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Role of Trisodium Citrate and Nanominerals in Mastitis Management in Dairy Animals: A Review

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ABSTRACT

Mastitis is the most common disease of dairy cattle across the globe including India and cause huge economic losses to dairy farmers. Mastitis is also the most common reason for antibiotic usage in dairy animals, which is an important reason for antimicrobial resistance development in human and veterinary important pathogen. Optimization of nutrition and improvement of immunity of lactating cows are important strategies to reduce the susceptibility to mastitis and thereby reduction of antibiotic use. Copper, Zinc and Manganese are important trace minerals for maintaining udder health and immunity but, their contents and bioavailability is affected by several factors. Citrate, the precursor for milk synthesis is an important determinant of udder health but, the role of citrate in udder health is not clear as milk citrate level is affected by various factors. This review discusses about the role of trisodium citrate and various forms of trace minerals in mastitis management in dairy animals.

Key words: Bioavailability, Dairy animals, Mastitis, Nano minerals, Trisodium citrate.

Mastitis is the most common and costly disease of dairy cattle across the globe including India. Among the animal diseases affecting the profitability to Indian dairy farmers and industry, mastitis is considered to be one of the most expensive diseases next to foot and mouth disease (Bardhan, 2013) through reduced milk production, milk quality, treatment and culling cost. Varshney and Naresh (2004) reported that the incidence of subclinical mastitis (SCM) is more common in India (10-50%) than clinical mastitis (CM: 1-10%) in cows and an annual loss to Indian dairy industry due to mastitis is about 2.37 thousand crore rupees of which, SCM accounted for approximately 70% of the loss. Other reports also estimated more incidence and economic loss due to SCM (58-72%; 4150-4365 crores) than CM (28-42%; 1700-3000 crores per annum) in India (Dua, 2001; Bansal and Gupta, 2009). Rathod *et al.* (2017) reported loss of INR 21,677- 88,340 per lactation due to SCM. Singh *et al.* (2014) reported more losses due to mastitis in high yielding crossbred (INR 1,314) than indigenous cows (INR 868) and buffaloes (INR 1, 272) per lactation. The importance of SCM is due to its more prevalence, it remains as the source of infection for herd mates, its longer duration of infections, further proceeds into CM, its difficulty of detection and it reduces the milk production and its quality (Tuteja *et al*., 1993; Seegers *et al*., 2003). Besides, the mastitis is the top most reason for antibiotic usage, which is an important public health concern due to antimicrobial resistance development in pathogen (Pol and Reugg, 2007). In order to reduce the antibiotic use, the optimization of housing, nutrition and improvement of immunity of lactating cows are important strategy to reduce the susceptibility to mastitis (Werven, 2018).

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Several studies reported the importance of Copper (Cu), Zinc (Zn) and Manganese (Mn) for maintenance of udder health and immunity in dairy animals (Sordillo *et al*., 1997; Bruno, 2010). In general, when mineral status is declined in animal body, first system to be compromised is immune system, followed by growth and fertility, before appearance of overt clinical symptoms (Wikse *et al.*, 1992). Several studies reported that supplementation of trace minerals during peripartum period resulted in improved functionality of neutrophil with lesser frequency and duration of mastitis (Mutoni *et al*., 2012; Dang *et al*., 2013). But the micronutrients contents vary in feedstuff and water; their absorption and utilization are also affected by their source and patho-physiological status of animals (*e.g*., disease, rumen environment, mineral antagonism, *etc*.,) (Srinivas, 2012) and thus the current global recommendation of microminerals may not be sufficient (Socha *et al.*, 2006). In this situation, increased level- or alternative source- of supplementation are options to increase the bioavailability; of which the former concept may not be a suitable strategy as several minerals have lesser levels of safety margin. For example, Cu toxicities reported in Jersey cattle at dietary levels of 37 ppm, three times the NRC (2001) requirement (Olson *et al*., 1999). On the other hand, nano formulations of trace minerals can effectively fulfil their requirement at reduced doses due to their more bioavailability (Rajendran *et al*., 2013).

