Short communication: Evaluation of α -linolenic acid–based intramammary nanosuspension for treatment of subclinical mastitis*

Rajnish K. Yadav,¹ Manjari Singh,¹ Subhadeep Roy,¹ Swetlana Gautam,¹ Jitendra K. Rawat,¹ Lakhveer Singh,¹ Mohd Nazam Ansari,² Abdulaziz S. Saeedan,² and Gaurav Kaithwas^{1†}

¹Department of Pharmaceutical Sciences, School of Biosciences and Biotechnology, Babasaheb Bhimrao Ambedkar University (A Central University), Lucknow 226 025, India

²Department of Pharmacology, College of Pharmacy, Prince Sattam Bin Abdulaziz University, Al-Kharj 16278, Saudi Arabia

ABSTRACT

The current study investigates the therapeutic efficacy of an α -linolenic acid (ALA, 18:3n-3)–based intramammary nanosuspension (ALA-NS) for treatment of subclinical mastitis. After confirmation of mastitis with the help of field-based testing, a total of 9 mixed-breed cows (23 udder quarter samples) were divided into 3 groups and treated with ALA-NS and cefoperazone intramammary suspension for 10 d. Subclinical mastitis on d 1 was confirmed through field-based tests such as pH, California Mastitis Test (CMT), Whiteside test (WST), and bromothymol blue test (BBT) scores. Treatment with ALA-NS (F1 and F2) exhibited significant effects on field-based parameters, along with curtailment of total microbial count [28 ± 3.16 (mean $\pm$ standard deviation) and 25 ± 4.24 cfu/50 μ L] and somatic cell count (SCC; 3.9 and 2.8 log SCC cells/mL), respectively for ALA-NS F1 and F2, after 10-d treatment. The efficacy of ALA-NS was further affirmed using more stringent markers for inflammation (nuclear factor kappa-light-chain-enhancer of activated B cells, NF κ B-p65), milk quality (sterol response element-binding protein-1c, SREBP-1c), and bacterial resistance (ubiquitin carboxyl-terminal hydrolase-1, UCHL-1) in milk samples. Treatment with ALA-NS (at 2 concentrations of ALA, F1 and F2) significantly decreased expression of NF κ B-p65, SREBP-1c, and UCHL-1 after d 10 of treatment. Apparently, anti-inflammatory, antibacterial, peripheral analgesic properties of ALA could account for the therapeutic efficacy of the proposed regimen.

Key words: bovine mastitis, α -linolenic acid, inflammation, NF κ Bp65, UCHL-1

Short Communication

Bovine mastitis is an inflammatory condition of the mammary glands of lactating animals, characterized by pain, edema, swelling, and polymorphic neutrophil infiltration. Mastitis is a curse for the dairy industry, as it decreases the productivity and quality of milk and increases the cost of herd management. Currently, antibiotics, either alone or in combination with nonsteroidal anti-inflammatory agents (NSAID) are most commonly prescribed for clinical management of bovine mastitis. However, long-term use of antibiotics causes bacterial resistance and has negative effects on consumer health (Li et al., 2013). Therefore, alternative, safer drugs with universal effectiveness, lasting benefits, and fewer side effects are requisite in the area of mastitis management.

Omega-3 (n-3) fatty acids may be among the best examples of how diet may affect inflammation. These fats exert a remarkable variety of biological responses, including inflammation and related clinical conditions (Yadav et al., 2018). α -Linolenic acid (ALA; 18:3n-3) is an n-3 PUFA and is transformed to class-3 and class-5 eicosanoids through sequences of desaturation and elongation processes. Previous study has affirmed the relationship between ALA (18:3n-3) supplementation and anti-inflammatory effects (Anand and Kaithwas, 2014). It is interesting to note that *Linum usitatissimum* fixed oil, containing 57.38% ALA (18:3n-3), has been found to display anti-inflammatory, antimicrobial activity and efficacy against subclinical cases of bovine mastitis (Kaithwas et al., 2011a,b). The present study was designed to explore ALA (18:3n-3) as a complementary therapeutic agent, with efforts to provide a ready-to-use intramammary formulation (pre-filled syringes) of ALA (18:3n-3) and cefotaxime nanosuspension. For this purpose, ALA (18:3n-3) was taken as oil phase, with Tween-80 as surfactant and polyethylene glycol-400 (PEG-400) as co-surfactant. The formulations were optimized and evaluated for particle size, size distribution, and stability parameters. Before proceeding

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†Corresponding author: gauravpharm@hotmail.com

to the field-based study on cows, preclinical efficacy of ALA-based nanosuspension (**ALA-NS**, **F1** and **F2**) was validated against lipopolysaccharide-induced mastitis in rats (Yadav et al., 2019). The current study investigated the effects of ready-to-use ALA-NS for treatment of subclinical mastitis in cows.

