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MICROBIOLOGY

Efficacy assessment of an intramammary formulation based on soluble polypyrrole in cows with experimentally induced mastitis

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ABSTRACT: Given the need for alternative therapies for bovine mastitis, an infectious disease of high prevalence and significant economic impact in dairy farms, this study evaluated the *in vivo* antimicrobial efficacy of an intramammary formulation based on soluble polypyrrole (PPy), a promising conductive polymer for biomedical applications, especially as an effective antimicrobial agent. In this assay, mammary quarters of healthy Holstein Friesian cows (n = 8) were inoculated with *Staphylococcus aureus* and treated, via the intramammary route, with an experimental formulation based on soluble PPy (5%) and a commercial formulation based on gentamicin sulfate. The effect of these treatments was evaluated based on milk cultures, Total Bacterial Count (TBC), Somatic Cell Count (SCC), and milk composition analysis of mammary quarter samples in seven experimental moments. In addition, hematological evaluations of the animals were also performed. The intramammary application of three levels of the experimental formulation based on soluble PPy resulted in higher log/mL of the TBC and SCC compared to a positive control group and a group that received gentamicin sulfate. The experimental formulation did not induce changes in milk composition or in hematological parameters. Certain factors related to pharmacokinetics, such as the type of carrier used in the formulation and the pharmacodynamics of soluble PPy, may have contributed to the antimicrobial ineffectiveness of the experimental formulation. The results of this study neither define nor decrease the antimicrobial potential of soluble PPy. Further research is required to develop formulations that enable the antibacterial action of soluble PPy in the intramammary environment of dairy cows.
Key words: bovine mastitis, *Staphylococcus aureus*, alternative treatment, conductive polymer.

Avaliação da eficácia de uma formulação intramamária à base de polipirrol solúvel em vacas com mastite experimentalmente induzida

RESUMO: Considerando a necessidade de terapias alternativas para a mastite bovina, uma inflamação, normalmente de causa infecciosa, de alta prevalência e de alto impacto econômico nas fazendas leiteiras, avaliou-se a eficácia antimicrobiana, *in vivo*, de uma formulação intramamária à base de polipirrol (PPy) solúvel, um polímero condutor promissor para aplicações biomédicas, especialmente como potente antibacteriano. Neste ensaio, quartos mamários de vacas holandesas sadias (n = 8) foram inoculados com *Staphylococcus aureus* e tratados, por via intramamária, com formulação experimental à base de PPy solúvel (5%) e com formulação comercial à base de sulfato de gentamicina. O efeito desses tratamentos foi avaliado com a realização de lactoculturas, da Contagem Bacteriana Total (TBC), da Contagem de Células Somáticas (SCC) e da análise da composição do leite das amostras obtidas de quartos mamários, em sete momentos experimentais. Avaliação hematológica dos animais também foi realizada. A aplicação intramamária de três doses da formulação experimental à base de PPy solúvel resultou em maiores log/mL da TBC e da SCC quando comparadas ao grupo controle positivo e ao grupo que recebeu sulfato de gentamicina. A administração da formulação experimental não induziu alterações na composição do leite e nos parâmetros hematológicos. Alguns fatores farmacocinéticos e farmacodinâmicos do PPy solúvel podem ser atribuídos a ineficácia antimicrobiana da pomada experimental. Outras pesquisas devem ser realizados em prol do desenvolvimento de formulações que permitam a atuação antibacteriana do PPy solúvel no ambiente intramamário de vacas leiteiras.
Palavras-chave: Mastite bovina, *Staphylococcus aureus*, tratamento alternativo, polímero condutor.

INTRODUCTION

Mastitis is the most prevalent disease in dairy herds, consisting of mammary gland inflammation that reflects production indices and profitability of agricultural production. These factors are affected by reduced milk production (ADRIAENS et al., 2021), high costs of treatment and prevention (HE et al., 2020), and diminished reproductive performance (DAHL et al., 2020; DALANEZI et al., 2020; RANASINGHE et al., 2021).

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Since, in most cases, this disease is caused by bacteria, antimicrobials are still the primary therapeutic strategy to treat mastitis (RUEGG, 2017) through drug administration via the intramammary route (MCALOON et al., 2021; TOMAZI & DOS SANTOS, 2020). This method provides high concentrations of the substance applied and reduces the consumption of antimicrobials (GUARDABASSI et al., 2010).

However, the indiscriminate use of conventional antimicrobials has promoted the selection of resistant microorganisms (CHEN et al., 2021; DORNELES et al., 2019; MECHESSO et al., 2021), decreasing therapeutic efficacy in intramammary infections (FREITAS et al., 2018). In this scenario, *Staphylococcus aureus*, one of the main pathogens of bovine mastitis, has shown a growing resistance pattern to the antimicrobials most frequently used worldwide to treat this disease (MOLINERI et al., 2021).

Many studies have been conducted to search for new antimicrobial compounds with a diversified nature (LOPES et al., 2020; TING et al., 2020; ZAFAR et al., 2021; ZDUŃCZYK & JANOWSKI, 2020) and to develop formulations to treat bovine mastitis. However, there are still no reports regarding studies that employed conductive polymers as antimicrobial therapeutic agents against this disease, especially soluble polypyrene.