Beside pathogenic factors, interference with availability of precursors for milk synthesis (*e.g*., citrate) affects udder health, particularly during early lactation period. Citrate is the main constituent of the buffer system in healthy udder for maintenance of milk pH at 6.5 and regulation of the homeostasis between Ca^{2+} and H^{+} ions. Citrate is also essential for sequestration of soluble Ca^{2+} in milk and thereby maintenance of milk fluidity (Shennan and Peaker, 2000). On the other hand, mastitic milk contained significantly lesser amount of citrate (Dhillon and Singh, 2013). Hence, deficiency of citrate in the udder would lead to the clumping of Ca^{2+}, which manifests as "flakes" in the mastitic milk and these flakes may cause injury and subsequent inflammatory reactions to udder parenchyma including changes in milk-blood barrier permeability. Based on this concept, several researchers evaluated the effects of trisodium citrate (TSC) supplementation on mastitic animals and found encouraging results. Dhillon *et al*. (1995) administered TSC and found decrease in bacterial count along with raised milk citric acid level and restoration of normal milk pH in dairy animals. They also found that administration of TSC replenished citrate deficiency and restored milk constituents on acute or subacute mastitic cows (Singh *et al*., 1997). TSC alone or with minerals also been recommended for prevention of mastitis in dairy animals by National Dairy Development Board (NDDB) (NDDB Annual Report, 2018). Under this program, they reported that feeding of 10 g of TSC for 10 days in feed or water for California Mastitis Test (CMT) positive animals resulted in reduction of pooled milk CMT positivity from 55% to 20% after seven rounds of TSC supplementation and testing. The reduction of CMT positivity of pooled milk sample was from 60% to 25% and 49% to 11% in cattle and buffalo, respectively (Kumar, 2016). On the other hand, several researchers reported that milk citrate level varies with stage of lactation, milk composition, season and diet and mammary epithelium is impermeable to citrate in both directions (Linzell *et al*., 1976; Holt and Muir, 1979; Braunschweig and Puhan, 1999). Therefore, it warrants further studies to understand the role of citrate in lactating dairy animals. In this review, we discussed about the role of TSC and various forms of trace minerals in mastitis management in dairy animals.

Effect of trisodium citrate on mastitis affected dairy animals

Citrate is an important constituent of milk buffer system and its fluidity. Citrate, an important intermediate molecule in tricarboxylic acid (TCA) cycle, is negatively correlated with ketone bodies level in milk and thus it can be an indicator of energy status in cows (Baticz *et al*., 2002). Milk citrate level varies with lactation stage, level of milk constituents (*e.g*., fatty acid levels), season and diet (Braunschweig and Puhan, 1999). Baticz *et al*. (2002) reported drop in citrate level during the metabolically critical period of first four weeks of lactation. In contrast, some researchers reported that variation in milk citrate with lactation stage is related to de novo synthesis of fatty acids and it is independent of diet and milk yield (Garnsworthy *et al*., 2006). Altogether, it indicated that further studies are required to understand the factors influencing the milk citrate level in lactating dairy animals.

Among the three immune responses of udder during mastitis, such as i) recruitment of PMN cells, ii) altered permeability of blood-milk barrier and iii) impaired synthetic and secretary activity of alveolar epithelial cells, one or more mechanism affects the milk composition. Normally, milk constituents like citrate, milk proteins, lactose, phosphate and calcium transportation across the mammary epithelium occurs *via* "exocytosis" process, while transportation of ions (Na^{+}, K^{+}, Cl^{-}), water and small molecules like glucose, amino acids occur *via* "trans membrane" pathways. Paracellular pathway allows direct transfer of interstitial fluid and serum components into milk via leaky tight junctions and it is activated during different physiological (*e.g*., colostrogenesis time, during late lactation or involution period, higher dose of oxytocin-mediated contraction of myoepithelial cells, *etc*.) and pathological conditions like mastitis. All these pathways are generally affected by the functional state of the mammary gland and regulated by hormones and growth factors (Shennan and Peaker, 2000; McManaman and Neville, 2003). Mammary epithelium is impermeable to citrate in both directions and thus level of milk citrate concentration is an indicator of mammary gland activity rather than general metabolism (Linzell *et al*., 1976). On the other hand, mastitic milk contained significantly lesser amount of citrate (Dhillon and Singh, 2013). Milk citrate is synthesized from glucose and acetate in mammary gland. About 130-160 mg of citrate per 100 ml of milk of cows and goats normally occur with function of maintaining lactogenesis. The increase of citrate concentration by 46 times in cows and 10 times in goats during 1-2 days prepartum to 2-3 days postpartum period, indicated the onset of secretion of milk (Peaker and Linzell, 1975). On the other hand, mastitis affected animals had lesser milk citrate concentration of about 33 mg/100ml (Oshima and Fuse, 1981; Dhillon *et al*., 1995). Oshima and Fuse (1981) reported decreased citrate values in SCM and the extent of decrease was proportional to the degree of mastitis. The citrate content in milk is reduced by its transfer into blood via passive diffusion pathway through paracellular route (Faulkner and Peaker, 1982). Dhillon *et al*. (1995)