We purchased ALA (CAS no. 463-40-1) from TCI (Portland, OR). All other chemicals and reagents were of analytical grade and were acquired from Genetix Asia Private Ltd., New Delhi, India. We formulated ALA-NS using ALA as oil base. Cefotaxime was suspended in Tween-80 and PEG-400. Subsequently, we added the ALA to the mixture with continuous sonication. A total of 8 formulations of ALA-NS were prepared, evaluated, and optimized for the study; only 2 were found stable and used in the present study (Supplemental Table S1; <https://doi.org/10.3168/jds.2019-16239>). Nine lactating mixed-breed mastitic cows with no other clinical illness were selected for the study from different places in Deoria, India. Cows were divided into the following treatment groups: (1) cefoperazone 2.5 mL/udder (concentration 25 mg/mL per udder quarter daily); (2) ALA-NS F1, 2.5 mL/udder (concentration 20 mg/mL per udder quarter daily); and (3) ALA-NS F2, 2.5 mL/udder (concentration 20 mg/mL per udder quarter daily). All treatments were administered once a day for 10 d. The study was approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India (Institutional Animal Ethics Committee approval no. SDCOP and VS/AH/CPCSEA/01/0039).

For sampling of milk, teat orifices of mastitic cows were washed with 70% ethanol, and 50 mL of milk was flushed from the teat orifice before collecting 50 mL of milk in a sterile collecting flask. Milk was sampled on d 1, 5, and 10 of the experimental period and stored at 4°C for further analysis. Milk pH was measured by pH meter (HI 98107, Hanna Instruments, Woonsocket, RI), and milk color and consistency were visually assessed. For the Whiteside test (**WST**), 5 drops of milk were placed on a glass plate (underside painted black) and mixed with 2 drops of NaOH (2%). The mixture was rapidly stirred for 20 to 25 s. The results were scored as follows: mixture remained particle-free = 0 (–); fine dispersed particles on close inspection = 1 (+); a milky whey and white particles with definite thickening = 2 (++); formation of white flakes = 3 (+++; Fthenakis 1995).

The bromothymol blue test (**BBT**) was performed by dividing Whatman filter paper (7 cm diameter; GE Healthcare Life Sciences, Marlborough, MA) into 4 different quadrants, denoted as right front, right back, left front, and left back. A drop of BBT solution was placed

in each quadrant, followed by a drop of milk. The color changes were scored as follows: pale green = normal, 1 (+); moderate green = 2 (++); dark blue green = 3 (+++; Marschke and Kitchen 1985).

The California Mastitis Test (**CMT**) was executed using the commercial CMT reagent. The test was implemented by rapidly mixing equal quantities (3 mL) of milk and CMT reagent (Kaithwas et al., 2011b). Results were scored as follows: no or slight precipitation formation, which dissolves with paddle rotation = normal milk (0); no gel formation, distinctive precipitation forms and does not dissolve after paddle movement = suspicious milk (1); gelatinization occurs = infected milk (2); concentrated gel formation that sticks to the paddle = severely infected milk (3).

We estimated SCC via direct microscopy of milk samples, using the methylene blue staining procedure (Kaithwas et al., 2011b). Total microbial count (**TMC**) of milk was estimated in aseptic conditions. Briefly, 50 µL of milk was stirred into 20 mL of sterile molten Muller Hinton agar medium, and the mixture was transferred to sterile Petri plates. The inoculated Petri plates were incubated at 37°C for 24 h. Microbial colonies were estimated in cfu, using a colony counter (Kaithwas et al., 2011b).

