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Efficacy Prediction of Autologous Platelet-Rich Plasma (PRP) against Sub-clinical Bubaline Mastitis

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Key words
Bubaline mastitis, Riverine-type buffaloes, SCM, PRP, Antibiotic, SCC

ABSTRACT

The current study was conducted on riverine-type buffaloes suffering from sub-clinical mastitis (SCM). The domestic water buffalo (*Bubalus bubalis*) provides milk, meat, hides, and draught power and gives a significant share to the agricultural economy of the globe. The river buffalo and the swamp buffalo are two distinct types of water buffalo that are present in different places of the world. River buffalo have been preferred for their superior ability of milk production. Mastitis is among the topmost health concerns of farm animals, causing massive financial damage to the agro-livestock sector globally. A total of 96 udder quarters/teats of buffaloes with SCM were arbitrarily allotted to three different groups, i.e., platelet-rich plasma (PRP) group (n=32), antibiotic group (n=32) and PRP plus antibiotic group (n=32). Whole blood was taken from the animal's jugular vein, and PRP was prepared by double spin open method and infused intra-mammary to the same animal from which the blood was taken. An intra-mammary antibiotic was administered intra-mammary to the animals of the antibiotic group. PRP plus antibiotic was allocated to the animals of the combined associated treatment group. Platelets significantly increased ($p<0.05$) and decreased WBCs and RBCs values in PRP. A significant decrease ($p<0.05$) was observed in the somatic cell count (SCC) of milk of animals treated with PRP. The use of PRP revealed 9.67 times higher chances of recovery compared to antibiotic alone, whereas the combination of PRP plus antibiotic was 4.33 times more effective than antibiotic only. Moreover, PRP was 55% more productive than the combination of PRP plus antibiotics. The area under the curve calculated by receiver operating characteristic (ROC) analysis predicted an acceptable area under the curve (0.73) regarding the efficacy of autologous PRP alternative to the antibiotic in sub-clinical bubaline mastitis. A significant ($p<0.05$) difference was observed in the mean rank difference among the three treatment groups through the non-parametric Kruskal-Wallis test. It was concluded that PRP plays a crucial role in tissue repair and regeneration of the injured mammary gland of buffaloes.

INTRODUCTION

Bovine mastitis is a remarkable confront worldwide menacing the dairy industry, distressing the quality and quantity of milk, and causing considerable monetary losses (Abebe *et al.*, 2016; Kovačević *et al.*, 2021). Mastitis is comprised of two main categories, i.e., clinical and sub-clinical. Clinical mastitis can be identified by the microbiological, chemical, and physical changes in milk, such as the presence of pathogenic microorganisms, a change in the milk's composition, and alterations in the udder's color and firmness (Brennecke *et al.*, 2021). SCM is symptomless, distinguished by increased somatic cell count. It can merely be analyzed by laboratory tests such as the California mastitis test (CMT), somatic cell count (SCC), Electrical conductivity, and white side tests (Radostits *et al.*, 2007; Hoque *et al.*, 2015). It is noteworthy that economic losses caused by sub-clinical mastitis are 3-4 times more than clinical mastitis and 17.2% milk production decrease in sub-clinical mastitis besides the manifestation of any apparent clinical signs (Mungube *et al.*, 2005). *Staphylococcus aureus* is a significant cause

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of anxious stern mastitis in livestock (Liu *et al*., 2022). *S. aureus* is becoming progressively resistant, which poses a threat in curative areas to the dairy business worldwide (Javed *et al*., 2021).

At present, mastitis is managed by the use of antibiotics (Royster and Wagner, 2015). Antibiotics utilized carelessly to cure *S. aureus* bovine mastitis may harm the immune system of the mammary glands and cause bacterial resistance (Dad *et al*., 2022). The pecuniary impact of mastitis and antibiotic resistance force the quest for non-antibiotic treatment options (Ijaz *et al*., 2021). Dairy cattle mastitis is cured alternatively by platelet concentrate (PC) (Constant *et al*., 2023). Blood-derived platelet-rich plasma (PRP) is a biological substance utilized in the regenerative cure of several pathological conditions. It is distinguished by an elevated concentration of platelets (Minimum three while the physiological quantity), which holds several growth factors with bactericidal, anti-inflammatory, and regenerative capabilities. PRP has been manifested to be helpful in the cure of bovine mastitis with an effect similar to that of antibiotics (Lange-Consiglio Anna *et al*., 2021). A platelet mass in plasma higher than the "usual" physiologic amount in whole blood is called PRP (Blair and Flaumenhaft, 2009).

