APPLICABILITY OF PLATELET- AND EXTRACELLULAR VESICLE-RICH PLASMA IN MEDICINE

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Abstract

Platelet-rich plasma is a blood-derived product with proven favourable effects after a local application in various healing disorders. It is also rich with extracellular vesicles - a heterogeneous group of nano- to micro-sized membranous structures -that are considered as the main mediators of regenerative effects. Hence, the prepared blood product can be suitably named »platelet- and extracellular vesicle-rich plasma«. Platelets and platelet-derived extracellular vesicles are not only important in haemostasis, but also in the immune response. Platelets are the most numerous blood immune cells. They are also the main source of blood-derived extracellular vesicles. Extracellular vesicles play an important role in intra- and intercellular communication, therefore they can be utilised in diagnosis and treatment. Platelet- and extracellular vesicle-rich plasma is being used for almost three decades in different fields of medicine, especially in surgery, due to its favourable regenerative properties. However, extracellular vesicles are seldom described in clinical studies that consider the platelet-rich plasma. Based on the molecular mechanisms of the healing process, functions of platelets and platelet-derived extracellular vesicles, platelet- and extracellular vesicle-rich plasma offers an important therapeutic solution in different diseases. Application of platelet- and extracellular vesicle-rich plasma is inexpensive and safe, however its preparation requires advanced laboratory skills. An article contains a description of this blood product and reported experiences on its use. We also present our recent advances which are a product of a collaboration of researchers from medical and biomedical fields. This collaboration leads to an advancement in the treatment modalities in different fields of medicine, also otorhinolaryngology and cervicofacial surgery.

1. Roles of platelets in immune response and tissue regeneration

Platelets are 2-5 µm sized blood cells without nuclei which are formed as a result of fragmentation of megakaryocytes in the bone marrow or lungs. Their lifespan is relatively short (7-10 days) due to the lack of the nucleus (1). According to the standards, their concentration in healthy human subjects is in the range of (1.5-4) x 10^{11} per litre of blood. Concentration in blood qualifies platelets in the second place, after red blood cells (2). Platelets have an essential role in haemostasis, that is why preparations with high platelet concentrations have been used (since approximately 1970) for the treatment of bleeding and for haemorrhagic diathesis. Back then the haematologists have named the preparation "platelet-rich plasma". Platelets are important not only in haemostasis but also in the immune response. They are the key cells of an innate and of an acquired immune responses and are therefore important in tissue regeneration (2). Due to a concentration greater than the blood concentration of leukocytes, platelets are the most numerous immune cells in blood. Platelet-derived extracellular vesicles (EVs) that are formed after platelet activation (1), are also important in providing haemostasis and immune responses. Knowledge on the roles of platelets and platelet-derived EVs has led to the rise of preparations with high platelet concentrations, especially platelet-rich plasma (PRP), which also contains high EV concentrations. This is why the preparation is called »platelet- and extracellular vesicle-rich plasma« (PEVRP) (2–5).

2. Extracellular vesicles

2.1. Description and classification

Extracellular vesicles (EVs) are a heterogeneous group of cell membrane structures that can arise from any cell, including plant cells and bacteria (6–8). EVs were isolated from various body fluids and cell culture media and subsequently examined by different microscopic techniques (9–11). EVs in blood isolates are a dynamic material derived from blood cell fragments (12) and surrounding solutions. Shear forces in the process of isolation are also important (13). Because most erythrocytes and leukocytes are removed from the blood sample in the first steps of EVs isolation, megakaryocyte- and platelet-derived molecules are often present in isolates (14). Standard laboratory tests do not currently cover the measurement of EVs in isolates, but the expected concentrations of EVs in isolates may be high (15).

