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Clinical Study

To Evaluate Antimicrobial Properties of Platelet Rich Plasma and Source of Colonization in Pressure Ulcers in Spinal Injury Patients

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Background. Exposure of pressure ulcers (PrUs), particularly to urine and feces, leads to increased colonization of wounds. The aim of the present study was to evaluate the source of microbial colonization and antimicrobial properties of autologous platelet rich plasma (PRP) in controlling it in PrUs. *Methods*. Twenty-five patients of spinal cord injury (SCI) with at least two PrUs were taken for the study. Local application of autologous PRP on one PrU (case) was compared with saline dressing on the other PrU (control). Urine cultures, urethral meatus, PrUs, and perineal swabs were taken at weekly interval for five weeks. *Result*. Colonization rate of PrUs (case) decreased from 92% at enrollment to 24% at the 5th week but did not significantly decrease in PrUs (control) from enrollment (84%) to the 5th week (76%). Association between PrU (case) and perineal cultures was observed for *Staphylococcus aureus* at enrollment 41% ($\chi^2 = 6.76$, $P < 0.01$) and at the 2nd week 47% ($\chi^2 = 5.83$, $P < 0.05$). 47% association between PrU (control) and perineal cultures at enrollment ($\chi^2 = 4.11$, $P < 0.05$) and 29% association at the 2nd week ($\chi^2 = 8.41$, $P < 0.01$) were observed for *Staphylococcus aureus*. There was association between bacteria present in perineum/urine and those colonizing PrUs. *Conclusion*. There is a significant association between PrUs colonization and bacteria present in local environment (urine and feces). Local application of autologous PRP changes the "biological milieu" of the PrUs through its antimicrobial properties leading to reduction in bacterial colonization.

1. Introduction

Pressure ulcers are frequent problem and often difficult to heal because they lack the growth factors necessary for the healing process and are frequently complicated by super added infections [1]. Bacterial infection is one of the most serious complications impairing wound healing and tissue regeneration. Even when applying strict disinfection, bacteria can infiltrate and colonize the underlying tissues of the wound. The combination of proteolytic enzymes, toxin-rich bacterial exudates, and chronic inflammation can alter the ratio of growth factors and metalloproteinases, thereby affecting the cellular machinery needed for cell proliferation and wound healing [2, 3]. Decreasing exudates and infection risks is beneficial for the patient, the wound, and the clinician and should improve the healing rate [4]. Developing approaches and strategies that may help to control or prevent the problem of wound infections would have considerable clinical, social, and economic effects [5].

The regenerative potential of platelet concentrates (PCs) has been explored considerably during the last two decades. However, only a few studies have evaluated the antimicrobial activity of platelet materials especially in chronic wounds [5–7]. PCs comprise a high concentration of platelets containing platelet growth factors, that is also abundant in concentrated leukocytes, neutrophils, monocytes, and lymphocytes [8]. Neutrophils and monocytes are rich in granules containing myeloperoxidase, which catalyzes the oxidation of chloride to generate hypochlorous acid and other reactive oxygen derivatives that act as potent bactericidal oxidants toxic to microorganisms and fungi [9, 10]. Platelets participate in antibody-dependent cell cytotoxicity functions to kill

protozoal pathogens. Finally, platelets release an array of potent antimicrobial peptides [11, 12].

Platelet concentrates can be autologous or heterologous. Allogenic platelet gel had been used by Scevola et al. to treat PrUs in a pilot study [13]. There is a potential risk of infection transmission via allogeneic blood derivatives; all safety procedures, namely, strict screening of donors. Pre- and posttreatment screening of recipients, are required if heterologous platelet concentrates are used.

The aim of present study was to evaluate antimicrobial properties of PRP in PrUs and to know the source of colonization of PrUs by correlating with the urine, urethral meatus, and perineal cultures. It was hypothesized that PrUs are colonized by bacteria present in the local environment (urine and perineum) and local application of autologous PRP to these PrUs may help in minimizing this colonization.

