Evaluation of Ascorbic Acid Treatment in Clinical and Subclinical Mastitis of Indian Dairy Cows

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ABSTRACT : A study was carried out to assess the therapeutic effect of ascorbic acid in mastitis of dairy cows. The herd with a population of 250-275 lactating cows was screened for clinical and subclinical mastitis for a period of 5 months. Based on inclusion and exclusion criteria, eighteen animals each with clinical and subclinical mastitis in one quarter only were selected as study population. Twelve cows (group A) with normal udder and health were also selected as a healthy control. Clinical mastitis cows were grouped as B (n=12) and C (n=6). Cows of group B were treated with ascorbic acid at 25 mg/kg, subcutaneously for 5 consecutive days and intramammary infusion (Ampicillin sodium 75 mg and Cloxacillin sodium 200 mg/infusion) based on antibiotic sensitivity test, till complete recovery. Group C cows received only intramammary infusion till the complete recovery. Eighteen subclinical mastitis cows were divided in group D (n=12) and E (n=6). Cows of group D were treated with ascorbic acid at 25 mg/kg subcutaneously for 5 consecutive days while group E did not receive any treatment. California mastitis test (CMT), somatic cell count (SCC), physical changes of udder and milk were used to diagnose and classify the mastitis. Evaluation of the therapy was based on CMT score and physical changes of udder and milk. Sample size calculation was also performed but was not followed for control groups due to scarcity of cases. Adequate blinding was done when and where required to avoid the biases. Confounding variables like herd, age of the cow, stage of the lactation, season and geographical region were duly considered and adequate blocking was followed. Ascorbic acid was administered in clinical and subclinical cases even after cure considering its immunostimulatory and healing inducing effects. The recovery rate was faster in cases of clinical mastitis treated with ascorbic acid along with an intramammary infusion (group B) than the quarters of group C cows. Quarter wise the average duration/number (3.16±0.11 days) of antimicrobial intramammary infusion was significantly ($p<0.01$) less in group B than that of average duration/number (5.33±0.20 days) of group C. Subclinical mastitis cows treated with ascorbic acid showed 83.33% recovery while 16.77% did not respond to treatment till last day of study. Cows of group E (untreated) did not recovered from the mastitis. Subjective parameters viz. swelling, pain reflex of udder and physical changes in milk from quarter of ascorbic acid treated cows (group B) disappeared earlier than that of group C cows. It is concluded from this study that the ascorbic acid might be useful as an adjunct in case of clinical mastitis to get quick recovery with less number of intramammary infusions. High recovery rate in subclinical mastitis quarters of group D cows is appreciable and opens a new avenue to conduct further trials in a larger population in various field conditions. However, the pharmacology of ascorbic acid with particular reference to health of mammary gland needs to be investigated. ***(Asian-Aust. J. Anim. Sci. 2002. Vol 15, No. 6 : 905-911)***

Key Words : Ascorbic Acid, Clinical Mastitis, Dairy Cows, Subclinical Mastitis and Treatment

INTRODUCTION

Mastitis continues to be a problem of serious concern for dairy farmers as well as scientific community throughout the world. The annual incidence of the disease in Indian dairy cows is 44 and 6.8% for subclinical and clinical mastitis, respectively (Singh and Singh, 1994). Subclinical form of the disease causes 18 and clinical form causes 50% milk loss in dairy cows (Radostits et al., 1994). Multidisciplinary approaches have been undertaken in recent years to find out a suitable remedy. None of the available antibiotics have been proven to have more than 60 percent efficacy in field conditions against the major pathogens of mastitis (Radostits et al., 1994). Vitamin E and selenium feeding, as an antioxidant, in dairy cows have shown appreciable protective effects by reducing the incidence of mastitis (Aseltine, 1991). Decreased concentration of ascorbic acid has been recorded (Antila and Antila, 1979; Steffert, 1993) from mastitis milk of cows. The low plasma ascorbic acid content was found to be associated with high yielding cows and sickness of the animals (Haag and Hofman, 1987). The number of the leukocytes per ml blood was correlated positively with vitamin C content of plasma (Haag, 1985). Curca and Fotache (1985) found low ascorbic acid content in cattle liver affected with fascioliosis. Ascorbic acid along with cupric ions was found successful to prevent and treat the mastitis of dairy cows as teat dip or intramammary infusion (Upton, 1988). Intramammary infusion of antibiotics has been reported to suppress the various activities of infiltrated polymorphnuclear leukocytes (PMNL) in mastitis udder of dairy cows (Hoeben et al., 1997; Lintner and Eberhart, 1990).

