ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 17, Issue 2, 2024

Online - 2455-3891
Print - 0974-2441

Research Article

PROSPECT OF UTILIZING EXPIRED HOMOLOGOUS PLATELET CONCENTRATES AS AN ALTERNATIVE SOURCE OF GROWTH FACTORS AND CYTOKINES IN CLINICAL SETUP FOR BETTER WOUND CARE AND HEALING

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Received: 14 August 2023, Revised and Accepted: 26 September 2023

ABSTRACT

Objectives: The purposes of this study were: (1) To evaluate the possibility of preparation of human platelet lysate (hPL) from expired platelet concentrate, its storage – qualitative assessment in terms of growth factor content between freshly prepared hPL and preserved hPL (in liquid nitrogen for up to 6 months) and (2) to evaluate regenerative potential of the stored hPL on the 2° burn wound as compared to standard topical treatment for burns (1% silver sulfadiazine [SSD]).

Methods: hPL was prepared by three rounds of freeze-thaw cycle; concentration of growth factors was estimated by enzyme-linked immunosorbent assay method and compared between fresh hPL and stored hPL. For clinical assessment, patients with 2° burn injury were randomly allocated to SSD and hPL treatment group. Wound status was compared on 3rd week by clinical assessments and based on histopathological findings. Immunohistochemistry was performed using cell proliferative markers to corroborate the extent of healing with the number of proliferating cells that give strong positive reactions to the markers.

Results: Concentration of growth factors in tested samples (hPL fresh - <72 h, hPL-3 month and hPL-6 month) was almost same. No significant decrease in concentration was observed in any of the tested growth factor up to 6 months. p-values (analysis of variance) for concentration variations of platelet-derived growth factor-AA, basic-fibroblast growth factor,and transforming growth factor beta were 0.8981, 0.6417, and 0.9540, respectively. Healing of 2° deep-dermal burns was better for hPL group than SSD treatment at 3rd week and the observed tissue regeneration pattern was corroborated well with the expression of cell proliferative markers.

Conclusion: Our findings suggest that hPL produced from expired platelet concentrates can be used as a potential source of biological factors that could serve its therapeutic need in the field of regenerative medicine, while on the other hand avoiding the waste of valuable human physiological resources.

Keywords: Human platelet lysates, Growth factors, Long-term preservation, Wound healing, Immunohistochemistry, Ki-67, P63, CD-71, Regenerative medicine.

 DOI: http://dx.doi.org/10.22159/ajpcr.2024v17i2.49138. Journal homepage: https://innovareacademics.in/journals/index.php/ajpcr

INTRODUCTION

Platelets play a central role in hemostasis and are also critically involved in complex cellular events of healing. Various growth factors and other signaling molecules are released from the platelets that play central roles in tissue regeneration.

However, globally, non-utilization of platelet due to short expiry period (5–7 days between the date of preparation and before transfusion) results in wastage of 10–20% of total collected platelet units [1-4].

To avoid the inevitable and conventional loss of expired platelets which are usually discarded by the blood banks, could alternatively be utilized as a robust source of growth factors and cytokines in a form of human platelet lysates (hPL). *In vitro* studies have reported that expired hPL could promote the growth and differentiation of adipose tissue-derived stromal cells [5] and mesenchymal stem cells (MSCs) [6]. Keeping this in mind, we presume that hPL could also be utilized as a source of important growth factors to promote tissue regeneration and wound healing.

Platelet contains α-granules which is an intercellular storage of a few fundamental protein factors that include platelet-derived growth factor (PDGF), vascular endothelial growth factor, epidermal growth factor, insulin-like growth factor, transforming growth factor beta (TGF-β), fibrin, fibronectin, and vitronectin [7,8]. Following activation, α-granules fuse with the platelet cell membrane that results in the onset of series of events. Firstly, a few secretary proteins are transformed into their bio-active form and released from the α-granules. Later, those active proteins attach themselves to the transmembrane receptor of the target cells, followed by activation of intercellular signal molecules which directs the complex events of wound healing [9].

Over the past few years, these growth factors are being utilized in the form of autologous platelet-rich plasma (PRP) for a wide range of clinical applications [9-16]. However, reportedly, in few cases, clinical outcome of autologous PRP has been controversial [17,18] which is probably due to variable quality of PRP in terms of platelet count, growth factor concentration [19,20], and physiological conditions of the patients [12,18,21].

Contrast to these backgrounds, hPL could be utilized to overcome the inherent drawbacks of the autologous PRP. Because hPL is a derivative of platelet concentrate which is isolated from healthy individual in relatively large volumes and thus the qualitative variation would not be of much concern. Furthermore, the use of hPL holds few advantages over autologous PRP. Firstly, it does not require a procedure of harvesting a large volume of patient's own blood and thus eliminating the additional health burden on the patients as well as saves time involved in that

procedure. Secondly, unlike autologous PRP, use of hPL would allow a clinician for recurrent and frequent clinical application of it. However, so far utilization of hPL for wound healing is rarely investigated and reported with negligible immunogenicity [22].