2. Material and Methods

The present study was conducted on 25 patients of SCI with at least two pressure ulcers admitted for treatment and rehabilitation in our tertiary care center. This was a prospective open label controlled interventional study to evaluate the effect of treatment with local application of autologous PRP on one PrU and to compare it with saline dressing on the other in the same patient. Pressure ulcer over which PRP was applied was designated as PrU (case) and PrU over which saline dressing was done was designated as PrU (control). The largest among all PrUs was selected as PrU (case). Hundred percent compliance was observed during the study period with no withdrawal. The patients were given detailed information about the purpose of the study and written consent was obtained from all the participants. The complete history of patients was taken to rule out any other occult medical or neuropsychological problem, and the complete general physical and neurological examination was done. X-rays of the injury site as well as of PrUs sites were done. Routine haematological investigations, namely, haemoglobin, bleeding time, clotting time, blood urea, blood sugar, serum sodium ion, serum potassium ion, total serum protein, and serum albumin, were done. Urine cultures and swabs taken from PrUs urethral meatus and perineum were sent for culture and sensitivity. The swab cultures were obtained from deepest part of the base of the ulcers.

After evaluating both PrUs, any necrotic tissue or any debris in the wound such as pieces of dead skin tissue or other material was removed before first application of PRP and saline dressing. Pressure ulcers were graded according to the European pressure ulcer advisory panel (1999) [14]. Only PrUs in the stage of critical colonization were included in the study. A PrU was considered to be in the stage of critical colonization on the basis of delayed wound healing, increased pain and exudates levels, discoloration, and odor [15].

Every patient was told about the nature and etiology of the lesion and advantages of proper position and periodic postural turning. As per the institutional protocol,perineal area was cleaned after every bowel/bladder procedure with water and thoroughly dried. Antibiotic solutions were avoided unless recommended by skin specialist for perineal complications. No topical antibiotic was used in control or case groups' ulcers. Systemic antibiotics were used after culture and sensitivity if patient had systemic signs of infection like pyrexia/foul smelling discharge from the ulcer.

3. Methodology

3.1. Pressure Ulcer Dressing with PRP. Platelet rich plasma was prepared in the Department of Blood Transfusion taking all standard aseptic preparation techniques on the day of application from the patient's own blood. Platelet rich plasma preparation was performed in a sterile environment. Platelet rich plasma was created by using a Cryofuge 6000i (Thermo Fisher Scientific, Germany). A total of 30 mL of blood was drawn from the patient's antecubital vein. Blood was anticoagulated with citrate phosphate dextrose adenine (CPDA) with a ratio of 1 : 9 (CPDA : blood). After a ten-minute centrifugation at 2000 rpm, the blood was layered in three basic components: red blood cells, platelets, and platelet poor plasma (PPP).

The red blood cells layer was at the lowest level because of different sediment coefficients. The platelet layer was in the middle and the PPP layer was at the top. Red cells layer was drawn from the tube. The remainder was agitated for several seconds and underwent a second centrifugation at 2000 rpm for ten minutes; the blood was then centrifuged into two layers; the supernatant was PPP while the lower layer was concentrated platelets. About three quarters of the supernatant was discarded and the residual PRP (approximately 6 mL) was introduced into two 5 mL Vacutainer tubes. Calcium chloride (10%) was taken into a 2 mL syringe and injected into both Vacutainer tubes in a ratio of 6 : 1 (PRP: 10% calcium chloride) to activate the PRP. Vacutainer tubes were agitated for 5 to 10 seconds to initiate the gel formation. After cleaning the wound with normal saline and debridement (if needed), activated PRP was applied to the ulcer under all aseptic precautions. When activated PRP was spread over the wound, it transformed into a gel. Nonabsorbent Vaseline gauze was applied over the wound after application of PRP and then dry cotton gauze and cotton paid were applied to absorb any discharge from the wound. A transparent drape was used to seal the wound. A twice weekly dressing was done for a minimum of 10 dressings. The PRP gel was prepared afresh for each dressing. The dressing was changed earlier if contaminated by urine or faeces.

3.2. Pressure Ulcer Dressing with Saline. Another pressure ulcer was dressed daily with saline dressing as a control and sealed with a transparent drape as done in case PrUs.