Infiltration of large amount of neutrophils in mastitis udder has been suggested as the primary defense to eliminate the infectious agents from the site of infection

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(Radostits et al., 1994). To accomplish a host defense role, neutrophils must be able to perform and complete a series of sequential functions i.e. migration, adherence, phagocytosis and cytocidal actions. These cells require higher concentration of ascorbic acid than any other vitamins to perform these functions (Basu and Schorah, 1982). Besides immunomodulatory effects, ascorbic acid is also well known for its other properties viz. hydroxylation of lysine and proline to synthesize procollagen and collagen a healing inducing substance (Basu and Schorah, 1982; Zubay, 1993), potent antioxidant (Niki, 1991) and anti-inflammatory agent (Davis et al., 1990). Craven (1987) suggested that the immune mechanism of the udder could be stimulated to enhance spontaneous elimination of infection. The present study was carried out in lactating cows to evaluate the therapeutic role of ascorbic acid alone in subclinical mastitis, and to assess its therapeutic advantage (if any) of its incorporation in conventional treatment of clinical mastitis with sensitive antimicrobial agents.

MATERIALS AND METHODS

Animals

The herd of lactating cows of dairy farm of Livestock Production Research Section of Indian Veterinary Research Institute was located as source of study animals with a variable population of 250-275. None of the special/recommended control programme for mastitis (Radostits et al., 1994) was practiced during the period of study. Cleaning and washing of milking byre and washing of udder with tape water before each milking were performed regularly. Hand milking was performed twice daily and there was no machine milking. Animals were fed on balanced ration prepared at the Feed Technology Division of the Institute. There was no additional feeding of antioxidant such as vitamin E and selenium. Teat dipping was not practiced regularly. Other routine health programme like deworming and vaccination (other than mastitis) were practiced regularly.

Selection of study animals

The prevalence of mastitis was considerably high in the Dairy farm and in a 5 months period 70 cases of mastitis (subclinical=40, clinical=30) were diagnosed. From 70 mastitis cows eighteen cows each with subclinical and clinical mastitis, and twelve cows separately with normal udder and health were selected as study animals based on inclusion (Age - 4 to 7 years, Lactation - 2nd to 5th, Stage of lactation- 15th day to 4th month, Milk yield - >10 lit/high yielder, Quarter affected-only one, Breed-crossbred of Jersey/Holstein Friesian with indigenous breed, Clinical signs of mastitis-only for clinical mastitis) and exclusion (Quarter - >1, First 15 days or later stage of lactation, History of prior infection in any quarter or any major health problem in present lactation, Presence of skin lesions on teat, Chronic or Peracute form of mastitis) criteria. Important blocking factors like herd, stage of lactation, season and geographical region were duly considered. The study was conducted in one herd during late summer and rainy season.

Chemicals

L-ascorbic acid and intramammary infusion, containing ampicillin as ampicillin sodium, I.P. 75 mg and cloxacillin as cloxacillin sodium I.P. 200 mg (Tilox) per infusion were procured from Spectrochem Pvt. Ltd., Mumbai, India and Wockherdt Pvt. Ltd., respectively.

Experimental design

Table 1 shows the protocol for therapeutic evaluation of ascorbic acid in subclinical and clinical mastitis. Twelve healthy animals with normal udder and milk served as healthy control (group A). Thirty six mastitis (clinical=18,

Table 1. Therapeutic protocol to evaluate ameliorative efficacy of ascorbic acid in clinical and subclinical mastitis in dairy cows

