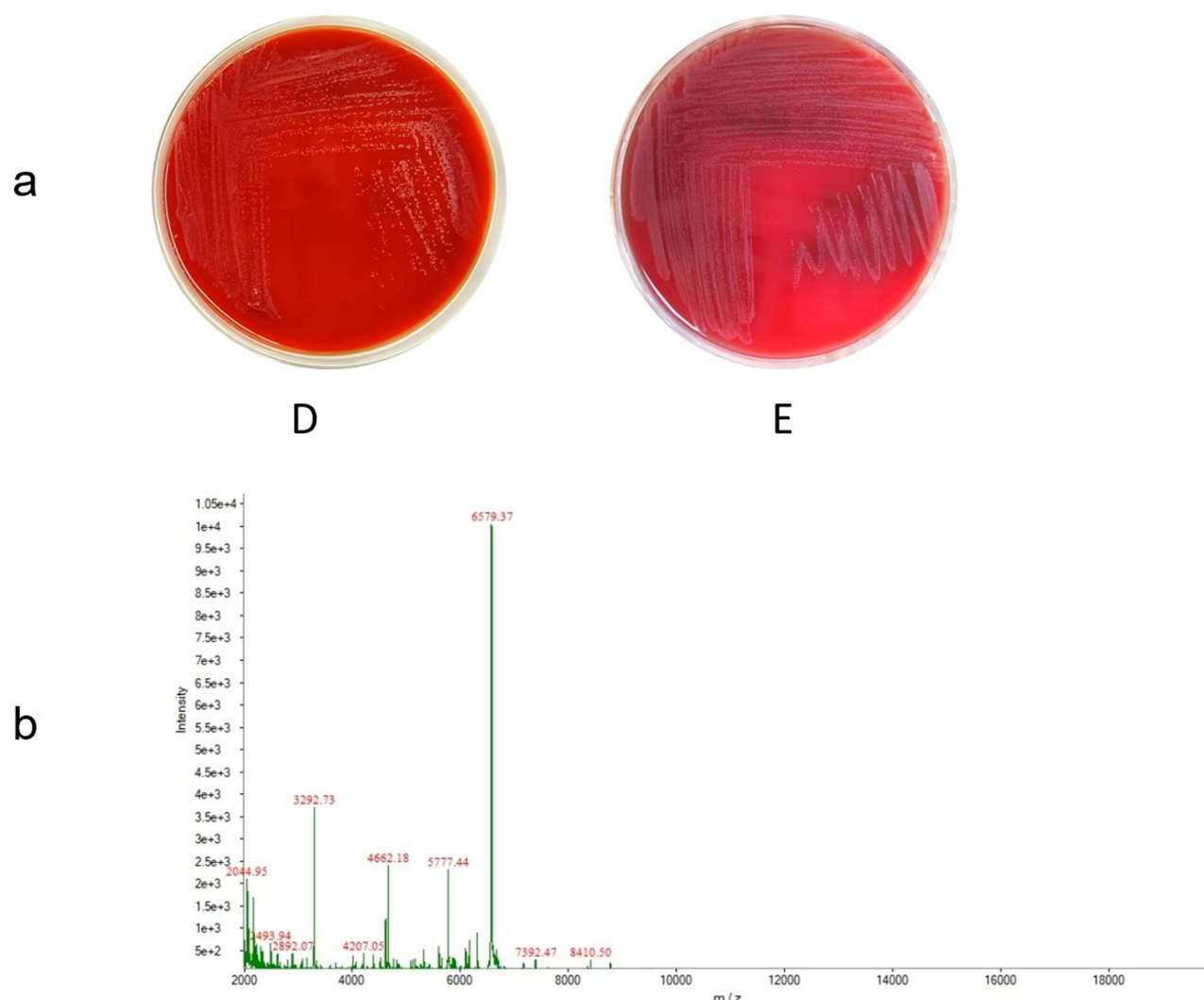


Figure 5 (a) Morphological observation of pus isolates from groups D and E in vitro incubation. (b): The mass spectrogram of pus isolates identified by MALDI-TOF MS.