Total protein from milk samples was extracted for the Western blot analysis. Briefly, milk samples were centrifuged at $9,257 \times g$ for 15 min, and the separated pellet was resuspended in HBSS buffer. The procedure was repeated 3 times. Protein was extracted by adding 100 µL each of phenylmethylsulfonyl fluoride and radio-immunoprecipitation assay lysis buffer. The mixture was sonicated (10 s) and centrifuged at $900 \times g$ for 20 min. The supernatant was acetone precipitated, and protein was quantified through the Bradford method (Dang et al., 2013). Expression of nuclear factor kappa-light-chain-enhancer of activated B cells (**NFκB-p65**), ubiquitin carboxyl-terminal hydrolase isoenzyme L1 (**UCHL-1**), and sterol response element-binding protein (**SREBP-1**) in the milk sample was then performed. Milk proteins were separated on SDS-PAGE gel (12.5%) and transferred to polyvinylidene fluoride membrane using the semidrying transfer method. Consequently, alienated proteins on the polyvinylidene fluoride membrane were blocked using 5% BSA and skim milk in Tris buffer saline with Tween-20 for 3 h and then incubated with primary antibody overnight at 4°C. β-Actin was used as a loading control. Further procedure was performed as described by Roy et al. (2017). For Western blot analysis, protein was loaded according to the following criteria: lane 1 represented samples from d 10 of treatment for group 1 (cefoperazone treatment only); lane 2 was d-1 milk sample of all

groups; and lanes 3 and 4 represented d 10 of treatment with ALA-NS F1 and F2, respectively.

Statistical analysis of data was performed using Graph Pad Prism 5.0 (Graph Pad Software, San Diego, CA). All data are presented as mean $\pm$ standard deviation and analyzed by one-way ANOVA followed by the Student-Newman-Keuls multiple comparison test.

Milk color changed from pale yellow to white, and milk consistency changed from watery to thick, after treatment with ALA-NS F1 and F2. Milk pH was normalized significantly ($P < 0.05$) on d 10 after intramammary treatment with both ALA-NS F1 and F2. Scores for CMT significantly decreased by d 10 (2 ± 0.00 , $P < 0.05$; and 1.5 ± 0.50 , $P < 0.001$) with treatment of ALA-NS F1 and F2, respectively, compared with d 1 of treatment (3 ± 0.00 ; Table 1). Both ALA-NS F1 and F2 significantly ($P < 0.001$) reduced WST and BBT scores after 10 d of treatment (Table 1). In the same way, SCC was also decreased nearly to normal by both ALA-NS treatments, with more favorable effects from F1 (Table 2). Initial TMC at d 1 of treatment was 195 ± 21.98 , 173 ± 46.10 , and 218 ± 29.56 cfu/50 μ L in groups 1, 2, and 3, respectively. After completion of treatment at d 10, TMC was reduced to normal levels: 37 ± 11.4 , 28 ± 3.16 , and 25 ± 4.24 cfu/50 μ L for groups 1, 2, and 3, respectively. Groups 2 and 3 showed higher percentages of microbial count inhibition (83.81 and 88.53%) compared with group 1 (81.05%; Table 1).

Blot analysis revealed significantly decreased expression of NF κ B-p65 and SREBP-1c on d 10 ($P < 0.001$) compared with d 1. We found that F1 reduced the expression of NF κ Bp65 and SREBP-1c more profoundly compared with F2 and cefoperazone. Expression of UCHL-1 was also significantly ($P < 0.001$) diminished after treatment with ALA-NS F1 and F2 (Figure 1).

Bacterial infection, as from *Streptococcus aureus* or *Escherichia coli*, is one of the foremost causes of mastitis in lactating animals. Previous findings have reported elevated milk pH in cases of subclinical mastitis (Sena and Sahani, 2001). Additionally, field-based tests, including WST, BBT, and CMT, are also used to confirm subclinical mastitis. All the animals selected for the present study scored positive for subclinical mastitis on the basis of field-based tests. Treatment with marketed cefoperazone and ALA-NS (F1 and F2) demonstrated significant effects on milk pH along with WST, BBT, and CMT scores.