Group	Type of animals	No. of animals	Treatment schedule		
			Drug	Dose	Duration
A	Healthy (Non-mastitic)	12	-	-	-
B	Clinical mastitic (Treated)	12	Ascorbic acid+ Ampicillin	25 mg/kg by S/C route	5 consecutive days 3-5 days*
			Cloxacillin	75 mg I/mam 200 mg I/mam	3-5 days*
C	Clinical mastitic (Control)	6	Ampicillin	75 mg I/mam	3-5 days*
			Cloxacillin	200 mg I/mam	3-5 days*
D	Subclinical mastitic (Treated)	12	Ascorbic acid	25 mg/kg by S/C route	5 consecutive days
E	Subclinical mastitic (Control)	6	-	-	-

(The evaluation of therapy was based on CMT score and physical examination of udder and milk in clinical and subclinical mastitis).
* Ampicillin and Cloxacillin was given in a combination (Commercial preparation “Tilox” Wockherdt, India Ltd) Based on CMT score till complete recovery or 5 days whichever is less but not less than 3 days.

subclincal=18) cows were divided into group B (n=12, clinical mastitis), group C (n=6, clinical mastitis), group D (n=12, subclinical mastitis) and group E (n=6, subclinical mastitis). The group C and E were served as control for the respective treatment group. The sample size of treatment groups (B and D) was within the range (Schukken and Deluyker, 1995). However the sample size for control groups (C and E) could not be followed and was cut off to half of the treatment groups due to the scarcity of the cases.

A simple randomization (Schukken and Deluyker, 1995) strategy was followed to treat the animals of group B, C and D. Animals of group B received Ampicillin sodium I.P. 75 mg and cloxacillin sodium I.P. 200 mg with suitable base as intramammary infusion (Tilox, Wockherdt, India Ltd.) and ascorbic acid at a daily dose of 25 mg/kg subcutaneously for 5 consecutive days at morning hours. The animals of group C received only intramammary infusion as in case of group B. None of the animals under study with clinical mastitis were left untreated on ethical grounds. Twelve animals of group D with subclinical mastitis were given ascorbic acid at 25 mg/kg, subcutaneously for 5 consecutive days. Six cows with subclinical mastitis of group E were left untreated. The dose of ascorbic acid was decided on the bases of report of Roth and Kaeberle (1985).

The cows were treated at the time of morning milking (05 to 08 h). Adequate blinding during treatment, evaluation and statistical analysis was followed.

Screening for mastitis and evaluation of treatment

Diagnosis and classification of mastitis were based on physical examination of the udder; physical and chemical changes in the milk, California Mastitis Test (CMT) score and somatic cell count (SCC) in milk of affected quarter. Subclinical mastitis was diagnosed on the basis of CMT score and SCC. The CMT is a highly suitable indirect test, cheaper, quicker than cell count and as accurate as a method of diagnosis (Radostits et al., 1994). Teepol (Merck Ltd.) 10 ml, Bromocresol (1:100) (RDH, India) 1 ml and Distilled water up to 100 ml were mixed together to prepare the CMT reagent. The CMT was carried out at cow side at the time of milking by mixing 3 ml of fresh milk with equal volume of CMT reagent. The milk and CMT reagent were mixed together by swirling the paddle or by stirring with a glass rod and the results were read after 10-15 seconds. The CMT reaction was standardized as 0 (No change in milk consistency), + (Gel is formed), ++ (Gel becomes thick and lumpy) and +++ (Gel adheres to the bottom of the paddle) by performing on various quarters. Milk samples showing 3 + CMT reactions with SSC (>2,700,000 cells per ml) but no visible abnormality of milk or udder, and milk samples with SSC≥2,700,000 cells per ml with visible abnormality of milk or udder were classified as subclinical and clinical mastitis, respectively (Radostits et al., 1994). Standard procedures (Coles, 1980) were followed to collect the milk for SCC and CMT. For SCC, 0.01 ml (10 µl) of milk was spread in 1 cm^2 area of a clean grease free slide. Smear was fixed and stained with Newman-Lampert stain within an hour of collection of samples. Fifty oil-immersion fields were examined from each smear. The final result was calculated by multiplying the cells per field (average) by working microscopic factor to get the number of leukocytes per ml of milk (Coles, 1980). SCC was used only to diagnose the subclinical mastitis not for evaluation while CMT was used for diagnosis and evaluation both. The CMT reaction was recorded on days 0, 3, 4, 5, 6 and 15 of the study.

