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Evaluation of the Effects Induced by Near Infrared Low Intensity Laser Radiation on Bovines Affected by Subacute Mastitis: A Single-Quarter Foremilk Sampling Study

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Abstract: Here we report the results of a 2-step study of the effects induced by near infrared low intensity laser radiation on bovines affected by subacute mastitis. In the first step, the correlation between Somatic Cells Count (SCC), Lactose and Chloride ion foremilk content in healthy and mastitic quarters of Italian Friesian cows, were studied and compared with the literature data. In the second step, the effects of the Near Infrared Low Intensity Laser Radiation (NIRLILR) on the cow affected by subacute mastitis, were evaluated in terms of 2 average quarter population parameters, the "average Somatic Cell Count" (SCC) and the "average Restoration Factors" (RF). It was revealed that the NIRLILR radiation treatment, in the experimental conditions adopted, caused an effective beneficial response of the cows against the mastitis.

Key words: Cow mastitis, somatic cell, lactose, chloride anion, near infrared low intensity laser radiation

INTRODUCTION

Mastitis constitutes a universal disease in dairy cattle. It is recognized as the most costly disease for the producers because of the limitations imposed by the high quality milk regulation adopted by most of the country and by the milk losses associated either to clinical (Haas *et al*., 2002; Hortet and Seegers, 1998; Houben *et al*., 1993; Rajala-Schultz *et al*., 1999) and subclinical mastitis (Fetrow *et al*., 1991; Hortet *et al*., 1999) or to mastitis treatments (Bennedsgaard *et al*., 2003; Hillerton, 1998). Decreased milk production account for about 70% of the total cost of mastitis and about 10-26 % of total milk loss occurs in quarters with subclinical Intramammary Infection (IMI). Antibiotic treatments account for about 85% of the cost in clinical mastitis. Such cost is associated to discarded milk and decreased production (Hurley *et al*., 2003).

Treatments, other than antibiotics, should have to be considered and tested. Actually, the most diffused alternative therapy, adopted in several conventional farms, make use of homoeopathic remedies (Anderson, 1997; Holmes *et al*., 2005).

Until now, there are no scientific evidences regarding the efficacy of such treatments (Holmes *et al*., 2005). Therefore, there is a need for non-chemical approaches to mastitis therapy.

In our study we explored an alternative, non-pharmacological, therapeutic agent: the near infrared low-level laser radiation.

Well-established mechanisms of action of the laser radiations have been described in the literature and several uses are being done in humane medicine (Tuner and Hode, 1999; Walker, 2002).

In veterinary medicine, low-level laser therapy has been used by the mid 1980's. The lasers used cover the power range from 20-1 W in the frequency range from the visible light to the infrared radiation. The most common wavelengths used are 650-660 nm in the visible spectrum and 808-830 in the near infrared spectrum.

Here we present the results of an individual quarter sampling study performed on a local Italian-Friesian dairy herd. The objective was to provide evidences on the efficacy of the 890-980 nm wavelength range laser treatment of bovine mastitis. For what being in our knowledge, such frequency range has never been applied in veterinary medicine as therapeutic agent against cow mastitis. In a previous work, a low-energy He-Ne-laser (630 nm) was used to irradiate the mammary glands (Stoffel *et al*., 1989). It was not observed any beneficial effects of such a treatment on the affected cows.

This study, in contrast, provides objective data on the effectiveness of the near infrared laser radiation mastitis treatment.

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MATERIALS AND METHODS

Cows: This study was performed in a local dairy, the Coretto farm, located in Rende next to Calabria University, in which a herd of 800 Italian Friesian cows were present.

California Mastitis Test (CMT): A small sample of milk (~2 mL) from each quarter was collected into a plastic paddle that has four shallow cups marked A, B, C and D. An equal amount of CMT reagent was added to the milk. In order to mix the content, the paddle was rotated and after a few seconds (~20 s) the score was read. The test was daily performed to support the data obtained by a precise somatic cells counting.

