Invited lecture/Scientific contribution/Original research

Surface-Based Total Blood Volume Calculation for Platelet and Extracellular Vesicle-Rich Plasma and Gel Preparation by Using a Mathematical Model

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Abstract: Platelet- and extracellular vesicle-rich plasma (PVRP) and platelet- and extracellular vesicle-rich gel (PVRG) are blood-derived products gaining attraction in regenerative medicine. However, despite their reported good efficacy, their preparation protocols are too time-consuming. Moreover, patient-tailored preparation protocols are desired to optimise platelet and extracellular vesicle (EV) count in PVRP and PVRG. This scientific contribution presents a clinical implementation of mathematical model for calculation of the desired total blood volume for PVRG preparation. PVRG was prepared according to previously derived mathematical model of blood cell and EV sedimentation during centrifugation based on the patient's erythrocyte sedimentation rate (ESR) (Božič et al., 2021). We estimated the volume of blood required for the treatment of the surface area of the individual patient, prepared the PVRG accordingly and applied it to the patient's wound. After six applications of 13 mL to 65 mL of PVRG, the osteoradionecrotic surface area decreased from 46 cm^2 to 18 cm^2, and infection was eradicated. The mathematical modelling of total blood volume needed to prepare PVRG proved useful to prepare the therapeutic amount of PVRG and also optimized time for the PVRG preparation protocol.

Keywords: Extracellular vesicles; Platelet-rich plasma; Regenerative medicine; Osteitis; Osteoradionecrosis; Osteomyelitis; Temporal bone

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1. Introduction

Significant concentrations of extracellular vesicles (EVs) have been measured and inspected in the autologous blood-derived platelet-rich liquid product, which was named platelet- and extracellular vesicle-rich plasma (PVRP) (Božič et al., 2021; Vozel et al., 2021). PVRP is defined a blood-derived product with concentrations of platelets and EVs higher than blood concentrations. Therefore, it presents an upgrade in autologous regenerative therapeutics (Vozel et al., 2020). PVRP, prepared by a two-step-plasma-based protocol, proved effective in treating chronic postoperative temporal bone cavity inflammation (Vozel et al., 2021). However, this time-consuming preparation protocol, which took on average 80 minutes, resulted in an approximately 10 % yield of PVRP from withdrawn blood (Vozel et al., 2021).

To overcome these obstacles, patient-tailored PVRP treatment has been proposed based on the mathematical model describing the movement of the cell constituents in the tubes during sedimentation in the centrifuge. Subjected to systemic centrifugal force, larger particles (i.e. erythrocytes) sediment faster and in such way to push smaller and less dense particles (platetets and EVs) in the opposite direction. As the latter accumulate above the erythrocyte boundary, they are enriched in plasma. However, after the erythrocyte boundary reaches the hematocrite level, platelets and EVs are sedimented from the plasma towards the bottom of the tube. It was indicated that the choice of optimal time for given centripetal acceleration of the centrifuge rotor is therefore key for preparation PVRP (Božič et al., 2021). Also, it should be taken into account that the concentration of platelets and EVs in PVRP strongly depend on the patient's erythrocyte sedimentation rate (ESR) (Bozič D et al., 2021). Here, we used the model (Bozič et al., 2021) to prepare PVRP according to two different protocols 1.) the "high platelet and EV" protocol aimed at creating a plasma with the highest possible platelet and EV concentration that can be obtained by a single spin preparation and EVs and 2.) a "half volume" protocol where we calculated the time needed for the upper border of erythrocytes to arrive down to one half of the length of the blood sample. We derived equations for exact calculations of blood volumes needed to treat the wound of the individual patient and provided a calculator to alleviate calculations (Steiner et al., 2022).

Then, PVRP has been successfully transformed to the semi-solid (i.e., gelatinous) state to create platelet- and extracellular vesicle-rich gel (PVRG) by the procedure previously described in a preclinical in-vitro pilot study (Vozel et al., 2020).

With the clinical implementation of described clinical protocols, we discovered that knowing the exact volume of blood needed to prepare PVRG proved beneficial. This scientific contribution shows the derivation of the novel equations for calculating the total volume of blood needed to prepare the desired volume of PVRG and reports on the effect of the applied preparation in a patient treated at the Department of Otorhinolaryngology and Cervicofacial Surgery, University Medical Centre Ljubljana, Ljubljana, Slovenia for osteoradionecrosis of the temporal bone.