Some of the important subjective parameters suggested by Schukken and Deluyker (1995), pertaining to udder health were also observed to diagnose and evaluate the treatment of clinical mastitis. The subjective parameters included in this study were swelling of affected quarter, pain reflection on pressure of quarter and physical changes in milk from clinically infected quarters. An adequate blinding was followed strictly to avoid diagnostic and evaluation bias during the study. The changes in all these three parameters were recorded as + - mild change, ++ - moderate change, +++ - severe change. Observing on other quarters before the start of actual experiment standardized these parameters. Subjective parameters were recorded from day '0' to day 6 of the study only.

The quarter was considered normal when CMT reaction returns to 0 along with no swelling, pain reflex and normal physical appearance of milk. In this study the number of quarters and cows in each group were equal. So there is no difference in quarter wise or cow wise evaluation.

Statistical Analysis

The treatment duration of intramammary infusion was analysed for significance with the help of paired T test for unequal sample size (Snedecor and Cocharan, 1967).

RESULTS

Clinical mastitis

CMT reaction on various days and treatment duration are shown in table 2. Quarter of animals of group B (n=12) with clinical mastitis received ascorbic acid and antimicrobial infusion showed faster recovery and required less number of intramammary infusion than the quarter of animals of group C. The effect of ascorbic acid is highly apparent as on 3 d of treatment, the milk from the quarters of group B cows showed mild (+) while quarters of group C cows showed sever (+++) reaction to CMT. Meanwhile the CMT reaction on 4 d was mild (+) in 2 quarters and was nil on 5 d, 6 d and 15 d in all the quarters of group B cows. In cows of group C the CMT reaction was severe (+++),

Table 2. CMT score on various days and duration of ascorbic acid and antibacterial intramammary treatment in mastitic cows

Group	Type of quarter	CMT score (days post treatment) in out of 12 quarters						Treatment duration (days)	
		0	3	4	5	6	15	I/mam infusion*	Ascorbic acid (25 mg/kg) s.c. in neck region
A (n=12)	Healthy -	-	-	-	-	-		-	-
B (n=12)	Clinical	12 (+++)	12 (+)	2 (+)	0 -	0 -	0 -	3.16±0.11**	5 consecutive days
C (n=6)	Clinical	6 (+++)	6 (+++)	6 (++)	6 (+)	1 (+)	- -	5.33±0.20	-
D (n=12)	Subclinical	12 (+++)	12 (+++)	4 (+)	2 (+)	2 (+)	2 (+)	-	5 consecutive days
E (n=6)	Subclinical	6 (+++)	6 (+++)	6 (+++)	6 (+++)	6 (+++)	6 (+++)	-	-

- Nil

n=Number of quarters.

* Combination of Ampicillin 75 mg and Cloxacillin 200 mg (Tilox, Wockherdt India Ltd.); Infusion was discontinued when nil CMT reaction was observed from the quarter.

** Significant at $p<0.01$.

CMT score is shown with in parenthesis.

moderate (++) and trace (+) in milk from the quarters of all six cows on days 3, 4 and 5, respectively. One quarter of group C cows showed trace CMT reaction while others did not react on 6 d. No CMT reaction was seen on day 15. The ascorbic acid treatment was not stopped even after getting cure and showing nil reaction to CMT, due to the healing inducing and immuno-enhancing properties of ascorbic acid and was given for 5 consecutive days to group B cows. Quarter wise, the average duration or number of antimicrobial intramammary infusion (3.16±0.11) was significantly ($p<0.01$) less in group B than average duration or number of antimicrobial intramammary infusion (5.33±0.20) of group C.

As per table 3 the cure rate was higher in group B than group C. In cows of group B the quarter wise cure rate was 83.33% (10/12) on 4 d and 100% (12/12) on 5 d, 6 d and 15 d of the study. However, it was 0% (0/6) till 5 d and was reached to 83.33% (5/6) and 100% (6/6) on 6 d and 15 d, respectively.