3.3. Followup. Weekly swabs were taken from PrUs, urethral meatus, and perineum and sent for culture and sensitivity for five weeks. Weekly urine cultures were also done. The PrUs were observed for healing and any complications.

3.4. Statistical Analysis. Data analysis included the mean and standard deviation (SD) according to data distribution. Chi-square test with Yates correction and McNemar's test were applied for comparison of culture results. A P value of 0.05 or less was considered statistically significant. Demographic and clinical data were compared by a two-tailed paired t test which calculate the group difference and pairing effectiveness. A P value of 0.05 or less was considered statistically significant.

3.5. Statement of Ethics. We certify that all applicable institutional and governmental regulations concerning the ethical use of human in our country were followed during the course of this research.

4. Results

Table 1 shows demographic, injury, and pressure ulcer profile of the study population. Neurological deficits as per ASIA Impairment Scale were as follows: A in 13; B in 4; C in 7; D in 1; and E in none of the patients. All the patients had bladder and bowel involvement after injury. They were on clean intermittent catheterization (CIC). Nine patients had anaemia at the time of presentation. Mean duration of PrUs was 72.76 ± 22.59 (range: 27–195) days. All PrUs (case) were grade IV. PrUs (control) were grade II in 11 patients, grade III in 4 patients, and grade IV in 10 patients. Figure 1 shows the incidence of bacterial colonization of the pressure ulcers during the study period. The colonization rate of PrUs (case) decreased from 92% at the time of enrollment to 24% at the 5th week. The colonization rate of PrUs (control) decreased from 84% at the time of enrollment to 76% at the 5th week. Table 2 shows the results of the cultures from the urine, urethral meatus, and perineal cultures. All patients revealed bacterial colonization of urine at the time of enrollment which decreased to 20 patients (80%) at the 5th week (P: 0.063). Similarly, the percentage of patients having bacterial colonization on perineal cultures decreased from 100% at the time of enrollment to 92% at the 5th week (P: 0.250). Table 3 shows the result of bacterial colonization of the pressure ulcers. Bacterial colonization of PrUs (case) was present in 24 patients (92%) at the time of enrollment which decreased to 6 patients (24%) at the 5th week after application of PRP (statistically significant, P value < 0.001). Bacterial colonization of PrUs (control) was present in 21 patients (84%) at the time of enrollment which decreased to 19 patients (76%) at the 5th week (statistically nonsignificant, P value 0.72). At the end of the study (5th week), 6 PrUs (case) revealed positive cultures in comparison to 19 PrUs (control) and the difference was statistically significant (P value 0.007).

Association between the growths of *Staphylococcus aureus* in perineal cultures and cultures from PrUs (case) was observed. At enrollment, association was 41% ($\chi^2 = 6.76$, $P < 0.01$) and at the 2nd week was 47% ($\chi^2 = 5.83$, $P < 0.05$). Association between the growths of *Staphylococcus aureus* in perineal cultures and PrUs (control) was observed at enrollment 47% ($\chi^2 = 4.11$, $P < 0.05$) and at the 2nd week it was 29% ($\chi^2 = 8.41$, $P < 0.01$). Thereafter, association between the growths of *E. coli* in PrUs (control) and urethral meatus was observed. At the 3rd week, association between

Table 1: Demographic, injury, and pressure ulcer characteristics of the study population.