With the development of conductive polymers with a high degree of solubility in various solvents, it has become possible, through chemical polymerization, to synthesize highly soluble polypyrrole in water (SILVA JUNIOR et al., 2016). Furthermore, the soluble form of polypyrrole has increased the possibility of its biomedical applications, especially as a potent antimicrobial (DA SILVA JR. et al., 2017; KHAN et al., 2019; PIRES et al., 2018).

The antimicrobial behavior of soluble polypyrrole against *S. aureus* isolates from mastitis cases has also been evaluated in previous studies, with satisfactory results characterized by low Minimum Inhibitory Concentration (MIC) values (ACOSTA et al., 2020; SILVA et al., 2020). From this perspective, this study aimed to evaluate the efficacy of an intramammary formulation based on soluble polypyrrole in cows affected by experimentally induced mastitis caused by *S. aureus*.

MATERIALS AND METHODS

Preparation of experimental formulation

An intramammary formulation based on soluble polypyrrole was prepared at the Laboratory of Pharmaceutical Techniques of the Federal University of Vale do São Francisco (UNIVASF), Central Campus. Initially, a base (emulsion) for the formulation was prepared by conventional emulsification techniques through phase inversion consisting of the following cycles: separate heating of phase 1 (oil phase) at 75 °C and phase 2 (aqueous phase) at 80 °C; then, phase 2 was slowly poured on phase 1 while being vigorously and constantly stirred. After that, the stirring speed was gradually reduced, and the mixture was cooled until reaching 40 °C. Next, a complementary phase (silicone mixture) was added, and the components were mixed until reaching complete homogenization and ambient temperature.

The components of the oil phase included Lanette WB®, propylparaben, isodecyl oleate, and butylhydroxytoluene, whereas the aqueous phase consisted of methylparaben, double-distilled glycerin, propylene glycol, and purified water. Finally, soluble polypyrrole was gradually added to the base through stirring, thus obtaining the final 5% concentration of the active ingredient.

In vivo experimental assays: Efficacy assessment of formulation based on soluble polypyrrole

Location and experimental animals

Efficacy assessments were conducted at the facilities of the Dairy Cattle Sector of UNIVASF. Clinically healthy Holstein Friesian cows (n = 8) of different ages and between the first and third lactations were used in this study. Their diet consisted of roughage (elephant grass) and a concentrate made of maize, soybean meal, and a mineral supplement, balanced for the lactation period. The cows were milked once per day using a mechanical milking unit. Animals returning three consecutive negative milk cultures were selected for the assay.

Inoculum preparation

Intramammary infection was induced in the cows using a *Staphylococcus aureus* isolate obtained from a case of subclinical bovine mastitis. The isolate was classified as multi-resistant and as a strong biofilm producer according to KREWER et al. (2015). The *S. aureus* isolate was previously stocked in 40% glycerol at -80 °C. Then, using this stock, 5 µL of the isolate was added to a Falcon tube containing 5 mL of Brain Heart Infusion (BHI) broth (Ionlab, Araucária, Brazil). Subsequently, the Falcon tube with the inoculum was incubated in a rotary shaker (NT712, Novatecnica, Piracicaba, Brazil) at 90 RPM and 37 ºC for 18 hours. After growth, the

bacterial inoculum optical density was measured at 600 nm (K37 UV/VIS, Kasvi, São José do Pinhais, Brazil) to prepare a bacterial suspension with 1.3×10^4 Count Forming Units (CFU)/mL. This suspension was washed twice with 10 mL of an 0.85% saline solution (Dinâmica, Indaiatuba, Brazil) and centrifuged for 10 minutes at 1,800 × *g* (TDL80-2B, Centribio, cidade, país), after which the mixture was resuspended in the same volume of saline solution. Next, a 10 × dilution was prepared to obtain a bacterial suspension of 1.3×10^3 CFU/mL in saline solution. Then, a 5 mL sterile syringe (Descarpack, Manesar Gurgaon, India) was used to aspirate 1 mL of the final bacterial suspension, after which the syringe was coupled to a urethral probe (no. 08, Medsonda, Arapoti, Brazil). Control mammary quarters were established by preparing syringes containing only the 0.85% saline solution. Finally, the bacterial suspension at 1.3×10^3 CFU/mL was plated on BHI agar (Ionlab) to confirm the size of the bacterial population. This entire procedure was conducted in a biological safety cabinet (Becnermed, Colombo, Brazil) to ensure sterility of the materials.

Experimental design and infection

After negative milk cultures were confirmed, three mammary quarters from each cow (n = 8) were inoculated with an *S. aureus* suspension (1.3×10^3 CFU/mL). One mammary quarter in each animal was established as a positive control (infected but untreated). The other two infected quarters were part of the treatment group (infected and treated). One mammary quarter was used as a negative control (receiving only the 0.85% saline solution), as shown in figure 1. Each mammary quarter was considered an experimental unit since each quarter is anatomically independent.

After inoculation, the strip cup test, the California Mastitis Test (CMT), and milk cultures were performed every 24 hours to confirm intramammary infection. All animals responded positively to the CMT, with some of these individuals exhibiting physical changes in the cup test. As a result, some animals manifested subclinical mastitis, whereas others showed the subacute clinical form of the disease. These clinical pictures of mastitis remained stable until the beginning of treatment.