Recently several in vitro studies have been reported that indicate benefits and advantages of utilization of hPL as a replacement of fetal bovine serum (FBS, widely used growth factor supplement for the in vitro cell culture) in clinical grade cell expansion to reduce chances of zoonotic contamination, potential risk of immunization, batch-to-batch variations of FBS quality as well as due to animal welfare concerns [18]. Moreover, reportedly, clonogenicity and differentiation potential of bone marrow-derived MSCs are better maintained in hPL as compared to FBS [18,23]. Pre-clinical evidences of the regenerative and cell expansion supporting features/functions of hPL are predominantly reported on MSCs.

However, recently, according to the European Pharmacopoeia (Eur. Pharm. 9.0 Chapter 5.2.12) hPL is classified as the "raw material of biological origin;" thus traceability, purification and safety concerns are guaranteed if the initial platelet concentrate are sourced from an established blood bank. However, quantitative differences between individual products (platelet concentrate unit) are obvious, therefore, pooling of products is generally applicable to rule out batch-to-batch quality variations [18] and indicated for large scale or commercial productions.

On the other hand, according to a guideline from the Paul Ehrlich institute, Germany, pooling without viral inactivation has been proven to have potentially higher risk of blood-derived infectious transmissions. However, virus inactivation is usually required when the pool size exceeds 16 units; alternatively hPL can be used as a smaller pooled product (<16 units) to meet the safety considerations. There are sufficient evidences that recommend possible utilization of expired platelet products for direct clinical use; the matter was well discussed in a forum of international society of blood transfusion on the current use of hPL in regenerative medicine [18].

Hence, in this pilot work, we have tried to find out the baseline prospects of utilizing hPL in clinical set-up to evaluate the possibility to prepare hPL from expired, non-pathogen inactivated platelet concentrate (collected on day 6) and to evaluate regenerative potential of the stored hPL on the second-degree burn wounds.

METHODS

Preparation of hPL, qualitative analysis, and microbial safety tests

15 units of platelet concentrates were collected on day 6 (earliest after expiration) from the institutional licensed blood bank, hPL was prepared to form expired, non-pathogen inactivated, and single unit platelet concentrate by three rounds of freeze-thaw cycle [5]. Every unit of hPL was then dispensed equally into three sterile 50 mL falcon tubes under aseptic condition. Two parts of hPL were utilized for clinical applications and the remaining one part of that hPL was further divided into three equal aliquots, each of which were subjected for qualitative analysis and microbial safety tests (screening for routine bacterial culture). All samples were stored at liquid nitrogen until further use. Quality analysis and microbial safety tests were done at three different time points as within 72 h (considered as fresh hPL), at 3rd months, and at 6th months, respectively. Stored hPL units were thawed at room temperature an hour before the quality analysis to estimate growth factor concentrations (PDGF-AA, basic-fibroblast growth factor [b-FGF] and TGF-β) by enzyme-linked immunosorbent assay (ELISA) method and an adequate volume of each sample were subjected for screening of bacterial contamination.

Estimation of Growth Factors and cytokine

All samples were estimated by ELISA method, human PDGF-AA (ELH-PDGFAA-1) kit, b-FGF (ELH-bFGF-1) kit, and TGF-β (ELH-TGFb2-1) kit of RayBiotech (USA) were used following manufacturers' protocol. Each test was performed in triplicate for each sample.

At respective time points, samples were thawed at room temperature an hour before the estimation. All 15 fresh hPL (1 of 3 aliquot) samples were estimated within 72 h of preparation. Remaining two2 aliquots of hPL were estimated on 3rd month (15 samples) and 6th month (15 samples), respectively.

Microbial safety test

All samples were thawed at respective time points as mentioned above and subjected for routine bacterial culture following standard protocol.

Clinical applications and evaluation of wound healing

This study was conducted on the burn victims who were treated in a Burn Unit of a Tertiary Care Hospital at Kolkata, India, and the study was approved by the Institutional Ethics Committee.

Eighteen age- and sex-matched patients with second-degree deep dermal thermal burn injury extending 10–20% of total body surface area were randomly selected and allocated equally (patients with pregnancy and any comorbidity such as diabetes, kidney disease, and cardiac disorders were not included in this study) into the comparative treatment groups; nine patients were recruited each in test and control group. Additional one patient had identical burn injuries on both the hands; the right hand was treated with hPL while the left hand was treated with silver sulfadiazine (SSD). Scheduled topical application of either hPL or SSD was given to all the patients at every alternate day. A thin layer of sterile gauze was socked adequately with hPL and applied over the wound surface. Outer surface of the gauze was covered by a double-layer jelonet to prevent outflow of hPL into the secondary dressing.

