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A New Medical Device for Platelet Rich Plasma Filler

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Introduction- Injectable fillers are used in aesthetic medicine to reduce visible signs of facial aging.

It involves loss of volume in the skin, muscle and superficial and deep fat compartments, superficial wrinkles and deep folds (1-2).

There are different products that vary on filling capacity, longevity, potentiality for causing allergic reaction, safety, indication.

Physicians select the most suitable agent for each patient by considering the advantage and disadvantage.

In fact, hyaluronic acid (HA) has the property to increase the skin's water-binding capacity (3-5).

Calcium hydroxylapatite (6) and poly-L-lactic acid (7) have been proved to stimulate autologous collagen (8-9).

Keywords: Growth factors, PRP, regenerative medicine, face wrinkles, perioral wrinkles, dermal matrix.

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I. Introduction

Injectable fillers are used in aesthetic medicine to reduce visible signs of facial aging.

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In fact, hyaluronic acid (HA) has the property to increase the skin's water-binding capacity (3-5).

Calcium hydroxylapatite (6) and poly-L-lactic acid (7) have been proved to stimulate autologous collagen (8-9).

Although fillers have lower immunogenicity and a good safety profile, several scientific articles have showed side effects (10).

Those can vary from allergic reaction (11), inflammatory nodule formation (12), infection (13), vision loss (14) and the limited use in human immunodeficiency (15).

Platelet rich plasma (Prp) has proved to achieve regenerative capacity on human tissues (16) and positive effect on facial dermal fibroblasts (17-18).

Autologous plasma filler was first introduced by Krajcik et al. in 1999 (19) and used for the treatment of different types and grades of facial wrinkles (20-22).

The main issues of the clinical use of plasma filler is its poor filling effect and duration.

In fact, the volume achieved just after the injection lasts only few hours because the plasma is reabsorbed.

The goal of our study was the preparation of a new medical filler device able to fill soft tissues for a reasonable period of time and also containing the regenerative Prp property.

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Our first step was the formulation of a thermosensitive gelableto embed Prp, the second to evaluate the behavior of the formulation alone, in particular, its dispersibility and the homogeneity and the third the study of platelets and the growth factors behavior inside the formulation.

After a detailed research on the scientific literature (23,24) poloxamer 407 was chosen for our purpose.

Multiple reasons have corroborated this choice: first of all, the family of "poloxamer" polymers is listed in the US and European Pharmacopoeia (25) and is approved by FDA for parenteral use in humans and this ensure safety, biocompatibility and tolerability required for parenchymal use.

Furthermore, poloxamer 407, being a non-ionic block copolymer, does not negatively interact with the biomolecules embed in the gel, such as platelets and growth factors.

Again, it allows to develop thermosensitive formulations without the need of excipients in preparations.

II. Materials and Methods

1. *Preparation of poloxamer formulation*

Aqueous solutions of poloxamer 407, purchased from Sigma-Aldrich (Milano, Italy), were prepared by the so-called "cold method".

Poloxamer powder was dissolved in bidistillate water at 277.15 K (4°C) under gentle stirring to facilitate the copolymer dissolution (26).

Solutions at poloxamer concentration of 15% (w/v) were prepared with this method and stored at 277.15 K (4 °C).

Differential scanning calorimetry (DSC) was used to determine the micellization and the gelation point.

Measurements were performed using a calorimeter Mettler 821^e (Mettler-Toledo, Greifensee, Switzerland) equipped with a cooling system with liquid nitrogen.

Scans were recorded from 273.15 (0 °C) to 313.15 K (40 °C) at a heating rate of 5 K/min (27).

Measurements were performed in duplicate and the results were expressed as the mean of the two measurements.

2. Analysis of the Platelet

The analysis of the platelet contained inside the formulation could not be address by normal hemochrome automatic analysis systems.

For this reason, platelet numbers were firstly estimated by using a Burker's chamber and the Breker – Cronkite method.

Observations were performed with a Nikon Eclipse 80i microscope provided of a digital camera Nikon Coolpix 8400.

Breker – Cronkite solution was prepared adding 0,01 g of brilliant cresyl blue to 100 mL of 1% (w/v) ammonium oxalate aqueous solution.

Medical Device with Prp sample was diluted with Breker – Cronkite solution (dilution 1/100) in a Thoma pipette.

After five minutes of pipette manual shaking, a drop of diluted solution was charged in the Burker's chamber and observed at microscope with magnification 40X.

We counted platelets presents in a big square of 1 mm^2 (Figure 1).

To obtain the number of platelets within 1 μL of PRP, the average of platelets counts in three squares was corrected for the initial dilution (100) and for the height of chamber (10) (28).

For this reason, the final formula is: Platelet number counted X 1000.

Prp platelet size and number were also determined using an AccusizerTM 770 Optical Particle Sizer (PSS Inc., Santa Barbara, CA, USA), using the technology "single particle optical counting", coupled with an auto-dilution system AccusizerTM 770A Autodiluter^{PAT} (PSS Inc., Santa Barbara, CA, USA).

100 μL of solution with Prp were injected in the system and measurements were performed for 30 minutes to allow the analysis of all the particles present in the sample.

Saline (0.9% NaCl) was used for the analysis and results were expressed as total particles size and mean volume diameter (Figure 2).

3. HPLC analysis of growth factors

We analyzed the Epidermal Growth Factor (EGF) which is centrally involved in the regulation of key processes of the epithelia.