2. Materials and Methods

2.1. The patient

A patient with osteoradionecrosis of the temporal bone was recruited in July 2021 at the Department of Otorhinolaryngology and Cervicofacial Surgery, University Medical Centre, Ljubljana, Slovenia, for the treatment with PVRG prepared by the novel patient-tailored ESR based preparation protocol. Patient signed written informed consent.

2.2. Autologous PVRG preparation protocol

2.2.1. Blood withdrawal prerequisites

Before blood withdrawal, a patient was ensured to meet the following criteria: the patient should not perform strenuous physical activity within 24 hours of treatment, the patient should be in a fasting

state at blood sampling (i.e., no food or beverages intake except water within 8 hours before treatment), the patient should not had high-fat dietary intake or alcohol consumption one day before the treatment.

2.2.2. Blood withdrawal procedure

The blood was withdrawn from a peripheral artery. Citrated 4.5 ml evacuated blood tubes (9 NC sodium citrate 0.105 M, BD Vacutainer, Becton Dickinson, San Jose, CA, United States) that were kept at room temperature were filled as indicated by the producer, to provide appropriate blood to anticoagulant ratio. Tubes were turned upside down 3-4 times immediately after sampling to achieve an adequate blood and sodium citrate mixture. The blood withdrawal volume was estimated as described by using mathematical model as described below. According to previous findings, blood yield from a citrated blood specimen yields is approximately 30-50 % of PVRP (e.g., from 10 ml of blood, about 3-5 ml of PVRP is produced in patients with normal ESR) (Božič et al., 2021). After withdrawal, blood was stored and transported to the centrifuge at room temperature (i.e., 22 °C) immediately taking into account the platelet concentrate storage guidelines (Božič et al., 2021).

2.2.3. Blood centrifugation

Before centrifugation, the blood tubes were again inverted 3-4 times to resuspend the samples. The blood was then centrifuged at the relative centrifugation force (i.e., RCF) of 300×g at 18 °C. The centrifugation time (t) was calculated individually from the patient's erythrocyte sedimentation rate (i.e., ESR) according to the mathematical model (Božič et al., 2021). The model estimates the volume of plasma and concentration of platelets and EVs as plasma forms during centrifugation. Following the possibilities considered in (Božič et al., 2021), we considered two options: The first option was the "high platelet and EV" protocol aimed at yielding plasma with the highest possible platelet and EV concentration that can be obtained by a single spin preparation without pelleting platelets and EVs for which the time $T_{\text{high platelet and EV}}$ is given by (Božič et al., 2021),

$$T_{\text{high platelet and EV}} = x_{\max} \ln(x_{\max}/x_{\min})/(2\varepsilon \text{ ESR RCF}) \quad , \qquad (1)$$

where $x_{\max}$ and $x_{\min}$ are the distances from the centrifuge rotor axis to the bottom and the level of the blood sample, respectively, ε is the adjustable constant, which was set to 0.13 (Božič et al., 2021) and RCF is the multiplicity of the gravitational constant that expresses the centrifugal force (in our case 300). The second option was the "half volume" protocol where we calculated the time needed for the upper border of erythrocytes to arrive down to one half of the length of the blood sample (Božič et al., 2021),

$$T_{\text{half volume}} = x_{\max} \ln((x_{\max} + x_{\min})/2x_{\min})/(\varepsilon \text{ ESR RCF}) \quad . \qquad (2)$$

By inserting the respective times of centrifugation into the expression for the concentration of platelets and EVs, the calculated platelet- and EV - enrichment factor in PVRP is higher than 2 (in the case of high platelet and EV protocol) or equal to 2 (in the case of "half volume" protocol).

2.2.4. PVRP harvesting

After the centrifugation, blood was divided into two distinctive fractions: red, which contains mainly erythrocytes, and yellow, which is PVRP. Sometimes an opaque whitish layer (i.e., buffy coat) is present between layers. PVRP is gently and slowly aspirated with a pipette (ref.: 225-1S, Termofisher scientific, ZDA) above the red and white layer without disturbing them. PVRP extracts from all tubes were merged in another sterile plastic tube or container, and the total volume was measured. PVRP was then activated to form a PVRG immediately or as soon as possible after PVRP harvesting.