Growth factors and cytokines are essential to the healing process and are known to be abundant in PRP (Farghali *et al*., 2017). Cytokines are censorious to countless basic homeostatic and pathophysiological progressions like inflammation, fever, tissue repair, wound healing, and fibrosis. They control cellular processes like migration, proliferation, and matrix synthesis (Gharee-Kermani and Pham, 2001). Platelets detain around 50–80 α-granules, comprising various growth factors and a century of bioactive proteins (Blair and Flaumenhaft, 2009). Platelet-derived growth factor (PDGF), connective tissue growth factor (CTGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), transforming growth factor-beta 1 (TGF-β1), hepatocyte growth factor (HGF), insulin-like growth factor (IGF) and vascular endothelial growth factor (VEGF) are the supreme significant growth factors in this framework (Neumüller *et al*., 2015). Growth factors are stowed in the platelet's α-granules, and a rise in platelet mass increases growth factors' mass. Many *in-vitro* investigations have revealed that numerous growth factors have a direct dose–response effect on the proliferation of cells, migration, and matrix production (Abrahamsson, 1997; Thomopoulos *et al*., 2005; Costa *et al*., 2006; Dhurat and Sukesh, 2014). The infection of the bovine udder persuades an assortment of alterations in the expression of genes coding for various growth factors; therefore, it may propose their likely character in glandular tissue defense or healing progresses (Lange-Consiglio *et al*., 2014). In this context, it has been suggested that administering these growth factors locally using PRP could improve the local curative environment and the capacity of pathologically impaired tissues to produce a restoration response (Marx, 2004).

Enriched plasma has been chiefly employed in the field of veterinary to cure ulcers of the skin, profound wounds, chronic wounds, and canine fistulas (Kim *et al*., 2009) and help to restore enteric ailments in pigs (Arshdeep, 2014). PRP is used to cure tendon diseases in sports horses (Georg *et al*., 2010) and bovine mastitis (Lange-Consiglio *et al*., 2014).

Currently, there is lacking published data regarding the autologous intra-mammary treatment of PRP in riverine-type buffaloes. This disease is being managed with antibiotics causing animal and human health hazards with antibiotic residues and resistance. PRP promotes the regeneration of secretory tissue of the udder by delivering a significant amount of growth factors and cytokines to that anatomical location. Therefore, these studies will help favor the prognosis of SCM in dairy buffaloes by using intra-mammary PRP therapy.

MATERIALS AND METHODS

Experimental animals

The lactating buffaloes were enrolled in these studies. The inclusion criteria for animals were those who have not received antibiotic treatment for at least 14 days before the start of the experiment. A total of 60 animals (96 udder quarters/teats) from lactating buffaloes were included in the analyses. The study was conducted in six different nearby located private buffalo dairy farms in the district Lahore-Pakistan. The Lahore district is situated at 31.5204 N, 74.3587 E, with an altitude of 217m. The feeding and drinking system of all animals in the dairy farms was the same.

Diagnosis and confirmation of sub-clinical bubaline mastitis

Initially, the milk samples were screened through California mastitis test (CMT) Kit (Portland, ME, USA) for sub-clinical bubaline mastitis (Ahmed *et al*., 2022). The positive samples were divided into three different treatment groups, i.e., PRP group (19 animals), antibiotic group (21 animals), and PRP plus antibiotic group (20 animals), each having 32 udder quarters/teats.

Further diagnosis of CMT-positive samples was confirmed by somatic cell count (SCC) values of more than 200,000 cells/ml of milk and bacterial growth on 5% sheep blood agar and MacConkey agar plates.

The somatic cells were stained by Newman's Lampert stain and were counted by the microscopic count method described by Schalm (1971).

Bacteriological culture

For bacteriological colture the milk samples were cultured according to Guha *et al*. (2012), instantaneously. SCM positive was defined as an animal culturally positive for at least one quarter.

Treatment of mastitis

The treatments were administered at a dose rate of PRP=5ml/teat, antibiotic=10 ml/teat, and PRP plus antibiotic (5ml+10ml)/teat to the identified udder quarters having SCC of more than 200,000 cells/ml of milk and culture positive through intra-mammary infusion into the teat canals once daily for three consecutive days, immediately after routine afternoon milking. Milk samples from buffaloes were obtained following National Mastitis Council standard practices (Adkins *et al*., 2017). After removing the initial three streams, about 40 mL of quarter milk was procured into 50 mL sterile tubes and kept on ice until delivery to the laboratory for evaluation. For further testing, entire milk samples were split into portions, as described by Yang *et al*. (2019).