Calibration curve for RP-UHPLC analysis was obtained using an HPLC Jasco LC-2000 plus, equipped with the quaternary gradient pump PU 2089 Plus, the UV-vis detector diode array MD 2010 plus and injector Rheodyne.

Controlled EGF was purchased from Sigma-Aldrich (Milano, Italy).

The column used was a C18 Ascentis Express Peptide 150 × 4.6 mm (L × I.D.), 2.7 μm and analysis were made combining two different mobile phases: H_2O/CH_3CN (95/5 %) + 0.1 % TFA (solvent A) and CH_3CN/H_2O (95/5 %) + 0.1 % TFA (solvent B), with a flow of 2.0 mL/min (29).

The mobile phases were degassed prior to use; cell and column temperature at 313.15 K (40 °C).

The calibration curve was performed in the concentration range of 0.086-0.4 mg/mL, with an injection volume of 5 μL.

4. Rheological analysis

Poloxamer rheological characterization was performed with the aim of better understanding the thermal behavior of poloxamer formulation.

A Stresstech HR Rheometer (Rheologia Instruments AB, Milano, Italy), equipped with a Peltier device for temperature control, was used for the rheological characterization.

Viscosity was measured with a cone-plate geometry (cone angle 1°, cone-plate diameter 40 mm).

Poloxamer formulation was analyzed in duplicate at different temperature (278, 283, 288, 293, 298, 303 and 308 K corresponding to 5, 10, 15, 20, 25, 30 and 35 °C).

For formulation in sol state, measurements were performed applying a stress range from $3.044 \cdot 10^{-3}$ to 1 Pa, while for temperatures at which the formulations were already at the gel state, measurements were carried out applying a stress range from $3.044 \cdot 10^{-3}$ to 10 Pa. 12.

5. Spreadability

The gel was tested by two independent persons for the spreadability.

The gels stored at 277.15 K (4 °C) were withdraw from the recipients and spread on the skin surface.

6. Preparation of Prp

For the preparation of the Prp, Plasma Active system (CE0373 – Medical Device s.r.l. via artigianato n.6, 52022 Meleto, Craviglia (AR) – Italy) was used.

It is composed of two vacutainer tubes (*BD Vacutainer® Brand SST II)* of 9 mm each with separator gel and anti-coagulant for harvesting 18 ml of peripheral blood.

Tubes were centrifugated for 5 minutes at 1800 rpm (*Sorvall Legend XTR Centrifuge - Thermo Fisher Scientific Robert-Bosch-Straße 1 D - 63505 Langenselbold Germany*) and 8 ml of Prp were collected from the upper part of the tube in a sterile matter and placed inside the Medical Device.

7. Preparation of poloxamer containing PRP

Five mL of Prp were added to 10 mL of thermosensitive gel.

The ratio of Prp/gel was chosen by considering the future application of the thermosensitive gel and the size of the final device (30).

Results have shown that 5 mL of Prp could be easily dispersed within 10 mL of the hydrogel by stirring the cold gel (4 °C) or by agitation of the closed container.

III. Result

1. Differential scanning calorimetry (DSC) results

DSC data show broad enthalpic transitions in the formulation due to poloxamer micellization.

In fact, in aqueous solution, with increasing temperature, poloxamer aggregates in micelles to minimize the free energy of solution.

Micellization temperatures decrease with increasing poloxamer concentration (Figure 3).

Also, micellization energy transition (ΔH_{mic}) per amount of poloxamer increases with increasing concentration.

Data of micellization temperature and ΔH_{mic} of poloxamer preparation is reported (Table 1).

Very interesting, in DSC thermograms is possible to see also a little enthalpic transition at temperature slightly higher than micellization point.

This peak is more evident in solutions with higher poloxamer concentrations and it has been previously attributed to the gelation transition (Figure 4).

In agreement with this little peak, gelation energy (ΔH_{gel}) is very small (Table 2).

Gel temperatures found from this approach agree with results reported in the literature (27).

This DSC analysis give a first information about poloxamer solution thermal behavior.

In fact, the decrease of micellization and gelation temperature with increasing poloxamer concentration clearly shows how formulations with high poloxamer concentration (e.g. 25-30% w/v) pass to gel state at lower temperatures compared to the formulations with a low poloxamer concentration.

Micellization and gelation temperatures are plotted in Figure 5.

These results demonstrate that gelation point is strongly temperature and concentration dependent.

2. Rheological analysis result

In Figure 6 is shown the behavior of poloxamer formulation viscosities as a function of temperature.

In the first part of curves, solution viscosity decreases slightly on warming but, reached a certain temperature, is possible to see a steep increase in viscosity.

This viscosity increase can be attributed to the sol-to-gel transition.

Very interesting, results obtained with rheological characterization agree with gel temperatures obtained from the DSC data (Figure 3).

3. Spreadability results

The best spreading was individuated for the formulation containing 15% of poloxamer that did not gelify immediately after contact with the skin.

However, once the gel was spread it remained on surface as a thick gel.

4. Result of poloxamer containing PRP

Prp dispersed homogeneously into the matrix without forming lipid globules but changing the appearance of the gel from completely transparent to a light-yellow color (Figure 7A).

The formulation pH did not change with respect to the control formulation (blank gel).

The smell of the formulation containing PRP was slightly different from the blank gel, but it was still pleasant.

