



Treatment with platelet- and extracellular vesicle-rich plasma in otorhinolaryngology-a review and future perspectives

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Contents

1. Extracellular vesicles	120
1.1 Classification and biogenesis of extracellular vesicles	121
1.1.1 Microdomain formation	121
1.1.2 Binding of cytosolic molecules	122
1.1.3 Budding	122
1.1.4 Fission	122
1.1.5 Exocytosis	122
1.2 Mode of action of extracellular vesicles	122
1.3 Isolation of extracellular vesicles	122
1.4 Applicability of extracellular vesicles isolates in clinical practice	123
2. Platelet- and extracellular vesicle-rich plasma (PVRP)	123
2.1 Platelets and platelet-derived extracellular vesicles	124
2.2 Platelet- and extracellular vesicle-rich plasma preparation protocols	127
2.2.1 Plasma-based protocols	128
2.2.2 Buffy coat-based protocols	128
2.3 Composition of platelet- and extracellular vesicle-rich plasma	129
2.3.1 Platelet concentration	130
2.3.2 Leukocyte concentration	130

2.4 Roles of anticoagulants in blood sampling and platelet- and extracellular vesicle-rich plasma activation type	130
3. Treatment with platelet- and extracellular vesicle-rich plasma	132
3.1 Face, nose and paranasal sinuses	132
3.2 Lips and oral cavity	134
3.3 Pharynx and esophagus	136
3.4 Salivary glands	136
3.5 Ear and lateral skull base	136
3.5.1 The external ear	136
3.5.2 The middle ear	136
3.6 Platelet- and extracellular vesicle-rich plasma adverse effects	137
3.7 Future of platelet- and extracellular vesicle-rich plasma in otorhinolaryngology	137
3.7.1 Treatment of chronic middle ear infection after cholesteatoma surgery with platelet- and extracellular vesicle-rich plasma	138
4. Future of extracellular vesicle isolates in otorhinolaryngology	142
Acknowledgments	143
References	143

Abstract

Extracellular vesicles (EVs), also known as microvesicles, exosomes, nanovesicles, micro-particles etc. are a heterogeneous group of nanometrically sized cell-derived membranous structures which can originate from any cell, including plant cell or bacteria. They were found in many body fluids including platelet-rich plasma (PRP). PRP is a blood-derived product with immune, hemostatic and regenerative effects. PRP is expected to contain important concentrations of EVs which could be important contributors to PRPs effects. For that reason, we call the preparation from PRP a platelet- and extracellular vesicle-rich plasma (PVRP). PVRP has been used in various medical fields including orthopedics, traumatology, wound care surgery, plastic, reconstructive and esthetic surgery, hand surgery, maxillofacial and oral surgery, gynecology, urology, ophthalmology, cardiothoracic surgery, neurosurgery and otorhinolaryngology. Otorhinolaryngology is a field in which PVRP has a great potential in the treatment of diseases of the face, neck, nose, paranasal sinuses, anterior skull base, lips, oral cavity, pharynx, esophagus, salivary glands, ear and lateral skull base. To present this potential and also hitherto gathered results in the field of otorhinolaryngology, this chapter describes the role of platelets and EVs in diagnostics and treatment with an emphasis on otorhinolaryngology. Also, we discuss future perspectives of EVs and PVRP in treatment of moist radical cavities after cholesteatoma surgery.



1. Extracellular vesicles

According to Théry et al. [1] International Society for Extracellular Vesicles endorses extracellular vesicle (EV) as a generic term for particles naturally released from the cell that are delimited by a lipid bilayer and

cannot replicate, i.e. do not contain a functional nucleus. This definition encompasses any cell, including plant cell or bacteria which can present a source of EVs [1–4]. First, it was believed that EVs serve in transport of cell waste only [2], but later their vital role in intercellular communication in health and disease became apparent [4–7].

EVs have been classified as microvesicles and exosomes in the majority of reviewed studies, therefore this chapter involves this classification also [2]. However, there is no consensus for specific markers of EV subtypes, so far and EVs should not be described as microvesicles or exosomes, unless their reliable specific markers of subcellular origin can be established within an experimental system. According to the minimal requirements of MISEV2018 publication [1] EVs should be described based on their:

(a) physical characteristics:

- i. size: small EVs (sEVs) for < 200 nm and medium/large EVs (m/lEVs) for > 200 nm.
- ii. density: low, middle, high.

(b) biochemical composition.

(c) descriptions of conditions or cell of origin.

1.1 Classification and biogenesis of extracellular vesicles

As already mentioned, EVs have been classified as microvesicles and exosomes also. This classification is based on EV formation mechanisms, size, composition and source. Van Niel et al. [2] classifies microvesicles as 50–500 nm-sized membranous particles which are formed by ectocytosis (i.e. plasma membrane budding) and exosomes as 50–150 nm-sized membranous particles which are formed by exocytosis of intracellular compartments [2,4]. Some molecular mechanisms of EV formation have been identified, therefore formation can be arbitrarily divided into stages [2,7].

1.1.1 Microdomain formation

An initial step of EV formation is the aggregation of proteins and lipids in the plasma membrane or multivesicular endosome (MVE) to form microdomains (i.e. aggregates of membranous molecules). Formation of microdomains on plasma membrane precedes the formation of microvesicles and formation of microdomains in MVE precedes the formation of intraluminal vesicles (ILV). Understandably, MVE is an intracellular compartment which contains vesicles formed by inward budding of MVE [2].

1.1.2 Binding of cytosolic molecules

Microdomains bind cytosolic molecules (e.g. proteins, RNA) which are later encapsulated within microvesicle or ILV [2].

1.1.3 Budding

Aggregates of microdomains and cytosolic molecules begin to bud outward (i.e. ectocytosis) from the plasma membrane in extracellular space to form microvesicle or inward of MVE to form ILV [2].

1.1.4 Fission

Microvesicles are finally formed by cleavage (i.e. fission) of plasma membrane bud and ILV by cleavage of MVE bud. On this stage, exosomes are encapsulated within MVE but called ILV [2].

1.1.5 Exocytosis

The final step of exosome formation is presumably exocytosis of MVE which releases ILV in extracellular space. On this stage ILV are named exosomes [2].

According to described mechanisms of EVs formation, it is expected that exosomes involve more steps of formation, are smaller and carry different cargo than microvesicles [2].

1.2 Mode of action of extracellular vesicles

EVs exert a physiological or pathological response when reaching a target cell. Target cell can be adjacent, distant or an origin cell. The latter implies an important role of EVs in autoregulation. There are three modes of action of EVs:

1. binding with plasma cell membranous receptors which induces cellular signaling;
2. the fusion of EV and plasma membrane which causes a cytosolic release of EV cargo
3. uptake of EV by plasma membrane with endocytosis [2].

