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RESEARCH ARTICLE

Dynamics of milk leukocytes in response to the polysaccharide fraction of Tinospora cordifolia in bovine sub clinical mastitis

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Abstract

Somatic cell count (SCC), phagocytic activity and percent neutrophil and lymphocyte count in milk were evaluated after intramammary infusion of polysaccharide fraction of *T cordifolia* (PFTC) in bovine sub clinical mastitis (SCM). Intramammary infusion of PFTC treatment significantly reduced the SCC and neutrophil count, conversely to the above the phagocytic activity of milk polymorphonuclear cells (PMNs) and lymphocyte count enhanced ($P<0.05$). The results suggest that the PFTC possesses antiinflammatory and immunomodulatory properties. In the present study the biological activity of the PFTC at standardized dose against bovine sub clinical mastitis is reported for the first time.

INTRODUCTION

Mastitis is the most costly disease in dairy cattle usually caused by bacterial infections and eventually damages the udder tissues (Mukherjee *et al.,* 2010). Neutrophils and lymphocytes play important role in the defense against mastitis in dairy cows, however poor functional activities of these cells has been observed with mastitis (Cai *et al.,* 1994) furthermore antibiotic therapy reduces the anti bacterial capacity of these immune cells (Hoeben *et al* ., 1997). Strategies aimed at improving the immune cells of the diseased udder during immunosuppressive stages would greatly impact the ability of the animal to resist the pathogenic infection.

Medicinal plants are used since dawn of civilization to combat diseases of man and animals. The herb *Tinospora cordifolia* (*T. cordifolia,* Menispermaceae) grows abundantly in India. It is a large glabrous climber, different parts of the herb is extensively used in the Indian System of Medicine. It is known for its immunomodulatory (Paharia and Sharma, 2003) antioxidant (Kapil And Sharma, 1997; Subramanian *et al.,* 2002) and antibacterial properties (Upadhayay *et al.,* 2011).

Development of such therapy will be of great help in such dairy farming system where the use of antibiotics are not desirable and also to those farmers who cannot afford conventional means of therapy.

Material and methods

Collection and extraction of polysaccharide fraction of Tinospora cordifolia (T.cordifolia) (PFTC)

T. cordifolia was procured from the institute campus and identified at the Department of Botany, Ruhelkhand University, Bareilly, India. The stems were cut into small pieces washed, shade dried and pulverized by a mechanical grinder, passed through mesh sieve. The powdered content was weighed and used

for the isolation of polysaccharide as per the method described by (Chintalwar *et al.,* 1999; Desai *et al.,* 2002). The polysaccharide fraction was tested for the presence of phytosteroides, tannins, flavonoides, saponins, alkaloide, glycoside, triterpenoides, carbohydrate and proteins following the standard procedures (Khandelwal, 2005).

Selection of cows and experimental protocol

Twenty four lactating cows were selected from an organized dairy farm (Cattle and Buffalo), Indian Veterinary Research Institute, Izatnagar. These cows were maintained in the animal shed of the institute under identical environmental conditions and were divided in 4 equal groups having 6 animals in each group. Cows of Group I clinically healthy cows, negative for California Mastitis Test (CMT) and Somatic Cell Count (SCC) less than 0.5 million cells/ml of milk sample and udder secretions were negative for pathogenic isolates served as control (group I). Eighteen cows in Group II, III and IV positive for sub clinical mastitis (SCM), screened on the basis of CMT positive reaction, somatic cell count more than 0.5 million cells/ ml of milk and positive for intramammary infection (IMI) were taken for the drug trial. Two hundred and fifty mg of sterile PFTC was infused per teat with a sterile antibiotic dispensing canula, in Group II cows, after diluting the drug in 10 ml sterile phosphate buffer saline, once a day for 5 days, similarly Enrofloxacin was infused by intramammary route in Group III cows for 5 days or clinical recovery, cows of group IV were kept as infected control and not given any treatment. Observations were made up to 30 days after initiation of treatment. The milk was discarded for 7 days in all the treatment groups except the healthy control group.