Subsequently, the animals were randomly divided into two experimental groups of four animals each. The first group received, via the intramammary route, 10 grams of the experimental formulation based on soluble polypyrrole in the two infected left mammary quarters. Likewise, the other group received 10 grams of the commercial

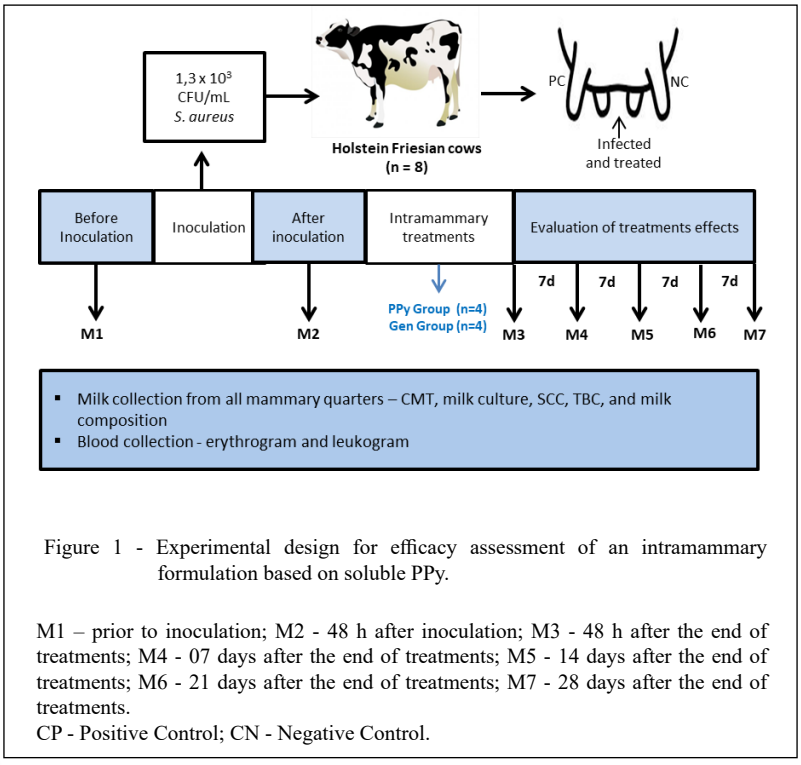

Figure 1 - Experimental design for efficacy assessment of an intramammary formulation based on soluble PPy.

M1 – prior to inoculation; M2 - 48 h after inoculation; M3 - 48 h after the end of treatments; M4 - 07 days after the end of treatments; M5 - 14 days after the end of treatments; M6 - 21 days after the end of treatments; M7 - 28 days after the end of treatments.
CP - Positive Control; CN - Negative Control.

formulation based on gentamicin sulfate (Mastifin, Ourofino, São Paulo, Brazil). The therapeutic formulations were administered over three consecutive days with a 24 hour interval between applications (Figure 1).

Evaluation of treatment effects

Responses to the intramammary treatments were evaluated by observing the presence/absence of bacterial growth in plates (milk culture) through the TBC, SCC, and analysis of milk composition based on milk samples from each mammary quarter. These evaluations were performed at seven different experimental moments: before inoculation (M1), after inoculation (M2), and at five moments after intramammary treatments (M3, M4, M5, M6, and M7). All guidelines of the National Mastitis Council (2004) were followed when collecting milk samples. Moreover, at the experimental moments mentioned, the hematological analysis of the animals was also performed (Figure 1).

Milk cultures

Milk culture was performed using 10 µL aliquots from each milk sample obtained from the individual mammary quarters. These aliquots were streaked in each quadrant of a Petri dish containing 5-8% sheep blood agar. The presence of mastitis was determined using the parameters established by the National Mastitis Council (LOPEZ-BENAVIDES et al., 2020). The criterion of at least 1 CFU/10 µL per milk sample was used to consider the mammary quarter positive for intramammary infection. Three milk samples were collected from each mammary quarter on alternate days.

TBC, SCC, and milk composition

Determination of TBC, SCC, and composition of the milk samples was performed at the Laboratory 'Clínica do Leite', located in Piracicaba-SP. For TBCs (× thousand CFU/mL), milk samples were collected in sterile containers containing the preservative azidiol and kept under refrigeration at 10 °C. Flow cytometry was employed in this analysis using Bactoscan equipment (Foss). The samples used for the SCC (× thousand cells/mL) and milk composition analyses (g/100g) were collected in containers containing the preservative bromonata and kept at ambient temperature. These parameters were evaluated using flow cytometry (SCCs) and infrared (milk composition) techniques, respectively, with the equipment CombiFoss 7 (Foss). The milk composition variables obtained included the contents of fat (g/100g), protein (g/100g), lactose (g/100g), total solids (g/100g), and defatted dry extract (g/100g).

Hematological evaluation

Hematological parameters of the animals were also monitored throughout the study. For that purpose, blood samples were collected via the coccygeal vein at the different experimental moments (Figure 1) and sent to the Microscopy Laboratory of UNIVASF, where an Hematoclin 2.8 Vet hematology analyzer (Bioclin) was used to determine erythrograms and leukograms. Blood smears were prepared to obtain differential granulocyte counts and for cell morphology analysis.

Statistical data analysis

The absence of normality in the SCC and TBC results was confirmed by the Shapiro-Wilk test and the values obtained for these variables were subjected to base-10 logarithmic transformation ($\log_{10}$). SCC and TBC values were compared between experimental moments by analysis of variance (ANOVA – repeated measures), whereas comparisons between groups were performed by one-way ANOVA.