2.2.5. PVRP activation (i.e., PVRG formation)

PVRP, autologous serum (in the ratio of 1:5 to PVRP) and 1M $CaCl_2$ (in the ratio of 1:100 to PVRP) were mixed to transform PVRP to PVRG. Additional blood was withdrawn in a 4 ml plastic blood

tube without anticoagulant (e.g., Z Serum, Vacutube, LT Burnik, d.o.o., Slovenia) and stored at room temperature. This blood was used to prepare serum, i.e., activator, which transforms PVRP (i.e., liquid) into a gel (i.e., PVRG). After about 5-10 minutes, blood clot was formed within the tubes, and then the tubes were centrifuged for 10 min at 1260×g and 18°C. The supernatant above the red blood clot is serum, which was gently aspirated without mixing layers and transferred by a sterile pipette into a sterile polypropylene tube. The volume of autologous serum needed to prepare PVRG depends on the desired volume of PVRP that will be transformed to PVRG. A mixture in the ratio 1:5 with PVRP is needed (for activation of 5 ml of PVRP, 1 ml of serum is needed). The blood yield for serum harvesting was calculated from the haematocrit. For instance, in 35 % haematocrit, the serum presents 65 % of blood volume.

To start the PVRP activation process (i.e., transformation of PVRP to PVRG) 1M $CaCl_2$ (14.7%, Pharmacy of University Medical Centre Ljubljana, stored at room temperature) in the ratio 1: 100 with PVRP (i.e., 1%; e.g., 170 µL to activate 17 ml of PVRP) was applied to the petri dish with the sterile pipette. Then, an autologous serum in the ratio 1: 5 with PVRP (i.e., 20 %; e.g., 4 ml to activate 20 ml of PVRP) was applied over the $CaCl_2$. Finally, Autologous PVRP was administered with the sterile pipette over the mixture of $CaCl_2$ and autologous serum. At least 5 minutes elapsed for the activation process to occur and PVRG was formed. It was expected that some of the mixture would not turn into a gel.

3. Results

3.1. Total blood volume calculations for PVRG preparation

In our case, a higher PVRG volume was required due to higher wound surface area. For that reason, a "half volume" centrifugation protocol was used (Eq. (2)).

The required PVRG volume was calculated from the wound surface area,

$$V_{PVRG} = S * h \quad , \tag{3}$$

where V_{PVRG} is PVRG volume, S is wound surface area, and h is the thickness of the layer of PVRG. In external wounds, as in our case, h = 1 cm was used for the calculation to cover the wound thoroughly. However, some of the mixture (PVRP, calcium chloride and serum) remains untransformed (i.e., liquid), and PVRG height is expected to be less than 1 cm.

PVRG application can be repeated several times, depending on the healing process. The time between applications can vary 1 to 14 days according to previous blood-derived products therapy regimens (Hu et al., 2019). After applying PVRG, a glassy, water-resistant layer is formed over the wound. Wound healing (e.g., granulation tissue formation) can be observed through this layer. For that reason, we suggested at least 14 days interval between each application.

The total blood volume ($V_{T,blood}$) needed for the PVRG preparation was calculated as follows. The total volume of blood needed consisted of contributions used for obtaining plasma and serum,

$$V_{T,blood} = V_{blood\ for\ PVRP} + V_{blood\ for\ serum} \quad , \tag{4}$$

where

$$V_{PVRP} = \eta * V_{blood\ for\ PVRP} \tag{5}$$

and η is the proportion of PVRP in blood. For the “half volume” protocol, η = 0.5 (Steiner et al., 2022). Volume of serum can be expressed by haematocrit Ht,

$$V_{serum} = (1\text{-}Ht) * V_{blood\ for\ serum} \quad , \tag{6}$$

whereas it is estimated that

$V_{serum} = 1/5 * V_{PVRP}$. (7)

Volume of PVRG is composed of contribuitions from PVRP and from serum,

$V_{PVRG} = V_{PVRP} + V_{SERUM}$. (8)

Volume of $CaCl_2$ is taken to be negligible and was excluded from the volume calculations. Blood for PVRP and for serum should be withdrawn into different tubes (citrated 4.5 ml blood tubes for PVRP and 4 ml plastic tubes without anticoagulant for serum), therefore patient-customized blood volume needed for PVRP ($V_{blood\ for\ PVRP}$) and for serum ($V_{blood\ for\ serum}$) should be calculated. It follows from Eqs. (7) and (8) that

$V_{PVRP} = 5/6 * V_{PVRG}$, (9)

from Eqs. (5) and (9) that

$V_{blood\ for\ PVRP} = (5/6\ \eta) * V_{PVRG}$ (10)

and from Eqs. (6), (7) and (9) that

$V_{blood\ for\ serum} = V_{PVRG}/(6 * (1\text{-}Ht))$. (11)

However, the volume of blood taken from the patient according to Eq.(3) and Eqs.(4), (10) and (11) would be

$V_{T,blood} = (5/6) * S * h * (1/\eta + 1/(5 * (1 - Ht))$. (12)