Collection of milk samples

Fifty ml of milk from each cow was collected in sterile tubes after cleaning the teat orifice with 70% ethyl alcohol and after discarding few streams of milk. The milk was collected on day 0, 7, 15 and 30 of the study period.

The SCC of the milk samples was done as per standard method (Schalm *et al.*, 1971). The identification of causative organism in collected milk samples was carried by spreading 10 µl of milk over 5% bovine blood agar plate, further the growth of the organism on selective media. The organisms were identified on the basis of colony morphology, characteristic hemolytic pattern and Gram's staining and further processed for biochemical tests (Balows *et al.*, 1991). SCC was done on day '0' and thereafter on day 7, 15 and day 30. Similar observations were also taken from the normal healthy cows.

Isolation of polymorphonuclear cells (PMNs) from milk samples

The isolation of PMNs from the milk samples was carried out as per the standard method (Daley *et al.*, 1991). Viability of the cell was checked by trypan blue (Sisco Research Lab, Mumbai, India) exclusion technique.

Assay of Phagocytosis

For candidacidal assay, the cell pellet formed at the bottom were washed with Hanks balanced salt solution (HBSS, pH 7.2) and resuspended in HBSS and final cell concentration was adjusted to 5×10^6 cells/ ml.

Candidacidal assay

Candidacidal assay was performed as per the standard method (BOYNE and ARTHUR, 1979). A suspension containing 0.2 ml milk leukocytes (10^6 cells), 0.2 ml *C. Albicans* ($2x10^6$ cells), 0.2 ml HBSS and 0.15ml fresh autologous pooled bovine serum was incubated for 65 minutes at 37°C with intermittent shaking. Then 0.25 ml methylene blue (2×10^{-4} mol) was added and further incubated for another 10 minutes. A sample of the final incubation mixture was placed in the haemocytomete. The phagocytic activity was calculated by counting the number containing ingested *C. albicans* (alive or dead), studied on day 0 and on day 7.

Differential Leukocyte Count (DLC) in milk

Differential leukocyte count in milk was done by the method as described by DULIN et al. (1982**).** Neutrophil and lymphocyte count was studied on day 0 and on day 7of study period.

Statistical Analysis

The data were analyzed applying One-way analysis of variance (ANOVA) to determine the level of significance between the groups, and Duncan's Multiple Range Test (DMRT) was applied to determine the level of significance within the group at different time interval by using statistical software package (SPSS Version 10.1 ,South Asia, Bangalore, India).

Results

Qualitative analysis of the extract

The qualitative analysis of plant extract revealed the presence of carbohydrate in the polysaccharide fraction.

SCC in response to therapy

The SCC of normal milk ranged from 2.284 ± 0.274 to 2.730 ± 0.232 x 10^5 cells/ml of milk in the collected milk samples from 5 healthy cows of group I. The SCC in group II and group III reduced non-significantly on day 7, however the significant (P<0.05) reduction was observed in the SCC on day 15 and 30 of the treatment trial period with respect to 0 day value (Table 1)

The SCC in group IV cows positive for SCM ranged from 11.516±1.891 to 16.650±4.145 in the collected milk samples infected for SCM. No difference in SCC could be observed till day 30.

Phagocytic activity (PA) of milk PMNs

The average number of milk leukocytes showing PA ranged from 20.80±0.583 to 20.80± 0.374 percent in group I. The phagocytic activity was significantly (P<0.05) augmented in the cows of group II, treated with PFTC on day 7 of treatment trial period.

However the PA increased non-significantly in group III, treated with enrofloxacin. Whereas the average number of milk leukocytes showing phagocytosis ranged 10.40 ± 0.748 to 10.60±0.509 in group IV and no changes in PA activity was observed during the study period. (Table 2)

Percent milk Neutrophil and Lymphocyte count

The neutrophil percent ranged between 25.000±0.707 to 25.400±0.748 percent in group I healthy cows. The neutrophil percent significantly (P<0.05) reduced to 33.73% and 44.98% on day 7 and day 15 respectively in the cows of group II, treated with PFTC. . However in group III, treated with antibiotic, there was only non-significant reduction in the neutrophil percent on day 7 and 15 of treatment trial period. Whereas the neutrophil percent ranged between 52.400±01.077 to 53.400±1.122 percent in milk drawn from cows infected for SCM, no difference could be observed till day 15 in group IV (Table 3).

