



160 of 172

# Review

# Role of Erythrocyte Sedimentation Rate (ESR) in preparation of Platelet and Extracellular Vesicles Rich Plasma

Arko M1, Romolo A2, Šuštar V2, Kralj-Iglič V2

- 1. Administration of the republic of Slovenia for Food Safety, Veterinary Sector and Plant Protection, Novo mesto, Slovenia
- <sup>2</sup> University of Ljubljana, Faculty of Health Sciences, Laboratory for Clinical Biophysics. Ljubljana, Slovenia

\* Correspondence: Kralj-Iglič Veronika; kraljiglic@gmail.com

# Abstract:

Uses of platelet and extracellular vesicles rich plasma (PVRP) are in many fields of medicine as it was found that PVRP has regenareative properties. Preparation of PVRP is performed by separation of erythrocytes from the liquid they are immersed in. Erythrocytes are the most numerous blood cells; they are also relatively large and dense as they are filled with haemoglobin. Therefore they sediment due to gravitation or systemic centrifugation force thereby pushing plasma that carries smaller particles in the opposite direction. This mechanism that takes place during processing determines the composition of plasma. Its tuning is therefore key in acquiring a preparation with desired properties. In particular, it is of interest to study the effect of erythrocyte sedimentation rate (ESR) on composition and volume of acquired plasma. Different animals have different ranges of ESR values which may enable insight into mechanisms of plasma preparation. In this contribution we present the basic mechanisms of plasma preparation and properties of blood of different animals.

**Keywords:** Erythrocyte Erythrocyte sedimentation rate; Platelet rich plasma; Horse blood, Cattle blood, Goat blood, Sheep blood

**Citation:** Arko M, Romolo A, Šuštar V, Kralj-Iglič V. Role of Erythrocyte Sedimentation Rate (ESR) in preparation of platelet and extracellular vesicles rich plasma. Proceedings of Socratic Lectures. **2022**, *7*: 160-165.

https://doi.org/10.55295/PSL.2022.D23

**Publisher's Note:** UL ZF stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



**Copyright:** © 2022 by the authors. Submitted for possible open access publication under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/ by/4.0/).







161 of 172

#### 1. Plasma rich with platelets and EVs

Application of blood derived products such as plasma (blood devoid of erythrocytes) to a damaged tissue area has hitherto proved to accelerate wound healing and help regenerate injured or damaged ligament and tendon fibers (Uršič et al., 2016, Vozel et al., 2020a). The preparation is made from patient's own blood following a procedure that is straightforward and easy to perform and can be injected directly to the damaged area. In this way, the healing substances can be delivered also to places that are not easily reached otherwise. With autologous application there is low risk of complications such as infections or immune rejection (De Pascale et al., 2015).

Initially, healing capacity of plasma preparations was attributed to platelets (**Figure 1 A,B**); it was found that activated platelets (**Figure 1B**) release different growth and inflammation factors to the extracellular millieu, which have been regarded as vectors of the healing process (Etulain et al, 2018). Later on, other features were considered, i.e. platelet membrane receptors, their effects on the immunomodulatory actions of the innate and adaptive immune system, effects of leukocytes and of mesenchymal stem cells (reviewed in Everts al., 2020), indicating complex interactions between cells in the healing process.

Substances that were considered as possible promoters of regeneration in PVRP are growth factors and inflammation markers. Previous research outlined following growth factors: platelet-derived growth factor-BB (PDGF-BB), transforming growth factor  $\beta$ -1 (TGF- $\beta$ 1) (Lee et al., 2013), insulin-like growth factor-1 (IGF-1) (Lubkowska et al., 2012), vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF) (Taniguchi et al., 2019). Inflammation factors Tumor necrosis factor alpha (TNF-alpha), interleukin 1 beta (IL-1 beta), Cholinesterase (ChE), erythrocyte Glutathione Stransferase (GST) and Interleukin 6 (IL-6) were already proved to be connected to concentration of EVs in blood-derived samples (Jan et al., 2021). TNF-alpha was originally described as a circulating factor that can cause necrosis of tumors, but has since been identified as a key regulator of the inflammatory response (Bradley, 2008). IL-6 has a role as an inflammatory mediator (Mendham, 2011). Potential marker of inflammation is activity of plasma Butyrylcholinesterase and erythrocyte Acetylcholinesterase. Both Cholinesterases may enhance inflammation. ChE is believed to modulate immune and anti-inflammatory response via the cholinergic system. Key factor in the anti-inflammatory cholinergic pathway is supposed to be oxidative stress (Villeda-Gonzalez, 2020). Erythrocyte GST activity has been proposed as the potential marker for oxidative stress (Neefjes, 1999) and EVs formation is considered to be provoked by oxidative stress (Borras, 2020) whereas inflammation processes in human body have impact on vesiculation (Chaar 2011).