Initial status of wounds on the 1st assessment day and thereafter progress of healing was evaluated by bed-side clinical examinations. End-point assessment of wound healing was evaluated by histopathological examination of skin biopsy obtained on 3rd week; epidermal thickness and the presence or absence of surface keratinization were considered as parameters of this study. An ocular micrometer consisting of an eyepiece graticule was used to measure the epidermal thickness from the outermost layer of epidermis to the dermoepidermal junction. The mean epidermal thickness derived from three different points was considered for data analysis.

Immunohistochemistry (IHC) was performed using cell proliferative markers (P63, Ki-67, and CD-71) to justify the extent of healing with the number of proliferating cells that give strong positive reactions to the markers. The number of cell showing immunoreactivity was counted from 500 keratinocytes and expressed as percentage. Results were compared between test (hPL) and control (SSD) treatment groups.

Tissue samples obtained by surgical skin biopsies (5 mm) were taken from the wound margin and fixed in 4% formaldehyde solution for routine histopathological examination and IHC study. The primary antibody of P63 (DAKO, Denmark), Ki-67 (DAKO, Denmark), and CD-71 (BioGenex, Netherland) was applied with a certain dilution according to manufacturer's instruction. Universal detection kit containing secondary antibody (HiDefDetectionTM, Cell Marque, USA) and DAB (3, 3'-diaminobenzidine) (Cell Marque, USA) was used for final immunostaining. Reaction was stopped by washing the sections with deionized water followed by counter staining with hematoxylin.

Statistical analysis

Data for numerical variables, counts, and percentages for categorical variables have been summarized as mean±standard deviation (SD). Student's independent samples t-test was employed to compare Numerical parameters including epidermal thickness. The Chi-square test was used to compare the demographic data between the study groups, while the Fisher's exact test was employed to compare the proportions of histopathologic parameters. Analysis was two-tailed,

and p<0.05 were considered as statistically significant. GraphPad Prism 5.00 (Inc. San Diego, CA, USA) software was used to analyze data.

RESULTS

Qualitative analysis

All 15 hPL samples were estimated for the growth factor concentrations; the median concentration (marked red in respective table) of PDGF-AA, b-FGF, and TGF-β were 24.9 ng/mL, 69.2 pg/mL, and 684.5 pg/mL, respectively, whereas red boxes indicate best 10 samples, those were chosen for clinical applications (Table 1).

For assessment of bioavailability/stability of growth factors during cryopreservation, concentrations of each factor were estimated and compared on 3rd and 6th month with their fresh counterpart. Dunnett's multiple comparison test was performed to evaluate quality of growth factors at said time points, that is, hPL fresh (72 h) VshPL 3 month and hPL fresh (72 h) VshPL 6 months. No significant decrease in concentration was noticed in any of the tested growth factor up to 6 months. p-values (analysis of variance [ANOVA]) for concentration variations of PDGF-AA, b-FGF, and TGF-β were 0.8981, 0.6417, and 0.9540, respectively. The mean concentrations of tested growth factors at every evaluating time points are depicted in the Fig. 1.

Microbial safety test

Bacterial growth was not reported from any samples at any assessment time point.

Clinical study

Patients' demographic data of both the treatment groups are shown in Table 2. Patients in the hPL group ranged between 20 and 48 years in age, with mean and SD of 30.7±9.97 years. Age range in the SSD group was also 20–48 years with mean and SD of 32.1±10.2 years. There was no statistically significant difference between groups in age and gender distribution as shown in Table 2.

Histological assessment of wound healing

Micrographs of hematoxylin and eosin (HE) stained sections were evaluated. Neo-epithelium formation was found in all samples in both treatment groups. The mean epidermal thickness of hPL-treated cases and SSD-treated cases was 65.6 µm and 52.3 µm, respectively, the difference was found to be statistically significant (p=0.018). However, surface keratinization was not statistically comparable in the test and control groups (p=0.3498) (Table 3). Epithelial regeneration was regular, compact, and homogenous with uniform keratinization of overlying stratified squamous epithelium and less number of inflammatory cells were observed in HE section of hPL group (Fig. 2a) whereas stromal edema and infiltration of acute inflammatory cells were more in SSD group. Alongside epithelial regeneration and keratinization were less homogenous as compared to hPL group (Fig. 2b). Histopathological findings corroborate well with the photographs of clinical outcomes (Fig. 3a-d). Although the cosmetic outcome of hPL treatment was better than that of SSD group, the rate of healing was not found to be significantly different between said treatment groups (Table 3).