Prp loaded thermosensitive gel was stored at 4 °C for 1 month and its characteristics were evaluated 7 and 30 days after preparation.

After 7 days of storage, the gel color and smell were unchanged and, more important, Prp was still homogeneously dispersed (Figure 7B).

After 1 month of storage, the smell was unchanged while a slight phase separation was observed (Figure 7C).

This phase separation was reversible by simpler agitation.

In fact, by gently shaking the closed container, the gel assumed the same aspect and homogeneity of a fresh prepared formulation (Figure 7D).

This finding is of particular interest for the final use and we store the formulation at: 2-8 °C andshake if before use.

5. Platelet behaviour

We analyzed 10 different samples of solution of poloxamer containing PRP after 12, 23, 73, 96, 120, 144, 168 hours from the preparation and expressed the results as mean.

Platelet remained intact for 72 hours and then numbers dropped rapidly as shown on Figure 8.

6. HPLC results of growth factors

Analysis made on the 10 different samples of solution of poloxamer containing Prp after 12, 23, 73, 96, 120, 144, 168 hours from the preparation showed that the EGF concentration increased progressively and reached his peak after 4 days and then decreased progressively.

The Egf concentration remained above 2,5 μg/mL after 7 days (Figure 9).

IV. Discussion

Fillers are among the most performed cosmetic medicine procedures worldwide (31-32).

They are used mostly on the face for the reduction of superficial and deep wrinkles (32-34), for

increasing the volumes of lips (35) and cheekbones (36) and for the redefinition of the mandibular profile (37).

There are different materials used for fillers including hyaluronic acid (38), collagen (39), calcium hydroxyapatite (40-41), and polycaprolactone (42), polymethylmethacrylate (43).

They vary depending on the filling capacity, due to their ability of recalling water (44), or even for the ability to stimulate collagen (45).

The advantages of commercial fillers are the simplicity of use, as they are supplied in single-use vials and the very low incidence of major complications as embolisms (46-48).

The disadvantages are the high cost, since to obtain appreciable results, several vials are required, and because of the limited duration, the treatment must be repeated periodically (49-50).

Again, possible side effects, although minor, create discomfort to the patients and stress to the doctors (51).

And definitely, fillers do not have regenerative activities.

Prp is used in dermatological clinic for its regeneration effects on dermal cells (17).

Different studies have already shown the positive effects on the treatment of facial wrinkles.

"Plasma filler" has always meant the simple injection of Prp into subcutaneous tissues (18, 20, 21).

But the main limitation of plasma fillers is the fact that it does not generate filling effects, if not for a few hours.

For this reason, we set up a new filler device able to combine volumetric and regenerative effects.

We faced different problems: first of all, the Prp is a fluid and to be well mixed inside the new filler device it is necessary that this is also in the liquid state.

Moreover, once injected into the tissues, this liquid would have to become a gel to guarantee the volumetric effect.

Furthermore, since both the platelets and the Prp have a low half-life outside the plasma, we had to guarantee the new filler device a chemical and physical composition that allows the survival of platelet and growth factors for several days.

So, we have chosen a polymer (poloxamer 407) that contains all these characteristics (23-24).

In fact, at low temperatures (2-8 C °) it is found in the liquid phase and this allows the simple mixing of the Prp inside it.

When the temperature reaches 32-37 ° C, the liquid passes to the gel state.

This ensures either the volumizing effect and the progressive release of growth factors within the tissues.

We performed precise measurements on both the number of platelets and EGF present in the new filler device at regular time intervals for 7 days.

EGF is known to be a potent stimulator of cell proliferation of various cells including keratinocytes, fibroblasts and vascular endothelial cells.

EGF stimulates the migration of keratinocytes and also stimulates fibroblasts and endothelial cells to promote the formation of granulation tissue (52-54).

These measurements were also difficult because platelets and the EGF could not be measured with the common automatic measurement systems.

The results found were encouraging for a clinical point.

In fact, the platelets were maintained vitality until 72 hours and then their number felled by freeing the granules containing the growth factors.

EGF, progressively increased its concentration with a peak after 72-96 hours and then slowly decreased until 7 days.

This new filler device could open new scenarios in facial rejuvenation and revolutionize the treatment of face wrinkles.

In fact, the advantages are evident: it would have volumizing and regenerative capacities.

This would allow wrinkles and grooves to be filled and regenerated at each application.

Furthermore, being autologous growth factors, there would be no side effects related to allergic reactions or inflammatory nodules.

Again, since the immune system is also present inside the plasma, this would help prevent the infections.

Finally, the cost of treatment would be cheaper than normal commercial filler, since for each session of treatment 8 ml of Prp are added to 16 ml of hydrogel to obtain a 24 ml filler.

The disadvantage lies on the Prp preparation.

V. Conclusions

Our study has shown that it is possible to obtain a new type of filler able to have both filling capacity, due to the gelling effect of the material used, and a regenerative effect due to the presence of Prp.

Therefore, new clinical trials will be necessary to assess the duration of the volumizing effect in the different areas of the face.