The hemogram results were analyzed by ANOVA for the evaluation throughout experimental moments, whereas comparisons between groups were performed by one-way ANOVA. The absence of normality was confirmed by the Shapiro-Wilk test for milk composition results. In that case, Friedman's ANOVA was used for comparisons, whereas the Kruskal-Wallis test was employed for comparisons between groups using Dunn's test to compare ranks.

The bacterial culture results were analyzed between experimental moments by the Q test of Cochran, whereas comparisons between groups were performed using Fisher's exact test. Data analysis was performed using the Statistical Package for the Social Sciences (SPSS), v.20.0 for Windows.

RESULTS AND DISCUSSION

Mammary quarters infected with *S. aureus* and treated with the intramammary experimental formulation based on soluble PPy continued to show high CFU/mL counts in milk during the post-treatment

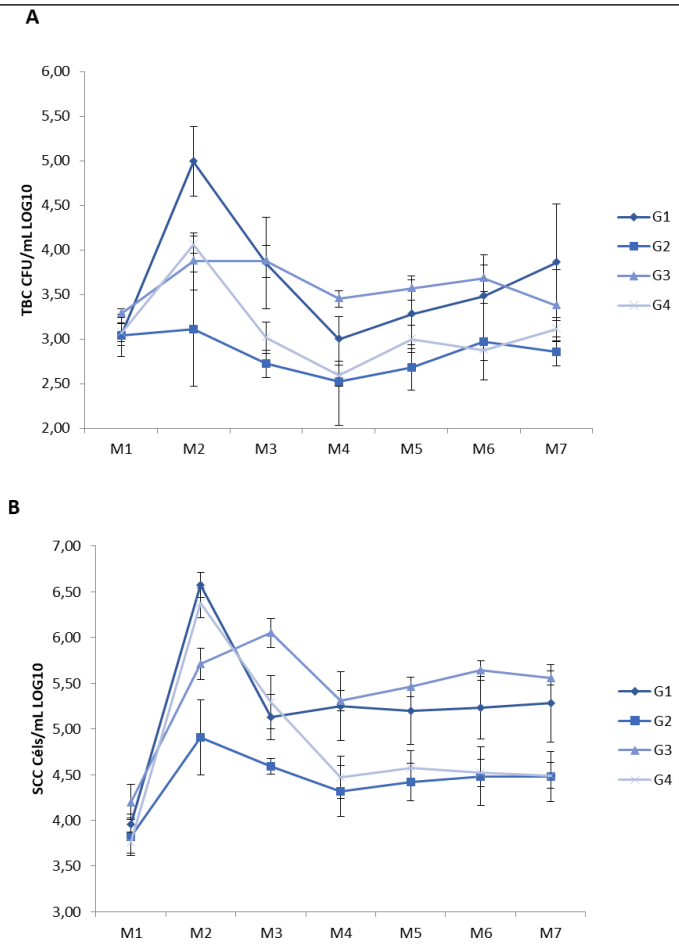

Figure 2 - Total Bacterial Count (A) and Somatic Cell Count (B) of milk from mammary quarters with experimentally induced mastitis caused by *S. aureus* and treated with intramammary formulations based on soluble PPy (G3) and gentamicin sulfate (G4). Infected and untreated mammary quarters (G1, positive control); non-infected mammary quarters G2, negative control.

M1 - prior to inoculation; M2 - 48 h after inoculation; M3 - 48 h after the end of treatments; M4 - 07 days after the end of treatments; M5 - 14 days after the end of treatments; M6 - 21 days after the end of treatments; M7 - 28 days after the end of treatments.

moments compared to mammary quarters treated with the commercial formulation based on gentamicin sulfate and the positive control quarters (Figure 2A).

The same dynamic was observed in SCC values of milk samples obtained from mammary quarters that received the PPy-based treatment, with higher means for the PPy group during the post-treatment moments compared to the positive control group (significant difference at M3 – $P < 0.05$) and the GEN group (significant difference at M6 and M7 – $P < 0.05$). See Figure 2B.

Furthermore, in the milk culture analysis between groups, there were statistical differences in the second, fourth, and fifth week after the end of treatments, in which periods the PPy group showed higher positivity in the milk cultures than the GEN group. As relevant parameters for the efficacy assessment of intramammary products in bovines (EMA, 2017), the TBC, SCC, and milk culture results indicated that the experimental formulation based on PPy did not display antimicrobial efficacy under the conditions of the present study.

A number of studies have already verified the *in vitro* antimicrobial activity of polypyrrole against *S. aureus* isolates (ACOSTA et al., 2020; SILVA et al., 2020). However, it is well known that *in vitro* antimicrobial activity does not imply *in vivo* efficacy since drug action within bovine mammary glands depends on various pharmacokinetic characteristics of the formulation tested (PYÖRÄLÄ, 2009). These characteristics include lipid solubility, the degree of ionization, the extent of the bond to whey and udder proteins, and the type of carrier used (LAINESSE et al., 2012).

Drugs highly soluble in water would remain confined to the milk if administered by the intramammary route only, whereas lipid-soluble drugs can penetrate the mammary gland tissues more effectively (GRUET et al., 2001; RONCADA et al., 2000). Therefore, as a substance highly soluble in water, PPy administered via the intramammary route would be restricted to the milk, which could hinder its action against *S. aureus*, a microorganism that can remain viable within leukocytes and mammary epithelial cells (ALGHARIB et al., 2020; SAEED et al., 2021).