evaluated the effect of TSC (12 g/day for 7-10 days) on acute and sub-acute mastitis affected buffaloes and found significant reduction of bacterial colonies, pH and replenishment of milk citric acid. They also evaluated the effect of TSC (15 g for 6-8 days) on acute or sub-acute mastitis affected crossbred cows by CMT method and found improved milk citrate level, milk fat, milk protein and lactose content with simultaneous reduction of milk pH (Singh *et al*., 1997). Sarfaraz *et al*. (2009) reported that supplementation of TSC (30 mg/kg b.w. for 8 days) alone resulted 69% cure rate than TSC and levamisole combined treatment in SCM affected buffaloes. But, in term of bacteriological count, combined treatment group showed better responses (Yousaf *et al*., 2010). Some studies indicated that TSC was even superior to antibiotics in regards to the restoration of milk pH and other milk constituents in mastitis affected cows with increase of milk yield, fat, and SNF (Prakash *et al*., 2010). Sindhu *et al*. (2018) reported that milk pH and chloride levels were significantly reduced while milk citrate, lactose and calcium were improved after combined administration of TSC (30 mg/kg, five days) along with antibiotic (three to five days). Above clinical studies diagnosed the SCM and evaluated its treatment outcome often using CMT and pH strip methods, which are lesser sensitive methods. They also used various duration of TSC treatment and did not mention about role of patho-physiological factors on treatment outcome. Therefore, further studies are required to understand the therapeutic efficacy of TSC in SCM cows.

Reddy *et al*. (2017) observed that supplementation of coated TSC (20 g for 10 days, N: 48) in CMT positive cows, increased milk yield and fat content. Kumar (2016) reported increase of about one litre of milk per day and become CMT negative after TSC supplementation. Santoshi *et al*. (2018) evaluated combined supplementation of TSC (25 g) and Vitamin E (1000 IU/animal/day) in transition Sahiwal cows and found significantly higher body condition score (BCS) and dry matter intake (DMI) in treated cows. They also found more milk yield, milk fat and lactose with reduced milk SCC in supplemented cows. Stumpf *et al*. (2013) found no influence of sodium citrate (100 g/cow/day) and sodium bicarbonate ($NaHCO_3$) supplementation on the metabolic parameters, milk yield and its composition, milk SCC, milk quality, body weight and BCS of the cows. Mbonwanayo *et al*. (2016) reported no significant effects of TSC (30 mg/kg b.w. for 7 days) on daily milk yield, fat, and protein. But they found marginal decrease of milk SCC in TSC supplemented cows. Shaikh *et al*. (2019) observed that administration of TSC powder (30 mg/kg, BID for five days) was found to be more efficacious for prevention of SCM in cows maintained under conventional housing system than in loose housing system. Recently, we reported that supplementations of TSC alone cause no significant changes in milk yield and its composition, but reduced milk SCC and CMT score with more bacteriological cure rate in SCM affected cows (Sahu *et al.,* 2022).