Expression pattern of Ki-67 and P63 were visibly higher in all the samples of the hPL group than that of SSD group. Percentage of Ki-67 and P63 positivity varied between 20% and 45% and 25% and 85%, respectively, in the hPL group, while the expression pattern of the said parameters varied between 15–35% and 25–65%, respectively, in the SSD group (Table 4). No negative staining was found for the said markers in any of the samples of comparative groups. The p values for variances of immunoreactivity of Ki-67 & P63 between the two groups were statistically significant (p<0.05). However, percentage of CD71 positive staining was not measurably different and did not show a significant difference, percentage varied from 50–90% to 50–75% (p=0.0891) between the respective groups (Table 4).

The expression patterns of all parameters in both the groups were depicted in the IHC photomicrographs (Fig. 4-6). Comparative differential expression patterns of the IHC markers between two treatment groups are depicted in Fig. 7.

DISCUSSION

Conventionally platelets are stored at 22±2°C with continuous and gentle agitation in polyvinyl chloride bag plasticized with di-(2 ethyl hexyle) phthalate for 5 days [24,25]; extended storage of platelet at this ambient temperature potentially increases risk of bacterial and viral contamination and also may lead to occurrence of structural lesions or morphological changes that impairs functionality of platelets [26].

Low-temperature storage of platelet could minimize the risk of microbial contamination but hypothermic storage alters the structural and functional properties of platelet [26]. However, main disadvantage of cold-stored platelet is that they rapidly disappear from the circulation of the recipient and thus such platelets are not suitable for transfusion [27].

Table 1: Qualitative analysis of hPL samples

Growth factor concentrations in fresh hPL (estimated within 72 h) samples				Values are arranged in descending order. Best 10 samples are within the red box		
S. No.	PDGF (ng/mL)	b-FGF (pg/mL)	TGF-β (pg/mL)	PDGF (ng/mL)	b-FGF (pg/mL)	TGF-β (pg/mL)
1	24.8	60.9	645.6	63.2	116.2	988.8
2	18.6	64.6	634.8	41.8	84.2	840.6
3	25.1	69.2	698.4	29.6	81.9	728.8
4	25.3	76.6	672.4	29.2	76.6	710.2
5	7.2	36.1	490.6	27.2	74.2	698.4
6	20.8	72.4	684.5	25.3	72.4	690.8
7	63.2	116.2	988.8	25.1	72.4	688.8
8	29.6	68.9	688.8	24.9	69.2	684.5
9	5.8	40.1	412.8	24.8	68.9	672.4
10	24.2	64.8	620.4	24.2	64.8	654.6
11	24.9	74.2	728.8	20.8	64.6	634.8
12	9.6	46.1	614.8	18.6	60.9	620.4
13	27.2	72.4	690.8	9.6	46.1	614.8
14	29.2	81.9	710.2	7.2	40.1	490.6
15	41.8	84.2	840.6	5.8	36.1	412.8

Growth factor concentrations in 15 fresh hPL samples were listed in the left site columns. In the right side columns, values were arranged in descending order; median values were marked in red while blue and green marked values indicate the upper and lower range of the distributions respectively. Yellow marked values within the red border indicate the values which were closer to the median value and the corresponding best 10 samples were (left side column) selected for the clinical applications. hPL: Human platelet lysate, PDGF: Platelet-derived growth factor, b-FGF: Basic-fibroblast growth factor, TGF-β: Transforming growth factor beta, PDGF: Platelet-derived growth factor, b-FGF: Basic-fibroblast growth factor

