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Topical gel formulation and stability assessment of platelet lysate based on turbidimetric method

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Abstract

Platelet-rich growth factors have attracted attentions of scientists and clinical practitioners who are involved in wound healing and regenerative medicine extensively, according to their unprecedented potential of promoting and catalyzing healing process. Platelet-rich growth factors are cost-benefit, available and more stable than recombinant human growth factors. These appealing characteristics have converted PRGF to one of the popular candidates for treatment of variety of wounds. According to these valuable properties, we decided to formulate and assess the effect of different excipients on the stability of such valuable protein based formulations. Different excipients have been chosen according to their effectiveness on the stability of proteins and their application in other similar formulations. The stabilizing effect of excipients was evaluated by measuring heat-induced aggregation of growth factors by turbidimetric assay. Glycerol, glycine and dextrose were chosen as stabilizing excipients for these formulations. The results show that dextrose has more stabilizing effect on prevention of heat induced aggregation of the platelet lysate growth factors than glycerol and glycine. All of the formulations also contained antioxidant, chelating agents, preservative and carbopol934 in order to form appropriate gel.

Keywords: Carbopol, Dextrose, Platelet-rich growth factors, Turbidimetric assay, Wound healing.

1. Introduction

Platelets are anucleate planar cells that are derived from megakaryocytes in the bone marrow. Platelet structure is functionally constructed proper for its crucial role in hemostasis and healing process (1).

Platelets, according to histological properties, contain two distinguishable parts: hyalomere and granulomere. Hyalomere is a part that plays a role in dynamic of the cell and granulomere is a part that contains valuable granules (2). Each platelet contains three kinds of granules; alpha, dense granules and lysozyme. Alpha-granules contain more than 300 kinds of proteins that each of them plays an important role in hemostasis and healing process. For instance PDGF, TGF-β, VEGF and IGF-I, II are some of the most important growth factors which have been known for their role in cell proliferation, intercellular matrix synthesis, angiogenesis and chemotaxis properties (3, 4). Dense granules contain vasoactive amines and other bioactive molecules that have some roles in vasoconstriction, increasing permeability of capillaries and other important functions in tis-

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sue healing and clot formation (5), but the most appealing characteristic of platelet growth factors is their healing property.

In order to understand this property, we should take a look at healing process. Healing process consists of five overlapping phases; hemostasis, inflammation, proliferation, remodeling and scar formation. Briefly, at the first phase, microvascular damage will be the result of injury that leads to coagulation cascade and attraction of inflammatory cells. At the second phase, polymorphonuclear leucocytes, monocytes and macrophages will be activated. At the third phase, fibroblasts will be activated, therefore, they are going to produce collagen and form intracellular matrix. At the fourth phase, there will be equilibrium between fibroblasts and extracellular matrix, and at the end of this phase a new epithelium will be formed (scar). There is no exaggeration if we claim that growth factors control these complicated process and at the top of this growth factors are PDGF, VEGF, TGF-β and FGF, and it is clear that these growth factors are available in α-granules of platelets. So, it seems that platelet growth factors play a crucial role in healing process and wound healing (4, 6). According to this fact, platelet and its dosage form, platelet-rich plasma (PRP), can act as a promising new therapy for wound healing and regenerative medicine.

Platelet-rich plasma was applied adventurously in almost all fields of medicine that were correlated to regenerative medicine (7). For instance, PRP clinically applied in dentistry, plastic surgery (8), oral and maxillofacial surgery (9) orthopedic surgery, ophthalmology, cardiac surgery and burns (1, 10).

In the field of cutaneous wound healing, several studies have been conducted in order to evaluate the effect of PRP on chronic, diabetic, surgical and other types of wounds. Almost in all of these studies PRP was accounted as an effective treatment for these wounds (11-18). PRP is a condensed suspension of platelets in plasma, but PRP needs to be activated in order to release its growth factors and manifest its healing properties eventually. There are some methods such as thrombin, calcium and collagen type I adding or applying freeze and thaw cycles which are effective for this

purpose (19). After the release of growth factors into the plasma, PRP will not be practically applicable until being prepared as a pharmaceutical dosage form.

There are plenty of formulations that have been designed for PRP delivery. The basic concepts behind these formulations is prolonging the release profile of growth factors, extending the residential time of growth factors on the site of action and increasing the physiochemical stability of growth factors or giving a specific characteristic to the formulation according to the site of action (mucoadhesion, thermosensetive property and *etc*.)(20-28).

Despite the effectiveness of these formulations in the short term studies, there is limited stability-oriented study evaluating physicochemical properties of growth factors during the long period of time. In this project, we aim to conduct a study in order to evaluate the effect of excipients on the physiochemical stability of platelet growth factors and attempt to suggest an appropriate topical formulation for these growth factors.