Subjective parameters like swelling of udder, pain reflex on palpation/pressure of udder and physical changes in milk from clinically infected quarters of group B and C were also recorded to assess the treatment (table 4). Swelling of udder was moderate on 2 d and turned to mild on day 3 and disappeared completely on 5 d in all the quarters of group B cows. However, it was severe (+++) till the 3 d and turned to moderate and mild on days 4 and 5 respectively in quarter of group C animals. All six quarters were in normal shape on 6 d of the study in group C. The pain reflex was measured by pressing the udder with the help of fingers. In quarters of group B, reflex to pain was severe (+++) on day 0, 1 and 2. However, it was moderate (++), mild (+), and nil on days 3, 4 and 5, respectively. Severe pain reflex was recorded from all quarters of group C cows till 3 d and became moderate, mild and nil on 4, 5 and 6 d respectively. This finding indicated that ascorbic acid was of little use to reduce the pain reflex in mastitis quarters. Physical changes of milk were mild on 3 d and disappeared in milk of 10 quarters on 4 d and milk from all quarters was apparently normal on 5 d onwards in group B. However, moderate physical changes in milk from quarters of group C cows were extended from 3 d to 5 d and all quarters were normal on 6 d in group C.

Subclinical mastitis

The milk CMT reaction and cure rate of various quarters with subclinical mastitis is recorded in table 2 and 3 respectively. The duration of ascorbic acid treatment was for 5 consecutive days, which was completed even after getting cure. It is evident that ascorbic acid treatment, brought a surprising change in CMT reaction on 4 d, as all the 12 quarters from 12 cows showed + reaction to CMT. Eight (66.66%) and 10 (83.33%) of 12 quarters recovered completely from subclinical mastitis on days 5 and 6, and did not show any reaction to CMT till last day (15 d) of the study. Two (16.66%) of 12 quarters showed mild (+) CMT reaction till the last day (15 d) and could not be cured in present study. Six untreated subclinical mastitis quarters of group C were showed severe (+++) reaction to CMT throughout the period of study.

DISCUSSION

Average number of intramammary infusion reduced

Table 3. Cure rate in subclinical and clinical mastitis with ascorbic acid alone and along with antimicrobial treatment

Group	Type of animals	n	Number of cured quarters on days					Total number of cured quarters
			3	4	5	6	15	
B	Clinical	12	0 (0%)	10 (83.33%)	12 (100%)	12 (100%)	12 (100%)	12 (100%)
C	Clinical	6	0 (0%)	0 (0%)	0 (0%)	5 (83.33%)	6 (100%)	6 (100%)
D	Subclinical	12	0 (0%)	0 (0%)	8 (66.66%)	10 (83.33%)	10 (83.33%)	10 (83.33%)
E	Subclinical	6	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)

n=Total number of affected quarters.
Figures with in parenthesis show percent cure rate.

Table 4. Udder health status before, during and after treatment with antimicrobial intramammary infusion alone and along with ascorbic acid in clinical mastitis of cows

	Days post treatment						
	0	1	2	3	4	5	6
Swelling							
Group B (n=12)	12	12	12	12	2	12	12
	+++	+++	++	+	+	-	-
Group C (n=6)	6	6	6	6	6	5	6
	+++	+++	+++	+++	++	+	-
Pain reflex							
Group B (n=12)	12	12	12	10	2	12	12
	+++	+++	+++	++	+	-	-
Group C (n=6)	6	6	6	6	6	2	6
	+++	+++	+++	+++	++	+	-
Physical changes in milk							
Group B (n=12)	12	12	12	12	2	12	12
	+++	+++	+++	+	+	-	-
Group C (n=6)	6	6	6	6	6	2	6
	+++	+++	+++	++	++	+	-

Group B-Antimicrobial intramammary infusion+Ascorbic acid 25 mg/kg, sc.
Group C-Antimicrobial intramammary infusion.
- Normal or no change.
+ - Mild change; ++ - Moderate change; +++ - Severe change.
Note: Adequate blinding was done at all the required levels.

significantly in group B received ascorbic acid in comparison to group C not received ascorbic acid. It could be due to the established properties of ascorbic acid. Animals including ruminants have the ability to synthesise vitamin C from D-glucose or D-galactose through the glucuronic acid pathway (Basu and Schorah, 1982). High yielder dairy animals are most prone to hypoglycemia either during advance stages of pregnancy or at the peak of lactation (Radostits et al., 1994). As per the authors knowledge no study has been reported on the relationship between glucose and ascorbic acid in general and ascorbic acid and diseases in dairy cows in particular.