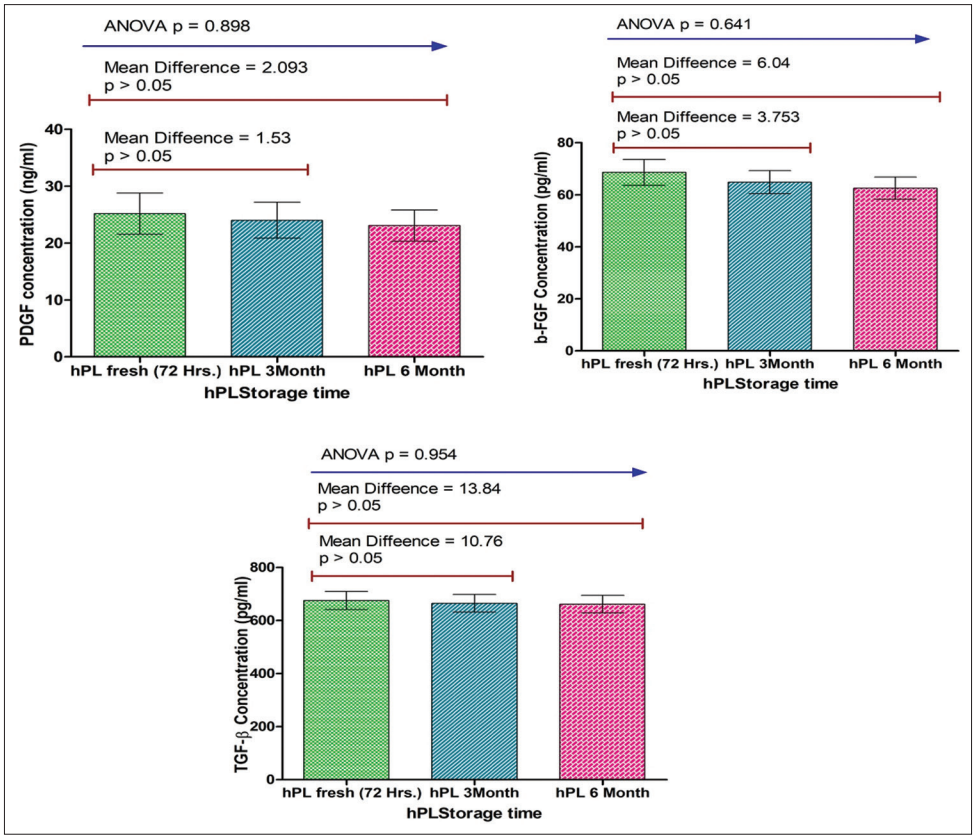

Fig. 1: Variation of growth factor concentrations in hPL at 3 and 6 months of storage in liquid nitrogen compared to baseline fresh hPL (tested within 72-h post-preparation). Bars represent mean (n=15)±SD. p<0.05 indicates a significant difference between groups with different storage time, using repeated measures ANOVA with "Dunnett's multiple comparison test" that indicates differences among the means of three groups were not significant for PDGF (p=0.8981), b-FGF (p=0.6417), TGF-β (p=0.954). hPL: Human platelet lysate, SD: Standard deviation, PDGF: Platelet-derived growth factor, b-FGF: Basic-fibroblast growth factor, TGF-β: Transforming growth factor β, ANOVA: Analysis of variance

Table 2: Demographic data of burn patients

Parameter	hPL group (n=9 ± 1*)	SSD group (n=9 ± 1*)	p-value (Student t-test and Chi-square test)
Age in years (Mean±SD)	30.7±9.97	32.1±10.2	0.76
Sex			0.62
Male	8	9	
Female	2	1	

*Indicates the same patient who had burns of identical etiology in both hand, one hand was treated with hPL while another hand was treated with SSD. Data showing the sex and age distribution of the burn patients allocated in the two comparative treatment groups; *P*<0.05 was of statistical significance.
hPL: Human platelet lysate, SSD: Silver sulfadiazine, SD: Standard deviation

Table 3: Comparison of the outcomes of two given treatments

Parameter	hPL (n=10)	SSD (n=10)	p value (t-test and Fisher's exact test)
Endpoint assessment			
Epidermal thickness in μm (Mean±SD)	65.6±12.44	52.3±10.53	0.018
Surface keratinization			
Present	7	2	0.3498
Absent	3	8	
>50% healing by 3 weeks			
Yes	8	7	1.000
No	2	3	
Complete healing by 3 weeks			
Yes	6	2	0.168
No	4	8	

Assessment on the rate of wound healing and quality of epidermal regeneration in comparative treatment groups. hPL: Human platelet lysate, SSD: Silver sulfadiazine, SD: Standard deviation

Alternatively, expired platelet may be utilized for *in vitro* cell expansion and also for therapeutic benefits. The scientific rationale why platelet derivatives have been of clinical interest is to utilize the growth factor cocktail derived from platelets for regeneration purpose. However, meta-analysis on published PRP studies has not demonstrated consistent clinical efficacy that may be due to the lack of standardization of production method and quality control. In most of the cases, autologous PRP is produced using point-of-care devices that are far from standard and regulatory oversight seen in transfusion medicine. Thus, utilizing homologous platelet concentrate from a licensed blood establishment would be a right step to produce standardized clinical-grade hPL that may be used for regenerative purpose. Due to high expiration rate of 10–20%, availability of expired platelet concentrate from a blood bank would be good enough for preparation and consistent supply of hPL in a clinical setup that may circumvent those drawbacks of autologous PRP.