C, F, and G, with statistically significant differences compared to groups B, F, and G. There were no statistically significant differences between groups D and E themselves (Table 2).

Discussion

Since the disease was only named separately internationally after 2011, the number of clearly reported cases is still low. In recent years, there has been an increase in reported studies on CNGM, and the number of cases of CNGM is significantly higher than before.^{3,9,17} Recent studies indicate that a lack of awareness among some clinicians and pathologists about the existence of this entity may contribute to the low number of CNGM cases currently reported. For example, a study by Shao et al¹⁷ demonstrated that out of 202 patients with non-puerperal mastitis, 104 cases met the pathological diagnostic criteria for CNGM. The results of the study suggest that patients with CNGM exhibit more severe clinical manifestations and require longer treatment durations, highlighting the necessity to distinguish them as an independent disease. We believe that further research into the pathogenesis of this condition may also assist in enhancing our ability to recognize CNGM as a distinct diagnostic entity.

More recent studies have demonstrated that CNGM differs significantly from GM in terms of clinical manifestations, especially the detection rate of CK.^{8,18} Therefore some studies suggest that patients with CNGM may be empirically treated with rifampicin, clindamycin, and other fee penicillins.³ The method of constructing an animal model of GM and

Table 2 Comparison of CRP (mg/L) and IL-1 β (pg/mL) Levels Among Groups

| Indicators | Time | Group A | Group B | Group C | Group D | Group E | Group F | Group G |
|--------------|--------|--------------------------------------|---------------------|------------------------------------|--------------------------------------|--------------------------------------|---------------------|-------------------------------------|
| CRP | Day 3 | 0.30 \pm 0.09 | 0.31 \pm 0.13 | 0.23 \pm 0.04 | 0.27 \pm 0.08 | 0.32 \pm 0.08 | 0.22 \pm 0.16 | 0.39 \pm 0.01 |
| | Day 7 | 0.37 \pm 0.12 | 0.27 \pm 0.14 | 0.28 \pm 0.10 | 0.31 \pm 0.08 | 0.33 \pm 0.12 | 0.23 \pm 0.04 | 0.27 \pm 0.09 |
| | Day 14 | 0.42 \pm 0.23 ^{fg} | 0.34 \pm 0.09 | 0.40 \pm 0.04 | 0.35 \pm 0.10 | 0.49 \pm 0.09 ^{fg} | 0.20 \pm 0.00 | 0.20 \pm 0.00 |
| IL-1 β | Day 3 | 889.70 \pm 480.41 | 796.40 \pm 386.49 | 541.95 \pm 308.76 | 1648.05 \pm 389.80 ^{abc} | 1676.40 \pm 312.99 ^{abc} | 919.17 \pm 266.61 | 1409.17 \pm 164.15 ^{abc} |
| | Day 7 | 1411.95 \pm 410.34 ^{bcdg} | 266.40 \pm 228.02 | 283.63 \pm 199.22 | 1842.50 \pm 588.25 ^{bcdg} | 1359.45 \pm 427.82 ^{bcdg} | 436.65 \pm 174.61 | 551.95 \pm 193.84 |
| | Day 14 | 1469.53 \pm 576.48 ^{bcdg} | 304.27 \pm 282.58 | 625.40 \pm 275.59 ^{afg} | 794.90 \pm 201.28 ^{abfg} | 780.52 \pm 164.82 ^{abfg} | 260.40 \pm 133.51 | 201.40 \pm 61.20 |

Notes: ^a indicates a statistically significant difference compared with group A, ($P < 0.05$). ^b indicates a statistically significant difference compared with group B, ($P < 0.05$). ^c indicates a statistically significant difference compared with group C, ($P < 0.05$). ^d indicates a statistically significant difference compared with group D, ($P < 0.05$). ^e indicates a statistically significant difference compared with group E, ($P < 0.05$). ^f indicates a statistically significant difference compared with group F, ($P < 0.05$). ^g indicates a statistically significant difference compared with group G, ($P < 0.05$).