1.3 Isolation of extracellular vesicles

Although the applicability of EVs is promising and mechanisms of their formation are thoroughly studied, there is presently no standard isolation protocol to harvest EVs and prepare EVs isolates so far. Different body fluids and cell-culture media have already been used to harvest and inspect EVs microscopically [8–10]. It is well-known that blood-derived EVs are a dynamic

material formed by blood-cell fragments [11]. Additionally, blood-derived EV isolation protocols are based on red blood cell (RBC) and leukocyte elimination, therefore harvested pellet contains EVs in addition to platelet- and megakaryocyte-derived molecules [12]. However standard laboratory tests do not detect EVs exclusively, concentrations of EVs in body fluids, culture media or EVs isolates can be high [13].

1.4 Applicability of extracellular vesicles isolates in clinical practice

EVs contain different molecules which are important in intercellular communication, therefore they could be applied in diagnosis or therapy of numerous diseases. For that reason, it is appropriate to term them as therapeutic tools. They could be used as biomarkers in the diagnosis of infectious, neurodegenerative, autoimmune diseases or tumors. Therapeutically, they could be used as vaccines to prevent infectious diseases or tumors, as immunosuppressive or regenerative therapy or as drug-delivery tools [6].

Although EV isolates still have not been used for therapy in daily clinical practice, they have been regularly used via platelet-rich plasma (PRP). PRP contains high concentrations of platelets and platelet-derived EVs (PDEVs), which possess PRP's main regenerative effects [12,14]. In fact EV isolates are expected to exert at least the same regeneration potential as PRP alone if not superior [12]. For that reason, the preparation can be appropriately named platelet- and extracellular vesicle-rich plasma (PVRP).



2. Platelet- and extracellular vesicle-rich plasma (PVRP)

The terminology of PVRP and its types is inconsistent (e.g. platelet-rich plasma, platelet-rich fibrin matrix, platelet-rich fibrin, platelet-rich growth factors, platelet concentrate, platelet gel) due to different observation methods, preparation and activation protocols [15]. Moreover, it was confirmed that PVRP's main regenerative functions are exhibited by PDEV derived from PVRP according to in-vitro and in-vivo animals studies [16]. An abundance of PDEVs and activated platelets prone to shed PDEVs in PVRP have been described and furthermore observed microscopically [14]. Fig. 1 shows a sample of PVRP, which contains large quantity of platelets (A) and sub-micron particles (B), believed to be PDEVs. Some red blood cells and leukocytes can usually be found as well. It can be seen that platelets are in activated state. It is known that upon activation, platelets are prone to

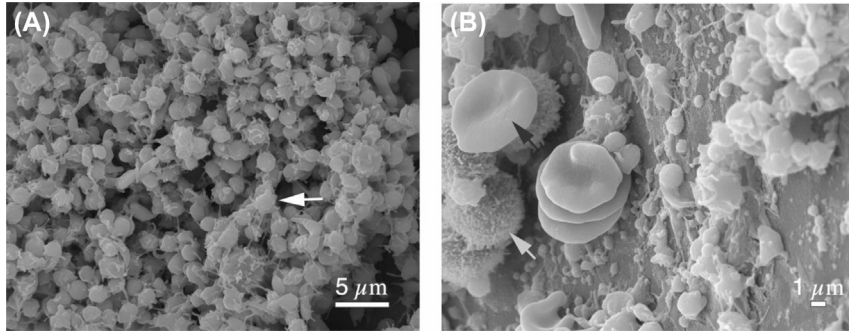


Fig. 1 Scanning electron micrographs of platelet- and extracellular vesicle-rich plasma. Platelets (Panel A) and sub-micron particles (Panel B) are present in high concentrations. White arrow (Panel A) shows activated, shedding platelet which is prone to produce extracellular vesicles by shedding (i.e. ectocytosis), black arrow (Panel B) shows a red blood cell and gray arrow (Panel B) a leukocyte.

shed vesicles [12]. Roles of PDEVs in PVRP additionally interferes the PVRP terminology. However, it is reasonable to define PVRP as a fraction of blood plasma with platelet and EV concentrations higher than in peripheral blood [17].

Additionally, it is of utmost importance to thoroughly describe the preparation and administration protocol to enable further analyses. Briefly, PVRP can be prepared whether by isolation of plasma or buffy coat from blood sample via distinctive centrifugation-based protocols. The following subchapters describe blood fractions, platelet and EV functions to better understand preparation protocols and biological roles of PVRP.

2.1 Platelets and platelet-derived extracellular vesicles

Fig. 2 shows a sample of blood of a healthy subject that underwent centrifugation 1000–2000 g for 20–30 min. It can be seen that erythrocytes which are colored red gather at the bottom of the tube and normally occupy approximately 55% of blood volume (i.e. hematocrit). Plasma, a yellowish liquid above the erythrocytes, of a healthy person normally occupies approximately 45% of blood volume. Between plasma and hematocrit is a thin layer called buffy coat which is consisted of leukocytes and platelets. Buffy coat normally occupies less than 1% of blood volume. To sum up, platelets are abundant in the buffy coat. However, due to a heterogeneous platelet size and shape which cause variable sedimentation rates, they can be found in plasma and hematocrit also. This should be taken into consideration when isolating platelets in PVRP preparation.

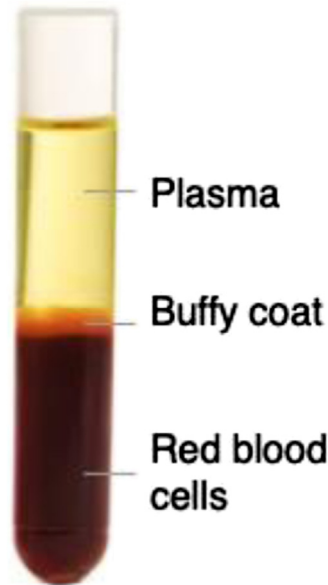


Fig. 2 Blood fractions after centrifugation at 1000–2000 g for 20–30 min. Upper yellowish layer is plasma and lower dark red are red blood cells. The ratio between the volume of red blood cells and total volume of blood is called hematocrit. Buffy coat, which occupies less than 1% of blood volume in a normal human subject, is observed as a greyish layer between plasma and red blood cells. Plasma or buffy coat are harvested from whole blood sample after the first centrifugation to produce platelet- and extracellular vesicle-rich plasma.