Somatic Cells Counting (SCC): Single-quarter foremilk samples were manually collected in 50 mL test tubes from each quarter cow. The somatic cell milk content was measured with the opto-fluoro-electronic counter FOSSOMATIC (Foss Electric, Hillerod, Denmark) in the laboratory of the Calabrian Milk Association (ASSOLAC). This instrument perform a specific, direct electronic count of the number of somatic cells in a milk sample preheated at 40°C and mixed with Ethidium bromide. It is able to count cells in suspension from a minimum of 50.000 cells/ml to a maximum of 10.000.000 cells mL^{-1}.

Lactose determination: Lactose concentration was determined, on each quarter sample, by infrared spectroscopy in accord with the published procedure, designated AOAC Official Method 972.16, that defines the following specific analytical wavelengths for the lactose C-OH groups: 9610 mm^{-1} and 1041 cm^{-1}. The determinations were made by a MilkoScan infrared spectrometer in the ASSOLAC laboratory.

Chloride anions concentration: Chloride ions concentration in the milk samples was determined by the standard Volhard analytical method (titration by $AgNO_3$) (Kolhtoff *et al*., 1989) on the filtrate resulting from the treatment of the milk sample with zinc ferric cyanide as described in the following procedure (Giuliano and Stein, 1992): In a 200 mL volumetric flask, 20 mL of milk, 100 mL of bi-distilled water and 2 mL of a 15 % ($w\ w^{-1}$) potassium ferric cyanide solution were mixed. Then, a 30 % ($w\ w^{-1}$) solution of zinc acetate was added and the new solution was exactly diluted to 200 mL. After 10-15 min a clear solution (filtrate) could be separated from the white precipitate just formed by a simple filtration procedure. All the results were expressed in $g\ L^{-1}$ NaCl.

Bacteriological analysis: Prior to samples collection for the bacteriological analysis, teats of the cows were cleaned with 95% ethanol and the first three streams of foremilk were discarded. From each quarter, 15 mL of milk sample were collected and stored at 2-4°C until bacteriological analysis. The sampling was made before the morning milking.

One hundreds microliters of each sample were plated on the following agar plates (Oxoid): Trypticase Soy Agar (5% washed sheep erythrocytes), Mac Conkey Agar and Baird Parker Agar. The plates were incubated aerobically at 37°C for 24 and 48 h. The identification of the different pathogens were made as follows:

Macroscopic estimation of the colonies; effectiveness on causing haemolysis on TSA, microscopic morphology evaluation on the Gram-stained samples and evaluation of the metabolic activities by simple methods (oxydase, catalase, coagulase) or commercial micromethods (API; BioMèrieux, Lyon, France). Bacteriological analysys was used only as one of the criteria used to select the quarter populations.

Criteria for the selection of the quarter populations: Each quarter population was formed selecting the udder quarters according to their SCC and to the response of the bacteriological analysis.

In the healthy group, only quarters with no evidences of bacterial infection and with SCC ≤300.000 in the milk were considered. On the other hand, the mastitic group was formed by quarters in which a true Intramammary Infection (IMI) was diagnosed by at least two-repeated isolation of the same pathogen in the last two months and by the high value of SCC (>500.000).

Moreover, in the evaluation of the effects induced by the laser radiation, two groups of mastitic quarters were compared: The laser irradiated and the control group. The mastitic control population was not left untreated, otherwise the work could not be considered ethic, but was treated by homoeopathic remedies.

Statistical analysis: Statistical analysis was performed by unpaired Student t-test when comparing the value of a parameter for two independent samples (mastitic vs. healthy; mastitic-control vs. mastitic-irradiated).

A paired student t-test was adopted when considering differences between two sample data belonging to the same quarter population before and after a treatment or when comparing the value of the same parameter in two different days during a treatment.

Differences between group means were considered significant at $p<0.05$.

The laser apparatus: The radiation experiments were carried out using the laser generator model STP-8, made by the MicroMedTech Society of Niznhj Novgorod (Russia). The apparatus comprises a rectangular box

(30×20 cm^2) in which four diodes generators were installed one at each corner. The laser irradiates near-infrared electromagnetic radiation in the frequency range between 890-980 nm. The average power supplied at the output of the device was less than 0.5 W. The generator head was provided with a control unit that monitors the operational state of the instrument and measured the irradiation time. A rechargeable battery (FIAMM-GS FG 10451 6V 4,5 AH) was connected to the generator head of the STP-8 apparatus in order to provide self sustained supply of electric current. A long handle (1 m) attached to the box was used to keep the instrument near the quarter of the cow.