plasma cell mastitis by locally injecting lesion tissue suspension of GM patients into the mammary glands of animals has gradually gained recognition. Some scholars have conducted studies based on this method, which has more fully revealed the pathogenesis of GM and the effects of drug treatment through the animal model.^{19–24} Recently, Liu R et al²⁵ conducted a series of studies related to *Corynebacterium parakroppenstedtii* and constructed a mastitis model with the help of *Corynebacterium parakroppenstedtii* suspension, which demonstrated the pathogenicity of *Corynebacterium parakroppenstedtii* in GM using Koch's postulates. The results of the study suggest that *Corynebacterium parakroppenstedtii* may chelate iron by releasing a new type of glycolipid (named corynekropbactins), cause the death of mammary cells and other mammary-gland- colonizing bacteria, and increase the levels of inflammatory cytokines. However, no modeling method has been reported for CNGM, which has hindered the study of this disease. Since 2011, Renshaw et al² have defined this unique histologic pattern of *Corynebacterium* infection in breast inflammation as “cystic neutrophilic granulomatous mastitis”. A growing body of research has demonstrated the close relationship between CNGM and *Corynebacterium*, particularly CK,^{11,12,26–28} which is a key point of differentiation between CNGM and GM. Studies have demonstrated that lipophilic antibiotics targeting CK have favorable therapeutic effects,^{29,30} further validating this viewpoint. Considering lactation stasis as the pathogenic basis of CNGM, postnatal SD rats were used to construct this model, aiming to simulate the pathogenic environmental conditions of CNGM as closely as possible. Building on the method of creating a GM model—through the local injection of GM patients' lesions into the mammary gland—and combined with literature on bacterial mastitis models induced by mammary injection of bacterial fluids,^{31–33} the present study attempted to construct an animal model of CNGM by adding CK suspension to the homogenate of human GM lesions. The feasibility of the modeling approach was then evaluated.

The results of the study showed that rats in both the tissue + low concentration bacterial suspension group and the tissue + high concentration bacterial suspension group developed breast lumps, redness, and swelling. In some cases, these symptoms progressed to rupture after implantation. Their localized mammary gland characteristics in these groups were consistent with those observed in clinical CNGM patients. The model was maintained over an extended period; by the 14th day, not only had the swelling not subsided, but local rupture had also occurred, building upon the swelling and redness observed on the 3rd and 7th days. This effectively simulated the clinical characteristics of GNGM patients, where swelling often progressed to abscess formation and rupture. In terms of pathological morphology, the CNGM group (tissue + low concentration bacterial suspension group and tissue + high concentration bacterial suspension group) differed from the GM group (tissue alone group). Microscopically, more granulomatous structures were observed in the CNGM groups, successfully replicating the unique histopathological features of CNGM in the presence of bacterial fluids: lipid vacuoles formed by neutrophilic encapsulation, with Gram-positive bacilli present within the vacuoles. After 14 days of implantation, breast lesions in both CNGM groups still showed bacilli on culture, verified as CK by MALDI-TOF MS. The increased levels of CRP and IL-1 β in the CNGM group were also consistent with the laboratory findings of clinical CNGM.¹⁷ As a sensitive indicator of inflammation, CRP recognizes and binds to pathogens and apoptotic cells, facilitating their clearance in innate immunity, which also can activate the classical complement pathway, enhancing pathogen clearance. Li et al³⁴ showed that elevated CRP levels are often accompanied by more severe clinical manifestations. The study conducted by Huang et al³⁵ suggested the association of serum CRP level and disease severity in GM patients. The increased synthesis of CRP can be induced by IL-1 β . In a randomized controlled trial, numerical reduction of serum IL-1 β , was found in GM patients after treated with a traditional Chinese herbal medicine compound, which suggested anti-cytokine therapies may be a promising treatment option to eliminate the inflammation in GM.³⁶