Platelets and platelet-derived EVs carry the most important regenerative functions of PVRP [12,14,16]. Resting platelets are 2–3 μm sized disc-like blood cells without nuclei, formed by megakaryocyte cytoplasm fragmentation in the bone marrow or lungs. Their lifespan is relatively short – 7–10 days [18]. Their blood concentration in healthy human subject is $150\text{--}400 \times 10^9/\text{L}$. Blood concentration qualifies them in the second place, after RBCs [19]. Platelets have an essential role in hemostasis, immune response and tissue regeneration. Their shape and size enable them to travel near the vessel wall and detect endothelial damage first [20]. They possess their functions via secretion of bioactive molecules contained in granules, cytoplasm or cellular organelles (i.e. extra-granular molecules) (Table 1). Alpha granules which are the largest and most abundant contain approximately 280 different types of proteins. Delta granules (i.e. dense granules) are smaller, less abundant and contain smaller molecules (Table 1). Lambda, the least abundant granules, are lysosomes, which contain mainly enzymes. T-granules have also been described

Table 1 Some molecules from platelets and platelet-derived extracellular vesicles in platelet- and extracellular vesicle-rich plasma with their functional roles in hemostasis, immune response and regeneration [16,21,23].

Molecule	Functions
<i>Alpha granules</i>	
bFGF	Chemotaxis, cell growth and differentiation, angiogenesis
CD63	Transmembrane adapter protein, leukocyte recruitment
CD40L	TNF- α family: B-cell response, endothelial cell and antigen-presenting cell activation
Cyclophilin A	Smooth muscle cell growth factor
MIP-1 α	Neutrophil, eosinophil activation, immunoglobulin formation
MMP-2, MMP-9	Extracellular matrix disintegration, platelet-leukocyte aggregate formation
PDGF	Cell growth and differentiation, monocyte/macrophage differentiation
PF4	Monocyte, neutrophil, T-cell recruitment and helper T-cell differentiation
Ppbp β -thromboglobulin NAP-2	Neutrophil activation and recruitment, macrophage phagocytosis activation
Protein S	Thrombin production and its regulation, angiogenesis
P-selectin	Leukocyte adhesion, complement activation
SDF-1	T-cell, monocyte and neutrophil chemotaxis
TGF- β	Cell proliferation, T-cell differentiation, B-cell and macrophage phenotype regulation
Thrombocidins	Bactericidal and fungicidal properties
Thrombospondins	Apoptosis, endothelial cell inflammation, platelet-macrophage aggregates formation
VEGF	Angiogenesis, adhesion molecule expression
vWF	Platelet adhesion, neutrophil extravasation
<i>Delta granules</i>	
ADP	Platelet, leukocyte and endothelial cell activation
Glutamate	T-cell regulation
Histamine	Vascular reactivity enhancement and degranulation, pro- and anti-inflammatory functions
Polyphosphates	Immune response stimulation
Serotonin	Dendritic cell and T-cell regulation, vasoconstriction and increased capillary permeability

Table 1 Some molecules from platelets and platelet-derived extracellular vesicles in platelet- and extracellular vesicle-rich plasma with their functional roles in hemostasis, immune response and regeneration [16,21,23].—cont'd

Molecule	Functions
<i>Extra-granular molecules</i>	
GPIIb/IIIa	Binding with leukocyte Mac-1
IL-1β	Acute inflammatory response, leukocyte and endothelial cell activation
Nitric oxide	Anti-inflammatory and antithrombotic effect
Thromboxane	T-cell differentiation, monocyte activation

ADP, adenosine diphosphate; *bFGF*, basic fibroblast growth factor; *CD40L*, CD40 ligand; *GPIIb/IIIa*, glycoprotein IIb/IIIa; *IL*, interleukin; *MIP*, macrophage inhibitory protein; *MMP*, metalloproteinase; *NAP*, neutrophil activating peptide; *PDGF*, platelet-derived growth factor; *PF*, platelet factor 4; *Pp100*, Pro-platelet basic protein; *SDF*, stromal cell-derived factor; *TGF*, transforming growth factor; *TNF*, tumor necrosis factor; *VEGF*, vascular endothelial growth factor; *vWF*, von Willebrand factor.

and contain molecules which are secreted via exocytosis or simply translocated to the extracellular surface of the plasma membrane [21]. Notably, some molecules from granules also provide antimicrobial effects of PVRP, identified in numerous in-vitro and in-vivo animal studies [22].

Understandably platelets are the most numerous blood immune cells due to a concentration greater than the blood concentration of leukocytes and important immunological and antimicrobial roles [19].

Platelet-derived extracellular vesicles (PDEVs) which are formed after platelet activation [18], possess cell functions of their origin, therefore they are important in hemostasis, immune response and tissue regeneration [19–21,24]. Additionally, platelet-derived exosomes isolated from PVRP possess a substantial role in the proliferation-, migration- and vessel formation-promoting effects of PVRP. These roles are mediated by composition of exosomes, which are rich in growth factors, especially bFGF, PDGF, TGF-β and VEGF (Table 1) [16].

2.2 Platelet- and extracellular vesicle-rich plasma preparation protocols

The knowledge of blood composition and characteristics of platelets described above enables us to prepare PVRP with different protocols. In fact, statistical review of 105 clinical studies describing the PVRP preparation protocols for later clinical application revealed that their repeatability is 10% [25].

At this point it is important to mention that platelets and leukocytes are also present in other blood fractions in addition to buffy coat. Therefore, it is impossible to completely separate platelets from other blood cells with centrifugation due to intra- and inter-subject blood components variability despite the protocol type. In general, there are 2 types of centrifugation-based PVRP preparation protocols: plasma-based and buffy coat-based protocols, differing in platelet yield, leukocyte concentration, volume of PVRP and consequent clinical applicability of prepared PVRP.

Additionally venepuncture technique (e.g. needle gauge, tourniquet use), materials used in PVRP preparation and staff skills are important in EV formation and therefore in PVRP preparation [11,26,27]. Blood donor and PVRP recipient are different in heterologous or the same in autologous in PVRP application.

2.2.1 Plasma-based protocols

These preparation protocols are based on the separation of blood plasma and platelets from leukocytes and erythrocytes. This is acquired by discarding hematocrit and buffy coat after the first centrifugation step (Fig. 3). Because buffy coat contains the majority of leukocytes and a considerable number of platelets due to their sedimentation rates, plasma-based protocols produce PVRP with low or minute leukocyte count at the expense of low platelet count. Yielded platelet concentrations are typically approximately 2–3 times higher ($300\text{--}500 \times 10^9$ platelets/L) than baseline whole blood platelet concentration [17]. To increase platelet count and to lower leukocyte count in PVRP, differential instead of single centrifugation can be utilized. Protocol shown in Fig. 3 is based on differential centrifugation of blood sample at low spin rate and short spin regimen (about 200–400 g for 4–6 min at 18 °C) to isolate RBCs initially, followed by centrifugation of harvested supernatant (i.e. plasma above buffy coat) at high spin rate and long spin regimen (about 600–800 g for 16–18 min at 18 °C) to concentrate platelets.