2. Materials and methods
2.1. Materials

Glycerol, glycine, dextrose, sodium hydroxide, sodium chloride, sodium metabisulfite and EDTA were purchased from Merck. Carbopol934 (BF Goodrich, USA) was used as a gel former and methylparaben (Sigma-Aldrich) was applied as a preservative that is proper for proteins formulations.

Platelet-rich plasma (PRP) was obtained from Fars Blood Transfusion Organization (Fars, Shiraz). PL was obtained by conducting freeze and thaw method on PRP. For this purpose PRP was frozen at -80°C for 5 hours and defrosted at 30 °C by water bath (19, 27).

2.2. Formulation development

In order to evaluate the effects of excipients on physical stability of platelet's growth factors, different formulations were prepared (Table 1). Each of the formulations was prepared by dissolving pre-determined percentages of EDTA, methylparaben, sodium metabisulfite, sodium chloride, glycerol, glycine and dextrose in 10

Table 1. Composition of the various PRP containing formulations.

Code of Formulations	Dextrose (M)	Glycine (M)	Glycerol (M)	Water up to
F1	-	1	-	100
F2	-	2	-	100
F3	-	3	-	100
F4	1	-	-	100
F5	1.5	-	-	100
F6	2	-	-	100
F7	-	-	2	100
F8	-	-	3	100
F9	-	-	4	100
F10	2	-	2	100
F11	-	2	2	100
F12	2	2	-	100
F13	2	2	2	100
F14	1.5	2	4	100

ml of deionized water. Carbopol934 was added at the end when the other excipients had been dissolved completely. At the end, the pH of the formulation was adjusted to 7 by 4 N sodium hydroxide solution (29-35).

2.3. Turbidity study

To assess physical stability of growth factors in different formulations, turbidity study was conducted for each formulation by UV-Vis spectrophotometer (Shimadzu, Japan) at 350 nm. Heat-induced aggregation was performed by water bath (Fater Rizpardaz, Iran) equipped by a thermometer at 53 °C (36). Each of the formulations samples was prepared by mixing 2.5 ml of PL to 2.5 ml of the vehicle. The blanks were prepared by mixing 2.5 ml of the vehicle to 2.5 ml of de-ionized water. The change in absorption at 350 nm was recorded every 30 sec till the rate of increase in optical density reached a plateau.
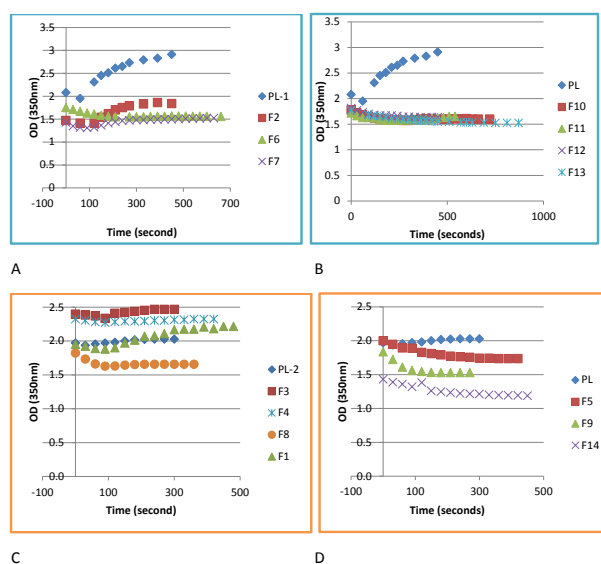

Figure 1. Turbidity profiles of different formulations contain platelet-rich plasma (PRP). A and B Heat-induced aggregation of proteins in PRP at 53 °C and absorbance determination at 350 nm versus platelet lysate from sample 1. C and D Heat-induced aggregation of proteins in PRP at 53 °C and absorbance determination at 350 nm versus platelet lysate from sample 2.

2.4. Determining the kinetics of proteins aggregation

In order to make heat-induced aggregation profile of formulations comparable, the kinetics of proteins aggregation was calculated. The order of reaction was determined by conducting graphical method (37).

3. Results and discussion

3.1. Turbidity study

With increase in temperature, proteins enthalpy and entropy will increase proportionally. Eventually, at a crucial point, entropy becomes greater than enthalpy. In this point, proteins are going to be unstable and tend to be unfolded and expose their hydrophobic portions. Proteins between this two states experience an intermediate state. In this state, proteins expose continuous hydrophobic patches that make suitable connection points for aggregation. In this situation, most of the proteins prefer to be aggregated. Aggregation is an unfavorable but stable condition that will usually happen as a consequence of physical and chemical instability of proteins (34).