However, compared to the clear benefits of hPL in expansion of MSCs *in vitro*, there is lack of scientific evidences supporting the efficacy of hPL in direct clinical applications. To overcome batch-to-batch qualitative variation, small-size pooling can be done for safety considerations. However, we have avoided pooling to bypass the chances

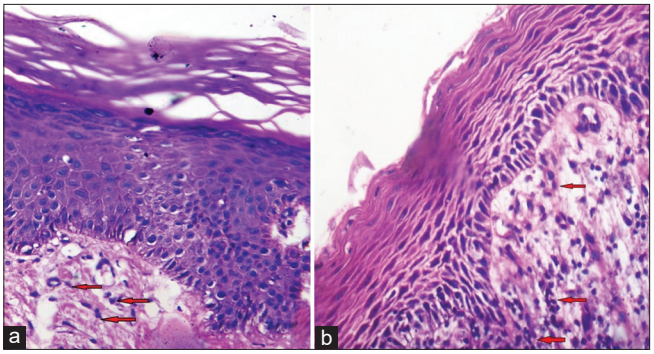

Fig. 2: Photomicrograph (40×, H and E) showing the histopathological findings in the hPL (a) and SSD (b) group. Homogenous and compact epithelial regeneration with uniform keratinization of overlying stratified squamous epithelium and less number of inflammatory cells (indicated by red arrow) were observed in hPL group as compared to SSD group. hPL: Human platelet lysate, SSD: Silver sulfadiazine

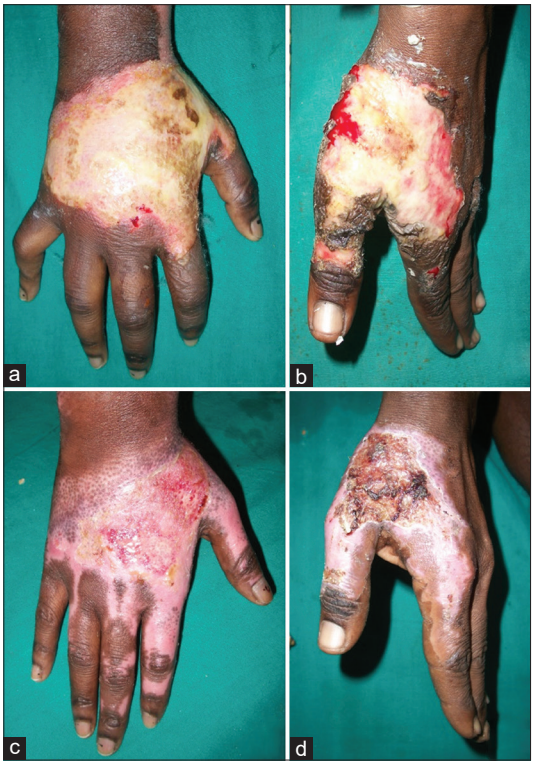

Fig. 3: (a and c) hPL and (b and d) SSD. Right and left hand of the same patient were treated with hPL and SSD respectively. Initial conditions of both the wounds were similar in nature. On the 3rd week, section (c) showing better regeneration as compared to section (d) in which presence of pseudo-eschar was evident. hPL: Human platelet lysate, SSD: Silver sulfadiazine

of contamination during manual handling of the samples; instead single donor hPL was used for each clinical evaluation. Platelet debris and micro-vesicles were also not removed from hPL as those have been reported to have functional roles in regeneration. After three rounds of freeze-thaw cycles, the whole hPL unit was opened once under aseptic condition to dispense the content of it into different aliquots which were letter subjected to quality control followed by clinical applications. Out of 15 hPL units, growth factor concentrations (PDGF-AA, b-FGF, and TGF-β) were similar (around the median range) in 11 samples, among which 10 samples were selected randomly for 10 clinical applications.

Chronic ulcers usually take several months to heal; thus to utilize hPL for regenerative purpose, relatively long-term preservation is essential that maintains its bioactivities of growth factors. Longer preservation of bioactive substances using liquid nitrogen or at −20°C/−80°C is recommended [28-30]. This study reports the possibility of 6 months long preservation of hPL in liquid nitrogen without causing degradation of growth factors and bacterial contaminations. As recommended in other studies that viral inactivation is not required for single donor hPL, thus samples were subjected for only bacterial screening. No growth was observed in any hPL samples during the study period that satisfying microbial safety consideration. Concentrations of PDGF-AA, b-FGF, and TGF-β were not differed significantly between fresh hPL than those of 3 months and 6 months cryopreserved hPL. Hence, this qualitative assessment on bio-availability of growth factors in hPL was favorable for clinical applications.

On the other hand, as delayed healing is usually associated with high treatment costs, prolonged hospital stays, and patient's quality of life, this study investigated the prospects of utilizing expired hPL on healing of burn wounds. Clinical outcomes were compared with 1% SSD treatment and justified further in terms of the degree of proliferation of epidermal cells through histopathology and immunohistochemical identification of P63, Ki67, and CD71 as the known markers expressed by the proliferating cells.