Initially, the belief was that EVs are only carriers of cell waste (6), but later it became evident that they play an important role in intercellular signalling (8,16) and thus have a significant impact on the course of many diseases (17,18). Some divide EVs into two groups; microvesicles and exosomes, in regard to their size and composition, as well as to their

assumed origin (6). Microvesicles are described as membrane-bound particles of 50-500 nm in size, which are formed by the process of ectocytosis or plasmalemma budding (8). Exosomes are described as membrane-bound particles of 50-150 nm in size, which are formed in the inner compartments (endosomes) of the cell and released into the extracellular milieu by fusion of the compartment membrane with the cell membrane in the process of exocytosis. Several molecular mechanisms are involved in the formation and secretion of exosomes and microvesicles (6,16). When an EV reaches the target cell, it triggers a physiological or pathological response. The target cell may be remote, adjacent, or the cell of EV's origin. This points out an important role of EVs in autoregulation. The EV's activity is dependent on its contact with the plasmalemma of the target cell. EV can act in three possible ways: via binding to membrane receptors, via coupling with plasmalemma and consequent release of cargo (carried by the EV) into the cytoplasm of the target cell and/or through the uptake of the EV by endocytosis (6).

2.2. Applicability of extracellular vesicles in diagnosis and treatment

EVs may serve diagnostic or therapeutic purposes because of their roles in intercellular communication. Their use as biomarkers of infectious, neurodegenerative, autoimmune diseases and tumours is promising. For therapeutic purposes, they could be used as vaccines against infectious diseases or tumours, as immunosuppressive or regenerative therapy, as carriers of active substances and even in the cosmetic industry to regulate skin pigmentation (17). The use of EV isolates for systemic treatment of tumours is at least in the stage 2 clinical trials. Platelet EVs are on the other hand the main effectors of regenerative effects of platelet-rich plasma - an already established treatment for various healing disorders (17). The effect of platelet EVs is also confirmed by researches stating that platelet EVs alone have the same or even better regenerative effect than platelet- and extracellular vesicle-rich plasma (14). However, efficient and reproducible EV isolation protocols are required to initiate and implement their use for diagnostic and therapeutic purposes (19).

3. Platelet- and extracellular vesicle-rich plasma (PEVRP)

3.1. Description and preparation procedures

The literature describes platelet-rich plasma (PRP) as part of blood plasma fraction with a platelet concentration higher than in peripheral blood (20). The process of PRP preparation also retains particles smaller than platelets in PRP, which is why PRP is also rich in EVs (Figure 1).

After centrifugation of the blood sample, platelets are mostly found in the "buffy coat", i.e. layer between hematocrit and plasma. Due to their heterogeneous sizes and shapes, they are also present in plasma and hematocrit, which should be taken into account in the

preparation of PEVRP. Because centrifugation does not enable complete separation of different cell types from one another, leukocytes and erythrocytes are always present in the PEVRP, while their amounts and ratios depend on the sample preparation. Many procedures were developed and described, but they are rarely reproducible (22).