2.2.2 Buffy coat-based protocols

These preparation protocols focus on platelet isolation. To achieve this, buffy coat is isolated from blood sample by centrifugation at high spin rates and long spin regimens. Namely, the platelet count is high on the expense of high platelet count. Typically buffy coat-based preparation protocols yield 3–8 times higher ($500\text{--}1.500 \times 10^9$ platelets/L) than baseline whole blood platelet concentration [17].

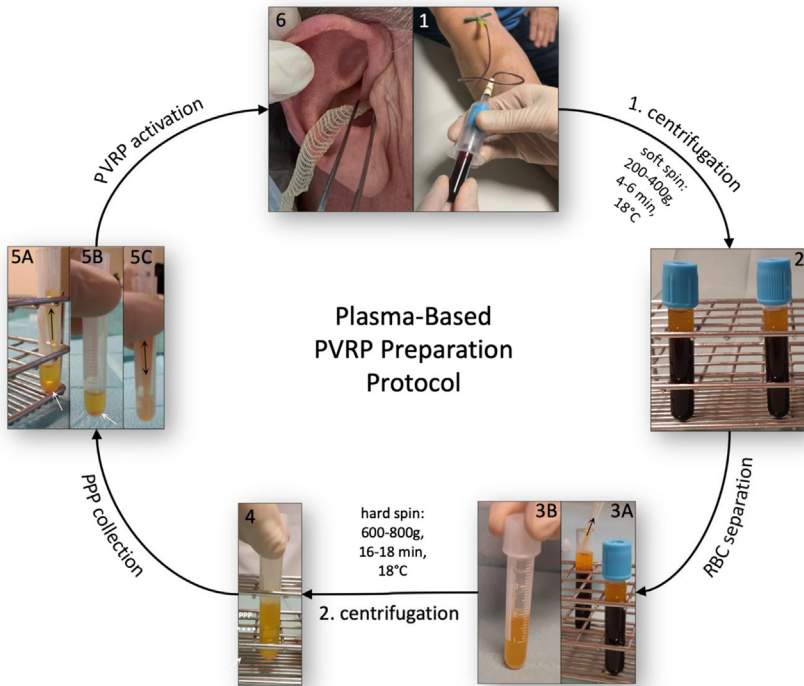


Fig. 3 A scheme of the plasma-based platelet- and extracellular vesicle-rich plasma preparation protocol. Blood samples are collected into tubes with anticoagulant (Panel 1) and are centrifuged at several hundred g and temperature close to room temperature for some minutes to prepare a product with distinctive layers (Panels 2 and 3A): red blood cells, plasma and buffy coat. Plasma that was harvested into citrated tubes (*black arrow* in Panel 3A) is transferred with a sterile pipette into a single tube (Fig. 2, Panel 3B). Accuracy is required to prevent buffy coat and red blood cell harvesting. Plasma is then centrifuged again (at about 500–1000 g for about 20 min at the same temperature) to produce a preparation with distinctive hazy pellet (*white arrows* in Panels 4, 5A and 5B). The upper half of this product (*bordered by the white dashed line* in Panel 4) that is called platelet-poor plasma (PPP) is discarded (Panel 5A) without prior resuspension. The remaining preparation (Panel 5B) is resuspended in the same tube (Panel 5C). From that point on, the preparation which is optionally exogenously activated prior autologous administration (Panel 6), is called platelet- and extracellular vesicle-rich plasma.

2.3 Composition of platelet- and extracellular vesicle-rich plasma

Ideal average concentrations of PVRP have been suggested according to healing effects. However, it is postulated that platelet concentrations above $1250 \times 10^3/\mu\text{L}$ could exhibit inhibitory effects [17]. Additionally, the

healing effects of other platelet concentrations in PVRP have been studied. PAW classification was published by Delong et al., which classifies PVRP according to platelet concentration (P), activation method (A) and leukocyte concentration (W). It provides guidance in PVRP preparation and standardization of data for subsequent meta-analyses of studies in various clinical settings. Delong et al., also proposed that these studies should include baseline whole blood platelet concentration [17].

2.3.1 Platelet concentration

Platelet concentration in PVRP is classified as low, moderate, high or super. Low means that platelet concentration in PVRP is lower than baseline and is termed as platelet-poor plasma. It has a minute effect; therefore, it is often used as a placebo. Moderate PVRP platelet concentrations are up to 4-times higher than baseline (i.e., up to $750 \times 10^9/\text{L}$). Majority of clinical studies that reported satisfactory effect used moderate platelet concentrations. If platelet concentration is 4- to 6-times higher than baseline (i.e., $750\text{--}1250 \times 10^9/\text{L}$), it is classified as high. This concentration is usually achieved with buffy coat-based PVRP preparation protocols. Several in-vivo clinical and animal studies report a beneficial effect of the high platelet concentration. Platelet concentrations in PVRP higher than 6-times baseline (i.e., above $1250 \times 10^9/\text{L}$) could be detrimental and could induce harmful effects due to paradoxical inhibition as excessive concentrations of platelets and growth factors lead to platelet apoptosis, receptor downregulation and desensitization [17].

2.3.2 Leukocyte concentration

Leukocyte concentration depends on the PVRP preparation protocol. In contrast to plasma-based protocols, buffy coat-based protocols yield leukocyte concentrations higher than baseline. High leukocyte concentrations, particularly neutrophils, promote scar formation, for that reason leukocytes are undesirable for these cases. In the prevention of infections and treatment of open wounds, high concentrations may be required [17].

2.4 Roles of anticoagulants in blood sampling and platelet- and extracellular vesicle-rich plasma activation type

When PVRP is prepared it can be administered to target tissue unchanged or administered as a gel.

PVRP gel is a result of an in-vitro (i.e. exogenous) platelet activation which can be simplified by blood clot formation. It can be achieved by using

various substances (i.e. activators), e.g. bovine thrombin, calcium chloride or both. Activators also dilute PVRP due to the increase of the volume. Side effects were observed after the application of the preparation; bovine thrombin was found to cause hypersensitivity reactions while calcium chloride induced pain or severe burning due to a decrease of pH in the preparation [17]. PVRP gel can be prepared also if blood is processed in a tube without anticoagulant. Indicatively, PVRP gel showed higher probability for adverse effects compared to native PVRP. Nomenclature of PVRP gel is inconsistent as it is often termed as platelet-rich fibrin, platelet-rich fibrin growth factors, platelet gel, platelet-rich gel etc. Therefore, classification of DeLong et al. suggests a description of activation type. Despite disadvantages, PVRP gel enables easier application in some cases due to mechanical characteristics (i.e. it can be sutured or manually applied to the target tissue). Additionally PVRP gel is supposed to release growth factors in a better controlled manner compared to native PVRP [17].