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Table 1: DSC data of micellization transition of poloxamer preparation

Poloxamer concentration (% w/v)	Micellization onset (K)			Micellization peak (K)			ΔH mic (-J/g)		
	Sample 1	Sample 2	Mean ± D.S.	Sample 1	Sample 2	Mean ± D.S.	Sample 1	Sample 2	Mean ± D.S.
15	287.02	286.22	286.62 ± 0.57	289.68	288.65	289.17 ± 0.73	3.58	3.79	3.68 ± 0.15

Table 2: DSC data of gelation transition of various poloxamer preparations

Poloxamer concentration (% w/v)	Gelation onset (K)			Gelation peak (K)			ΔH gel (-J/g)		
	Sample 1	Sample 2	Mean ± D.S.	Sample 1	Sample 2	Mean ± D.S.	Sample 1	Sample 2	Mean ± D.S.
15	295.95	-	295,95	296.36	-	296,36	0.00015998	-	0.00016

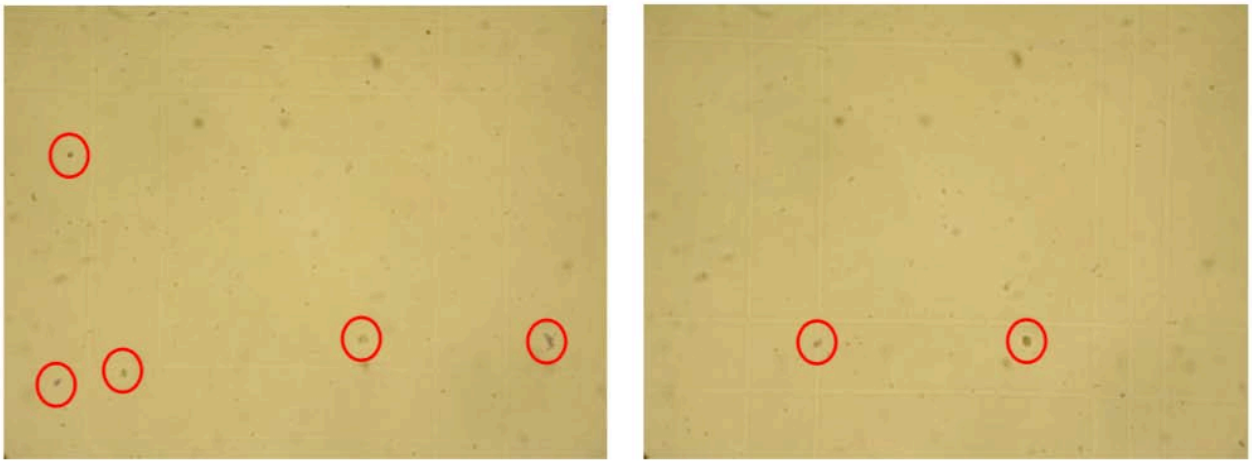

Figure 1: A schematic representation of Burker's chamber

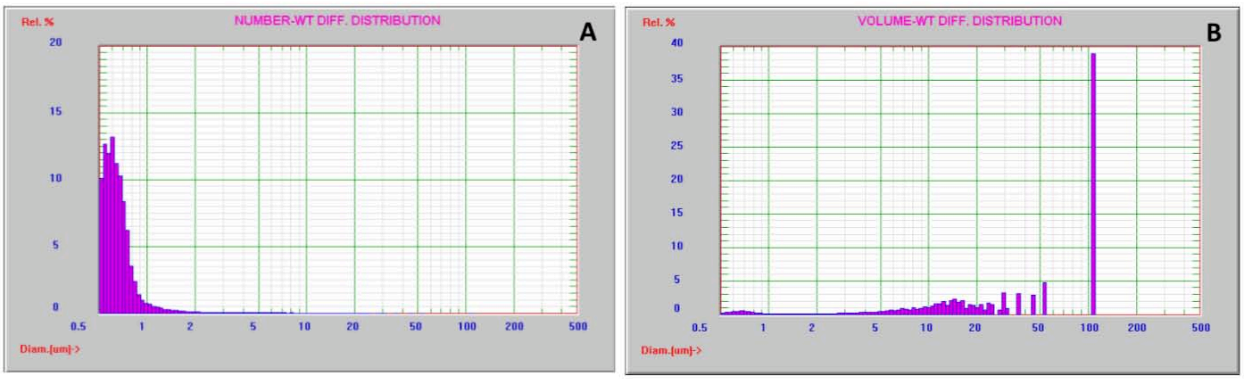

Figure 2: Optical Particle sizer.

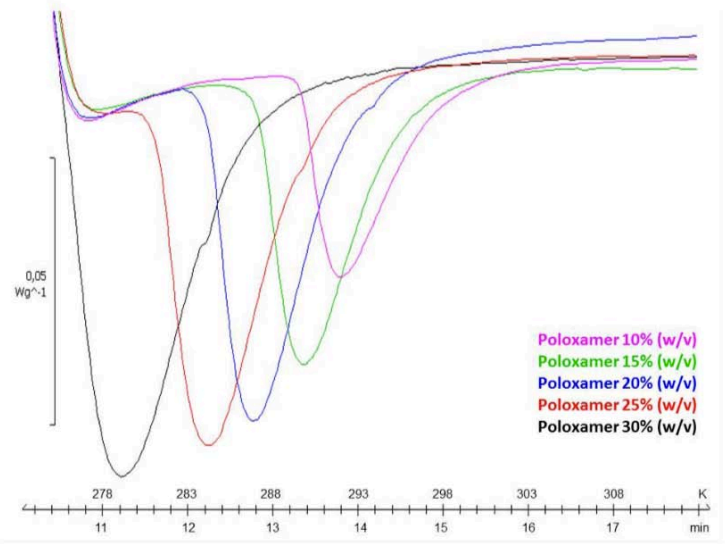

Figure 3: DSC thermograms of different poloxamer concentration samples. Micellization temperature (represented by the enthalpicpeak) decreases with increasing poloxamer concentration