Another critical aspect of the pharmacokinetics of an intramammary drug is its degree of ionization, which indicates the percentage of the drug that is ionized. The non-ionized form of a drug spreads more quickly through biological membranes compared to the ionized form (LAINESSE et al., 2012). Therefore, since the bioactivity of polypyrrole has been attributed to the resulting positive charges along synthesized chains (RAMIREZ et al., 2019), it is suggested that this characteristic may have hindered PPy diffusion through cell membranes of mammary epithelium.

Furthermore, another important aspect relates to the pharmacokinetics of soluble PPy, i.e., its biocidal action against *S. aureus*, especially in the intramammary environment. The antimicrobial mechanism of soluble PPy is attributed to the presence of positive charges formed for every three to five monomers along its main chain. As a result, a strong electrostatic interaction is established with species of opposite charges, e.g., the bacterial cell wall (SESHADRI & BHAT, 2005; VARESANO et al., 2013). The interaction of the charges of the polypyrrole molecule with those of the bacterial cell wall promotes rupture of the latter and leakage of intracellular contents, resulting in death of the bacterial cell (SILVA JUNIOR et al., 2016).

With regard to the nanoparticulate soluble PPy, its electrostatic interactions with the bacterial cell wall were more efficient under *in vitro* conditions (SILVA JUNIOR et al., 2016), which could result in more effective antimicrobial formulations. However, the experimental formulation based on soluble PPy did not exhibit *in vivo* efficacy. Unlike *in vitro* bioassays, in which interactions between bacteria and biocides are facilitated, it is possible that the formulation carrier and/or some other factors of the intramammary environment, e.g., electrolytes and milk proteins (CONSTABLE & MORIN, 2003), might have influenced the action mechanism as they bound and neutralized the positive charges of soluble PPy along the polymeric chain.

The effect of the intramammary treatments on milk composition was also evaluated in the present study (Table 1). Of all parameters analyzed, the contents of protein, total solids, and defatted dry extract showed no significant changes in the groups analyzed ($P > 0.05$) throughout the seven experimental moments ($P > 0.05$).

Lactose was thae parameter with the most significant changes among experimental groups, specifically at periods M2 (48 h after inoculation) and M3 (48 h after the end of treatments), in which the negative control showed higher means than groups G1 and G3 ($P < 0.05$). This decrease in lactose content of infected quarters could be due to the lower capacity of synthesis by epithelial cells, indicating that mastitis decreased lactose content in infected quarters and with the passage of lactose from milk into the blood (SANTOS & FONSECA, 2019).

The PPy-based formulation did not change milk components after intramammary treatments (M3 to M7). In the post-treatment period, the only change identified was attributed to the GEN group, whose mean fat content was significantly lower than the positive control group ($P < 0.05$) at M4. However, at the other moments (M3, M5, M6, and M7), the mean fat content of the GEN group was equivalent to the other experimental groups ($P > 0.05$).

Furthermore, unlike the gentamicin group, analysis within the PPy group throughout the experimental moments showed no relevant post-treatment differences ($P > 0.05$) for the milk components analyzed. These results suggested that the experimental formulation based on soluble PPy did not compromise milk components, which are quality indicators routinely used in the dairy industry (BRASIL, 2018).

With regard to hematological parameters, the administration of three intramammary applications of the experimental PPy formulation did

Table 1 - Means of seven replicates of milk composition analysis (g/100g) of infected mammary quarters treated with experimental intramammary formulations based on soluble PPy and gentamicin sulfate.