The histopathological features of both CNGM and GM show a granulomatous structure. The granulomatous structure of CNGM is composed of lipid vacuoles surrounded by neutrophils and an outer capsule of epithelioid histiocytes, and some of these vacuoles may contain gram-positive bacilli. GM mainly showed non-caseating granulomas centered on breast lobules with epithelioid histiocytes, without lipid vacuoles and gram-positive bacilli.³⁷ In this study, the simple bacterial fluid groups (B and C) were selected as controls. A localized breast lump appeared only on the day of implantation and gradually disappeared thereafter. No granulomatous structures were observed in the histopathology, and only a small amount of inflammatory cell infiltration was noted, which diminished over time compared to earlier stages. This outcome is inconsistent with the clinical and pathological features of GNGM. As a lipophilic and fastidious

bacterium, *CK* may not survive or multiply effectively in the breast due to environmental limitations,³ which is the likely reason for the bacterial fluid failed to replicate the disease without accompanying human lesion tissue.

In the PBS group, the mass gradually disappeared after implantation, and no inflammatory infiltration was observed in the histopathology, indicating that PBS did not affect the development of CNGM. In the CFA group, lumps were observed on the 3rd and 7th days after implantation, disappearing by the 14th day. Histopathology showed infiltration of both acute and chronic inflammatory cells on the 3rd and 7th days, with a significant reduction in inflammatory cell infiltration by the 14th day. However, no granulomatous structures were observed. This suggests that CFA can induce specific alterations in the immune response, such as promoting delayed hypersensitivity reactions against self-antigens.³⁸ CFA has been previously used in various chronic inflammation models, including inflammatory pain,³⁹ arthritis,⁴⁰ and autoimmune encephalomyelitis,⁴¹ showing some pro-inflammatory effects. However, its induction effect on CNGM is limited: first, the inflammatory response is not long-lasting and the swelling subsides too quickly; second, the degree of inflammation observed in pathology is mild, with no formation of granulomatous structures and no bacilli present, which is inconsistent with the pathogenesis of CNGM. In contrast, although the GM model group (tissue-only group) exhibited clinical features such as swelling, and redness, the pathomorphology lacked the characteristic manifestations of CNGM, and rod-shaped bacilli were not seen in the culture of the pus specimens, which did not align with the pathogenesis of CNGM.

The method of combining tissue with a bacterial suspension, based on traditional GM modeling and incorporating a certain amount of CNGM pathogenic bacteria-*CK*, was confirmed in our study to better simulate the pathogenesis and clinical manifestations of human GNGM and successfully induced the occurrence of a CNGM rat model.

Conclusion

The addition of *CK* to the traditional GM model can successfully induce a CNGM rat model in this study. The combination of GM lesion tissue and bacterial suspension better mimics the clinical symptoms, histopathology, immune response, and expression of inflammatory factors in GNGM patient. In addition, the model remained stable and persisted for two weeks, and pus cultures confirmed the continued survival of *CK*. To the best of our knowledge, there are no previous reports of CNGM model studies, this study provides a valuable platform for further exploration of the etiology, pathogenesis and potential drug options for CNGM. Unfortunately, we are still unclear about the GM lesion tissue and the specific mechanism of *CK*'s role in CNGM, and we will further investigate this.

Data Sharing Statement

The original contributions presented in the study are included in the article. Further inquiries can be directed to the corresponding authors.

Ethical Statement

The clinical samples were reviewed and approved by the Medical Ethics Committee of Beijing Hospital of Traditional Chinese Medicine, Capital Medical University with the approval number: No. 2023BL02-120-02, dated December 05, 2023. The animal experiment was reviewed and approved by the Animal Ethics Committee of the Institute of Chinese Materia Medica China Academy of Chinese Medical Sciences with the approval number: No. 2023D024, dated October 23, 2023.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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