Nucleoprotein P63 is well known to have an important role in the maintenance of epidermal stem cells (ESC) populations and their proliferation during tissue regeneration [31]. Some studies have reported that P63 is specifically expressed in the ESC which remain in the basal layer of skin epithelium but not in other types of keratinocytes such as transient amplifying (TA) cells and early differentiated (ED) cells [32]. On the other hand, reportedly, another protein Ki67 is strictly associated with cell proliferations and exclusively be detected within the nucleus during all active phases of cell cycle, that is, G1, S1, G2, and mitosis but not in resting cells (G0) [33].

However, in contrast to the reports of Pellegrini *et al*. [32], we have found that P63 was expressed by all proliferating progenitors of ESC and interestingly, Ki67 was expressed only in the proliferating cells of basal layer. Similar pattern of Ki67 expression was reported by Noszczyk and Majewski [34]. Thus by comparing the expression patter of both P63 and Ki67, we assume that P63 could be a marker for proliferating population of basal keratinocytes including both ESC and TA cells, whereas Ki67 can be used to distinguish between ESC and TA cells. Strong immonoreactivity of CD71 can be seen in all types of keratinocytes including ED cells,

Table 4: Expression patterns of the cell proliferative markers

Parameters	hPL treatment group (n=10)			SSD treatment group (n=10)		
	Range of positivity (%)	Mean positivity	SD	Range of positivity (%)	Mean positivity	SD
Ki-67	20–45	38.0	8.98	15–35	26.4	7.04
P63	25–85	63.0	20.06	25–65	47.1	12.05
CD-71	50–90	69.0	15.04	50–75	58.0	9.07

hPL: Human platelet lysate, SSD: Silver sulfadiazine, SD: Standard deviation

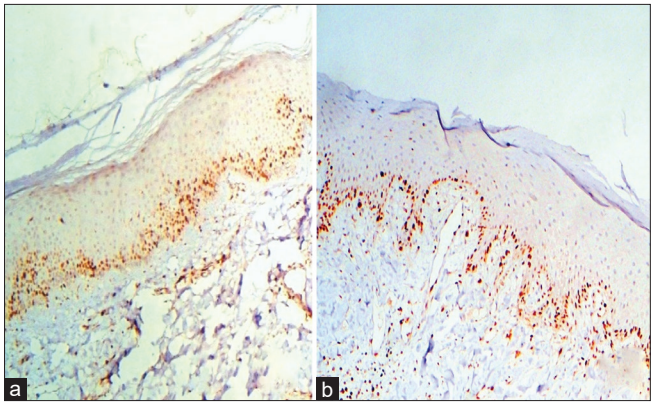

Fig. 4: (a and b) Immuno-staining (nuclear) with Ki-67 (a) (hPL group) - Ki-67 was detected abundantly in keratinocytes of both basal and parabasal layers. (b) SSD Group - Ki-67 was detected in a few keratinocytes, mostly in the basal layers but rarely in the parabasal layers. hPL: Human platelet lysate, SSD: Silver sulfadiazine

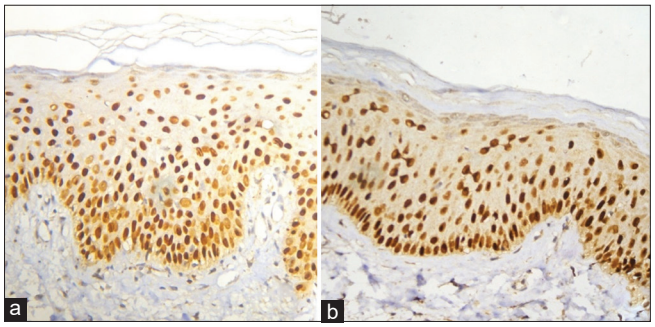

Fig. 5: (a and b) Immuno-staining (nuclear) with P63: Similar expression pattern of P63 was observed in the basal layer of both comparative IHC microphotographs (a) (hPL) and (b) (SSD). P63 positive cells were more abundantly present in supra-basal layer (spinous) of hPL group than that of SSD group. hPL: Human platelet lysate, SSD: Silver sulfadiazine