Characteristics	Values
Age (years), mean ± SD (range)	36.84 ± 12.67 (range: 20–60)
Gender, *n* (%)	
Men	19 (76)
Women	6 (24)
Mode of trauma, *n* (%)	
Fall from height	15 (60)
Road traffic accidents	8 (32)
Fall of heavy objects on back	2 (4)
Bony level of injury, *n* (%)	
Cervical	6 (24)
Dorsal (D1–D11)	6 (24)
Dorsolumbar junction (D12-L1)	11 (44)
Lumbar	2 (8)
Neurological level of injury, *n* (%)	
ASIA A complete	13 (52)
ASIA B incomplete	4 (16)
ASIA C incomplete	7 (28)
ASIA D incomplete	1 (4)
Body mass index, mean ± SD	
Enrollment	20.97 ± 2.96
Final	21.78 ± 2.97
Associated comorbidities, *n* (%)	
Anaemia	9 (36)
Muscle atrophy	10 (40)
Spasticity	1 (4)
Contractures	3 (12)
Pressure ulcer profile	
Incidence of PrUs per patient	2.32
Mean duration of PrUs at enrollment (days), mean ± SD (range)	72.76 ± 22.59 (27–195)
Number of PrUs, *n* (%)	
Two	20 (80)
More than two	5 (20)
Grade of PrUs ((EPUAP), *n* (%)	
PrUs (Case)	
Grade IV	25 (100)
PrUs (control)	
Grade II	11 (44)
Grade III	4 (16)
Grade IV	10 (40)
Sites of PrUs, *n* (%)	
PrUs (case)	
Sacrum	16 (64)
Trochanter	5 (20)
Ischial tuberosity	3 (12)
Malleolus	1 (4)
PrUs (control)	
Sacrum	1 (4)
Trochanter	18 (72)
Ischial tuberosity	2 (8)
Malleolus	1 (4)
Heel	2 (8)
Medial epicondyle of humerus	1 (4)

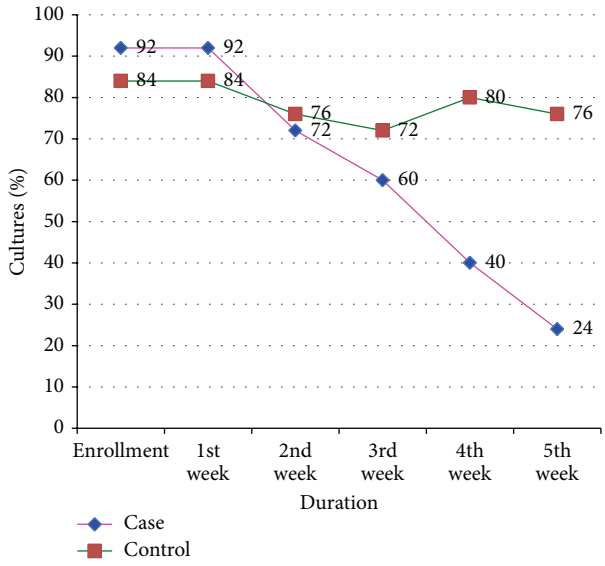

Figure 1: Result of cultures from PrUs.

the growths of *E. coli* in PrUs (control) and urethral meatus was 50%, at 4th week was 58%, and at 5th week was 81%.

Anemia was present in 9 patients at the time of enrollment which decreased to 4 patients after the 5th week of the study.

Figure 2 is the clinical pictures of the PrU (case) and PrU (control) after PRP and saline dressings.

5. Discussion

Platelet rich plasma therapy has been growing as a viable treatment alternative for a number of clinical applications and has potential benefit for use in chronic wounds. The sustained release of large quantities of autologous growth factors, cytokines, and other mediators found in PRP plus the favorable mononuclear cell profile of PRP may help in stimulating wound healing and resolving chronic inflammation [7].

The most common organisms identified from PrU (case) were *Staphylococcus aureus* and *E. coli* in the present study. In PrU (control) cultures, *Staphylococcus aureus* was the most common organism at enrollment (38.09%), at the 1st week (42.85%), and at the 2nd week (26.3%). *E. coli* was the most commonly cultured organism at the 3rd week (38.88%), at the 4th week (30%), and at the 5th week (47.36%). According to Campbell and Delgado, *Staphylococci*, *Streptococci*, *Pseudomonas aeruginosa*, and others were among the infecting organisms in PrU [16]. Salcido and Lorenzo reported in their study that the infection was the most common major complication of PrUs and the most common organisms isolated from PrUs were *Proteus mirabilis*, group D *Streptococci*, *Staphylococcus aureus*, *Pseudomonas*, and *Corynebacterium* organisms. But they did not find any synergy between various sources of infection and PrUs, as we had found in the present study [17]. A study by Mazzucco et al. showed that positive microbiological cultures in both study and control are difficult to heal wounds [18]. Gurgen reported high bacterial counts in chronic wounds as one of the causes of imbalance in wound healing process [19].