Subclinical mastitis is an inflammatory condition of the udder, leading to exudation and leakage of cellular components in milk. Such exudation and cellular infiltration increase SCC in milk, which is an important indicator of mastitis (Viguier et al., 2009). Augmented SCC is accompanied by diminutions of milk protein casein and lactose, as well as augmented enzymatic

Table 1. Effects of intramammary treatment with α -linolenic acid nanosuspension (ALA-NS), in 2 formulations (F1, F2), on field-based parameters and total microbial count (TMC) in mastitic cows¹

Item	d 1					d 5					d 10				
	pH	CMT	WST	BBT	TMC	pH	CMT	WST	BBT	TMC	pH	CMT	WST	BBT	TMC
Group 1	7.35	3	2	2	195	6.9	2	1	1	118	6.8	1.5	1	1	37
	± 0.35	± 0.50	± 0.00	± 0.00	± 21.98	± 0.14	± 0.00**	± 0.00***	± 0.00***	± 13.35*** (39.48%)	± 0.10	± 0.50**	± 0.00***	± 0.00***	± 11.4*** (81.05%)
Group 2	7.45	3	2	2	173	7.1	2	1	1	121	6.9	2	1	1	28
	± 0.21	± 0.00	± 0.00	± 0.00	± 46.10	± 0.00*	± 0.00***	± 0.00***	± 0.00***	± 39.81** (30.05%)	± 0.28*	± 0.00***	± 0.00***	± 0.00***	± 3.16* (83.81%)
Group 3	7.25	3	2	2	218	6.8	2	1	1	137	6.7	1.5	1	1	25
	± 0.21	± 0.00	± 0.00	± 0.00	± 29.56	± 0.14*	± 0.00**	± 0.00***	± 0.00***	± 16.42*** (37.15%)	± 0.21*	± 0.50*	± 0.00***	± 0.00***	± 4.24** (88.53%)

¹Values are presented as mean $\pm$ SD; each group consisted of 3 animals. Group 1 treatment = cefoperazone only; group 2 = ALA-NS F1; group 3 = ALA-NS F2. Values in parentheses represent percentage inhibition. Statistical analysis compared d 1 with other days using Student-Newman-Keuls multiple comparison test. CMT = California Mastitis Test; WST = Whiteside test; BBT = bromothymol blue test. Comparison with d 1: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Table 2. Effects of intramammary treatment with α -linolenic acid nanosuspension (ALA-NS), in 2 formulations (F1 and F2), on SCC after treatment¹

Item	d 1	d 5	d 10
Group 1	7.3 $\pm$ 0.3	6.4 $\pm$ 0.4*	3.3 $\pm$ 0.2*
Group 2	8.2 $\pm$ 0.4	7 $\pm$ 0.7*	3.9 $\pm$ 0.2*
Group 3	7.1 $\pm$ 0.3	5.9 $\pm$ 0.4*	2.8 $\pm$ 0.4*

¹Values are presented as mean $\pm$ SD, log SCC cells/mL; each group consisted of 3 animals. Group 1 treatment = cefoperazone only; group 2 = ALA-NS F1; group 3 = ALA-NS F2. Statistical analysis compared d 1 with other days using Student-Newman-Keuls multiple comparison test.

Comparison with d 1: * P < 0.001.

activity, with reduction of quality and quantity of dairy products (Forsbäck et al., 2010). Many reports suggest a positive correlation between SCC and CMT score (Kaithwas et al., 2011b; Guha and Guha, 2012). The CMT reagent reacts with leukocytes infiltrated into milk after bacterial infection and generates a thick gel. The intensity of gel formation is directly related to the number of leukocytes in milk. Thus, CMT results compare generally to SCC, with a higher CMT score pointing toward peculiarly increased SCC. In the current study, the SCC and CMT scores were consistent with previous studies and were normalized by treatment with ALA-NS (Kaithwas et al., 2011b). Treatment with ALA-NS F1 and F2 reduced the TMC in milk, suggesting a reduced bacterial load and indicating antimicrobial efficacy of the formulations. It is

important to note that ALA has been very well cited in literature as an anti-inflammatory, analgesic, and antipyretic by peripheral actions. In addition, ALA has also been reported to curtail vascular leakage and cellular infiltration, which could account for the improved udder condition of the treated animals. In addition, ALA has also been reported to have antibacterial properties particularly against mastitis-causing pathogens. All in all, NSAID-like and antimicrobial properties of ALA could be the key players for therapeutic management of mastitis.