Whole blood (30ml) was collected aseptically from animals (Khan *et al*., 2021) of the PRP group and PRP plus antibiotic group once daily for three consecutive days from the jugular vein of each identified animal through 16-18 gauge needles for PRP preparation by the double-spin open method according to Dashore *et al*. (2021) and Dhurat and Sukesh (2014), with minor modifications. On average, 5 ml PRP was harvested from 30 ml of whole blood. An Automatic Hematology Analyzer machine assessed the number of platelets in whole blood and PRP. The average 1 x 10^9 platelets/ml was found to be the concentration of platelets. PRP was aliquoted in 5 ml dosages that were ready to use. The PRP was infused intra-mammary through the teat canal into the same animal once daily for three consecutive days from which the blood was taken.

For treatment of mastitis SPECTRAMAST® LC, an intra-mammary sterile antibiotic (ceftiofur) suspension, was used to treat buffaloes with SCM (10 ml of suspension contains: active substance: 125 mg Ceftiofur equivalents (as the hydrochloride salt), 700 mg microcrystalline wax, 500 mg oleoyl polyoxylglyceride, cottonseed oil (q.s.), after routine milking, infused antibiotic through the teat canal into udder quarters at 24-h intervals for three consecutive days.

Statistical analysis

The unpaired student t-test analyzed the cellular values of whole blood and PRP by GraphPad Prism 9.5.1. SCC count was analyzed by two-way ANOVA using Minitab® 21.3.1. To analyze the impact of various treatment combinations on the outcome of sub-clinical bubaline mastitis (recovered or non-recovered), a binary logistic regression model was used through Minitab® 21.3.1. Moreover, the mean rank difference among the three treatments was analyzed through the non-parametric Kruskal-Wallis test, followed by Dunn's multiple comparison test. The P-value was considered significant if less than 0.05 (α= 0.05).

RESULTS

Figure 1 shows a highly significant ($p<0.05$) number of platelets recorded in PRP compared to whole blood. Conversely, a high number ($p<0.05$) of WBCs and RBCs were present in the whole blood. The somatic cell count did not show highly significant differences among the groups at initial sampling, including day 0 to day 3 (Table I). A substantial decline in the somatic count was recorded in the treated groups after day 7. Moreover, the PRP-treated group revealed a marked amelioration at days 14 and 28 compared with the PRP plus antibiotic and antibiotic treated animals (Table I). The binary logistic regression results showed that PRP was 9.67 times more effective than antibiotics alone. In contrast, the combined treatment of PRP plus antibiotic was 4.33 times more active than the antibiotic alone. Whereas PRP alone was 55% more dynamic than the combined treatment of PRP plus antibiotic (Table II and Fig. 2).

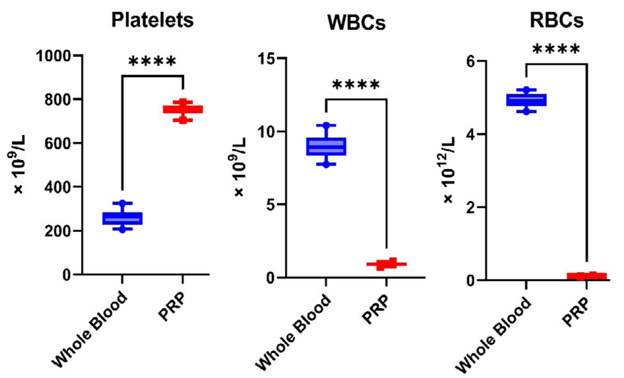

Fig. 1. Box and whiskers plot for cellular concentrations (platelets, WBCs, and RBCs) in whole blood and PRP.

There was no significant ($p<0.05$) mean rank difference between PRP and PRP + antibiotic, but a significant ($p<0.05$) difference was observed between PRP and antibiotic and PRP + antibiotic and antibiotic (Fig. 3).

Table I. The somatic cell count (Mean ± SEM) was recorded in each treatment group on different days and analyzed through two-way ANOVA using Minitab® 21.3.1. Cells that have different superscripts are significantly different (p<0.05).

Days	SCC × 10^4 per ml of milk		
	PRP	PRP + antibiotic	Antibiotic
0	266.969±27^{abc}	287.031±29.6^{ab}	245.531±26.2^{abc}
1	294.406±22.6^{ab}	327.188±23.1^{a}	281.344±21.7^{ab}
3	255.719±19.5^{abc}	216.687±19.5^{bcd}	208.562±17.5^{bcd}
7	187.094±17.7^{cdef}	202.5±15.3^{bcde}	181.5±19.8^{cdef}
14	105.625±8.7^{fgh}	114±23.1^{efg}	125.25±12.6^{defg}
28	19.969±4.28^{h}	38.062±12.3^{gh}	61.437±9.7^{gh}

Table II. Odd ratios for categorical predictors.