Association between perineal cultures and cultures from PrUs (case) was observed for Staphylococcus aureus growth at enrollment 41% ($\chi^2 = 6.76$, $P < 0.01$) and 2nd week 47% ($\chi^2 = 5.83$, $P < 0.05$). This indicated that infection in PrUs (case) originated from organism colonizing the perineal flora. There was an association between the growths of *Staphylococcus aureus* in perineal culture and culture from PrU (control) also at enrollment 47% ($\chi^2 = 4.11$, $P < 0.05$) and 2nd week 29% ($\chi^2 = 8.41$, $P < 0.01$). Thereafter, association between urethral cultures and cultures from PrUs (control) appeared for *E. coli*. This indicated that infection in PrUs (control) was originated from perineal flora in the first two weeks and after that organism colonizing the urethral meatus predominated to infect the PrUs (control). According to Ferrell et al. (1993), wound exposure to bacterial contamination, particularly from urine and feces, led to increased colonization of wounds and was associated with slower rate of healing [20]. Findings of the present study have implications in the management and rehabilitation of SCI patients with PrUs. Clinicians and nurses should stress the importance of high standards of patient's personal hygiene to avoid colonization of wounds.

There was significant decrease in the colonization rates of PrUs (case) (P: 0.001) during study period, but the decrease was not significant in PrUs (control) (P: 0.72). Knox et al. reported wound healing acceleration and infection-fighting properties of autologous PRP [21]. Yuan et al. observed *Staphylococcus aureus* infection in one of the three chronic wounds in 3 patients over which PRP was applied and demonstrated antibacterial function of PRP against *Staphylococcus aureus* [1]. Lacci and Dardik reported antibacterial activity of PRP against *Staphylococcus aureus* and *E. coli* [22]. Carter et al. reported that infection rates were lowered when PRP treatment was used as compared to control case [4]. Burnouf et al. reported *in vitro* antimicrobial properties of PRP against *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Staphylococcus aureus* [6]. Drago et al. reported that PRP inhibited the growth of *Enterococcus faecalis*, *Candida albicans*, *Streptococcus agalactiae,* and *Streptococcus oralis*, but not of *Pseudomonas aeruginosa* strains [23]. Frank et al. demonstrated the bacteriostatic effect of equine pure platelet rich plasma and other blood products against methicillin-sensitive *Staphylococcus aureus* in an *in vitro* study. The bacterial growth was significantly ($P = 0.01$) inhibited for pure platelet rich plasma (P-PRP), pure platelet rich gel (P-PRG), leukocyte poor gel (LPG), and platelet poor plasma (PPP) in comparison with heat inactivated plasma (IP) and the positive control group during the first 12 hours [24].

Critical colonization can result in failure of healing, poor-quality granulation tissue, increased wound friability, and increased drainage [25]. Optimal wound care to prevent progression from colonization to infection remains the foundation of good clinical practice [26]. The present study shows that local application of PRP in PrUs through its

Table 2: Results of urine, urethral meatus, and perineal cultures.

Duration	Urine culture		Urethral meatus culture		Perineal cultures	
	Number of patients found positive (%)	Common organisms (percentage of total positives at that time)	Number of patients found positive (%)	Common organisms (percentage of total positives at that time)	Number of patients found positive (%)	Common organisms (percentage of total positives at that time)
At the time of enrollment	25 (100)	*E. coli* (40), *Acinetobacter* (8), *Pseudomonas* (8)	24 (96)	*E. coli* (75), *S. aureus* (20.83), *Klebsiella* (4.16)	25 (100)	*S. aureus* (68) *E. coli* (20)
1st week	23 (92)	*E. coli* (78.26), mixed (17.39)	22 (88)	*E. coli* (59.09), *S. aureus* (22.72), *Klebsiella* (9.09)	24 (96)	*S. aureus* (50) *E. coli* (20.83)
2nd week	23 (92)	*E. coli* (69.56), *Klebsiella* (17.39)	23 (92)	*E. coli* (56.52), *S. aureus* (21.73), *Klebsiella* (17.39)	24 (96)	*S. aureus* (70.83) *E. coli* (16.66)
3rd week	22 (88)	*E. coli* (72.72), *Pseudomonas* (18.18), *Klebsiella* (9.09)	20 (80)	*E. coli* (70) *S. aureus* (10) *Klebsiella* (10)	24 (96)	*S. aureus* (70.83) *E. coli* (16.66)
4th week	21 (84)	*E. coli* (66.66), *Acinetobacter* (9.52)	20 (80)	*E. coli* (60) *S. aureus* (20) *Klebsiella* (15)	23 (92)	*S. aureus* (56.52) E. coli (34.78)
5th week	20 (80)	*E. coli* (65), *Klebsiella* (10), *Pseudomonas* (5)	19 (76)	*E. coli* (57.89) *S. aureus* (21.05) *Klebsiella* (10.52)	22 (88)	*S. aureus* (59.09) *E. coli* (27.27)
P value*	NS (0.063)		NS (0.07)		NS (0.250)	