The lymphocyte percent ranged between 19.200±0.583 to 19.400±0.748 percent in milk drawn from healthy cows. The lymphocyte percent significantly (P<0.05) increased to 50.79% and 90.47% on day 7 and day 15 respectively in the cows of group II, treated with PFTC. In group III, treated with antibiotic, the lymphocyte percent was increased significantly (P<0.05) to 17.54% and 54.38% respectively on day 7 and 15 of treatment trial period compared to 0 day value. The lymphocyte percent ranged between 11.600±0.509 to 12.000±0.548 percent in cows infected for SCM, however no changes could be observed on days 0, 7 and 15 of the trial period in the cows of group IV (Table 4).

Table 1

Somatic Cell Count (SCC) (1x 10^5 cells / ml of milk) in response to the treatment with polysaccharide fraction of *T. cordifolia* (group II) and Enrofloxacin (group III) and SCC in normal healthy and positive control animals (group I, group IV) (mean ± SE)

Groups (N=5)	Day of treatment (AT)			
	Day 0	Day 7	Day 15	Day 30
Gr. I	2.53± 0.12^{a}	2.28±0.27^{a}	2.50±0.23^{a}	2.73±0.23^{a}
Gr. II	13.21±3.50^{bB}	9.20±2.73^{bAB}	3.84±1.03^{aA}	3.25±0.82^{aA}
Gr. III	11.44±1.31^{bB}	8.73±1.44^{bB}	4.34±0.49^{aA}	3.25±0.32^{aA}
Gr. IV	16.65±4.14^{b}	14.44±4.48^{c}	11.51±1.89^{b}	2.33±1.68^{b}

* Superscripts in each row (A, B) and each column (a, b, c) differ significantly (p<0.05).

Table 2

Phagocytic activity of milk polymorphonuclear cells in response to the treatment with polysaccharide fraction of *T. cordifolia* (group II) and Enrofloxacin (group III) and SCC in normal healthy and positive control animals (group I, group IV) (mean ± SE)

Groups (N=5)	**Phagocytic activity** (% of phagocytic leukocytes) Day of treatment	
	Day 0	Day 7
Gr. I	20.80±0.583$^{a, x}$	20.80±0.374^{a,x}
Gr. II	12.00±0.447^{a,y}	23.00±1.095^{b,x}
Gr. III	10.80±0.374^{a,y}	13.80±0.583^{a,y}
Gr. IV	10.20± 0.748$^{a,\ y}$	10.60±0.509^{a,z}

* Superscripts in each column (x,y,z) and each rows (a, b) differ significantly ($p<0.05$).

Table 3

Percent Neutrophil count in response to the treatment with polysaccharide fraction of *T. cordifolia* (group II) and Enrofloxacin (group III) and SCC in normal healthy and positive control animals (group I, group IV) (mean ± SE)

Groups (N=5)	Neutrophil (%) Days of treatment		
	day 0	day 7	day15
Gr. I	25.000±0.707^{a}	25.200±0.860^{a}	25.400±0.748^{a}
Gr. II	49.800±1.463^{bC}	33.000±0.707^{bB}	27.400±0.927$^{a\ bA}$
Gr. III	50.800±0.860^{b}	47.400±0.678^{c}	45.400±0.678^{c}
Gr. IV	52.600±1.122^{b}	52.400±1.077^{d}	53.400±1.122^{d}

* Superscripts in each row (A, B, C,) and each column (a, b, c) differ significantly ($p<0.05$)

Table 4

Percent Lymphocyte count in response to the treatment with polysaccharide fraction of *T. cordifolia* (group II) and Enrofloxacin (group III) and SCC in normal healthy and positive control animals (group I, group IV) (mean ± SE)