----------Total Protein----------		M1	M2	M3	M4	M5	M6	M7
G1	Mean	3.40^{Aa}	3.51^{Aa}	3.60^{Aa}	3.58^{Aa}	3.54^{Aa}	3.53^{Aa}	3.57^{Aa}
	s.r.m.	0.13	0.13	0.12	0.13	0.12	0.10	0.10
G2	Mean	3.47^{Aa}	3.40^{Aa}	3.64^{Aa}	3.60^{Aa}	3.68^{Aa}	3.54^{Aa}	3.60^{Aa}
	s.r.m.	0.12	0.10	0.14	0.11	0.09	0.13	0.14
G3	Mean	3.37^{Aa}	3.55^{Aa}	3.70^{Aa}	3.65^{Aa}	3.61^{Aa}	3.50^{Aa}	3.52^{Aa}
	s.r.m.	0.10	0.13	0.07	0.09	0.07	0.11	0.13
G4	Mean	3.49^{Aa}	3.66^{Aa}	3.59^{Aa}	3.52^{Aa}	3.50^{Aa}	3.52^{Aa}	3.49^{Aa}
	s.r.m.	0.13	0.12	0.14	0.14	0.16	0.17	0.18
----------Lactose----------		M1	M2	M3	M4	M5	M6	M7
G1	Mean	4.27^{Aa}	3.34^{Aa}	4.01^{ABa}	3.82^{Aa}	4.00^{Aa}	3.93^{Aa}	3.83^{Aa}
	s.r.m.	0.07	0.17	0.18	0.22	0.22	0.25	0.23
G2	Mean	4.38^{Aa}	4.33^{Ba}	4.21^{Ba}	4.29^{Aa}	4.43^{Aa}	4.41^{Aa}	4.35^{Aa}
	s.r.m.	0.08	0.06	0.09	0.08	0.07	0.09	0.08
G3	Mean	4.31^{Aa}	3.51^{Aa}	3.62^{Aa}	3.70^{Aa}	4.10^{Aa}	3.91^{Aa}	3.79^{Aa}
	s.r.m.	0.11	0.28	0.16	0.39	0.10	0.19	0.21
G4	Mean	4.36^{Abc}	3.67^{ABa}	4.04^{ABab}	4.30^{Aabc}	4.34^{Abc}	4.40^{Ac}	4.38^{Ac}
	s.r.m.	0.05	0.22	0.09	0.08	0.08	0.08	0.09
----------Total Solids----------		M1	M2	M3	M4	M5	M6	M7
G1	Mean	9.72^{Aa}	10.55^{Aa}	9.59^{Aa}	10.21^{Aa}	9.92^{Aa}	10.13^{Aa}	10.37^{Aa}
	s.r.m.	0.35	0.41	0.27	0.49	0.35	0.39	0.50
G2	Mean	9.63^{Aab}	10.19^{Aab}	9.79^{Aa}	10.18^{Aab}	10.11^{Aab}	10.15^{Ab}	10.03^{Aab}
	s.r.m.	0.22	0.32	0.20	0.48	0.28	0.16	0.23
G3	Mean	9.82^{Aab}	10.39^{Aab}	9.26^{Aa}	9.49^{Ab}	9.99^{Aab}	10.26^{Aab}	9.50^{Aab}
	s.r.m.	0.22	0.59	0.09	0.29	0.29	0.42	0.33
G4	Mean	9.50^{Aab}	10.44^{Ab}	9.62^{Aab}	9.50^{Aa}	10.12^{Ab}	10.08^{Ab}	9.94^{Aab}
	s.r.m.	0.24	0.27	0.26	0.19	0.28	0.21	0.18
----------Fat----------		M1	M2	M3	M4	M5	M6	M7
G1	Mean	1.08^{Aa}	2.75^{Ab}	0.99^{Aa}	1.78^{Bab}	1.38^{Aab}	1.65^{Aab}	1.97^{Aab}
	s.r.m.	0.26	0.51	0.19	0.37	0.21	0.29	0.58
G2	Mean	0.78^{Aa}	1.52^{Aa}	0.92^{Aa}	1.24^{ABa}	1.02^{Aa}	1.17^{Aa}	1.07^{Aa}
	s.r.m.	0.15	0.35	0.17	0.36	0.20	0.12	0.14
G3	Mean	1.19^{Aa}	2.36^{Aa}	0.94^{Aa}	1.05^{ABa}	1.28^{Aa}	1.84^{Aa}	1.16^{Aa}
	s.r.m.	0.20	0.55	0.11	0.13	0.26	0.43	0.19
G4	Mean	0.66^{Aa}	2.14^{Ac}	0.99^{Aab}	0.68^{Aa}	1.27^{Abc}	1.15^{Aabc}	1.11^{Aabc}
	s.r.m.	0.12	0.51	0.19	0.09	0.18	0.14	0.12
--------Defatted dry extract-------		M1	M2	M3	M4	M5	M6	M7
G1	Mean	8.64^{Aab}	7.79^{Aa}	8.60^{Aab}	8.43^{Ab}	8.54^{Ab}	8.48^{Aab}	8.41^{Aab}
	s.r.m.	0.18	0.29	0.29	0.27	0.31	0.30	0.25
G2	Mean	8.85^{Aab}	8.68^{Aa}	8.88^{Aab}	8.94^{Aab}	9.10^{Aab}	8.98^{Ab}	8.96^{Ab}
	s.r.m.	0.16	0.14	0.13	0.16	0.13	0.20	0.20
G3	Mean	8.63^{Aa}	8.03^{Aa}	8.32^{Aa}	8.45^{Aa}	8.71^{Aa}	8.42^{Aa}	8.34^{Aa}
	s.r.m.	0.16	0.24	0.09	0.40	0.08	0.26	0.31
G4	Mean	8.84^{Aabc}	8.30^{Aa}	8.63^{Aab}	8.83^{Aabc}	8.85^{Abc}	8.93^{Ac}	8.83^{Ac}
	s.r.m.	0.19	0.31	0.24	0.20	0.24	0.24	0.27

G1: Infected and untreated mammary quarters (positive control); G2: non-infected and untreated mammary quarters (negative control); G3: mammary quarters infected and treated with soluble PPy; G4: mammary quarters infected and treated with the commercial formulation based on gentamicin sulfate;

Equal uppercase letters in the same column indicate the absence of statistical significance ($P > 0.05$);

Equal lowercase letters in the same row indicate the absence of statistical significance ($P > 0.05$);

M1 - prior to inoculation; M2 - 48 h after inoculation; M3 - 48 h after the end of treatments; M4 - 07 days after the end of treatments; M5 - 14 days after the end of treatments; M6 - 21 days after the end of treatments; M7 - 28 days after the end of treatments.

Table 2 - Means of six replicates of the hematological parameters of cows treated with an experimental intramammary formulation based on soluble PPy and with a commercial formulation based on gentamicin sulfate (GEN).