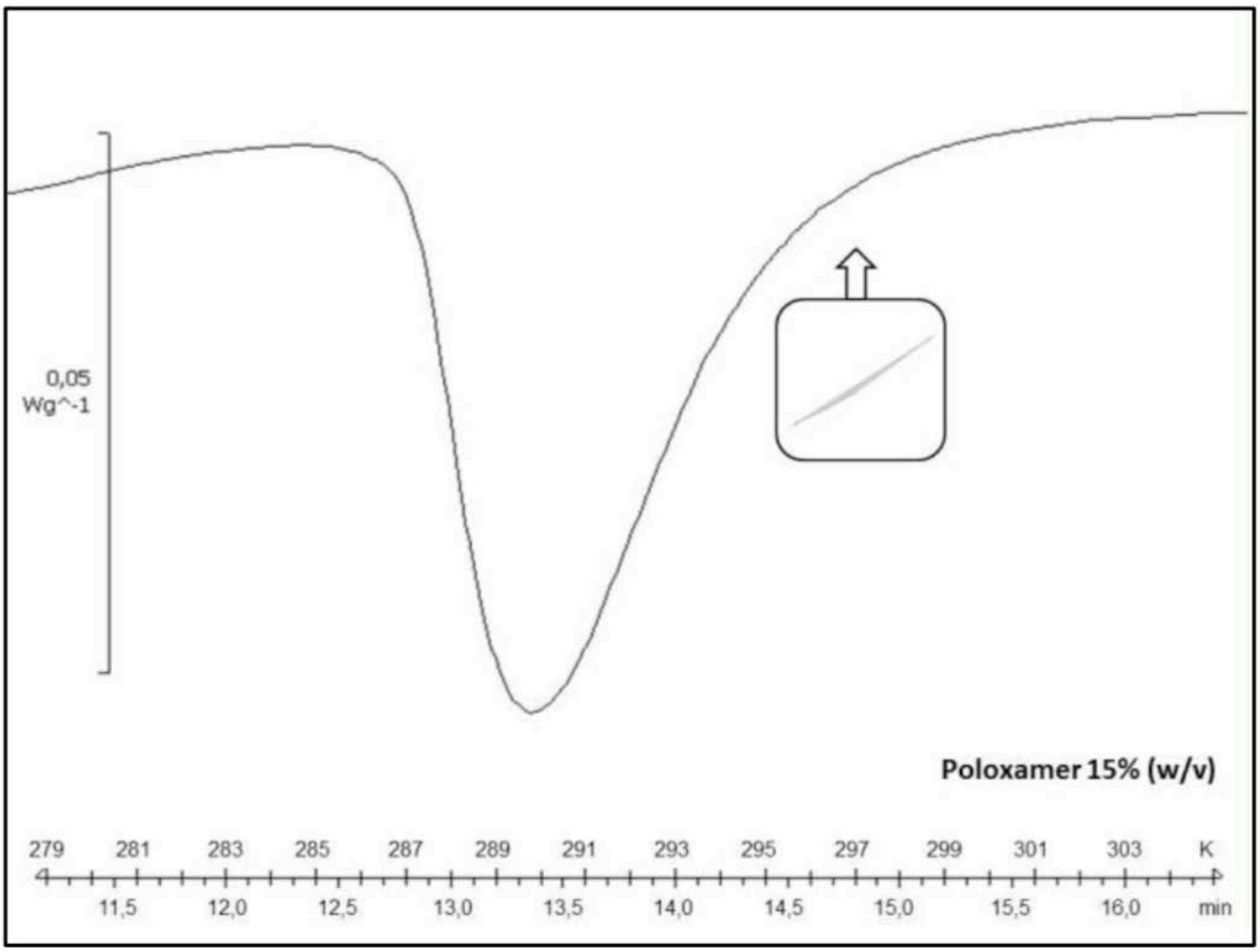

Figure 4: DSC thermogram of poloxamer concentration sample. In the pane there is an enlargement of gelation transition