Recent developments in the field of biology and medicine have outlined nanosized (ranging from 20 nm-1000 nm) membrane-enclosed cellular fragments (extracellular vesicles – (EVs)) which are abundant in preparations from processed plasma (Šuštar et al., 2011a,b, Božič et al., 2020) (**Figure 1G**) as important mediators of intercellular communication. It can be expected that these particles may be involved in the above features. EVs are formed by cells of all types during apoptosis, in the final stage of membrane budding (shown in platelets (**Figure 1B**), leukocytes (**Figure 1C**) and erythrocytes (**Figure 1F**)), by release from internal cell compartments or due to mechanical impact (Kralj-Iglič et al., 2020). Being free to move with body fluids, they can reach distant cells, interact with them and affect their biological function. Containing cell-type-specific signatures, EVs of selected cell types have been proposed as therapeutic agents in regenerative medicine, vaccination trials, and drug delivery (Fais et al., 2016, Lener et al., 2015). EV formation by cells is considered a physiological process (Su et al., 2018) and is accelerated as a result of oxidative stress (Borras et al., 2020) or inflammation process (Char et al., 2011).

Both platelets and EVs seem to be important bioactive substances in plasma. Importance of plateletderived EVs was previously outlined and it was indicated that the mechanism by which they are formed is a critical determinant of their phenotype and function (Ferreira et al., 2020). A question can be posed about what would be the optimal composition of plasma preparation regarding platelets and EVs.

#### Proceedings of 7th Socratic Lectures 2022









**Figure 1**. A: resting platelets, B: activated platelets, C: budding leukocytes, D: packed erythrocytes at the bottom of the centrifugation pellet, E: erythrocytes in physiologic in-vitro conditions, F: budding erythrocytes, G: isolate from blood showing numerous EVs (white arrow) and singular residual erythrocytes; there are no resting platelets and no leukocytes in the sample.

#### 2. Preparation of plasma rich with platelets and EVs

The gold standard method for preparation of PVRP is centrifugation of blood samples. The procedure is simple to perform, it requires besides a centrifuge only few basic laboratory tools and contact with foreign materials is minimized as not to introduce corrosion particles into the preparation. The exising PVRP protocols aim at depletion of plasma in erythrocytes and enrichment in platelets. Different settings have been suggested, based on empirical findings. Widely used are two-spin protocols (**Figure 2**) where relatively low centrifugation pulls are used in both spins (up to 1000 times the acceleration due to gravity of Earth (g)). In Spin 1, blood is centrifuged to pellet erythrocytes. In Spin 2, the Spin 1-supernatant that is abundant in platelets and EVs is centrifuged again to pull the particles with densities higher than that of the surrounding solution towards the bottom of the centrifuge. PVRP is constituted from the lower portion of the sample (**Figure 2**). The recovery of platelets (the number of platelets in the PVRP sample divided by the number of platelets in the blood sample) reaches about 80% (Hsin et al., 2017) while platelet concentration can reach values above its baseline level in whole blood (Božič et al., 2021).



**Figure 2.** Two-spin protocol for preparation of PVRP. Blood is centrifuged (Spin 1) to separate erythrocytes (pellet) from plasma (supernatant). The acquired plasma is centrifuged again (Spin 2) to concentrate platelets and EVs that are denser than the surrounding liquid into the lower part of the tube (PVRP).