----------ERITROGRAMA----------			M1	M2	M3	M4	M5	M6
Red Blood Cell Count (× 10^6/uL)	PPy	Mean	6.84^{Aa}	6.21^{Aa}	8.20^{Aa}	6.53^{Aa}	6.77^{Aa}	8.90^{Aa}
		s.e.m.	0.23	0.23	1.02	0.49	0.36	1.10
	GEN	Mean	6.08^{Aa}	5.87^{Aa}	7.89^{Aa}	6.38^{Aa}	6.78^{Aa}	6.60^{Aa}
		s.e.m.	0.41	0.37	0.87	0.43	0.55	0.62
Hemoglobin (g/dL)	PPy	Mean	8.65^{Aa}	9.10^{Aa}	10.08^{Aa}	9.48^{Aa}	9.40^{Aa}	10.55^{Aa}
		s.e.m.	0.24	0.26	0.48	0.38	0.37	0.61
	GEN	Mean	8.28^{Aa}	8.80^{Aa}	9.90^{Aa}	9.20^{Aa}	10.25^{Aa}	9.48^{Aa}
		s.e.m.	0.39	0.51	0.57	0.61	0.87	0.96
Hematocrit (%)	PPy	Mean	30.65^{Aa}	25.30^{Aa}	36.93^{Aa}	29.88^{Aa}	30.90^{Aa}	42.35^{Aa}
		s.e.m.	0.70	0.58	4.38	2.03	1.87	6.01
	GEN	Mean	27.73^{Aa}	24.38^{Aa}	36.45^{Aa}	29.90^{Aa}	31.98^{Aa}	31.30^{Aa}
		s.e.m.	1.47	1.31	3.64	1.79	2.72	2.99
Mean Corpuscular Volume (fL)	PPy	Mean	44.95^{Ab}	40.88^{Aa}	45.15^{Ab}	45.90^{Ab}	45.75^{Ab}	47.35^{Ab}
		s.e.m.	0.92	1.07	1.01	1.06	1.13	1.27
	GEN	Mean	45.88^{Aab}	41.60^{Aa}	46.48^{Ab}	47.00^{Ab}	47.25^{Ab}	47.55^{Ab}
		s.e.m.	0.82	1.17	0.89	0.78	0.98	0.89
Mean Corpuscular Hemoglobin (pg)	PPy	Mean	12.65^{Aa}	14.68^{Aa}	12.58^{Aa}	14.60^{Aa}	13.93^{Aa}	12.18^{Aa}
		s.e.m.	0.64	0.27	0.94	0.84	0.68	1.16
	GEN	Mean	13.63^{Aa}	14.98^{Aa}	12.78^{Aa}	14.38^{Aa}	15.05^{Aa}	14.28^{Aa}
		s.e.m.	0.46	0.11	0.96	0.24	0.21	0.18
Mean Corpuscular Hemoglobin Concentration (g/dL)	PPy	Mean	28.28^{Aa}	35.98^{Aa}	27.88^{Aa}	31.93^{Aa}	30.63^{Aa}	25.98^{Aa}
		s.e.m.	1.27	0.48	1.89	1.44	1.66	2.85
	GEN	Mean	29.85^{Aab}	36.08^{Ab}	27.55^{Aab}	30.65^{Aab}	32.03^{Aa}	30.18^{Aa}
		s.e.m.	0.78	0.76	1.74	0.26	0.57	0.46
Distribution Range of Red Blood Cells (%)	PPy	Mean	16.05^{Aa}	29.73^{Ab}	16.48^{Aa}	16.63^{Aa}	16.05^{Aa}	15.95^{Aa}
		s.e.m.	0.42	1.00	0.48	0.82	0.23	0.45
	GEN	Mean	15.65^{Aa}	28.23^{Ab}	15.65^{Aa}	15.23^{Aa}	15.15^{Aa}	15.35^{Aa}
		s.e.m.	0.24	0.90	0.35	0.25	0.39	0.31
Platelets (× 10^9/L)	PPy	Mean	532.50^{Aa}	274.00^{Aa}	590.25^{Aa}	782.25^{Aa}	315.25^{Aa}	384.50^{Aa}
		s.e.m.	33.15	44.20	72.86	309.99	67.38	55.08
	GEN	Mean	571.50^{Aa}	343.50^{Aa}	747.75^{Aa}	659.50^{Aa}	436.50^{Aa}	637.75^{Aa}
		s.e.m.	137.27	74.43	151.79	185.19	151.84	252.07

Equal uppercase letters in the same column indicate the absence of statistical significance (P > 0.05);
Equal lowercase letters in the same row indicate the absence of statistical significance (P > 0.05);
M1 - prior to inoculation; M2 - 48 h after inoculation; M3 - 48 h after the end of treatments; M4 - 07 days after the end of treatments; M5 - 14 days after the end of treatments; M6 - 21 days after the end of treatments; M7 - 28 days after the end of treatments.

not induce changes involving erythrocyte variables of cows, including erythrocytes, hemoglobin, hematocrit, Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH), Mean Corpuscular Hemoglobin Concentration (MCHC), and the distribution range of red and white cells until 28 days after treatment (P > 0.05) – table 2. The same behavior was observed after leukogram analysis (Table 3). A considerable increase was observed in granulocytes of the PPy group compared to the GEN group in the post-inoculation period. However, no significant differences were observed in the differential counts of neutrophils, eosinophils, and basophils.

Table 3 - Mean of six replicates of the hematological parameters of cows treated with an experimental intramammary formulation based on soluble PPy and with a commercial formulation based on gentamicin sulfate (GEN).