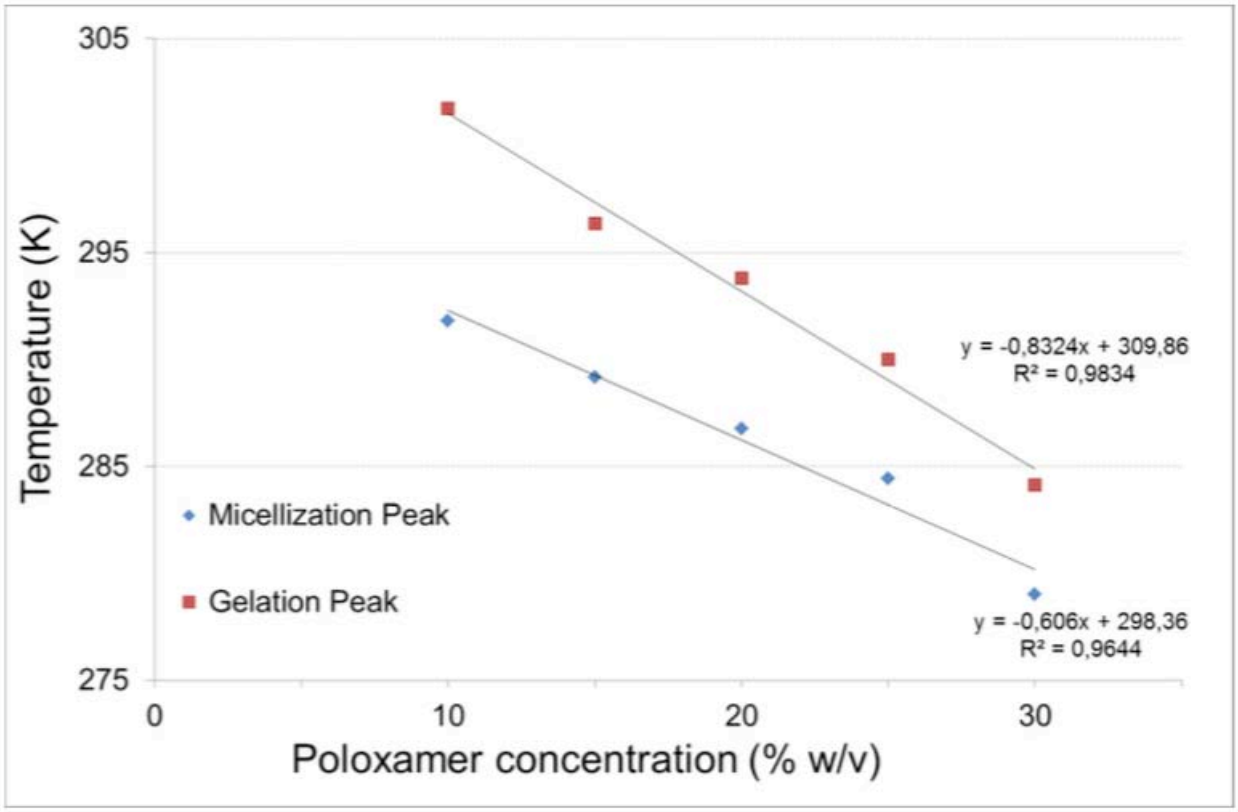

Figure 5: Micellization and gelation temperatures at different poloxamer concentrations

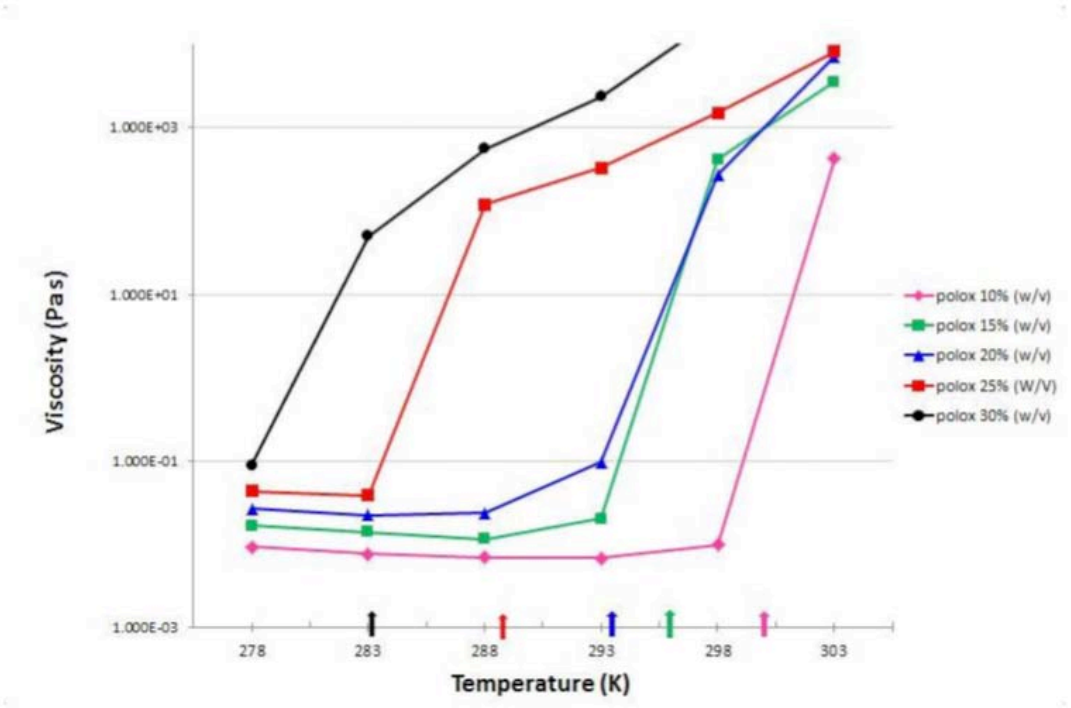

Figure 6: Trend of poloxamer formulation viscosity as a function of temperature. The arrows in the x-axis indicate the gel temperatures obtained from DSC measurements