The forces on a particle are gravitational/systemic centrifugal force, the buoyancy and the resisting force that is described by the Stokes law. In equilibrium, the sum of the forces vanishes so that

$$\Delta \rho_{\text{erc}} Xg \, 4\pi r \text{erc}^3/3 = 6\pi r \text{erc} \,\eta \, u \text{erc} \qquad , \tag{1}$$

where  $\Delta \rho_{\text{ERC}}$  is the difference between the density of the erythrocytes and the density of the fluid, Xg is the respective acceleration that drives sedimentation expressed as a multiplicity of the Earth gravity acceleration g and X is the multiplication factor (generally referred to as relative centrifugal force RCF in the centrifuge),  $r_{\text{ERC}}$  is the radius of the erythrocyte,  $\eta$  is the viscosity of plasma and  $u_{\text{ERC}}$  is the velocity of the erythrocyte. In gravitational field (X = 1), the velocity of sedimentation of erythrocytes is

$$u_{\rm ERC} = 2\Delta\rho_{\rm ERC}r_{\rm ERC}^2 g / (9\eta) \qquad (2)$$

In the centrifuge, the force depends on the distance of the particle from the rotor axis *x*. The factor *Xg* is defined by the distance of the bottom of the tube from the rotor axis  $x_{max}$  and the angular velocity of the centrifuge (expressed also by its frequency in rotations per minute,  $\omega = 2\pi \text{RPM}/60$ ), so that

$$X g = \omega^2 x_{\text{max}}$$





163 of 172

(4)

It follows from Eqs. (1) and (3) that the velocity of sedimentation of erythrocytes in the centrifuge is

$$u_{\rm ERC} = 2\Delta \rho_{\rm ERC} r_{\rm ERC}^2 Xg \, x \, / (9 \, \eta \, x_{\rm max}) \qquad .$$

We can see that the velocity of the particle is proportional to the difference in particle density and the liquid density and to square of the size of the particle, meaning that larger and denser particles will sediment faster. This is the basis of the separation of erythrocytes from platelets in gravitational field and also in centrifuge. Erythrocytes are bigger than platelets and EVs. Moreover, they tend to form aggregates called rolleaux (**Figure 3**) that travel as unities and thereby further promote the difference in sedimentation velocities between erythrocytes and smaller particles.



Figure 3. Rolleaux formation in normal in vitro conditions (A) and in the presence of anaesthetic (B).

It was found that the preparation procedure can significantly impact the blood cells and EVs (Šuštar et al., 2011, Božič et al., 2020) and therefore the volume and composition of PVRP (Everts, 2020). Besides EVs that are already present in plasma *in vivo*, the preparations contain EVs that are formed during the sample processing due to the shear and temperature stresses imposed upon the blood cells during processing of the sample (Šuštar et al., 2011, Božič et al., 2020). It is therefore of utmost interest to understand the effect of centrifugation on identity and formation mechanisms of EVs in plasma preparations as well as on their biological role.

#### 3. Enrichment of plasma with platelets and EVs by centrifugation

Blood can be described as composed of a mixture of particle types differing in density, size and shape. It was previously observed that in concentrated multiparticle-type suspensions, the creeping flow considerably hinders sedimentation of particles (Major, 1978); if species of particles in suspension greatly differed in density and size, the lighter and smaller particles could be withdrawn into the flow opposite to the flow of the heavier and larger ones (Major, 1978). This principle was recently applied to blood where due to the large size and higher density of erythrocyte lumen, erythrocytes and leukocytes move faster than smaller platelets and EVs and push those against the centrifugation pull into the plasma zone (Božič et al., 2021). Furthermore, when an erythrocyte moves in the direction of the gravitational (or corresponding centrifugal) force, a following erythrocyte is dragged into the left behind "cavity". As the concentration of erythrocytes in blood is high, the continuing action results in sequential ordering of the settling dispersed phase into erythrocyte channels and surrounding plasma zones (Pribush et al., 2010). The erythrocyte sedimentation is accelerated due to formation of the channels (Pribush et al., 2010), and is therefore faster than that expected from the relations considering movement of singular cells. Enrichment of plasma in smaller particles takes place as long as erythrocytes travel towards the bottom of the tube. When they pack in the pellet, smaller particles are no longer subjected to the drag of the erythrocyte counterflow and centrifugal pull reverses their direction toward the bottom of the tube. In this interval, plasma becomes depleted of smaller particles.