Steffert (1993) and, Antila and Antila (1979) reported decreased ascorbic acid concentration in milk of mastitis dairy cows. The dairy animals with mastitis of this study could have also developed the deficiency of vitamin C and its administration could be helpful to bring faster recovery in group B and D cows.

The pathogenesis of mastitis starts from an injury/damage to glandular tissue followed by invasion, colonization and infection. Infection results in further damage of mammary tissue, which should heal quickly by an effective therapy. Healing can take place only when sufficient amount of collagen is being synthesized by system. There is considerable evidence that reduced form of ascorbate is essential for proline and lysine 2-oxoglutarate dioxygenases enzymes reaction, responsible for hydroxylation of proline and lysine amino acid. The hydroxylation of these enzymes results into procollagen and finally into collagen fiber. In our study this property of

ascorbic acid could be of great value to bring the faster recovery and high cure rate with less number of intramammary infusion in group B and alone in group D.

Findings of this study might also be supported by the findings of other workers on the immunomodulatory aspect of ascorbate in animals. The cells responsible for immunological response contain 40-60 times higher ascorbic acid concentration found in the plasma. Acute disease, infection and trauma rapidly deplete these high concentrations within the leukocytes (Basu and Schorah, 1982). Antimicrobial intramammary infusion in bovine mastitis suppresses the functions of PMN Leukocytes of mammary gland (Hoeben et al., 1997; Pappe et al., 1991; Lintner and Eberhart, 1990; Nickerson et al., 1985). Meanwhile, Roth and Kaeberle (1985) reported interesting findings regarding ascorbic acid in bovine. Ascorbic acid at 20 mg/kg by s/c route in cattle resulted in enhancement of neutrophil oxidative metabolism and their capability to mediate antibody dependent cell mediated cytotoxicity. It was also in their findings that ascorbic acid administration augmented the *Staphylococcus aureus* ingestion by bovine neutrophils. Functionally suppressed neutrophils by dexamethasone treatment in cattle were reversed to normal functions after ascorbic acid treatment. In same line, other workers also suggested the immunostimulatory effects of ascorbic acid in animals (Thomas and Holt, 1978; Lewin, 1975; Cummins et al., 1992; Hidiroglou et al., 1995; Kobiesy and El-Ali, 1994).

Johnston and Lehmeyer (1977) observed that SOD is intracellular superoxide escaping from the neutrophil could damage surrounding tissue and cause inflammatory disease. Vitamin, unlike the enzyme, is not limited to the cell and could serve as a protective agent to surrounding tissue. After disclosing the importance of neutrophils and adverse effects of antibiotics on them and immunostimulatory and healing inducing effects of ascorbic acid, it has become further easy to accept that recovery in clinical and subclinical mastitis could be attributed to the immunostimulatory and healing inducing effects of ascorbic acid administration in cows of group B and D.

There are some other reports that further justify the administration of ascorbic acid as immunostimulator and the faster recovery. Antibiotics are less effective after the development of clinical signs as the multiplication of bacteria slowed down considerably (Craven and Anderson, 1982). In such situations immunomodulation likely to be provided by ascorbic acid, could be responsible for quick recovery in quarters of group B cows. Craven (1987) reviewed the interaction of antibiotics, bacteria and various components of immune system.

Recovery rate shown by subclinical mastitis quarters (group D) is highly appreciable and could be attributed to the immunostimulatory effects of ascorbic acid on mammary neutrophils. It is also assumed that ascorbic acid could be of great help to enhance the healing by enhancing the collagen synthesis that could have not permitted the bacteria to adhere and colonized in the mammary gland. However, in case of subclinical mastitis the self-recovery rate is high and near about 60% has been reported in dairy cows. Stimulated udder defense mechanism resulted in spontaneous recovery of udder infections (Hill et al., 1978).

We concluded from this study that the ascorbic acid might be useful as an adjunct in case of clinical mastitis to get quick recovery with less number of intramammary infusions. High recovery rate in subclinical mastitis quarters of group D cows is appreciable and opens a new avenue to conduct further trials in a larger population in various field conditions. However, the pharmacology of ascorbic acid with particular reference to health of mammary gland of ruminants should be investigated in detail.

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