Aggregated proteins are like particles that are recognizable by turbidimetric method. Heat-induced aggregation of proteins can be correlated to their innate stability and excipient-induced stability. Excipient can modify the stability of proteins by increasing or decreasing $\triangle G_{fold} \rightarrow_{unfold}$. The effect of the excipient and formulation additive on formulation stability would be recognized by the change in the slope of the line correspond to aggregation of proteins at constant temperature accordingly (34).

In order to assess the effect of excipient in different formulations, we have plotted UV absorption of formulations samples against time at constant temperature at 53 °C (Figure 1). Because we had two PRP sources from two different volunteers in this experiment, two sets of results were reported and compared. According to Figure 1, PL has the greatest slope compared to other formulations. These curves are not linear and we cannot compare their slope with each other before converting them to linear pattern by calculating aggregation kinetics.

3.2. The kinetics of aggregation

In order to compare rate of aggregation properly, it is necessary to determine the order of aggregation kinetics. With focusing on the absorption-time curve of PL more precisely, we will find out that there is a two distinct part in this graph (Figure 2). In the first part there is a descending curve whichis related to the processes that are decreasing the total optical density. These processes would be the result of some process such as lysis of the remaining platelets in the solution. Because these processes happen in very short time and have been almost disappeared before 60 sec of experiment, it doesn't seem that they confound the final
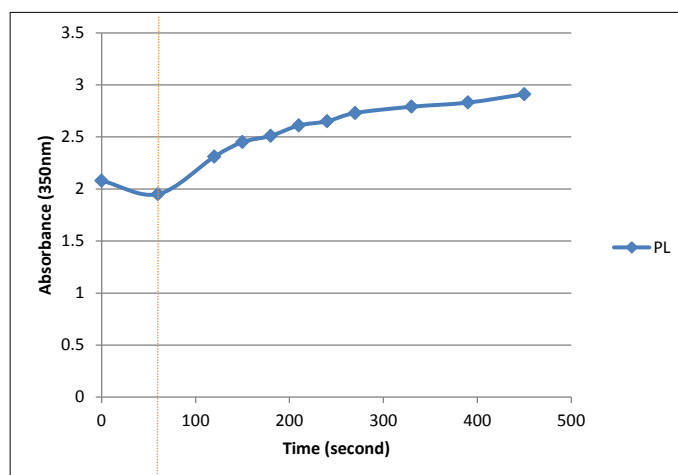

Figure 2. Optical density of platelet lysate sample versus time in 53 °C and absorbance determination at 350 nm with 2 distinct segments (A and B).

Table 2. Kinetic constants for different formulations having different excipients.

Formulations	K	$\frac{K}{K_{pl}}$
F1	0.0105	0.66
F2	0.0076	0.95
F3	0.0167	1.06
F4	0.0062	0.39
F5	No aggregation observed	-
F6	No aggregation observed	-
F7	0.007	0.87
F8	0.0197	1.25
F9	No aggregation observed	-
F10	No aggregation observed	-
F11	0.0049	0.16
F12	No aggregation observed	-
F13	No aggregation observed	-
F14	No aggregation observed	-

result of experiment. In this regard we ignored the first part of the graph and the kinetic of the aggregation was determinedconsidering the last part of the graph.

Protein aggregations may follow first or other order of kinetics (30). To determine the order of kinetic, we have applied graphical method (37). According to Figure3 and determining the correlation coefficient of the last part of the graph, aggregation kinetic was better fitted to first order kinetics because log (absorption) versus time related to PL have yielded a straight line.

If the aggregation follows first order kinetics the following exponential equation will be properly fitted to the absorbance-time curve (38, 39).

$$A = A_{\lim}(1 - \exp(-k(t - t_0)) \qquad (Eq.\ 1)$$

$$L_n \frac{A_{\lim} - A}{A_{\lim}} = -k(t - t_0) \qquad (Eq.\ 2)$$

Where A is absorbance, $A_{\lim}$ is the amount of A at $t \rightarrow \infty$ where curve reach to the plateau state. K is the rate constant and t_0 is the value of t when $A = A_0$.