*Shows the statistical analysis between the number of patients found positive at the time of enrollment and at 5th week. NS: not significant.

Table 3: Results of PrUs (case) and PrUs (control) cultures.

Duration	PrUs (case) cultures		PrUs (control) cultures		NS/SS (*P* value)**
	Number of PrUs found positive (percentage)	Common organisms (percentage of total positives at that time)	Number of PrUs found positive (percentage)	Common organisms (percentage of total positives at that time)	
At the time of enrollment	23 (92)	*S. aureus* (30.43) *E. coli* (30.43) *Pseudomonas* (13.04)	21 (84)	*S. aureus* (38.09) *E. coli* (33.33) *Pseudomonas* (14.28)	NS (0.66)
1st week	23 (92)	*S. aureus* (26.08) *E. coli* (26.08) Klebsiella (21.72)	21 (84)	*S. aureus* (42.85) *E. coli* (33.33) *Pseudomonas* (14.28)	NS (0.66)
2nd week	18 (72)	*E. coli* (44.44) *S. aureus* (33.33)	19 (76)	Mixed (31.57) *S. aureus* (26.31) *E. coli* (21.05)	NS (1)
3rd week	15 (60)	*E. coli* (26.66) *S. aureus* (20) *Klebsiella* (20) Mixed (20)	18 (72)	*E. coli* (38.88) *S. aureus* (27.77) Mixed (22.22)	NS (0.55)
4th week	10 (40)	*S. aureus* (30) *E. coli* (30) *Pseudomonas* (20)	20 (80)	*E. coli* (35) *S. aureus* (25) Mixed (25)	SS (0.009)
5th week	6 (24)	*S. aureus* (50) *Pseudomonas* (16.66) *Proteus* (16.66)	19 (76)	*E.coli* (47.36) *S. aureus* (36.84)	SS (0.0006)
NS/SS (*P* value)*	SS (0.001)		NS (0.72)		

NS: not significant, SS: statistically significant.
*Indicates the statistical analysis between values at the time of enrollment and at the 5th week in the same group.
**Indicates statistical analysis between values of two groups (cases and control) at one point of time.