Sodium citrate contains the sodium salt of citrate and it dissociates into sodium and citrate after its absorption. The citrate ions are further metabolized into bicarbonate ions, which act as blood buffering agent. Several studies investigated the effect of bicarbonate in dairy animals and found different results. For examples, Mckinnon *et al*. (1990) found no improvement in feed intake or milk production, fat percentage and rumen volatile fatty acid (VFA) production after bicarbonate supplementation. In contrast, Rogers *et al*. (1985) reported increase of milk yield, DMI and rumen VFA production in $NaHCO_3$ supplemented cows. Vicini *et al*. (1988) investigated the effect of bicarbonate supplementation along with *ad lib* or restricted ration and found increased DMI of cows fed ad libitum while restricted diet had lower milk yield, milk fat, protein and SNF. Feeding higher level of $NaHCO_3$ in diet not only increased DMI, milk yield, milk fat but also increased conception rate in early lactating Nili Ravi buffaloes during summer (Sarwar *et al*., 2007).

Effect of trace minerals on udder health of lactating dairy animals

Trace minerals such as Cu, Zn and Mn play an important role in maintaining the udder health and immunity of lactating dairy animals. Supplementation of Vitamin E, Se, Cu and Zn around peripartum period resulted in more rapid influx of neutrophil with its increased functionality in mammary gland and lesser duration of mastitis in Indian dairy cattle (Mutoni *et al*., 2012). Cortisol levels are also maintained at basal level in micronutrient supplemented animals (Dang *et al*., 2013). Supplementation of Vitamin E, Cu, Zn and combination of all these micronutrients significantly increased the phagocytic activity of neutrophils and lymphocytes proliferation during the peripartum period (Dang *et al*., 2013). Other studies, with Zn methionine supplementation showed reduced SCC (Kellogg, 1990). Whitaker *et al*. (1997) compared the effects of Zn (25 ppm) from organic and inorganic sources and found that source of Zn had no effect on intra-mammary infections (IMIs) and SCC. Cu supplementation during peripartum period (-60 to + 30 days) decreased the severity of *E. coli* induced mastitis and reduced the prevalence rate of IMIs due to major mastitis pathogens (Scaletti *et al*., 2003). Although, studies on the effect of Mn alone on immunological function are limited, increasing Mn status has been shown to enhance the killing ability of macrophages (Tomlinson *et al*., 2008). Since Cu, Zn and Mn are synergistically stimulating the superoxide dismutase activity and immune cell production, combined supplementation of these minerals is expected to improve the immune functions in dairy animals. For example, replacement of 30-33% inorganic Cu, Mn and Zn with organic form resulted in 34-45% reduction in milk SCC in dairy cows (Popovic, 2004).

Several studies conducted in Indian dairy animals revealed differential effects of micronutrients. Kumar *et al*. (2009) reported that Cu supplementation (5 g/ week for 45 days as Cu sulphate) in SCM affected buffaloes before calving had no impact on recovery from SCM after calving (based on CMT), while Zn supplementation during peripartum period had some beneficial impact. However, none of the animals in both the groups developed CM. Malhotra (2011) supplemented Cu (20 ppm) during peri-partum (-60 to + 60 days) period and found no significant effect on immunity, metabolic and endocrine profile of Karan Fries cows. Chandra *et al*. (2013) evaluated the effect of individual and combined supplementation of Vit. E (1,000 IU/cow/day) and Zn (60 ppm/ cow/day) during peri-partum (-60 to + 90 days) period and found better BCS, higher blood glucose and enhanced milk yield with lower non esterified fatty acid and lipid peroxidation level in combined group than the individually supplemented Sahiwal cows. Sharma *et al*. (2014) reported that nutritional supplementation ameliorated the SCM-induced metabolic, biochemical and milk compositional changes in cows. Similar to oral supplementations, injectable preparations were found to be differential effects. Warken *et al*. (2018) reported that subcutaneous mineral supplementation improved the immune response and minimized the oxidative stress in lactating dairy cows. In contrast, Ganda *et al*. (2016) reported that injectable trace minerals administration in cows with elevated SCC had no effect on milk yield, milk composition and milk SCC. Variations in beneficial effects of antioxidant minerals such as Cu, Zn and Mn supplementation in mastitic dairy cows could be due to supplementation levels, formulations, sources and subsequent differential bioavailability.