Level A	Level B	Odds ratio	95% CI
PRP	Antibiotic	9.6667	(2.4423, 38.2612)
PRP + antibiotic	Antibiotic	4.3333	(1.4052, 13.3627)
PRP + antibiotic	PRP	0.4483	(0.1017, 1.9760)

The odds ratio for level A relative to level B.

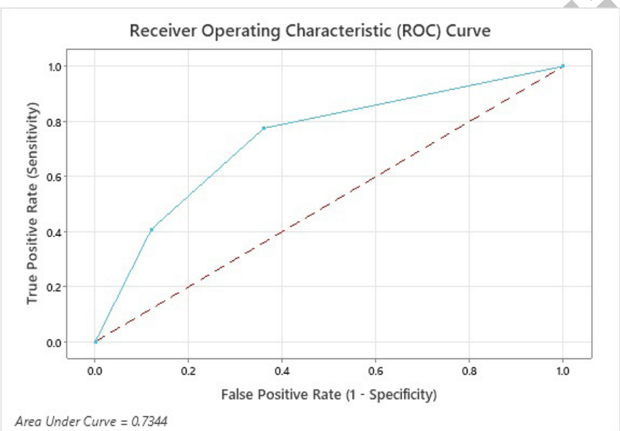

Fig. 2. Receiver Operating Characteristic (ROC) curve predictive of the therapeutic efficacy of PRP against Sub-clinical bubaline mastitis. The area under the curve (0.73) indicates the autologous PRP is an acceptable therapeutic.

DISCUSSION

The udders and milk of dairy animals are both still prone to bacterial infection, which constitutes a severe warning to the security and safety of food (Aqib *et al.*, 2022). In the present study, we prepared PRP by the double-spin open centrifugation method. An average of 5 ml PRP was produced by 30 ml of whole blood. Previous studies and the American Association of Blood Banks' technical manual have affirmed that the double spin procedure is the accepted way to prepare PRP (Sweeny and Grossman, 2002; Dhurat and Sukesh, 2014). Conversely, Harrison *et al*. (2020) reported that the single spin technique reduces platelet yield by 53% and is not favored. Depending on the individual baseline platelet count, the tool utilized, and the method, a 30 cc venous blood draw will produce 3-5 CC of PRP (Dhurat and Sukesh, 2014). To prevent untimely activation of platelets, the PRP elements were collected with the help of sodium citrate-like anticoagulant (Harmon and Rao, 2013; Patel *et al*., 2013). Preparing PRP for clinical or research purposes, sodium citrate is a typical anticoagulant (Arpornmaeklong *et al*., 2004; Ogino *et al*., 2005; Dallari *et al*., 2006; Plachokova *et al*., 2006; Sarkar *et al*., 2006).

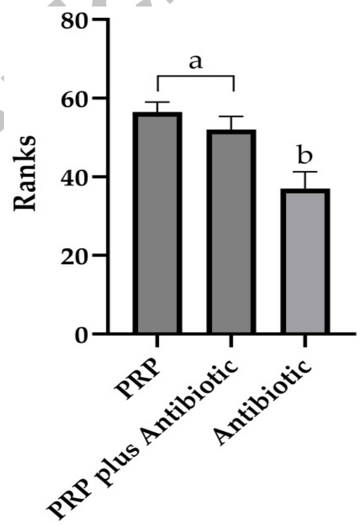

Fig. 3. Comparison of the mean rank difference of autologous PRP, combination of PRP + antibiotic and antibiotic against sub-clinical bubaline mastitis. The rank means having different superscripts are significantly different at (P < 0.05).