3.2. Calculation of the PVRG volume for patient with osteoradionecrosis of lateral skull-base

3.2.1. Patient profile and summary

84-year-old male with arterial hypertension, hyperlipidaemia, aortic stenosis and chronic atrial fibrillation on therapy with apixaban presented with a squamous cell cancer of the left auricle. The patient was primarily treated with tumour resection, suprafacial parotidectomy and left sided selective neck dissection. He received postoperative radiotherapy. One year postoperatively, he presented with a cancer recurrence in a left external ear canal and was treated surgically with complete microscopical cancer resection, including auricle amputation and subtotal petrosectomy. The tissue defect, which reached the superior part of the squama of the left temporal bone, could not be covered with a free flap due to the patient's age and vessel disease. Thus, the defect was entirely reconstructed with left extended musculocutaneous pectoralis major muscle flap. A few days postoperatively, the patient tested positive for COVID-19, which compromised the patient's general condition. The flap started to necrotise, and osteitis of the lateral-skull base developed. Moreover, the epithelialisation of exposed cortical bone was hampered due to osteoradionecrosis, and it was exposed on two areas of 0.4 cm^2 and 1 cm^2. The patient's general condition remained poor but stable.

3.2.2. Treatment regimen

Due to the patient's general healthcare status, which posed a high risk of general anaesthesia, surgical revision with flap reconstruction could not be performed. The patient was initially treated with intravenous antimicrobials and regular flap necrosis removal to prevent intracranial and systemic complications. Despite this treatment, the osteitis persisted, and epithelialisation did not occur. Therefore, outpatient salvage treatment, including multiple PVRG administration and necrosis removal, was offered to the patient. The wound was treated with six applications of PVRG and debridement on day 0 (baseline), 8, 17, 35, 56 and 108. The number of PVRG applications are strictly patient-specific and depend on the wound healing rate. It can be repeated as many times as needed until the wound reaches the desired degree of healing. Blood was withdrawn from the radial artery in citrated tubes due to the requirement of large PVRG volume and unsuccessful peripheral vein cannulation. Blood withdrawal volume was prepared with a "half-volume" centrifugation protocol.

Over the PVRG, a nonadhesive absorbent dressing, Mepilex® (Mölnlycke, Gothenborg, Sweden), was applied. Directed intravenous antimicrobial treatment for osteitis of six weeks duration was discontinued at the first PVRG application.

3.2.3. Determination of the PVRG volume

Table 1 shows the results on V_{PVRG} obtained during the treatment of the patient. Here the time of centrifugation was 5 minutes which yielded about the maximal possible volume of PVRG compatible with haematocrit (about half of the volume of the blood taken) (**Table 1**). It can be seen that the time of centrifugation should have been shorter than the one proposed by a general protocol. As the ESR of the patient changed decreased along the treatment, the centrifugation time should have been accordingly adjusted (Table 1). The best improvement in wound surface was achieved between day 35 and 56 (the wound surface decreased from 40 to 21 cm^2).

Table 1. Regimen of the treatment of the osteoradionecrosis of the lateral skull base with PVRG (Steiner et al., 2022).

Day	Wound size (cm^2)	ESR (mm/h)	$V_{T,blood}$ (mL)	V_{PVRG} (mL)	$T_{half\ volume}$ (min)
0	46	56	125	65	1.54
8	46	55	30	13	1.57
17	43	40	96	50	2.16
35	40	40	80	45	2.95
56	21	42	65	33	2.05
108	18	38	57	30	2.27

Centrifugation was performed at 300 * *g* and 18 °C. PVRG – platelet and extracellular vesicle-rich gel; ESR – erythrocyte sedimentation rate.

3.2.4. Treatment outcome

PVRG formed a protective biofilm over the wound after each application (**Figure 1, Panel C**). The infection healed, i.e., the antimicrobial swab was negative, and there was no discharge—fibrin and granulation tissue formed under the PVRG biofilm. Later epithelialisation began from the wound edges and its central parts. However, the epithelialisation did not cover the exposed cortical bone, not even after the cortical bone was drilled out and PVRG applied over the cancellous bone. One of the exposed areas of the bone surfaces was reduced from 1 cm^2 to 0.4 cm^2. The second one remained non-epithelialised (0.4 cm^2).