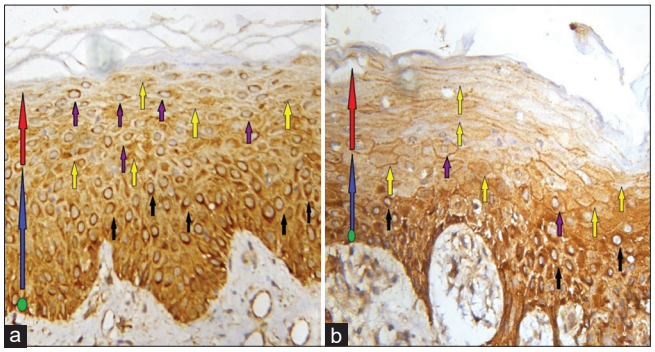

Fig. 6: (a and b) Immuno-staining (transmembrane) with CD-71: Red and blue arrow indicates the Spinous and Granular layer, respectively, green circle indicates the basal layer whereas both black and purple arrow indicates nucleated cells; purple arrow indicted cells are more differentiated (probably ED cells) than black arrow indicated TA cells. Yellow arrow indicates terminally differentiated cells (anucleated). More number of TA cells were present in the spinous layer of micro-photograph section (a) (hPL) than section (b) (SSD). Thickness and homogenecity of spinous layer was better seen in section (a). Thicker granular layer was observed in section (b) where ED and terminally differentiated cells were present abundantly. hPL: Human platelet lysate, SSD: Silver sulfadiazine, ED: Early differentiated, TA: Transient amplifying

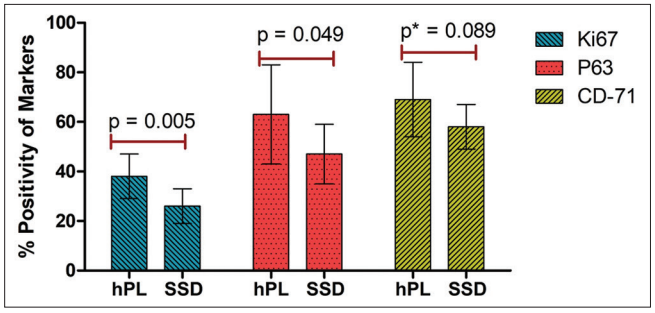

Fig. 7: Differential expression pattern of proliferating cell markers in comparative treatment groups. *p>0.05 was not statistically significant

ESCs are normally slow-cycling but have tremendous potential for cell division. On physical challenge, ESC gives rise to more rapidly dividing TA cell population which can proliferate for few rounds of cycle before entering into the post-mitotic stage within 4–5 days [35,36]. Gradual loss of proliferative potential occurs quickly for sub-population of TA cells which undergoes ED (ED cells). Amy Li *et al.* has demonstrated that tissue regeneration can be elicited by all types of keratinocytes but ED cells have limited regenerative potential [37].

Our experimental panel of IHC micrographs showing expression of CD71 was more pronounced in the basal and supra-basal keratinocytes of both treatment groups; however, the marker was expressed in all the layers of epidermis. Stratum spinosum layer was visibly thicker and strong immunoreactivity of CD71 seen in hPL group, whereas the stratum granulosum was thicker and stained more with the CD71 in SSD group. Most of the layer was covered by anucleated keratinocytes, which was probably due to the presence of more number of ED cells. Somehow ED of keratinocytes might have triggered by SSD and resulted in an undesirable aesthetic outcome finding out the exact reason behind that was beyond the scope of this research work.

Topical application of silver dressings are usually favored to inhibit bacterial infection locally, however, silver compounds (such as SSD) are known to exert cytotoxic effect on keratinocytes proliferation and migration [38]; moreover, SSD has been known to have serious disadvantages of pseudoeschar formation and associated delayed wound healing [39]. Following an injury, various overlapping phases of healing evolve in dynamic interaction between different cell types that include epidermal cells, dermal cells, and bone marrow-derived cells. Keratinocytes plays the role of "first line of defense" during tissue repair. With the release of inflammatory cytokines, keratinocytes interacts with the fibroblast which in turn activates keratinocytes in a double paracrine manner [40-42]. Activated keratinocytes migrate into the wound area, where their proliferation and differentiation continue until the cellular structure is re-established. Proper balance between proliferation and differentiation of keratinocytes are extremely essential; any dysregulation may lead to formation of scar tissue [43,44] which is undesirable from the esthetic point of view.

CONCLUSION

Expired platelet can be utilized alternatively as platelet lysate (hPL) that can be cryopreserved atleast up to 6 months without compromising microbial safety consideration and preserving the growth factors as well.

In clinical set-up, hPL can be utilized effectively as growth factor supplement for better regeneration of wounds. Rate of healing may not be faster than conventional therapeutic options but superior aesthetic outcome is expected as somehow hPL drives proliferation and differentiation of different cell types including keratinocytes in a better way.

Confirmation of these results in larger randomized controlled trials, with exploration of molecular events involved in better regeneration of tissue and pharmacological aspects of hPL utilization will be worthy areas for future study.

AUTHOR CONTRIBUTIONS

Dr. AbhishekAdhya: Concepts, design, literature search, experimental studies, data analysis, and manuscript preparation. Dr. Soumya Gayen: Clinical study. Dr. Monoranjan Sow: Clinical study. Kalyan Das: Clinical study, manuscript review, and editing. Arindam Sarkar: Reviewing concept and design of the study and monitoring clinical studies.

CONFLICTS OF INTEREST

None.

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