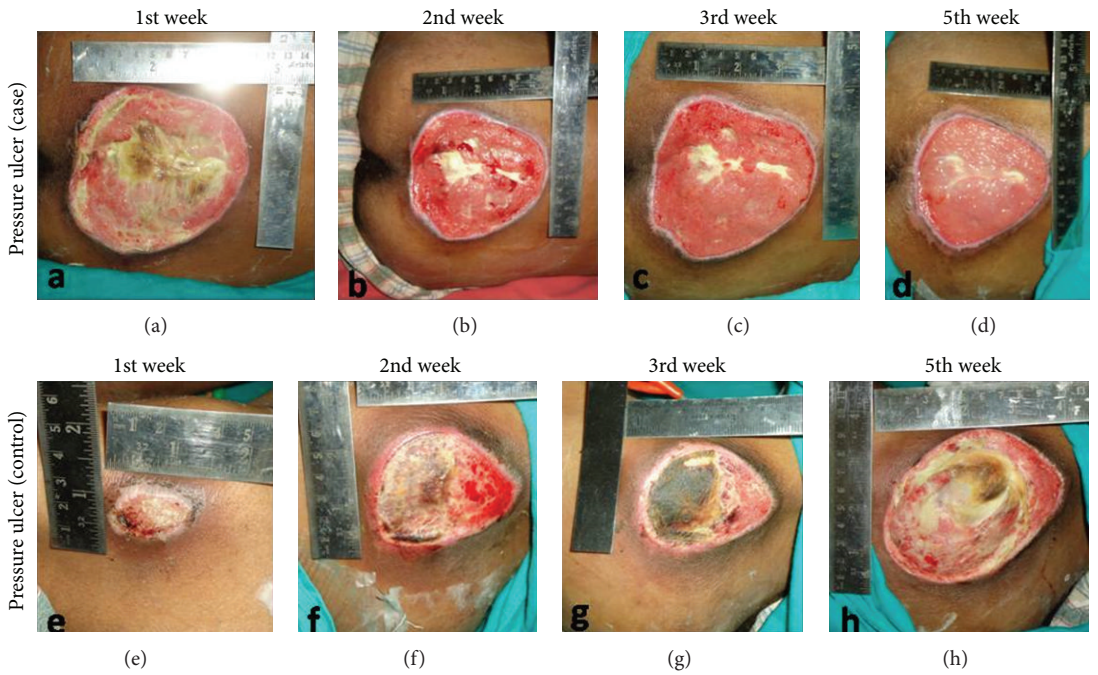

Figure 2: Clinical photographs of the sacral PrU (case) (a)–(d) and trochanteric PrU (control) (e)–(h) at 1st, 2nd, 3rd, and 5th weeks in a 27-year-old paraplegic patient. PrU (case) (a)–(d): (a) clinical photograph at first week. EPUAP grade IV PrU, wound size 54 cms^2, and PUSH score 14. (b) Clinical photograph after 2nd week of PRP application. Wound size 48 cms^2 and PUSH score 13. (c) Clinical photograph after the 3rd week of PRP application. Wound size 45 cms^2 and PUSH score 13. (d) Clinical photograph after the 5th week of PRP application. Wound size 38 cms^2 and PUSH score 12. PrU (control) (e)–(h): (e) clinical photograph at first week. EPUAP grade III PrU, wound size 8 cms^2, and PUSH score 8. (f) Clinical photograph after the 2nd week of saline dressing. Wound size 20 cms^2 and PUSH score 11. (g) Clinical photograph after the 3rd week of saline dressing. Wound size 26 cms^2 and PUSH score 13. (h) Clinical photograph after the 5th week of saline dressing. Wound size 34 cms^2 and PUSH score 13. PrU (case) is healthy, but PrU (control) has deteriorated after 5 weeks.

antimicrobial properties can decrease colonization and hence infection rate. In the another study, we have demonstrated that advanced wound therapy using local application of PRP seems to be a promising alternative to standard saline dressing in PrU healing [27].

No major complication was observed with local PRP therapy. Anaemia was present in 4 (16%) patients at the end of the study period and it was treated with blood transfusion and iron therapy. Mazzucco et al. (2004) showed that neither adverse reaction nor *in situ* recurrence was observed in 53 patients with chronic ulcers treated with platelet gel [18]. Anitua et al. reported four adverse events (ulcer bed infection: 3, anaemia: 1) in three patients after PRP treatment for chronic wounds in 5 patients, treated with oral antibiotics and oral protein energy supplements [8]. Gurgen reported infection with *Pseudomonas aeruginosa* in two chronic wounds after PRP therapy in 14 wounds [19]. Yuan et al. showed no adverse effect with PRP treatment in 3 patients with chronic wounds [1]. Singh et al. reported no systemic or wound site side effect after PRP treatment in 49 patients [27].

Limitations of the present study include that grades and anatomical distribution of the PrUs in controls and cases were not the same. However, choosing more sever PrUs as cases and showing reduction in colonization in these PrUs adds to the strength of the study; and majority of PrUs (control) were at greater trochanter (72%), which share almost same local environment as sacral ulcers. The authors did not perform any control on platelet gel preparation before application in the study. Another limitation was that we did not perform cell, bacterial, and factor estimation in the study. Future research directions include detecting what factors in PRP are responsible for these antimicrobial properties and whether improving local hygiene helps in decreasing colonization of PrUs.