Bioavailability of trace minerals in dairy animals

Although well-balanced dairy diet contained high levels of trace minerals, it has no nutritional use, unless they are absorbed and utilized for numerous metabolic functions including immune response, growth, production and reproduction of dairy animals. Trace minerals deficiency is rarely identified as most of the time the deficiency causes subclinical infections rather than clinical manifestation at their earlier stage. Immunity and enzyme functions are first to be compromised during trace mineral status deficiency (Wikse *et al*., 1992) and thus, it is very critical to maintain the adequate level for optimal performance and health of dairy animals. Blood concentrations of micronutrients are more reliable indicator of mineral status of the cow than assessing dietary mineral status, as blood concentrations are significantly correlated with nutritional status of trace elements (Herdt and Hoff, 2011). They reported normal range for serum Cu as 0.6 to 1.1 µg/mL, Zn as 0.6 to 1.9 µg/mL and Mn as 0.9 to 6.0 ng/mL. However, several studies observed that plasma trace minerals concentrations and neutrophil functionality remain unchanged in additional supplemented group (Nemec *et al*., 2012; Dietz *et al*., 2017). Kincaid (1999) reported that liver concentration is more suitable than blood level of Cu, Zn and Mn in dairy animals.

The traditionally used inorganic mineral formulation in dairy animals has differential bioavailability due to their different solubility, molecular mass, electrical charge, pH properties and chemical reactive states that affect the absorption in the dairy cow. Besides, patho-physiological states of cows also influence the absorption. Srinivas (2012) reported variable content and bioavailability of cationic and anionic minerals (Table 1). NRC (2001) estimation also revealed absorption of 1 to 5% for Cu, 0.5 to 1% for Mn and 5 to 15% for Zn in mature cattle. With such low levels of actual absorption and variability of this mineral content in feedstuffs and rations, it is very difficult to feed the optimum requirement to dairy animals. The actual absorption is always low in oral feeding irrespective of sources due to rumen environment and other dietary antagonists. Species and breed also has some effect on trace mineral absorption (Blezinger, 2008). Kumar *et al*. (2017) also reported lesser absorption values of Mn, Cu, and Zn from maize germ oil cake in Indian dairy animals.

Absorption of Cu is much lower in ruminants than in non-ruminants, due to modifications that occur in the rumen environment. For instances, high dietary molybdenum with sulfur and iron results in thiomolybdates and copper sulfide formation in the rumen respectively, which greatly reduce Cu absorption and its metabolism. Dietary factors that affect bioavailability of Zn in ruminants are not well defined, though phytate does not seems to affect Zn absorption in ruminants because of microbial phytase enzyme mediated degradation of phytate in the rumen. Mn is very poorly absorbed in ruminants, and it could be due to high dietary calcium and phosphorus (Spears, 2003). Therefore, current dairy NRC, 2001 requirements for minerals such as Cu, Mn and Zn are may not be sufficient to meet the needs of the cow (Socha *et al*., 2006). They also studied the relationship between Mn intake and apparent Mn balance in dry and lactating dairy cows and found that feeding Mn above NRC requirement is necessary for achieving positive balance. Dose-dependent increase of Mn absorption suggested that a non-saturable paracellular absorption is likely occurring in cattle at higher Mn intake, as in broilers compared to transcellular pathway absorption during lesser amount of Mn (Goff, 2018). Altogether, it indicates that increased level of supplementation or alternative source of highly bio-available formulation is required to meet the demand of

Table 1: Contents and bioavailability of trace minerals.

Minerals	Standard	Content	Bioavailability	Requirement (mg/kg DMI)
Cu	$CuSO_4$	25%	1-28%	4-15
Mn	$MnSO_4$	32%	4-12%	20-25
Zn	$ZnSO_4$	35%	10-50%	10-16