If blood was drawn into tubes without anticoagulant, a blood clot would form during the first centrifugation and the product is not defined as PVRP. Therefore, blood must be drawn into tubes containing anticoagulant [28]. The classification by DeLong et al. does not consider the type of anticoagulant in the preparation of PVRP, although it is important [17]. Based on a study comparing three anticoagulants: sodium citrate, ACD (acid citrate dextrose solution) and EDTA (ethylenediaminetetraacetate) in the preparation of PVRP, do Amaral et al. [28] recommended the use of sodium citrate as an anticoagulant in venepunction. Sodium citrate enables the preparation of more concentrated PVRP and stimulates the proliferation of mesenchymal stromal cells without interfering with their phenotype [28].

Also, PVRP can be administered to target tissue without prior intentional activation (i.e. native PVRP). Then activation process happens in the target tissue (i.e. endogenously) which mimics the physiological platelet activation mechanism via interaction with type I collagen. Type I collagen is even more potent activator than thrombin. Endogenous activation prevents oversaturation of growth factor receptors due to natural growth factor release pattern. Additionally, native PVRP can be injected to the target tissue [17].

Although an activation type is important, PVRP is always activated exogenously and endogenously. Namely, platelets are imperatively exogenously activated during venepuncture and centrifugation and also endogenously by type 1 collagen [17,26]. Activation of plasma is targeted at

transformation of platelets as regards the reactions that take place in the platelet membranes and their surroundings. However, as the local membrane composition is connected to the local curvature, changes in the platelet shape also take place. Activated platelets first exhibit tubular protrusions while the mother cell becomes globular. Eventually, the protrusions detach from the mother cell which is prone to fragment. Remodulation involving material from adjacent cells and/or fragments, including residual RBCs, may take place. Fragmented and reconstituted platelets and RBCs thereby form EVs in the preparation [14,19–21,24].

Basic knowledge of PVRP preparation protocols, PVRP composition, type of PVRP activation and desired PVRP administration technique allow a clinician to appropriately choose the most suitable PVRP for a patient. It is imperative to be aware of the importance of EVs in PVRP as mentioned above.



3. Treatment with platelet- and extracellular vesicle-rich plasma

Ten years after the first use of PVRP in hematology in 1970, it began to be used in maxillofacial surgery followed by the treatment of musculoskeletal injuries in athletes [15]. PVRP use has expanded to other fields of medicine especially to surgical treatment of various disorders (Table 2). Although PVRP's wide indication area, there is a deficit of systematic reviews or meta-analyses regarding its use for the majority of diseases.

There is a lack of meta-analyses and systematic reviews of PVRP application that would enable further validation of this treatment option for different diseases, especially in otorhinolaryngology [60]. However, the application of PVRP was thoroughly described according to an anatomical area in otorhinolaryngology due to its invaluable potential (Table 3).

3.1 Face, nose and paranasal sinuses

PVRP has been proved efficient in different methods of facial rejuvenation. It was administrated as a local injection on half of the face every 2 weeks for 3 months. The effect was compared to the contralateral half of the face treated with injected growth factors (i.e. mesotherapy). PVRP was more efficient in rejuvenation compared to mesotherapy according to patient-reported and objective measures [62]. Additionally, a recent systematic review

Table 2 A survey on platelet- and extracellular vesicle-rich plasma clinical application in different fields of medicine (except otorhinolaryngology).

Field of medicine	Medical indication, disease or surgical procedure associated with PVRP application
Orthopedics and traumatology	^a Plantar fasciitis [29] ^a Elbow epicondylitis [29] ^a Knee osteoarthritis [30,31] Hip osteoarthritis [32] Achilles tendinopathy [14] Patellar tendinopathy [14] Bone fracture [31] ACL rupture [31]
Plastic, reconstructive, esthetic and hand surgery	Reduction mammoplasty [33] Abdominoplasty [33] Carpal tunnel syndrome [34] Alopecia [35,36] Breast reconstruction with lipofilling [37]
Wound care surgery	^a Diabetic ulcers [38] Venous leg ulcers [39] Arterial ulcers [40] Pressure ulcers [39] Traumatic wounds [40] Chronic wounds [31,41]
Maxillofacial and oral surgery	^a Periodontal defects [42] ^a Periodontal disease [43,44] ^a Temporomandibular joint osteoarthritis [45] Odontogenic mandibular cysts surgery [46] Mandibular fracture surgery [46,47] Dental implant surgery [43] Maxillary sinus lift [43] Bisphosphonate related osteonecrosis of the mandible [43] Orthognathic surgery [48] Dental extraction [43]
Gynecology and urology	Caesarean section [37] Cervical ectopia [37] Vulvar dystrophy [37] Cancer vulvectomy [37] Vesicular, perianal, rectovaginal fistulae [37] Erectile dysfunction [49] Urinary incontinence [37,49] Infertility [37] Vaginal infections [37] Vaginal aging [37] Peyronie's disease [49]

(Continued)

Table 2 A survey on platelet- and extracellular vesicle-rich plasma clinical application in different fields of medicine (except otorhinolaryngology).—cont'd

Field of medicine	Medical indication, disease or surgical procedure associated with PVRP application
Ophthalmology	Hyposecretory dry eye [50] Dry eye syndrome after laser refractive surgery [51] Sjögren’s syndrome [52] Corneal ulcers [53]
Cardiothoracic surgery	Sternotomy wounds [54,55] Chylothorax post esophagectomy [56] Mediastinitis after cardiac surgery [55]
Neurosurgery	Post-dural puncture headache [57] Lumbar spine surgery [58] CSF leakage after cranial and spinal surgery [59]

ACL, anterior cruciate ligament; *CSF*, cerebrospinal fluid; *PVRP*, platelet- and extracellular vesicle-rich plasma.
^aDenotes systematic review or meta-analysis. Some fields of medicine share medical indications, diseases or surgical procedures listed in the table.

showed that PVRP injections are safe and may have modest benefit in the treatment of skin aging [61]. A meta-analysis of 7 studies showed that PVRP is efficient as a combination therapy with fractional carbon laser therapy or microneedling for atrophic acne scars [63]. PVRP has also been used in face and neck lift to accelerate surgical wound healing [33].

Wound healing was accelerated by PVRP also in rhinoplasty, especially in the early postoperative period [64]. PVRP was proved effective in increasing viability and keeping the form of inserted diced cartilage in nasal dorsum after rhinoplasty [65].

Administration of PVRP-soaked nasal packs reduced crusting, bleeding, complaints and accelerated healing after rhinosurgical procedures [66].