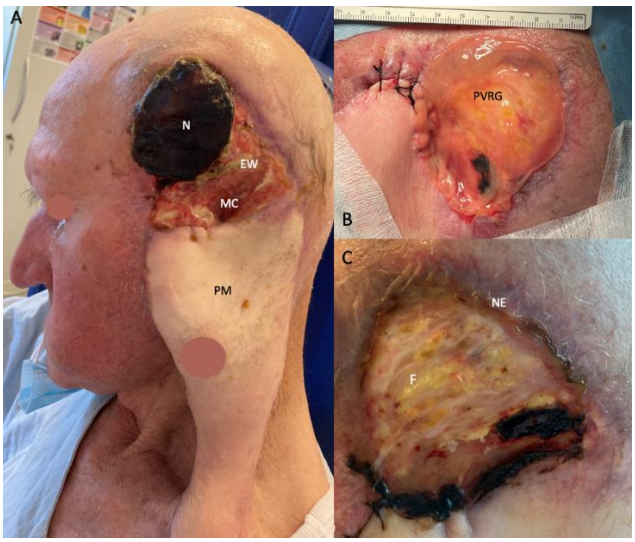

Figure 1. Photographs of a patient with an osteoradionecrosis and osteitis of left lateral skull-base treated with six applications of platelet- and extracellular vesicle-rich gel (PVRG). (**A**) pre-treatment wound in the area of necrosed (N) extended pectoral muscle flap (PM). Two areas of exposed bone (EW) are shown around the exposed mastoid cavity (MC) area. (**B**) PVRG is administered over the debrided wound. (**C**) on the 56th day after the first PVRG administration, NE is growing centripetal in the wound, which bleached due to inflammation regression. Dark areas are crusts formed after exposed cortical bone drilling. Fibrosis (F) is seen in centre.

4. Discussion

This clinical research presents for calculations of blood volume needed for PVRG preparation to cover a surface with targeted area. Using the equations helps to estimate the amount of blood needed to prepare 3-D model of PVRG that would cover the total wound surface. In our practice before the development of the model, it has happened that too much or too little blood was withdrawn. Repeated harvesting of too much blood from the patient can be harmful, and withdrawing too little results in an insufficient wound covering.

4.1. Patient-tailored, ESR-based PVRP and PVRG preparation protocols

The preparation protocol that is based on the mathematical model can be chosen according to the size and location of the target tissue and the patient's ESR (Božič et al., 2021). The model yields the time of centrifugation at chosen centripetal acceleration of the centrifuge rotor. In subjects with normal *Ht*, the "high platelet and EV protocol" (Eq. (1)) yields higher platelet and EVs concentrations in PVRP at the expense of lower PVRP volume (Božič et al., 2021). Thus, this protocol is appropriate in cases where the target tissue surface is small. The "half volume protocol" (Eq. (2)) yields higher PVRP volume (50% blood yield) at the expense of lower platelet and EVs concentrations in PVRP. However, these concentrations are in patients with normal *Ht* and ESR still above the baseline blood levels (Božič et al., 2021). The latter protocol was used to treat osteoradionecrosis of lateral skull-base, which was a sequela of radiotherapy of external ear canal cancer and the patient's overall health status post-COVID-19 was poor. It is known that severe and fatal complications of osteonecrosis in this area can develop due to drastic changes in bone tissue from post-radiation DNA damage and cell death (Lyons et al., 2008). This causes disorganisation of the extracellular matrix and prevents new vessel formation resulting in hypocellularity and tissue hypoxemia(Haubner et al., 2015). Irradiated bone becomes necrotic, exposes through the skin and may become infected (i.e., osteitic) (Beacher et al., 2018).

Since in the case presented the wound surface area decreased noticeably after multiple PVRG applications (from 46 cm^2 to 18 cm^2) and bacterial osteitis was treated successfully, PVRG could be considered a treatment modality for scalp osteoradionecrosis in further research. Furthermore, we observed that the calculated volume of PVRG with novel equations adequately covered the wound.

In a previous study of the effect of PVRP on chronic wounds in the middle ear (Vozel et al., 2020) the same two-spin protocol for PVRP preparation was used for all patients. The first spin was performed at 300g for 5 minutes. According to the model (Božič et al., 2021). The patient considered in this work had an increased ESR and the centrifugation time 5 minutes would be sufficient for the erythrocytes to reach haematocrit even before the spin were completed. As the platelets and extracellular vesicles in the plasma above the haematocrit are no longer subjected to counterflow, they would be sedimenting and therefore become depleted in plasma. Besides yielding higher quality plasma, estimating the time of preparation also shortened the preparation procedure, which was favourable.

5. Conclusions

Calculations of blood volume needed to prepare PVRG in order to cover the desired wound surface are expected to improve the quality of PVRG and optimize the time of the PVRG preparation protocol.

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