lactating cows. In the later strategy, feeding of chelated organic mineral has been practiced to increase the bioavailability of trace minerals (e.g., Zn-methionine, Cu-lysine, and Mn-methionine). Osorio *et al*. (2016) reported that supplementation of Zn, Mn, and Cu as amino acid complexes produced the positive response in milk yield and milk protein level with increased neutrophils and lymphocytes phagocytosis, and antioxidant capacity in dairy cows. Hackbart *et al*. (2010) reported that supplementation of organic trace minerals (Zn, Mn, Cu, and Cobalt) increased milk yield in mid-lactation, but did not affect postpartum follicular dynamics, embryo quality, or liver trace mineral concentrations. In general, it is commonly assumed that organic source is believed to improve the relative bioavailability than their inorganic counterparts (NRC, 2001). Meta-analysis study also showed that organic trace mineral supplementation could improve production and reproduction performances in lactating dairy cows (Rabiee *et al*., 2010). Apart from sources and formulations, supplementation of nanominerals to increase their bioavailability is a recently growing strategy as nanominerals have lesser antagonistic effects with other compounds at intestines with improved digestive efficiency, immunity and performance in livestock and poultry (Gopi *et al*., 2017).

Effect of nanominerals (Cu, Zn and Mn) on udder health and performance of ruminant animals

Rajendran *et al*. (2013) evaluated the effects of nano ZnO supplementation in SCM affected lactating HF crossbred cows and found increased milk yield and decreased milk SCC than other conventional formulations. The serum concentration of Zn was also found to be significantly higher in nano Zn supplemented cows than other group of cows. The results indicated that the more bioavailability and thus speedy recovery from SCM among nano Zn compared to other forms. Supplementation of nano ZnO, *in vitro*, showed an improvement in the growth of ruminal microorganisms, ruminal microbial protein synthesis, and energy utilization efficiency in the early phase of incubation (Zhisheng, 2011). Cai *et al.,* (2021) reported that nano ZnO supplementation improved Zn bioavailability without affecting milk yield, health status, and mammary gland permeability in dairy cows. Nano Zn also increased blood Zn levels and immunity in Zn-deprived goats (Song *et al.,* 2021). Similarly, nano Cu increased blood Cu content, antioxidant capacity, daily gain and wool yield in Cu-deprived sheep and goats (Shen *et al.,* 2021; Min *et al.,* 2022; Zhao *et al.,* 2023). Singh *et al*. (2018) also reported that supplementation of nano ZnO significantly improved the plasma Zn levels in pre-ruminant lambs without causing toxicity. They suggested that nano ZnO formulation could be a better source of supplementation with reduced cost in future. In contrast, Zaboli *et al*. (2013) also reported either feed intake or average daily gain was not affected in kids supplemented with nano ZnO.

Abdollahi *et al*. (2020) found that nano ZnO increased the post-weaning feed intake, digestibility and blood Zn concentration compared to normal ZnO in calves. Chang *et al*. (2020) observed ZnO and Zn-methionine mediated reduction of diarrhoea incidence in dairy calves.They recommended ZnO supplementation during first 3 days of life and Zn-Met supplementation for the subsequent period. Combined supplementation of nano Cu and nano Zn improved young dairy calves' health by improving immunity and antioxidant status (Pandey *et al.,* 2022). They also found nano Cu supplementation at reduced levels, reduced oxidative stress through increased antioxidants levels and upregulation of antioxidant encoding genes and immune variables in Sahiwal heifers (Kushwaha *et al.,* 2022). Riazi *et al.* (2019) reported that the higher level of nano ZnO supplementation had no advantages on *in vitro* rumen fermentation. Several studies reported that oral supplementation of ZnO or nano ZnO had no significant effect on Zn blood level in sheep (Smith and Embling, 1993; Najafzadeh *et al*., 2013). Alijani *et al.* (2020) indicated that nano ZnO had advantages over inorganic ZnO in terms of increasing the Zn absorbability, rumen and blood antioxidant activity, blood IgG levels and decreasing blood urea nitrogen. Shafi *et al.,* (2020) reported better efficacy of nano Zn on reduction of milk SCC than inorganic Zn in lactating Does.

CONCLUSION

The available literatures revealed that the supplementation of trisodium citrate had differential effects and further studies with more specific indicators of treatment outcome like milk SCC, bacteriological cure rate, *etc*., are required to understand the effects of trisodium citrate on udder health. Supplementation of trace minerals as organic form showed more beneficial effects than inorganic forms. Available studies revealed better efficacy of nanominerals than inorganic minerals on their bioavailability and improving udder health in dairy animals.

Conflict of interest: None.

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