Additionally, PVRP was efficiently applied concomitantly with graft materials for anterior skull base cerebrospinal fluid leaks [70], frontal sinus obliteration after surgical treatment of chronic frontal sinusitis [68,69] and fibrous dysplasia [67].

3.2 Lips and oral cavity

PVRP is efficient also in the pediatric population, in reducing scar width after cleft lip reconstruction according to randomized controlled study [71]. It

Table 3 A survey on treatment with platelet- and extracellular vesicle-rich plasma in distinct anatomical areas of otorhinolaryngology.

An anatomical area in otorhinolaryngology	Medical indication, disease or surgical procedure associated with PVRP application
Face, neck, nose, paranasal sinuses and anterior skull base	Facial rejuvenation [61,62] Neck rejuvenation [62] Facial acne scars ^a [63] Facelift [33] Neck lift [33] Rhinoplasty [64,65] Postsurgical nasal packing [66] Frontal sinus obliteration [67–69] Anterior skull base cerebrospinal fluid leaks [70]
Lips and oral cavity	Cleft lip [71] Oral mucosal lesions [72,73] Oral pemphigus vulgaris [74] Osteoradionecrosis of the mandible [75]
Pharynx and esophagus	Pharyngoplasty for obstructive sleep apnea [76] Traumatic esophagocutaneous fistula [77]
Salivary glands	Suprafacial parotidectomy [78,79]
Ear and lateral skull base	
External ear	Auricle amputation [80]
Middle ear	Acute eardrum perforation [81] Chronic eardrum perforation [82–91] Reconstruction of the posterior external ear canal wall [92] Mastoid reconstruction after CWD mastoidectomy [93,94]

CWD, canal wall down; PVRP, platelet- and extracellular vesicle-rich plasma.
^aDenotes systematic review or meta-analysis.

improves healing in the early postoperative period and significantly reduces pain when administered as a gel on oral defects after excision of potentially malignant lesions [72,73]. The efficiency of intralesional PVRP injections was comparable to corticosteroid injections for the treatment of oral pemphigus vulgaris, therefore PVRP was suggested as an alternative treatment of this disease [74]. Another disease treated with PVRP gel is mandibular osteoradionecrosis [75].

However, no systematic reviews or meta-analyses were found for the treatment of the diseases of lips and oral cavity with PVRP.

3.3 Pharynx and esophagus

In pharyngology, PVRP application was associated with pharyngoplasty for the treatment of obstructive sleep apnea. It was administered as a gel to the surgical wound before suturing. PVRP statistically significantly decreased the incidence of palatal wound breakdown, provided better pain management and reduced the time taken to return to the normal diet [76].

In esophagology, PVRP was used in the treatment of traumatic esophagocutaneous fistula in a single pediatric patient [77].

3.4 Salivary glands

PVRP enhanced healing process also when its application was associated with superficial parotidectomy due to benign parotid gland tumor. In a recent controlled trial, it was administered as a gel in the surgical wound which reduced complications rate, length of hospitalization and duration of drainage tube [78]. Another randomized controlled study reported a benefit for the same indication [79].

3.5 Ear and lateral skull base

The vast majority of studies in otology and lateral skull base surgery report the administration of PVRP for tympanic membrane perforations.

3.5.1 *The external ear*

An external ear was treated with PVRP in a patient suffering from traumatic complete auricle amputation. Replanted auricle was postoperatively repetitively treated with local injections of PVRP, polydeoxyribonucleotide and hyperbaric oxygen therapy. Although this is a case report, authors report efficacy of PVRP [80].

3.5.2 *The middle ear*

Multiple studies report the administration of PVRP for treatment of tympanic membrane perforations, acute or chronic. Acute traumatic perforations were treated efficiently with PVRP-gel only in a randomized controlled study [81]. The gel was also administered in chronic perforations during tympanoplasty due to better durability. It was administered as a plug lateral to the tympanic membrane after being reconstructed with tympanoplasty and perichondrium graft, due to central perforation. Success of graft taking was statistically significantly higher and infection rate lower in a group treated with PVRP compared to the control group [89]. In general, it

appears that PVRP is more efficient in the treatment of tympanic membrane perforations if administered concomitantly with grafts or packing material. Namely, PVRP improves the fascial [82,88] or fat graft uptake [90] and improves healing if administered as PVRP-soaked atelocollagen sponge [91] or gelfoam [85].

Furthermore, PVRP was administered as the hourglass-shaped gel graft to the small tympanic membrane perforations after freshening perforation margins. A successful perforation repair was achieved in 84% in this study [87]. Despite the promising results, a randomized controlled study reported the low success rate of an hourglass-shaped PVRP for treatment of tympanic membrane perforations [95]. Therefore standardization of PVRP preparation protocols and reporting guidelines are required to perform adequate meta-analyses to evaluate the effectiveness of PVRP without grafts in tympanoplasty.

Osteogenic potential of PVRP has already been identified, therefore it was successfully applied in bone reconstruction in otorhinolaryngology. It was administered with bone pate to obliterate mastoid after canal wall down mastoidectomy. The case series study reported more than 85% total success rate with no reported complications. An advantage of PVRP and bone pate obliteration is that a disease recurrence can be easier radiologically identified compared to other obliteration materials [94]. Additionally, PVRP was used with bone pate and titanium mesh to reconstruct posterior ear canal wall after canal wall down mastoidectomy [92]. To conclude there are no randomized controlled clinical studies of PVRP application in mastoid surgery.

3.6 Platelet- and extracellular vesicle-rich plasma adverse effects

Adverse effects related to the application of PVRP are very rare. The majority of adverse effects are related to the surgical procedure during which the PVRP is administered. The risk to transmit infectious or malignant disease is minute when using autologous PVRP. Heterologous PVRP application can cause a rejection reaction also [96].

3.7 Future of platelet- and extracellular vesicle-rich plasma in otorhinolaryngology

PVRP offers potential for its use also in the treatment of inner ear, retrocochlear, vestibular, facial nerve, laryngeal, tracheal and voice disorders. According to published animal studies, PVRP could be combined with skin

grafts for soft tissue reconstruction [97], as the treatment option for vocal cord paralysis [98], vocal cord injury [99], dysphagia [100], pharyngocutaneous fistula [101], anosmia [102], facial palsy [103] or hearing loss [104].

Due to a successful application and promising results of PVRP in the treatment of ear diseases, authors propose PVRP use in the treatment of chronic ear inflammation after canal wall down mastoidectomy required to treat cholesteatoma. For that reason, following subchapters describe cholesteatoma, its current treatment and new treatment options proposed by authors.