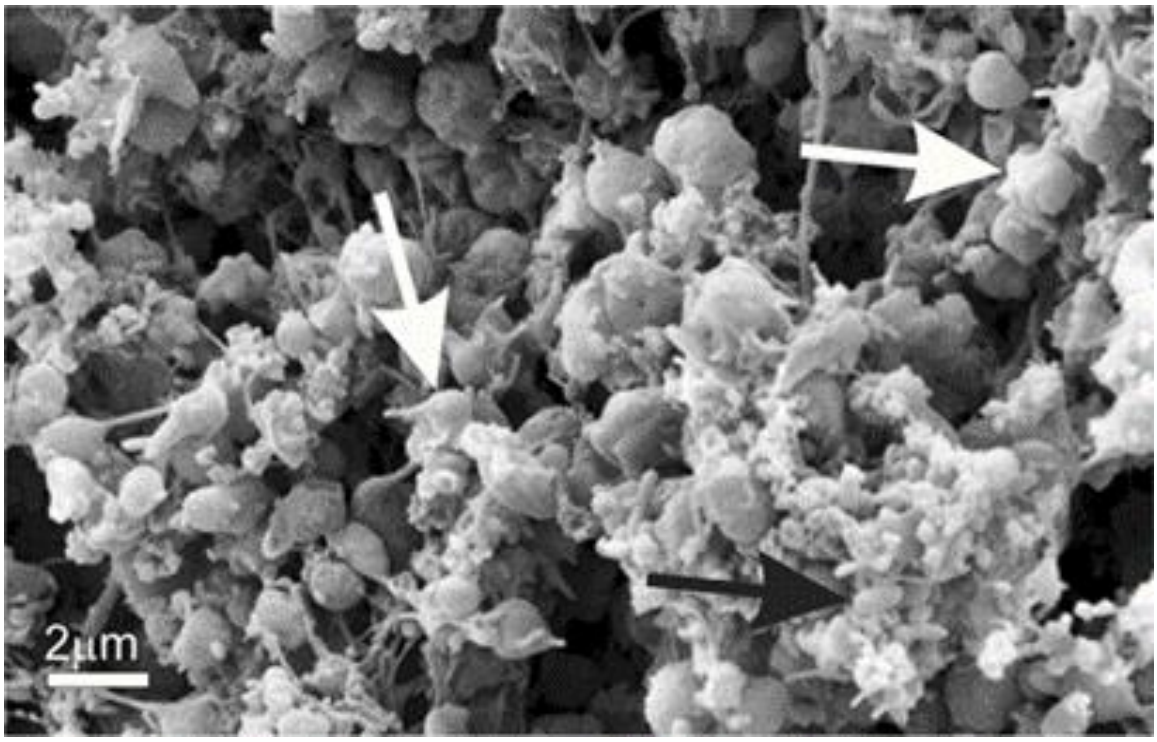

Figure 1: Electron microscopic image of activated platelets (white arrow) and extracellular vesicles (black arrow) in platelet-rich plasma. Adopted by Uršič et al. (21).

In general, procedures for PEVRP preparation by sequential centrifugation can be divided into blood plasma-based and buffy coat-based procedures, the blood plasma-based procedures being reported as the more effective ones (20).

The aim of the former is to separate blood plasma and platelets from leukocytes in the first centrifugation step. A leukocyte-poor preparation is formed at the expense of a lower platelet concentration because the buffy coat, which has a high platelet concentration, is discarded. The purpose of buffy coat-based procedures is to isolate "buffy coat", which results in PRP fraction with higher concentrations of platelets and leukocytes than obtained by other procedures.

The platelet concentration in PEVRP ideal for regenerative purposes has been determined in some studies and is approximately 5 times the average normal blood platelet count (i.e. baseline cocncentration: 150-350 x $10^3/\mu L$) (20). The preparation of PEVRP essentially depends on initial blood platelet count and on blood viscosity. As both of these parameters

are highly variable, it is complicated to prepare autologous preparations with the same concentrations for each patient. On the other hand, the concentrations in heterologous PEVRP are more easily adjusted. PEVRP may, according to DeLong et al. be divided into 4 types according to its platelet concentration; low, moderate, high and super. Low platelet concentration PEVRPs is considered when it is lower than the platelet concentration in blood, moderate when it is up to 4 times higher, high when it is 4-6 times higher and super when it exceeds 6 times baseline concentration in blood (20). PEVRP may contain different concentrations of leukocytes, depending on the preparation process. Buffy coat-based procedures yield a preparation with a high leukocyte concentration (higher than blood). Leukocytes in high concentrations, especially neutrophilic granulocytes, can inhibit healing, which is not preferred in the treatment of scars. In contrast, high leukocyte concentrations can have a beneficial effect in accelerating open wound healing and preventing infection. Blood plasma-based procedures result in PEVRP with a lower leukocyte concentration than that in blood (20).

Platelet activation in PEVRP can be triggered outside the body before administration (i.e. exogenously) or allowed to take place in the body after administration (i.e. endogenously). Endogenous activation is triggered by the type 1 collagen in tissues, which is a more effective activator than thrombin. The latter is used in exogenous activation. Exogenous activation creates a gel that results from the formation of a blood clot, allowing easier and more precise manual application for a more localized action on the damaged tissue. The gel is expected to have a longer duration of action, as it releases growth factors more gradually. On the other hand, endogenous activation supplies tissues with sufficient growth factors, while making their mechanism of action more physiological (20). In addition to the activation modes mentioned, platelets are also activated during centrifugation and later during the administration of PEVRP (20,23).

3.2. Applicability and adverse effects of platelet- and extracellular vesicle-rich plasma

Ten years after its first use in haematology in 1970, PEVRP started to be used in maxillofacial surgery and later in the treatment of musculoskeletal injuries in athletes (24). Its use has expanded to other fields of medicine for the treatment of various disorders (Table 1).