The current studies showed superior chances of recovery versus antibiotic alone, while the adjunct therapy by PRP + antibiotic was 4.33 times more potent than antibiotic alone. However, PRP alone was 55% extra productive than the adjunct therapy of PRP plus antibiotic. Recently, intramammary platelet concentrate administration was shown to be as effective as an antimicrobial agent in treating and preventing SCM recurrence in dairy cattle (Dal *et al*., 2019). Italian researchers reported the primary clinical use of PRP for mastitis through intramammary route administration PRP may be helpful for a fast recovery of the inflammatory retort by playing a part in restricting the tissue injury of the mammary gland parenchyma and lowering the reappearing rates. According to the results, PRP was effective in treating both acute and chronic clinical mastitis (CM) in cows caused by Gram-positive and Gram-negative bacteria; further, the combined effect of platelet concentrate and the antibiotic was significantly well executed versus the antibiotic only in recovering the afflicted mammary quarters or lowering the SCC (Lange-Consiglio *et al*., 2014). Platelet granules have growth factors and antimicrobial peptides and contain serotonin, catecholamines, osteonectin, proaccelerin, von Willebrand factor, and other chemicals. After platelet aggregation, they are released in large amounts and may have antimicrobial properties (Bielecki et al., 2007). According to Drago *et al*. (2013) and Li and Li (2013), PRP exhibits potent antibacterial action against group A streptococci and methicillin-resistant MRSA. The ideology behind the PRP application revolves around the regenerative abilities of its growth factors when deposited locally to specific tissues and cells (Dhurat and Sukesh, 2014; Chang *et al*., 2018). In addition to their hemostatic abilities, platelets are rich in cytokines and growth factors (GFs) that can impact cell proliferation, angiogenesis, and inflammation (Alves and Grimalt, 2018). An antimicrobial effect may promote faster wound healing when PRP is applied for wound therapy. It is well known that platelets have antimicrobial action against biofilms and individual bacteria. They quickly aggregate at the site of endothelium injury brought on by microbial colonization and serve as the body's paramount natural protection against infection. Growth factors (GF) and abundant cytokines in alpha granules promote an inflammatory response and recruit immune cells to the injury site to fight infections (Yeaman, 2010). Platelet-related blood derivatives are the significant origin of growth factors (GFs) like PDGF and TGF-β_1 amid other poly peptides essential for minimizing inflammation and wound relieving (Giraldo *et al*., 2015). When platelets come into contact with bacteria, they take part in bacterial co-adhesion, which causes bacteria to be sequestered and phagocytosed (Różalski *et al*., 2013). Neutrophils can connect with endothelial cells and leucocytes in cell-to-cell connections with platelets (Klinger and Jelkmann, 2002).

Antibiotics can be administered systemically by injection into the body or intra-mammary infusion by being pushed upward through the teat canal (Ibrahim, 2017). Ceftiofur, cephapirin, and pirlimycin antibiotics most frequently treat bovine mastitis in North America (Roy and Keefe, 2012). The third-generation cephalosporin antibiotic ceftiofur is regularly utilized in the dairy business. Ceftiofur, marketed for veterinary use in the United States and Europe, is the medicine of choice for treating mastitis on most dairy farms (Ganda *et al*., 2017; Durel *et al*., 2019). Ceftiofur is bactericidal by impeding the enzymes vital for synthesizing peptidoglycan, which results in bacterial cell lysis and thus inhibits bacterial cell wall synthesis (Hornish and Katarski, 2002). Oliver *et al*. (2004) assessed the effectiveness of intramammary therapy of extended ceftiofur in lactating dairy cows for the treatment of subclinical mastitis using the bacteriological treatment proportions established on 14 and 28 days negative culture after the last treatment and described that treatment effectiveness is increased by increasing the period of antibiotic therapy in *S. aureus*, *S. uberis*, and other environmental *Streptococcus* sp.; additionally, they stated that the cure rates for coagulase-negative *Staphylococcus* sp., *Streptococcus dysgalactiae*, *Corynebacterium bovis*, *S. uberis*, and *S. aureus* were 86%, 80%, 70%, 67%, and 36%, respectively, during an 8-day extended ceftiofur treatment.

Furthermore, the impurity of animal products with antibiotics and antibiotic residues in food has become a menace to public health with the rising demand for animal proteins (Zhang *et al*., 2020). Though, the overuse of antibiotics in treating bovine mastitis is a significant issue, and antibiotic therapy is often not curative (Breyne *et al*., 2017). Therefore, it is crucial to discover new therapeutic approaches and/or minimize the application of antibiotics for infections caused by bacteria in animals (Zhang *et al*., 2020).

CONCLUSION

In the present study, PRP revealed significantly greater efficacy against sub-clinical bubaline mastitis than antibiotic therapy. It could be concluded that PRP may subdue cytokine discharge, reduce inflammation, and thus help in tissue regeneration of the injured mammary gland of buffaloes.

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Ethical statement

The Ethical Review Committee, Office of Research Innovation and Commercialization, University of Veterinary and Animal Sciences, Lahore, Pakistan approved (Approval Letter No. DR/160 Dated: 05-04-2022) the studies etiquette.

Statement of conflict of interest

The authors have to declared no conflict of interest.

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