3.7.1 Treatment of chronic middle ear infection after cholesteatoma surgery with platelet- and extracellular vesicle-rich plasma

3.7.1.1 Cholesteatoma burden

Cholesteatoma is defined as the presence of squamous epithelial tissue in the middle ear and/or mastoid associated with chronic inflammation. The most common type of cholesteatoma arises from the tympanic membrane and is called an acquired cholesteatoma which is depicted in Fig. 4. The congenital type is very rare. The incidence of acquired cholesteatoma in the developed world is low, with an estimated annual incidence of 9.2 cases per 100 000 inhabitants [105]. However, the incidence may be higher in developing countries based on the higher incidence of chronic suppurative otitis media. Due to increased migration flows from the developing to developed countries, the incidence of cholesteatoma and chronic suppurative otitis media is rising in developed countries as well [106].

3.7.1.2 Cholesteatoma treatment

Cholesteatoma is a disease treated surgically by removing squamous epithelial tissue trapped in middle ear space. Many surgical options and approaches exist based upon the nature and extent of the disease. They range from simple tympanoplasty to radical mastoidectomy. The latter is called open technique surgery which is depicted in Fig. 5. Cholesteatoma extending into the antrum and mastoid and those extending medial to the head of the malleus may require complete mastoidectomy. Many patients with cholesteatoma will undergo multiple surgical procedures due to recurrence of the disease. Each subsequent procedure is more ablative and less concerned with hearing status as opposed to the eradication of cholesteatoma. Despite the advance in surgical techniques, an open technique surgery is still used in approximately 40% of cholesteatoma surgery [107]. These patients end up with large

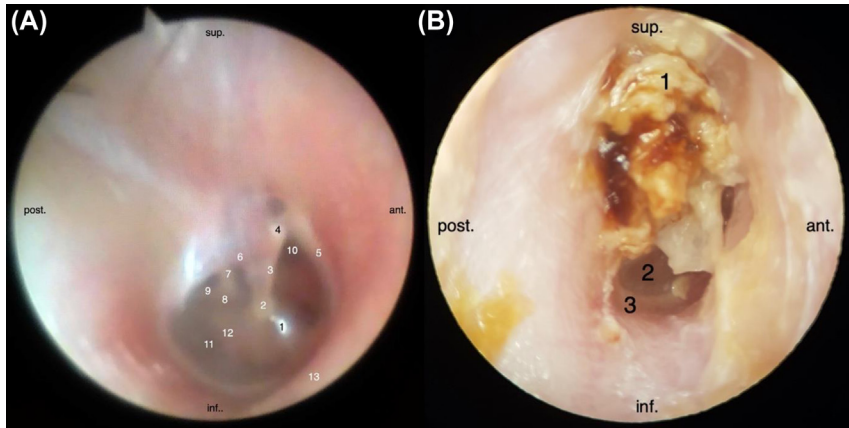


Fig. 4 Endoscopic photographs of the normal tympanic membrane (A) and cholesteatoma (B) of the right ear. Comment: A shows normal tympanic membrane, which is pale, gray and in neutral position (i.e. not retracted toward middle ear or bulging outwards), light reflection (1) is cone-shaped located in an anteroinferior quadrant of the tympanic membrane. Umbo (2), handle of malleus (3), lateral processus of malleus (4), tympanic annulus (5), chorda tympani (6), incus (7), incudostapedial joint (8), stapes (9), shadow of eustachian tube (10), shadow of round window niche (11) and promontory (12) are visible through transparent tympanic membrane. Anterior external ear canal wall is bulging posteriorly (13). B shows typical keratinizing masses (i.e. squamous epithelial tissue) of cholesteatoma (1) in posterolateral aspects of the tympanic membrane, remnant of pars tensa (2) and tympanic annulus (3). Posterior external ear canal wall and attic are probably eroded, which is a sign of a destructive chronic ear inflammation requiring surgical treatment.

postsurgical cavity due to removal of the posterior external ear canal wall (i.e. radical cavity).

Due to multiple suppurative periods from the radical cavity and formation of granulation tissue, a large spectrum of surgical and nonsurgical treatment options have been applied to reduce patient discomfort [108,109]. Granulations in a once-healed radical cavity arise when self-cleaning ability and ventilation are hampered and epidermal debris is allowed to collect and become infected with bacteria [110]. This causes a persistent discharge from moist radical cavities which is depicted in Fig. 6 [111].

The most common bacteria isolated from a moist radical cavity is *Staphylococcus aureus* [110]. Consequently, frequent cleaning and conservative treatment measures that include removal of the granulation followed by chemocautery with substances as 2% aqueous gentian violet are needed to control the discharge from moist radical cavities. However, frequently an

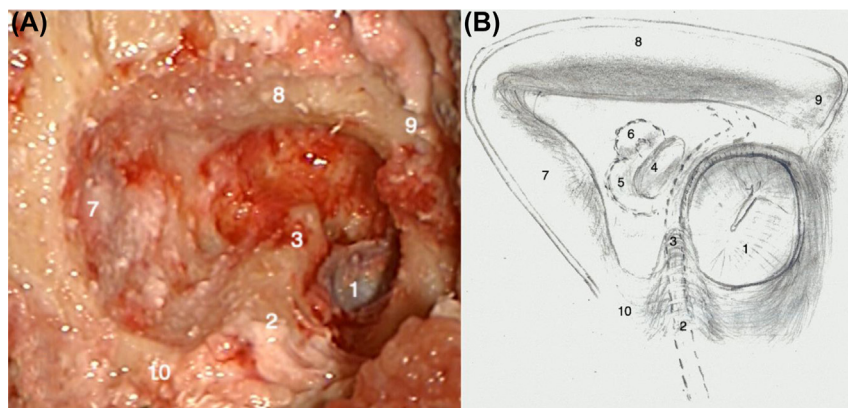


Fig. 5 Intraoperative otomicroscopical photography of right radical cavity after removal of cholesteatoma (A) and an illustration (B) with anatomical landmarks. Comment: The mastoid and middle ear with air cells are drilled and deepithelialised during surgery to remove the complete cholesteatoma and make a single cavity (i.e. radical cavity) which enables good postoperative ventilation and drainage of the middle ear. Important structures are identified during surgery: pars tensa of tympanic membrane (1), base of former posterior external ear canal wall (2), facial ridge (3), facial nerve (dashed), lateral (4), posterior (5) and superior semicircular canal (6), sigmoid sinus (7), middle cranial fossa dura (8), zygomatic root (9), mastoid tip (10). The radical cavity reepithelialises postoperatively.

area of granulation resists epidermisation owing to a small underlying focus of osteitic bone or sequestrum [112]. Although there is little published evidence to indicate the incidence of moisture or discharge following open technique surgery, two 10-year studies show that improvement in surgical techniques has reduced this complication [113]. Discharge is reported in 3%–20% of radical cavities [114,115].