From this prospective study, we reached an inference that there is association between PrUs colonization and bacterial presence in local environment (urine and feces). Local application of autologous platelet rich plasma changes the "biological milieu" of the PrUs through its antimicrobial properties leading to reduction in bacterial colonization.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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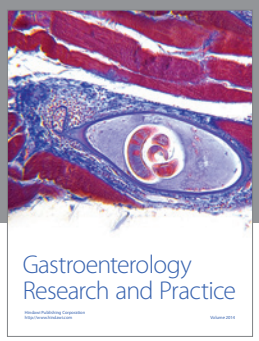

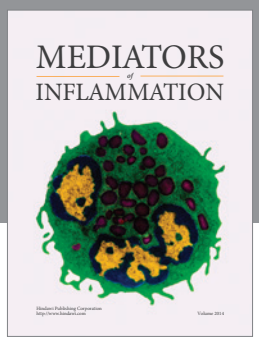

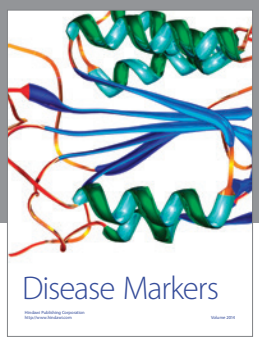

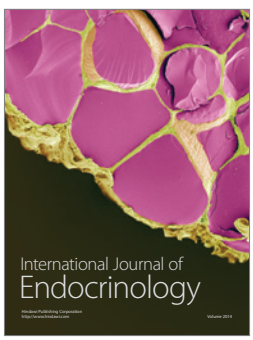

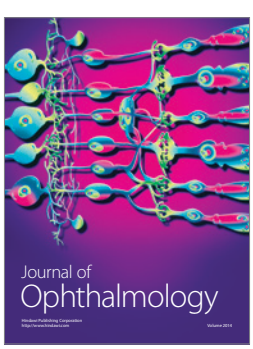

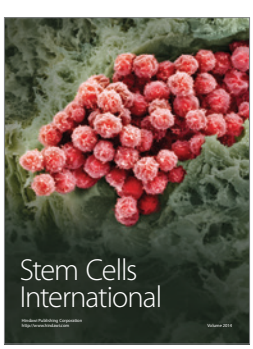

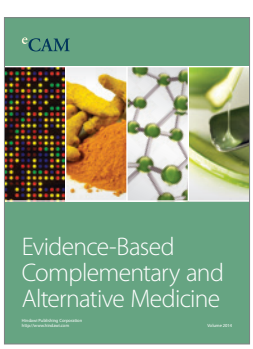

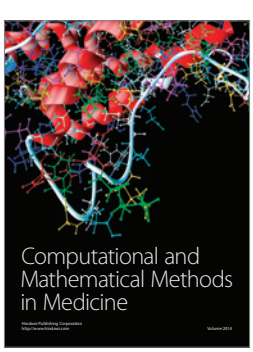

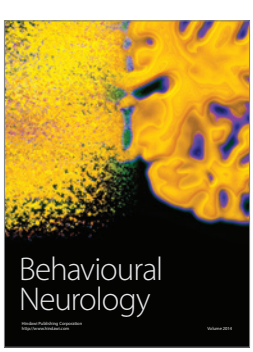

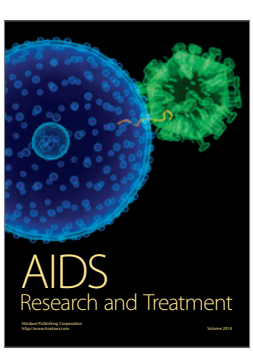

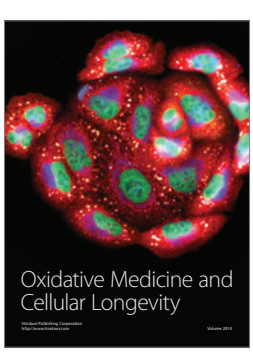

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