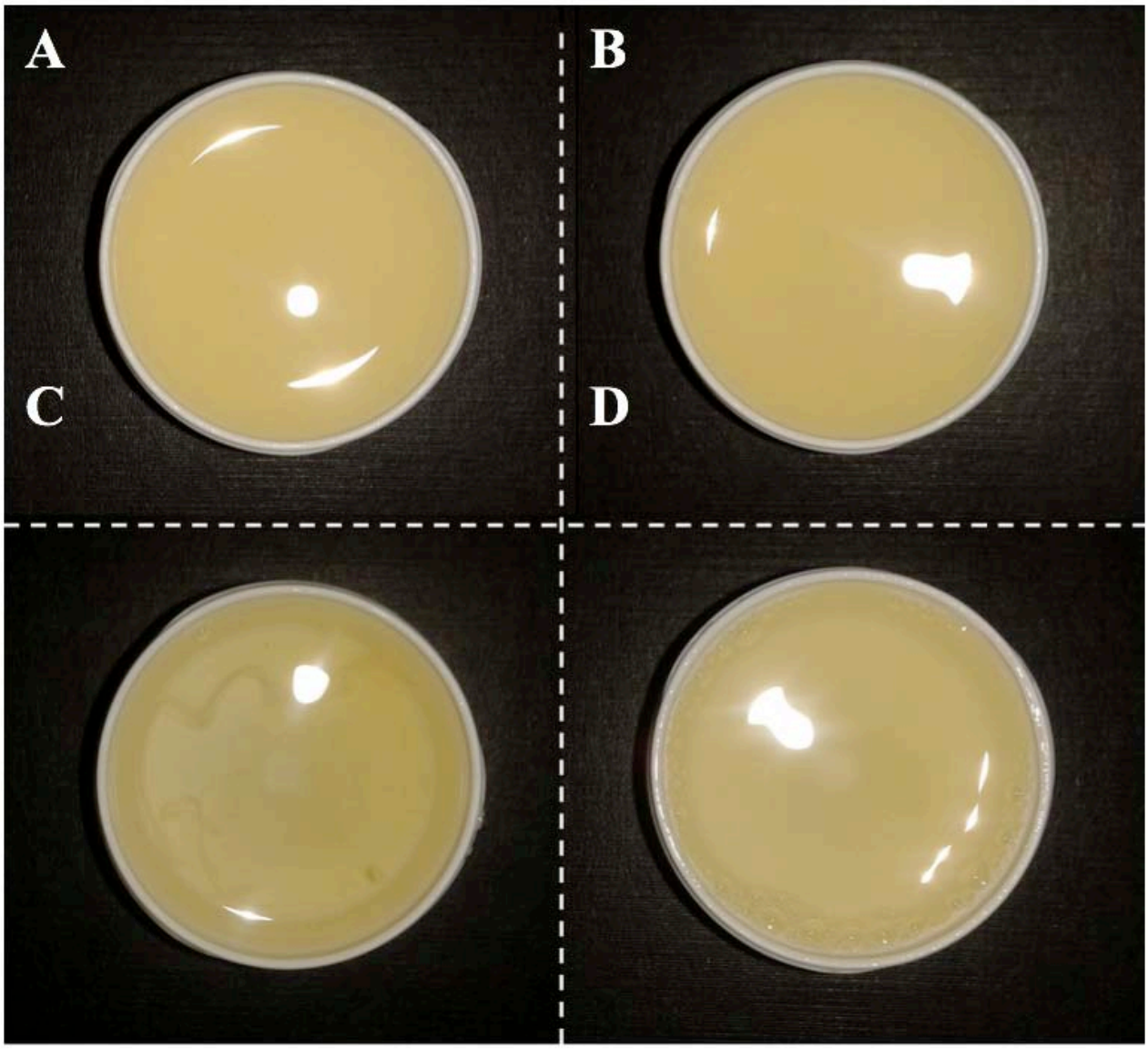

Figure 7: Thermosensitive gel containing PRP fresh prepared (A) and after 7 days from preparation (B). Formulation after 1 month of storage at ~ 4 °C before (C) and after (D) manual shaking