Groups	Lymphocyte (%)

(N=5)	Days of treatment		
	day 0 AT	day 7 AT	day 15 AT
Gr. I	19.200±0.583[b]	19.400±0.748[b]	19.200±0.734[b]
Gr. II	12.600±0.812[aA]	19.000±0.837[bB]	24.000±0.775[cC]
Gr. III	11.400±0.509[aA]	13.400±0.245[aB]	17.600±0.400[bC]
Gr. IV	11.600±0.509[a]	12.000±0.548[a]	11.600±0.509[a]

* Superscripts in each row (A, b, C) and each column (a, b, c) differ significantly ($p<0.05$).

Discussion

Appropriate elimination of pathogen from the infected site requires both the effectiveness of the drug and optimum functioning of the hosts' immune system. Neutrophil are the predominant cell type found in the mammary gland during inflammation and play vital role in udder defense mechanism (Sordillo *et al.*,1997). Leukocyte functions like diapedesis into the infected gland (Hill *et al.,* 1979) phagocytosis (Mehzard *et al.,* 2001) and respiratory burst activities are impaired in post partum lactating animals.

In the present study, the somatic cell count non significantly decreased on day 7, which means there was influx of leucocytes into the mammary gland indicated the chemoattractent property of the herb which could be due to the presence of bioactive substances like polysaccharide in the stem extract. After the initial enhancement of SCC, significant reduction in cell count (35.8%) was observed on day 15. The clinical recovery was observed from day 15. We recorded increased lymphocyte count to an extent of 50.79% and 90.47% on day 7 and day 15 and enhanced phagocytic activity of milk cells with PFTC treatment. Conversely to the above the neutrophil count reduced significantly ($P<0.05$). Clinical recovery and reduction in SCC indicates the clearance of bacterial load from the infected udder, which could be due to the enhanced leucosis and increased phagocytic activity of the milk PMNs. The enhanced phagocytic activity of the milk PMNs in the bovine mammary gland treated with the extract indicates the immunomodulatory properties of the herb. Upadhya and coworkers (2011) demonstrated antibacterial effect of *T. cordifolia* against pathogenic microbes like *Klebsiella pneumoniae, Escherichia coli, Micrococcus luteus, Streptococcus pneumoniae , Staphylococcus aureus, Bacillus cereus* and *Lactobacilus acidophilus.* Mukherjee and coworkers (2010) observed reduction in total bacterial count and increased phagocytic activity of milk PMNs with the treatment of *T. cordifolia* extract in bovine sub clinical mastitis. The chemical component of *Tinospora cordifolia* such as polysaccharide, glycosides, alkaloid and steroids are found to have anticomplimentary and immunostimulating activities (Kapil and Sharma, 1997). The immune competence of the phagocytic cells can be modulated by number of specific and non-specific mediators (Smith, 1994). Singh *et al.,* (2003) reported that *T. cordifolia* treatment resulted in significant leucocytosis and predominant neutrophilia in murine model, the authors also recorded an increase in the number and percent phagocytosis of *S. aureus* by peritoneal macrophages. Similarly, Nair *et al.,* (2006) observed activation of macrophages was with 1, 4-α-D-glucan treatment isolated from *T. cordifolia.* Siddalingappa *et al.,* (2012) studied the anti-inflammatory activities of aqueous extracts of *Tinospora cordifolia* in murine model, the author further explained that the analgesic and anti-inflammatory activities o0f the herb extract could be by the inhibition of the PG_3 synthesis.

Conclusion

The findings of our study indicate that upon intramammary infusion of PFTC of *T. cordifolia* stem shows reduction in SCC, neutrophil count and increased lymphocyte count and phagocytic activity. The polysaccharide fraction treatment indicate the anti-inflammatory and immunomodulatory potential of the treatment in bovine SCM.

T. cordifolia can be used as adjunct or alternative to antibiotic in order to reduce the antibiotic residue in food chain. Further work is continued in the laboratory on the chemical component responsible for the amelioration of sub clinical as well as its efficacy in clinical mastitis.

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