Adverse effects associated to the use of PEVRP and platelet gel are very rare, most of them are related to the process of drug administration or surgical procedure during which the preparations are used. The risk of transmission of infectious or malignant disease when applying autologous preparations is minimal. Heterologous preparation may provoke a rejection reaction (59).

Table 1: Examples of the use of platelet- and extracellular vesicle-rich plasma in different fields of medicine

FIELD	DISEASE OR INDICATION
orthopaedics and traumatology	lateral epicondylitis, knee osteoarthritis, achilles and patellar tendinopathy (21), anterior cruciate ligament reconstruction, knee arthroplasty, fracture healing (25)
plastic and cosmetic surgery	facial and neck rejuvenation (26,27), soft tissue reconstructions (28), facelift, reduction mammaplasty, abdominoplasty (29), breast reconstruction with lipofilling (30), alopecia (31,32)
wound care surgery	chronic wounds (25), venous leg ulcers, arterial ulcers, diabetic foot ulcers, traumatic wounds (33), pressure ulcers (34)
maxillofacial and oral surgery	odontogenic cysts of the mandible (35), tooth extraction wounds, periodontal disease (36), insertion of dental implants (36,37), maxillary sinus lift, bisphosphonate osteonecrosis of the mandible (35,36,38)
gynaecology	skin wounds after caesarean section and other procedures, cervical ectopia, vulvar dystrophy, cancer vulvectomy, vesicular, perianal, rectovaginal fistulae, urinary incontinence, premature ovarian failure, refractory endometrial thickening after artificial insemination, repeated vaginal infections, vaginal rejuvenation (30)
ophthalmology	corneal ulcers (39), dry eye syndrome after laser refractive surgery (40), Sjögren's syndrome (41)
cardiovascular surgery	prevention of sternotomy wounds infections (42)
otorhinolaryngology	acute eardrum perforation (43), reconstruction of the posterior wall of external auditory canal (44), auricular replantation (45), mastoid obliteration (46), chronic eardrum perforation (47–54), suprafacial parotidectomy (55,56), craniofacial reconstruction and frontal sinus obliteration (57), anterior cranial base fistulas (58).

4. Future applications of platelet- and extracellular vesicle-rich plasma

In the field of otorhinolaryngology, there are relatively few clinical studies on the use of PEVRP compared to the other areas, despite known disorders of healing in some diseases in this field. Chronic inflammation of the middle ear after surgical and standard conservative treatment presents a therapeutic challenge. Additionally, the use of PEVRP is promising in the treatment of pharyngocutaneous and orocutaneous fistulas (60), vocal cord diseases

(61,62) and facial nerve disorders (63). Based on ophthalmological experiences (40) the preparation could also be stored in appropriate containers and administered for the treatment of ear infections in the form of drops.

Before application, it is important to examine the blood cell concentrations in PEVRP as proposed by DeLong et al. (20). For further application of PEVRP in the sterile areas of human body it is also important to test PEVRP's sterility. Concentrations of EVs in PEVRP could be determined by flow-citometry, however the methods of isolaton and detection of EVs need improvements (61).

So far, we evaluated two different PEVRP preparation protocols during our preclinical research. Analyzes of blood cell concentrations using standard haematology tests and flow cytometry have resulted in our tailored protocol based on the protocol of Amable et al. (64). Flow citometry has also provided us concentrations of particles smaller than platelets, which also include extracellular vesicles. However, further sterility tests still need to be performed to validate the protocol of PEVRP preparation.

In the future, the PEVRP will be used for the treatment of chronic middle ear infections in the form of PEVRP-soaked ear wicks. PEVPR will be analyzed for the sterility, to use it for the treatment of sterile areas of the human body. In any case, adherence to the principles of asepsis and antisepsis in the preparation of PEVRP with experienced medical and biomedical staff is required. Knowing that that our product has an expectedly high concentration of extracellular vesicles and that EVs possess the main regenerative roles (14), our future goal is to produce isolates of extracellular vesicles from venous blood.

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