Another option to prevent moist radical cavity is to obliterate it with synthetic material as bioactive glass, hydroxyapatite, calcium phosphate ceramic granules, coral, demineralized bone matrix, titanium, silicone or with autologous tissue as musculoperiosteal flaps, bone chips, bone paste and cartilage [108,116].

3.7.1.3 Proposed platelet- and extracellular vesicle-rich plasma application in moist radical cavities after cholesteatoma surgery

Not only the sequelae of cholesteatoma and its surgical treatment are debilitating, above-mentioned long-lasting conservative measures of chronically inflamed moist radical cavities are significantly worsening the patients' quality of life and put a significant burden on health care. Due to the knowledge

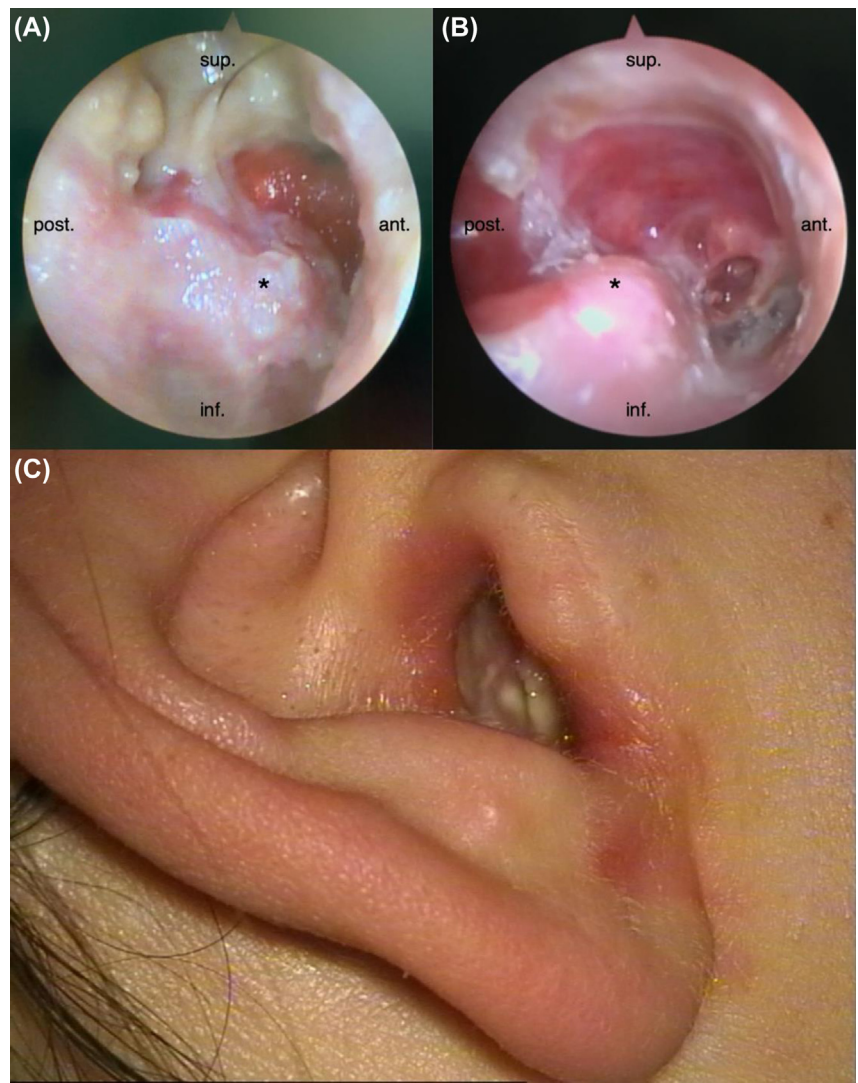


Fig. 6 Endoscopic photographs of inflamed, i.e. moist radical cavities after cholesteatoma surgery and photography of inflammation extending to auricle. Comment: A and B depict diffuse inflammation of radical cavities. An abundant discharge is visible on A. Facial ridges (*) are relatively spared from the inflammatory process. Inflammation is extending to auricle (C).

of local immune response mechanisms in radical cavities and exhausted treatment options, new conservative treatment options are being sought. We have identified PVRP as promising treatment option for moist radical cavities based on published preclinical and clinical studies. PVRP could be prepared by 2-step plasma-based centrifugation protocol and administered to moist radical cavity via PVRP-soaked ear wick (Fig. 3).



4. Future of extracellular vesicle isolates in otorhinolaryngology

Since PVRP is considered as effective treatment option in otorhinolaryngology and PDEV are its main effectors, there is a need to design a PDEV-based isolate in the future. PDEV isolates have to our best knowledge not been used in clinical settings for wound healing disorders so far.

However, other cell-derived EV isolates have already been proposed to be used either as diagnostic or therapeutic tool [6]. Namely, EVs have been studied thoroughly to clarify the pathogenesis of disease.

Pathogenesis of vestibular schwannoma-associated hearing loss has not been clarified yet. For that reason, EVs derived from vestibular schwannoma cells have been studied and identified as important mediators in the pathogenesis of sensorineural hearing loss in vestibular schwannoma. These findings enable further research of vestibular schwannoma diagnosis and treatment [117]. Pathogenetic role of exosomes was also identified in otitis media [118].

For the evaluation of inner ear status, exosomes could be used as biomarkers, since animal studies show an important role of inner ear exosomes in drug ototoxicity [119]. EVs, especially exosomes possess therapeutic potential also. They could be used as drug vehicles [120]. Exosome-associated adeno-associated virus (Exo-AAV) could be used to insert the deafness-responsible missing genes to inner ear hair cells. In animal studies, Exo-AAV transduced all inner ear hair cells (i.e. inner and outer) compared to adeno-associated virus which transduced inner hair cells only [120]. EVs isolated from inner ear hair cells may reduce the noise- and drug-related cochlear damage as they may incorporate pro-resolving mediators, anti-inflammatory drugs and may carry drugs. Therefore, these EVs could be used intracochlearly via concomitant cochlear implantation to reduce post-implantation inflammation in scala tympani [121].

Due to known pathogenetic roles of EVs in carcinogenesis [4,7], they possess a screening, diagnostic and prognostic roles in head and neck cancer

also. Salivary and serum exosomes with these roles were identified in oral squamous cell cancer [122–125] and laryngeal cancer [126]. Also, exosomes that negatively regulate radiosensitivity of laryngeal cancer cells were identified, therefore novel insights into laryngeal cancer treatment are under development [127]. However, to fulfill claimed expectations of EV isolates as promising diagnostic and therapeutic tools, more evidence on EV-based disease mechanisms should be gathered and methods of EV harvesting and assessment improved [128]. For further application of these preparations in the sterile areas of the human body, it is inevitable to test their sterility.

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