Laser radiation treatment: Each quarter of the cows was directly irradiated for 30 sec, keeping the head of the generator at a distance of about 10 cm from the mammary gland. On the rear quarters, the laser radiation was preferentially focused near the region of the supramammary lymph nodes.

One cycle of laser-treatment consisted of five radiation treatments, one per day, performed at the same our every day.

Homoeopathic treatment: *Calacarea Carbonica* was used as homoeopathic treatment. It was dissolved in sterile H_2O and diluted at 200 CH. Five mililiter of the solution were injected in the neck of the cow via subcutaneous puncture, two times per day, for three consecutive days.

RESULTS

Somatic cell number, lactose and chloride ion concentration in the foremilk of healthy and mastitic quarters: Figure 1a, b shows the dispersion plots between the lactose and the chloride ion content versus the Somatic Cell Number (SCC). The dispersion patterns were characteristic for both analytes and could be subdiv-ided into three regions. Two adjacent regions were not totally separated but were partially overlapped. Actually, one must take into account that it is impossible to make a net differentiation between the different regions choosen:

- Healthy region, with SCC <400 000, in which the NaCl concentration ranged in the restricted interval 1,6÷1,9 gL^{-1} and the lactose concentration ranged in the quite narrow interval between 4.7 and 5.3 g 100g^{-1} (Fig. 1a).
- Mastitic region with 400 000<SCC<2 000 000, in which the NaCl concentration was between 1.9÷2.6 gL^{-1} and the lactose one was between 4.7÷4.4 g 100g^{-1} (Fig. 1a).
- Mastitic region, with SCC >2 000 000, in which a wide dispersion pattern was found and the NaCl concentration ranged between 2.0 and 3.5 g L^{-1} and the lactose content ranged between 4.7 and 2.7 g^{-1} 100 g (Fig. 1b).

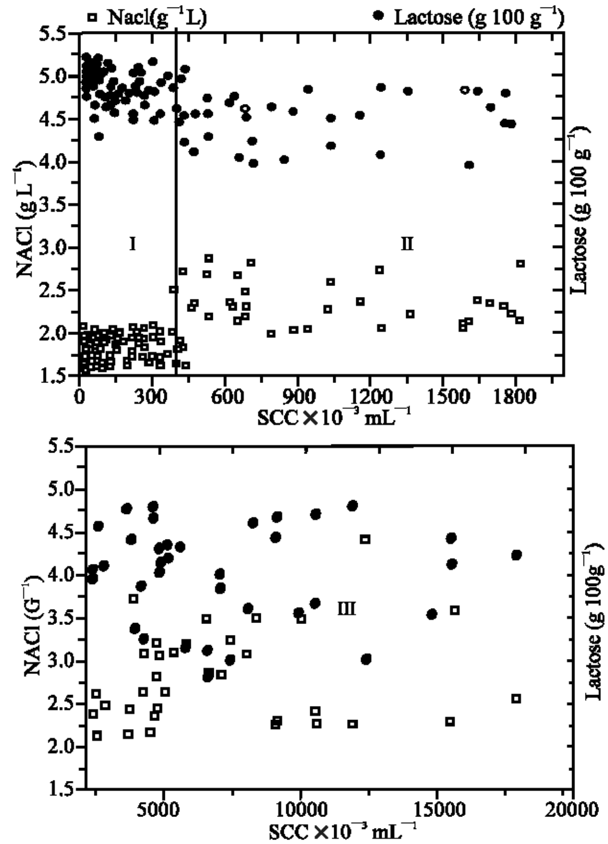

Fig. 1a, b: Dot plots for lactose (•) and chloride ion (□) concentration, as a function of SCC$\times10^3$ mL^{-1}, in the cow milk, a) for SCC$\times10^3$ mL^{-1}<2000 and b) for SCC$\times10^3$ mL^{-1}>2000