$A_{\lim}$ would be figure out from absorbance-time curve. In Figure 4 these curves and their relative
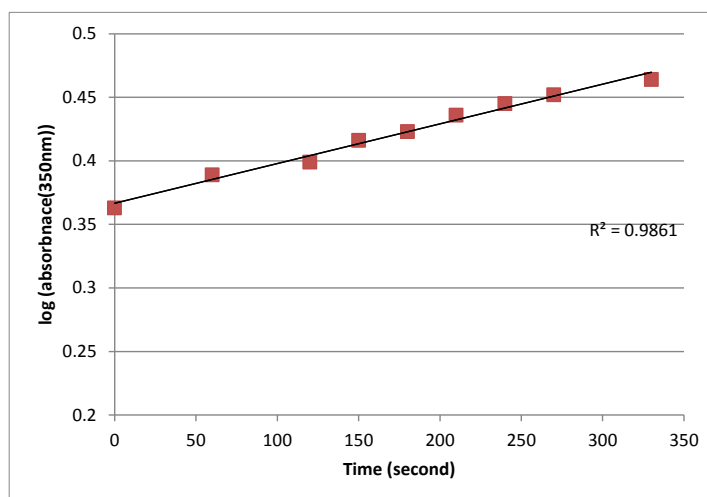
Figure 3. Logarithmic graph of optical density at 350 nm against time profile.
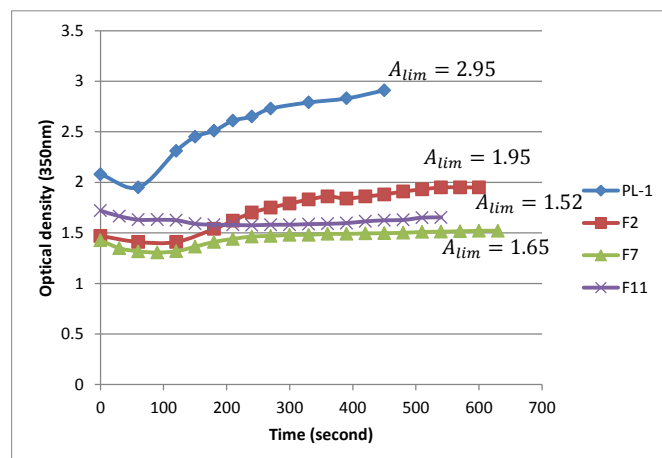
Figure 4. The ultimate optical density of different formulations (A$_{lim}$) and platelet lysate samples at 53 °C.

A_{lim} have been determined.

After determining A_{lim}, rate constant of first order kinetics (K) was calculated for different formulations by plotting $L_n \dfrac{A_{\text{lim}} - A}{A_{\text{lim}}}$ versus time where slope of this curve will give us K for each of the formulations. In Table 2 rate constants for different formulations have been calculated. As mentioned above we used two different sources of PRP in our experiments from two different volunteers that have two different growth factors contents profile. Although we will have two different results, but in order to make these results comparable, the stability ratio (the proportion of different formulations aggregation rate constant to PL aggregation rate constant) was calculated and reported in Table 2.

All of these formulations have a different rate constant which was related to the type and concentration of the various additives introduced in each formulation. All of these formulations contained equal amounts of carbopol934 as a gel former, EDTA as chelating agent, sodium metabisulfite as an antioxidant, methylparaben as a preservative and sodium chloride. Glycerol, glycine and dextrose were introduced in different quantities in various formulations to assess the stabilizing potentials of each additive in different concentration. Glycine as a protein stabilizing additive has been used in proteins formulations previously (39, 40)(40, 41). Although glycine could stabilize proteins through preferential exclusion theoretically, but in this study glycine didn't show any significant stabilizing effect on growth factors. On the other hand no significant aggregation protecting effect was observable in this study. Glycine was used in three different concentrations in this study (F1, F2 and F3) and at its lowest concentration (1M) showed the best stabilizing effect.

Glycerol also was used in three different concentrations in this study (F7, F8 and F9). Glycerol as a polyol has been used to stabilize proteins in previous studies (32). In this study glycerol showed best stabilizing effect at higher concentration (4M). According to the results of this study no aggregation was determined during the time profile of this study.

Dextrose showed significant stabilizing effect on PL growth factors. In concentrations above 1.5 M (F5 and F6), dextrose showed its best stabilizing effect and no aggregation was recorded though at 1M concentration (F4). Based on the results of this study dextrose was better stabilizing agent than glycine 2M (F2) and glycerol 3M (F8).

In the formulations that had glycerol, glycine and dextrose as a combination (F10, F12, F13 and F14), dextrose have played crucial role and stabilizedthese formulation more efficiently than each of these additive when used as single stabilizing agent. According to formulation 11 (F11), glycerol and glycine in combination showed synergistic effect in stabilizing growth factors.

4. Conclusions

In order to suggest appropriate topical for-

mulation of PRP, different formulations were designed and tested for physical stability. Although glycerol could significantly stabilize growth factors against the aggregation, but dextrose, in this experiment showed an important role in stabilizing these growth factors and can be applied individually or in combination with other appropriate excipients as a proper stabilizing additive. We should consider that this study did not assess growth factors according to their biological activity or chemical stability thus more complementary experiment

such as animal test or biological activity studies is suggested.

Conflict of Interest
None declared.

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