----------LEUCOGRAM----------			M1	M2	M3	M4	M5	M6
Total Leukocytes (× 10^9/L)	PPy	Mean	5.13^{Aa}	6.38^{Aa}	7.28^{Aa}	7.00^{Aa}	5.60^{Aa}	5.35^{Aa}
		s.e.m.	0.92	0.41	1.35	1.17	0.50	0.87
	GEN	Mean	4.85^{Aab}	5.69^{Aa}	7.93^{Ab}	6.13^{Aab}	10.20^{Aab}	6.75^{Aab}
		s.e.m.	0.66	0.54	0.57	0.54	2.64	0.79
Lymphocytes (× 10^9/L)	PPy	Mean	1.93^{Aa}	1.55^{Aab}	2.53^{Ab}	2.25^{Aab}	1.98^{Aab}	2.03^{Aab}
		s.e.m.	0.34	0.26	0.33	0.10	0.29	0.38
	GEN	Mean	1.38^{Aa}	2.00^{Aa}	2.68^{Aa}	2.35^{Aa}	2.65^{Aa}	2.10^{Aa}
		s.e.m.	0.05	0.36	0.45	0.38	0.21	0.22
Monocytes (× 10^9/L)	PPy	Mean	0.48^{Aa}	0.56^{Aa}	0.55^{Aa}	0.73^{Aa}	0.55^{Aa}	0.45^{Aa}
		s.e.m.	0.09	0.17	0.10	0.23	0.10	0.10
	GEN	Mean	0.48^{Aab}	0.90^{Ab}	0.63^{Aab}	0.53^{Aa}	1.30^{Aab}	0.60^{Aab}
		s.e.m.	0.08	0.06	0.03	0.03	0.60	0.09
Granulocytes (× 10^9/L)	PPy	Mean	2.73^{Aa}	4.27^{Ba}	4.20^{Aa}	2.53^{Aa}	3.08^{Aa}	2.88^{Aa}
		s.e.m.	0.53	0.37	1.00	0.65	0.21	0.51
	GEN	Mean	3.00^{Aab}	2.79^{Aa}	4.63^{Ab}	3.25^{Aab}	6.25^{Aab}	4.05^{Aab}
		s.e.m.	0.63	0.33	0.43	0.26	2.20	0.58
Neutrophils (× 10^9/L)	PPy	Mean	2.39^{Aa}	3.65^{Aa}	3.77^{Aa}	2.17^{Aa}	2.69^{Aa}	2.61^{Aa}
		s.e.m.	0.50	0.54	1.09	0.58	0.17	0.55
	GEN	Mean	2.78^{Aab}	2.18^{Aa}	4.17^{Ab}	2.87^{Aab}	5.42^{Aab}	3.71^{Aab}
		s.e.m.	0.56	0.33	0.34	0.28	1.70	0.61
Eosinophils (× 10^9/L)	PPy	Mean	0.33^{Aa}	0.59^{Aa}	0.43^{Aa}	0.34^{Aa}	0.37^{Aa}	0.27^{Aa}
		s.e.m.	0.12	0.25	0.18	0.17	0.16	0.09
	GEN	Mean	0.21^{Aa}	0.60^{Aa}	0.46^{Aa}	0.37^{Aa}	0.83^{Aa}	0.34^{Aa}
		s.e.m.	0.08	0.11	0.12	0.07	0.51	0.10
Basophiles (× 10^9/L)	PPy	Mean	0.00^{Aa}	0.04^{Aa}	0.00^{Aa}	0.02^{Aa}	0.02^{Aa}	0.00^{Aa}
		s.e.m.	0.00	0.04	0.00	0.02	0.02	0.00
	GEN	Mean	0.01^{Aa}	0.01^{Aa}	0.00^{Aa}	0.01^{Aa}	0.00^{Aa}	0.00^{Aa}
		s.e.m.	0.01	0.00	0.00	0.01	0.00	0.00

Equal uppercase letters in the same column indicate the absence of statistical significance ($P > 0.05$);
Equal lowercase letters in the same row indicate the absence of statistical significance ($P > 0.05$);
M1 - prior to inoculation; M2 - 48 h after inoculation; M3 - 48 h after the end of treatments; M4 - 07 days after the end of treatments; M5 - 14 days after the end of treatments; M6 - 21 days after the end of treatments; M7 - 28 days after the end of treatments.

CONCLUSION

Administration of an intramammary therapy based on soluble PPy in cows with mastitis induced by *S. aureus* did not decrease the TBCs or SSCs of milk compared to animals treated with gentamicin sulfate. The intramammary experimental formulation of soluble PPy was not effective against *S. aureus* under the experimental conditions employed in this study. On the other hand, no significant changes were observed in milk components and hematology of the studied animals associated with the intramammary administration of the PPy-based formulation. A better understanding of the pharmacokinetics and pharmacodynamics of soluble polypyrrole in the intramammary environment could result in improved formulations that will enable the use of this compound to treat infectious mastitis in bovines.

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DECLARATION OF CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

AUTHORS' CONTRIBUTIONS

APPA and MMC contributed with the projection and conception of the experiments. APPA, RFSS and MPA performed the in vivo animal experiments, APPA e DCNS carried out the laboratorial analysis. APPA, RMP e MMC supervised and coordinated the in vivo experiments. RMP was responsible for the statistical data analysis. APPA, RMP, MMC and RFSS prepared the draft manuscript. HPO e FAGSJ produced the PPy solution and SA formulated the experimental ointment. All authors critically reviewed the manuscript and approved the final version.

ETHICS AND BIOSAFETY COMMITTEE

All experimental procedures in this study were approved by the Ethics Committee on Animal Use (CEUA) of Universidade Federal do Vale do São Francisco (UNIVASF) under the register number 0001/130220.

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