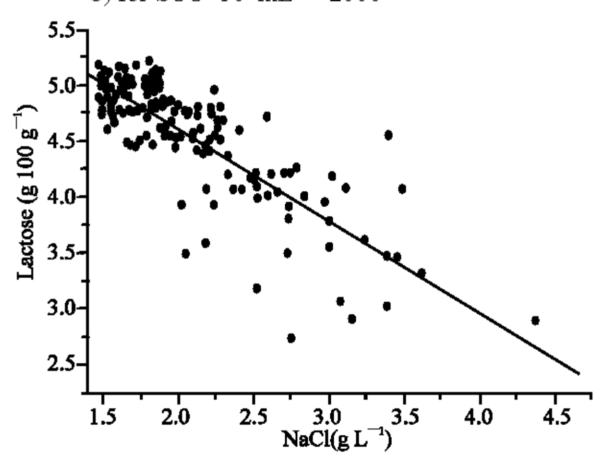

Fig. 2: Linear regression for NaCl (gL^{-1}) and Lactose (g 100 g^{-1}) levels in the foremilk of the Italian Friesian cows of the Coretto herd

Chloride anion and lactose content in the milk are negatively and linearly correlated: In Fig 2 a scatter graph was plotted between the lactose and the chloride ion milk content found in each foremilk sample. It was found that the two parameters are negatively and linearly correlated. The best linear fit of the data with the value of the Pearson product-moment correlation coefficient (r), the value of the coefficient of determination (r^2) and the confidence interval were also reported in Table 1.

Lactose and chloride anion concentration range in healthy and mastitic quarter populations: In Table 2 the calculated mean chloride and lactose concentration values found in the foremilk, were reported for a healthy quarter populations and for a mastitic one.Three important results could be extracted from Table 2.

- The mean NaCl content in the healthy quarter population was significantly lower than in the mastitic quarter one ($p<0.001$).
- The mean lactose content in the healthy quarter population was significantly higher than in the mastitic quarter one ($p<0.001$).
- A total differentiation between mastitic and healthy quarters based on the NaCl (or lactose) concentration is not possible, because the dispersion regions relative to the two populations are partially overlapped.

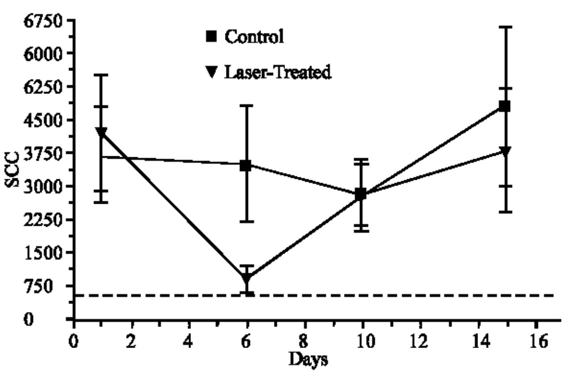

Fig. 3: Variation of mean SCC $\times10^{-3}$mL^{-1} in the milk of control (■) and laser irradiated (▼) quarter populations. *Points*, mean cell count. *Bar*, SEM

Table 1: Linear regression for lactose/NaCl

Data: Lactose = A + B × NaCl

Parameter		Value		Error
A		6.262		0.101
B		-0.825		0.047
r	r^2	SD	N	P
-0.81	0.65	0.32	168	<0.001

The kuestler number: The Kuestler Number (KN) has been defined as the (ratio Vanlandingham *et al.*, 1941):

$$\text{Kuestler}\,\frac{\text{NaCl}(\text{g L}^{-1})}{\text{Lactose}(\text{g L}^{-1})}\times 100 \qquad (1)$$

In Table 2 the calculated mean KN values for the two populations were also reported. In the calculus of the lactose concentration in g L^{-1} it was assumed that the mean density of the foremilk was 1.030 gm L^{-1} at 20°C.

It has been previously shown that when such ratio is greater than 4.5 an inflammatory process of the mammary gland has to be considered (Giuliano and Stain, 1992). Our statistical analysis showed a good agreement between our data and the literature ones (Table 2).