Distribution of different types of particles in the sample in dependence of time was modelled by applying laws of motion leading to a system of partial differential equations (Božič et al., 2021). Approximative simple and transparent solution was found. Model parameters were set by fitting to data from a cohort study including patients with chronic ear wounds and subjects without the record of disease (Božič et al., 2021) assuming that the movement of platelets and EVs is in the direction opposite to erythrocytes but with the proportional magnitude of velocity,



Univerza *v Ljubljani* Zdravstvena</del> fakulteta





164 of 172

(5)

(6)

 $u_{P2} = - \varepsilon u_{ERC}$ 

where P2 denotes platelets and EVs and  $\varepsilon$  is an adjustable constant.

It was found that Spin 1 (centrifugation of blood) was crucial in preparation of plasma. With appropriate choice of centripetal accelerations of the centrifuge rotor and appropriate centrifugation times, enrichment of plasma in the upper part of the centrifugation tube with platelets and EVs reached factor 2.3 (Božič et al., 2021).

### 4. Erythrocyte sedimentation rate (ESR)

ESR is a test that is routinely performed in clinical practice. It gives a rate (in millimetres per hour), at which red blood cells in the whole blood descend in a standardized Westergren tube in gravitational field during a period of one hour. With respect to model equations,

 $u_{\rm ERC} = {\rm ESR}$ 

so that it follows from (4)-(6) that

 $u_{\rm P2} = -\varepsilon \, \mathrm{ESR} \, X / x_{\rm max} \qquad . \tag{7}$ 

ESR is influenced by pro-sedimentation factors, mainly the presence of fibrinogen, and also physical properties of particles, e. g. the negative surface charge of erythrocytes. When an inflammatory process is present, the high level of fibrinogen enhances the formation of rouleaux. In humans, ESR was found connected to pathological states such as inflammation, infection, pregnancy, anemia, autoimmune disorders, some kidney diseases and some cancers. The rate of erythrocyte sedimentation is affected by both inflammatory and non-inflammatory conditions. In inflammatory conditions, fibrinogen, other clotting proteins and alpha globulin are positively charged, thus increasing the ESR. In non-inflammatory conditions, plasma albumin concentration, size, shape, and number of red blood cells and the concentration of immunoglobulin can affect the ESR. Non-inflammatory conditions that can cause raised ESR include anemia, kidney failure, obesity, ageing, and female sex. ESR is also higher in women during menstruation and pregnancy. An increased number of red blood cells (polycythemia) causes reduced ESR as blood viscosity increases. Hemoglobinopathy, such as sickle-cell disease, decrease ESR due to improper shape of red blood cells that impairs stacking. In animals like horses, cats and pigs, the rouleaux formation can be a normal physiological finding.

Westergren's original normal values (men 3 mm/h and women 7 mm/h) made no allowance for a person's age and were found higher in women than in men. Later studies indicated that ESR tends to rise with age (normal values were found to be 1 to 2 mm/h at birth, rising to 4 mm/h 8 days after delivery, and then to 17 mm/h by day 14). In human, normal values are now thought to be 3-10 mm/hour, they were found to be slightly higher in normal populations of African Americans than Caucasians of both genders and in anaemic individuals. ESR differs in different animal species an is especially high in the horse (120 mm/hour) while in the cow, goat and sheep it was found to be 1 mm/hour (Bottiger et al., 1967). It is proposed that ESR is higher in athletic mammalian species like pronghorn antelope (Antilocapra americana) (Popel et al., 1994).

**Funding:** This research was supported by Slovenian Research Agency through the core foundings No. P3-0388 and J3-3066.

Conflicts of Interest: The authors declare no conflict of interest.