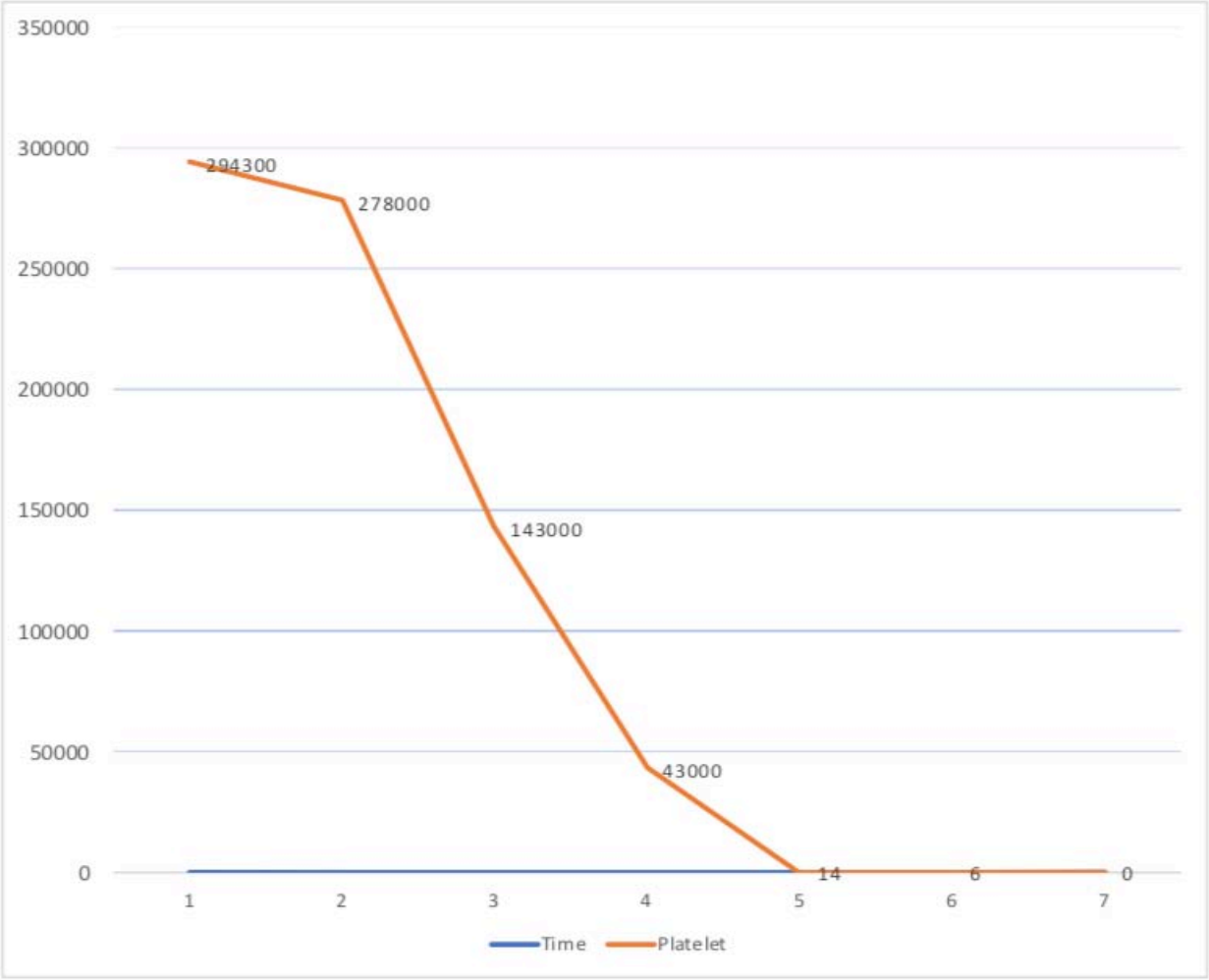

Figure 8: Platelet behaviour on preparation of poloxamer containing PRP

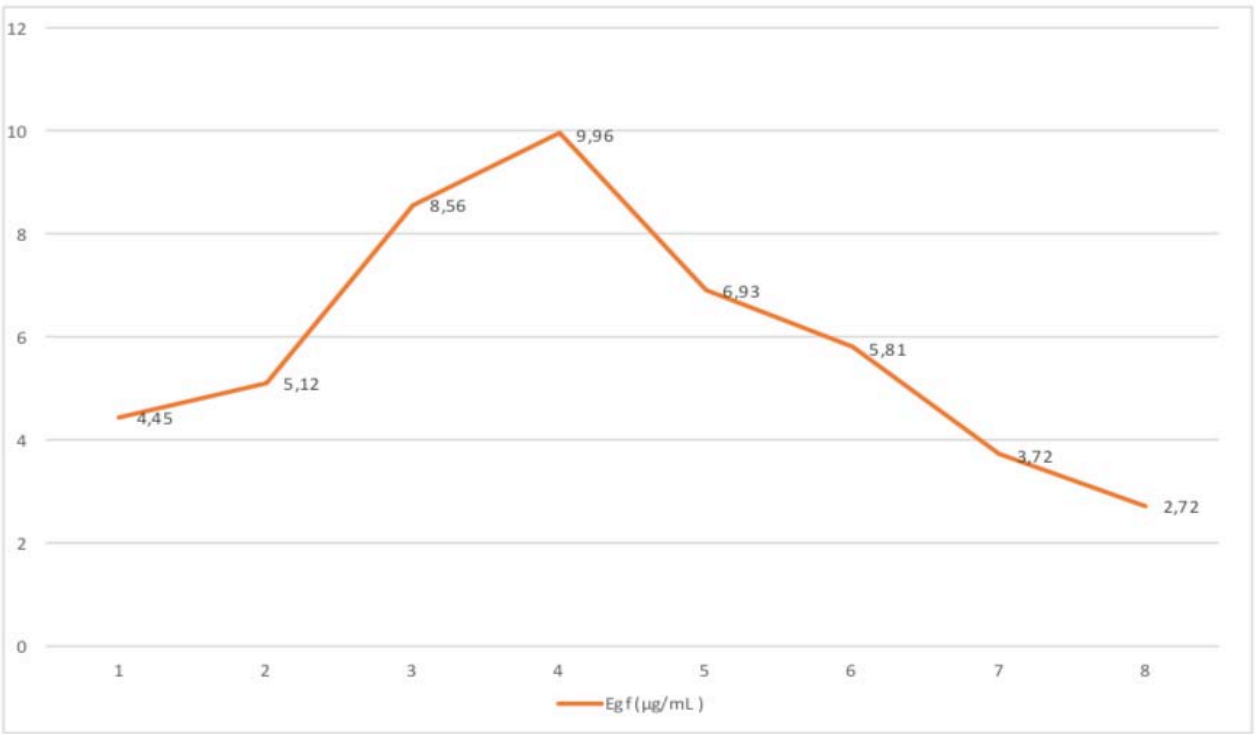

Figure 9: Egf behaviour on preparation of poloxamer containing PRP