Starting from the results of the first-step analysis, we evaluated the effects induced by the near-IR laser radiation treatment, on the quarters cow affected by mastitis, monitoring the mean somatic cell counting and the chloride ion level in the foremilk of two groups of mastitic quarters, one treated by the laser radiation and the other, the control, treated by homoeopathic remedies.

NIRLILR effects on the somatic cells number: In Fig. 3 data on the mean Somatic Cell Number (SCC) for the two groups of quarters were reported. At the beginning of the irradiation treatment (starting point) the two populations was chosen to have approximately the same starting values of this parameter (~ 4 000×10^3). After five days of radiation treatments (the end of a radiation-treatment-cycle), the mean somatic cell number in the laser-treated group, was reduced for about 78% with respect to the starting value ((873±323) × 10^3 vs (4 172±1 349) × 10^3; $p<0.05$); N=575). In contrast, no significant variation was observed in the non-irradiated-homoeopathically-treated control quarter population ((3 654±1075) ×10^3 vs (3 474±1290) ×10^3; N = 605).

Table 2: Concentration range of NaCl (gL^{-1}) and Lactose (g 100 g^{-1}) found in the foremilk of healthy and mastitic quarter populations of Italian Friesian Cows.Mean values of the Kuestler number for the same samples

Parameter	Quarter population	Mean	Min	Max
NaCl (gL^{-1})				
	Healthy	1.63±0.03		
		N = 81	1.46	1.85
	Mastitic	2.37±0.15		
		N = 91	1.88	4.17
Lactose (g 100 g^{-1})				
	Healthy	4.84±0.04		
		N = 81	4.31	5.3
	Mastitic	4.51±0.12		
		N = 91	3.35	5.11
Kuestler number				
	Healthy	3.37±0.07		
	Mastitic	5.25±0.36		

It must also be noted that the mean SCC values at the 6th day (2nd point in the graphs) in the laser-treated and in the control group were statistically different ($p<0.05$) from each other.

The mean cell number was also monitored 5 and ten days post-treatment (Fig. 3). In the irradiated group a significant increasing trend of its value was observed with respect to the minimum obtained after 5 laser treatments. Actually, the SCC starting value was completely restored 10 days post-treatment (15th day in Fig. 3). In the control population this parameter oscillates within the SEM though its tendency was to increase after 15 days.

NIRLILR effects on the NaCl foremilk concentration: In order to describe the average response of a quarter populations to an external stimuli, in terms of the NaCl content, we introduced an average parameter, called the "average restoration factor", RF. It was defined by the expression 2:

$$\overline{RF} = \sum_{i=1}^{N} \frac{RF_i}{N} \quad (2)$$

Where, RF_i, the current restoration factors, were defined by the Eq. 3:

$$RF_i = \frac{C_i - C_{min}}{C_{min}} \qquad i = 1,2,3...N \quad (3)$$

in which C_i is the NaCl concentration (gL^{-1}) in the i-th quarter; C_{min} is the minimum NaCl concentration ($g\ L^{-1}$) value found in the healthy quarters population; N is the number of quarters in the population.

Using Eq. 2 and 3 we can express the $\overline{RF}$ by the Eq. 4:

$$\overline{RF} = \frac{1}{N}\sum_{i=1}^{N} \frac{C_i - C_{min}}{C_{min}} \qquad i = 1,2,3...N \quad (4)$$

It can be argued that RF is always positive and goes to zero as the mean NaCl concentration value of a quarter population goes to the minimum NaCl value (C_{min}).

In Fig. 4 data on the "average Restoration Factor" (RF) for the two groups of quarters were reported. At the beginning of the radiation treatment (starting point) the two populations had approximately the same starting value of this parameter (~0.55). In the homoeopathically-treated-control group (N = 605), the value remained constant within the first ten days of observation and tended to increase after 15 days.