Encouraged by the above, the authors considered it worthwhile to analyze the effects of ALA-NS F1 and F2 on milk quality and bacterial resistance using more stringent markers. Several bacteria (gram-positive and gram-negative) actuate the inflammatory reaction through the release of significant amounts of pro-inflammatory cytokines (TNF- α , IL). Pro-inflammatory cytokines actuate NF- κ B pathway in epithelial cells present at the site of infection. Several studies have correlated the role of NF κ Bp65 homodimers in bacterially mediated chronic inflammation. In the current study, higher expression of NF κ Bp65 was observed in the milk of mastitis-affected cows. This observation is in line with a previous report that suggested correlation between increased expressions of NF- κ B in milk cells and development of mastitis (Boulanger et al., 2003). Consequently, it may be supposed that elevated NF κ Bp65 activity detected in milk cells from mastitis-affected cows is due to either direct or indirect invasion

Figure 1. α -Linolenic acid nanosuspension, formulations 1 and 2 (ALA-NS F1 and F2), and cefoperazone altered protein expression of nuclear factor kappa-light-chain-enhancer of activated B cells (NF κ B-p65), sterol response element-binding protein (SREBP-1c), and ubiquitin carboxyl-terminal hydrolase isoenzyme (UCHL-1). Lane 1 = d 10 of treatment sample of cefoperazone treatment alone; lane 2 = d 1 milk sample; lanes 3 and 4 = d 10 treatment samples of ALA-NS F1 and F2, respectively. β -Actin was used as loading control. Each protein expression was performed in triplicate. All data are presented as mean $\pm$ SD and analyzed using one-way ANOVA, followed by Student-Newman-Keuls multiple comparison test. Comparison with d 1 (* P < 0.05, *** P < 0.001) was considered significant.

(or both) of these pro-inflammatory cells by pathogens. Treatment with ALA-NS decreased NF κ Bp65 expression more than did treatment with cefoperazone alone. Furthermore, evidence indicates that bacteria alter milk fat synthesis in cases of mastitis by altering the stearyl-CoA desaturase 1 (SCD1), an important enzyme for fat catabolism. Expression of SCD1 is stimulated under lipid synthesis by transcription factors, notably SREBP-1 (Xu et al., 2016). The SREBP-1 is synthesized and reserved as a membrane-associated precursor in the endoplasmic reticulum and stimulates gene transcription for lipid synthesis (Eberlé et al., 2004). In the current study, we perceived upregulated SREBP-1c expression in mastitic milk, suggesting increased lipid biogenesis through bacterial infection. However, treatment with ALA-NS curtailed SREBP-1c expression in comparison to treatment with cefoperazone alone. All in all, ALA-NS demonstrated a more favorable effect upon inflammatory (NF κ Bp65) and milk quality (SREBP-1c) markers than did cefoperazone-only treatment, which is in line with previous finding (Pyörälä, 2003).

Multidrug resistance (MDR) to antibiotics is an important concern in treatment of mastitis. A previous study showed that proteins such as ubiquitin carboxy-terminal hydrolase-L1 (UCHL-1) and P-glycoprotein (P-gp) play important roles in MDR (Wang et al., 2016). For instance, P-gp is one of the important proteins of the ATP-binding cassette transporter, which acts as a physiological hurdle by expelling antibiotics and is responsible for suboptimum antibiotic response (Sharom, 2011). Thus, P-gp inhibitors enhance the activity of antimicrobial agents such as azithromycin and erythromycin (Seral et al., 2003). A previous study has suggested that overexpression of UCHL-1 is responsible for augmented activity of P-gp in cells, which could be correlated with MDR (Jin et al., 2015). In the present study, we observed overexpression of UCHL-1 in the milk of mastitis-affected cows, and treatment with ALA-NS decreased UCHL-1 expression. Thus, the authors postulate that ALA-NS formulations have an additional advantage in combating the chances of MDR.

We conclude that ALA-NS can curtail subclinical mastitis, with several advantages over conventional treatments. Currently, treatment of subclinical mastitis is associated with combination therapies of anti-inflammatory and antimicrobial agents. Use of ALA offers both of these components, as ALA, being a peripheral analgesic, also reduces pain, providing additional benefit. The proposed ALA-NS formulations have the ability to counter MDR and immediately improve milk quality, which are usually drawbacks of conventional treatments.

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ORCIDS

Mohd Nazam Ansari <https://orcid.org/0000-0001-8580-3002>

Gaurav Kaithwas <https://orcid.org/0000-0003-1649-5434>