# References

- Borras C, Mas-Bargues C, Sanz-Ros J, Roman- Dominguez A, et al. Extracellular vesicles and redox modulation in aging. Free Radic Biol Med. 2020; 149: 44-50. <u>https://doi:10.1016/j.freeradbiomed.2019.11.032</u>
- Böttiger LE. Erythrocyte Sedimentation rate and protein-bound carbohydrates in domestic animals. Acta Vet Scand.1967; 8: 279–286. https://doi.org/10.1186/BF03547833
- Božič D, Hočevarc M, Kononenkod V, Jerana M, Štiblerbet U, et al. Chapter Five Pursuing mechanisms of extracellular vesicle formation. Effects of sample processing. In: Bongiovanni A (Ed.), et al. Advances in biomembranes and lipid selfassembly. Vol. 32, Elsevier: Academic Press. 2020; pp: 113-155. https://doi:10.1016/bs.abl.2020.09.003







- 4. Bottiger LE, Svedberg CA. Normal erythrocyte sedimentation rate and age. Br Med J. 1957;2(5544):85-87. DOI:10.1136/bmj.2.5544.85
- 5. Božič D, Vozel D, Hočevar M, Jeran M, Jan Z, et al. Enrichment of plasma in platelets and extracellular vesicles by the counterflow to erythrocyte settling. Platelets. 2021; 33: 592 602. https://doi.org/10.1080/09537104.2021.1961716
- 6. Bradley JR. TNF-mediated inflammatory disease. J Pathol. 2008; 214: 149-60. https://doi:10.1002/path.2287
- 7. Chaar V; Romana M, Tripette J, Broquere C, et al. Effect of strenuous physical exercise on circulating cell-derived microparticles. Clin Hemorheol Microcirc. 2011; 47: 15-25. https://doi:10.3233/CH-2010-1361
- 8. De Pascale MR, Sommese L, Casamassimi A, Napoli C. Platelet Derivatives in Regenerative Medicine: An Update. Transfus Med Rev. 2015; 29: 52-61. <u>https://doi:10.1016/j.tmrv.2014.11.001</u>
- 9. Etulain J. Platelets in wound healing and regenerative medicine. Platelets. 2018; 29: 556-568. DOI: 10.1080/09537104.2018.1430357
- 10. Everts P, Onishi K, Jayaram P, et al. Platelet-Rich Plasma: New Performance Understandings and Therapeutic Considerations in 2020. Int J Mol Sci. 2020; 21: 7794. https://doi:10.3390/ijms21207794
- Fais S, O'Driscoll L, Borras FE, Buzas E, et al. Evidence-Based Clinical Use of Nanoscale Extracellular Vesicles in Nanomedicine. ACS Nano. 2016; 104: 3886-3899. https://pubs.acs.org/doi/10.1021/acsnano.5b08015
- 12. Ferreira PM, Bozbas E, Tannetta SD, Alroqaiba N, et al. Mode of induction of platelet-derived extracellular vesicles is a critical determinant of their phenotype and function. Sci Rep. 2020; 10: 18061. <u>https://doi.org/10.1038/s41598-020-73005-3</u>
- 13. Gillum RF. A racial difference in erythrocyte sedimentation. J Nat Med Assoc. 1993;85(1):47-50.
- 14. Kafner EJ, Nicol BA. Haemoglobin concentration and erythrocyte sedimentation rate in primary care patients. J Royal Soc Med. 1997;90(1):16-18. DOI:10.1177/014107689709000106
- Kralj-Iglič V, Pocsfalvi G, Mesarec L, Sustar V, et al. Minimizing isotropic and deviatoric membrane energy An unifying formation mechanism of different cellular membrane nanovesicle types. PLOS ONE. 2020. https://doi.org/10.1371/journal.pone.0244796
- 16. Lener T, Gimona M, Aigner L, Borger V, et al. Applying extracellular vesicles based therapeutics in clinical trials an ISEV position paper. J Extracellular vesicles. 2015; 4: 30087, https://doi:10.3402/jev.v4.30087