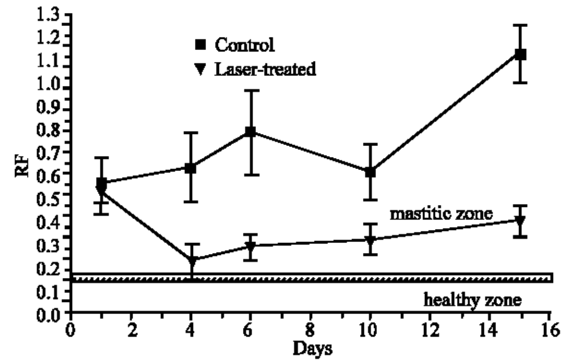

Fig. 4: RF of the control (■) and the laser-treated (▼) quarter population during (first 3 points) and after the treatment

By contrast, on the laser-treated quarter group (N = 575), a significant reducing effect was induced by the laser radiation, since the first three laser treatments. The effect on the mean chloride ion concentration was strong enough to bring the RF to the value 0.15. Such effect was also slightly retained after the end of the treatment cycle, though an increasing trend was observed. Actually, 10 days post-treatment, no significant difference was observed with respect to the starting value, in the confidence interval chosen. On the other hand, it must be observed that at the 15th day, the RF parameter assumed a significant lower value in the laser treated population than in the control one.

DISCUSSION

In a first stage of the research, we investigated significant chemical parameters linked to cow mastitis.

We studied the change in the levels of chloride ions and lactose in the foremilk collected from a single-quarter sampling, as a function of the Somatic Cell number (SCC). Such analysis showed that the NaCl and lactose concentration increased and decreased, respectively, in a step-wise manner, as the SCC rose above the value 400000 cell mL^{-1}. Rather than a so precise value, it should be reasonable considering a pertinent SCC range around 400000 cell mL^{-1}, that has a statistical meaning: We say 300000÷500000 cell mL^{-1}. Therefore, we should have to expect, with good approximation, that a net increase of the NaCl content will be verified above 500000 cell mL^{-1}.

Furthermore, we observed that the levels of chloride ions and lactose were negatively and linearly correlated in the entire range of composition experimentally determined. Moreover, substantial agreement with the literature data was found when we compared the composition of a healthy quarter population to that of a mastitic one (Schalm *et al*., 1975). Actually we found that in the former, the chloride ion concentration was significantly higher than in the last one and, conversely, that the lactose content was significantly lower in the healthy than in the mastitic population.

In a second stage, we could evaluate the effects produced by NIRLILR on the above mentioned significant chemical parameters characterizing the mastitic condition. The effect induced on the mastitic quarters, by the near infrared low intensity laser radiation, was to reduce the SCC as well as the chloride ion level in the foremilk collected from such quarters. Both parameters were monitored during and after the radiation treatment. It was observed that the reducing effect was significant at least after three treatments, but the effects did not have a long duration since just ten days post-treatment it quite vanished.

On the other hand, it must be underlined that the mean SCC in the laser-treated population, was reduced to a value close to 500000 cell mL^{-1}.

Further consideration could be made on the meaning of the RF, if we assume, as previously reported in this paper, that the step-wise increase in the NaCl content, from the range 1.6÷1.9 gL^{-1} to the wide dispersion range 1.9÷3.5 gL^{-1}, observed above the SCC value of 500000 cellm L^{-1}, identifies the throughout from a healthy quarter population towards a mastitic one. Around such value of the somatic cell count, we could identify a small interval of chloride ion composition in which there was a partial overlap between the two dispersion ranges. Such a region, in which it could not be done a net differentiation between mastitic and healthy quarters, could be considered to separate a healthy quarter population from a mastitic one. It seemed reasonable to take the NaCl concentration range 1.85-1.88 gL^{-1} as the overlapping region (Table 2). All the values comprised in the above defined region, were not considered in the statistical analysys. From simple algebra, the equivalent RF overlapping region was calculated and drawn in the graph of Fig. 4 (dashed rectangle). The radiation treatment induced the RF parameter to merge with the overlapping region, very close to the healthy zone.

CONCLUSION

We could conclude that the laser treatment induced a beneficial response of the animal against the mastitis because both the parameters monitored had a similar decreasing behaviour under the radiation treatment and assumed values very close to the milk composition region that characterizes a healthy quarter population.

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