- 17. Lubkowska A, Dolegowska B, Banfi G. Growth factor content in PRP and their applicability in medicine J Biol Regul Homeost Agents. 2012; 26: 3S-22S.
- 18. Major JJ. Sedimentology, Springer Berlin Heidelberg, 1978, https://doi:10.1007/3-540-31079-7
- 19. Mendham AE, Donges CE, Liberts EA, Duffield R et al. Effects of mode and intensity on the acute exercise-induced IL-6 and CRP responses in a sedentary, overweight population. Eur J Appl Physiol. 2011; 111: 1035-1045. https://doi:10.1007/s00421-010-1724-z
- 20. Neefjes VM, Evelo CTA, Baars LGM, Blamco CE. Erythrocyte glutathione S transferase as a marker of oxidative stress at birth. Arch Dis Child. 1999; 81: 130–133. https://doi.org/10.1136/fn.81.2.F130
- 21. Popel AS, Johnson PC, Kameneva MV, Wild MA. Capacity for red blood cell aggregation is higher in athletic mammalian species than in sedentary species. J App. Physiol. 1994. https://doi.org/10.1152/jappl.1994.77.4.1790
- 22. Pribush A, Mayerstein D. The mechanism of erythrocyte sedimentation. Part 1: Channeling in sedimenting blood. Colloids Surf B Biointerfaces. 2010; 75: 214–223. DOI:10.1016/j.colsurfb.2009.08.036
- 23. Shin HS, Woo HM, Kang BJ. Optimisation of a double-centrifugation method for preparation of canine platelet-rich plasma. BMC Vet Res. 2017; 26: 198. https://doi:10.1186/s12917-017-1123-3
- 24. Su Y, Luo C, Zhang Z, Hermawan H, et al. Bioinspired surface functionalization of metallic biomaterials. J Mech Behav Biomed Mater. 2018; 77: 90-105. DOI: 10.1016/j.jmbbm.2017.08.035
- 25. Šuštar V, Bedina-Zavec A, Štukelj R, Frank M, Bobojevic G, et al. Nanoparticles isolated from blood: a reflection of vesiculability of blood cells during the isolation process. Int J Nanomed. 2011a; 6: 2737-2748. https://doi.org/10.2147/IJN.S24537
- 26. Šuštar V, Bedina-Zavec A, Štukelj R, Frank M, Bobojevic G, et al. Lipids Health Dis. 2011b; 10: 47, https://doi:10.1186/1476-511X-10-47
- 27. Taniguchi Y, Yoshioka T, Sugaya H, Gosho M, et al. Growth factor levels in leukocyte-poor platelet-rich plasma and correlations with donor age, gender, and platelets in the Japanese population. J Exp Orthop. 2019; 6: 4. https://doi:10.1186/s40634-019-0175-7
- 28. Uršič B,Vozel D, Sustar V, Kocjancic B, et al. Extracellular Vesicles from Platelet-Rich Plasma as Conveyors of Regeneration Potential in Orthopedics. J Hematol Thromb Dis. 2014; 2: 5. DOI:10.4172/2329-8790.1000163
- Villeda-González JD, Gomez-Olivares JL, Baiza-Gutman LA, Manuel-Apolinar L. et al. Nicotinamide reduces inflammation and oxidative stress via the cholinergic system in fructose-induced metabolic syndrome in rats. Life Sci. 2020; 250: 117585. <u>https://doi:10.1016/j.lfs.2020.117585</u>
- 30. Vozel D, Božič D, Jeran M, Jan Z, Pajnič M, et al. the role of platelet-and extracellular vesicle-rich plasma in the treatment of temporal bone cavity inflammation: a randomized controlled trial. Socratic lectures : 3rd International Minisymposium, Ljubljana, 17. April 2020 : peer reviewed proceedings; pp: 41- 52. Available from: <u>https://repozitorij.uni-lj.si/Izpis-Gradiva.php?id=119124&lang=slv</u>
- 31. Westergren A. Diagnostic tests: the erythrocyte sedimentation rate range and limitations of the technique. Triangle; the Sandoz Journal of Medica Science. 1957;3(1):20-25.