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Review

Platelet-derived extracellular vesicles for regenerative medicine

Miquel Antich-Rosselló[1,2], Maria Antònia Forteza-Genestra[1,2], Marta Monjo[1,2,3]* and Joana M. Ramis[1,2,3]*

[1] Cell Therapy and Tissue Engineering Group, Research Institute on Health Sciences (IUNICS), University of the Balearic Islands (UIB), Ctra. Valldemossa km 7.5, 07122 Palma, Spain.

[2] Health Research Institute of the Balearic Islands (IdISBa), Palma, Spain.

[3] Departament de Biologia Fonamental i Ciències de la Salut, UIB.

* Correspondence: joana.ramis@uib.es (JMR), marta.monjo@uib.es (MM); Tel.: +034 971 25 96 07

Abstract: Extracellular vesicles (EVs) present a great potential for the development of new treatments in the biomedical field. To be used as therapeutics, many different sources have been used for EVs obtention, while only few studies have addressed the use of platelet derived EVs (pEVs). In fact, pEVs have been shown to intervene in different healing responses, thus some studies have evaluated their regenerative capability in wound healing or hemorrhagic shock. Even more, pEVs have proven to induce cellular differentiation, enhancing musculoskeletal or neural regeneration. However, the obtention and characterization of pEVs is widely heterogeneous and differs from the recommendations of the International Society for Extracellular Vesicles. Therefore, in this review, we aim to present the main advances in the therapeutical use of pEVs in the regenerative medicine field while highlighting the isolation and characterization steps followed. The main goal of this review is to portray the studies performed in order to enhance the translation of the pEVs research into feasible therapeutical applications.

Keywords: extracellular vesicles; exosomes; platelets; regenerative medicine.

1. Introduction

In recent years, extracellular vesicles (EVs) have emerged as potential therapeutic effectors in the biomedical field. EVs are membranous subcellular structures released by any cell type, which comprise different subpopulations that differ on morphology, size, composition and cellular origin [1]. In the past, EVs had been referred by many different names such as microvesicles, exosomes, microparticles or apoptotic bodies, among others; according to their size, their tissue or cell origin, their claimed function or even their presence outside the cell [2]. However, these EV subgroups presented a great diversity on the biomechanism behind their formation and the functions they perform, thus distinguishing them has proven not to be easy [3]. Therefore, a consensus has been reached and the most accepted classification is performed according to the characterization and the isolation methodology used [1].

In general, EVs present a huge interest for the development of new treatments. EVs enable cell to cell communication, which can prevent the development of diseases by promoting homeostatic physiology or lead to pathological states, depending on the nature of the producing cell and the stimuli that activated the EV production [4]. There exist different cellular mechanisms for EVs secretion and uptake, crucial for intercellular communication, that are still unknown [5]. For this reason, some research focus on the use of naturally produced EVs while other aim to understand the molecular functionality of EVs to design new bioengineered carriers for enhanced cell delivery treatments or the addition of alternative cargos [6,7].

Today, EVs are thought to be secreted by all cell types, being stem cells and immune cells some of the most studied EV sources for therapeutical approaches [8,9]. Nevertheless, clinical translation of cell cultured derived EVs has been hindered due to the high regulations requirements for *ex vivo* cell expansion [10]. Thereby, platelet EVs (pEVs) have emerged as a potential therapeutic source which overcome these limitations. Platelets can

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be obtained from blood donation and prepared as platelet concentrates, thus avoiding *ex vivo* cell expansion. Even more, the human origin and the lack of growth medium components diminishes concerns over contamination or immunological safety [10]. Therefore, platelet concentrates are being evaluated as feasible pEV sources for regenerative medicine.

Platelet concentrates such as platelet rich plasma are biological samples widely evaluated in regenerative medicine [11]. Platelets can release growth factors, cytokines and extracellular matrix modulators that promote revascularization, restoration of damaged tissue and activation of mesenchymal stem cells [12]. Even more, pEVs appear to be also important effectors for platelet regenerative function [13]. However, clinical use of platelet concentrates is still limited due to the high variability on the preparation and formulation techniques [11]. In addition, pEVs in contrast to platelets, can cross tissue barriers, extending their abilities beyond the blood [14]. Therefore, many studies have focused on the use of pEVs in order to surpass the platelet concentrates limitations. In this review, we will evaluate some of the most relevant advances of pEVs in the regenerative medicine field and analyze the basic requirements for their clinical translation.

2. Regenerative effects of pEVs

Recently, pEVs have been postulated to play a key role in homeostatic processes [15]. In fact, platelets and pEVs are natural mediators of different physiological processes and contribute to the immune system response functions [16]. However, only few articles have evaluated the potential of pEVs as regenerative tools (Figure 1). Therefore, in this review, we will introduce the main advances in the different regenerative approaches evaluated until now.

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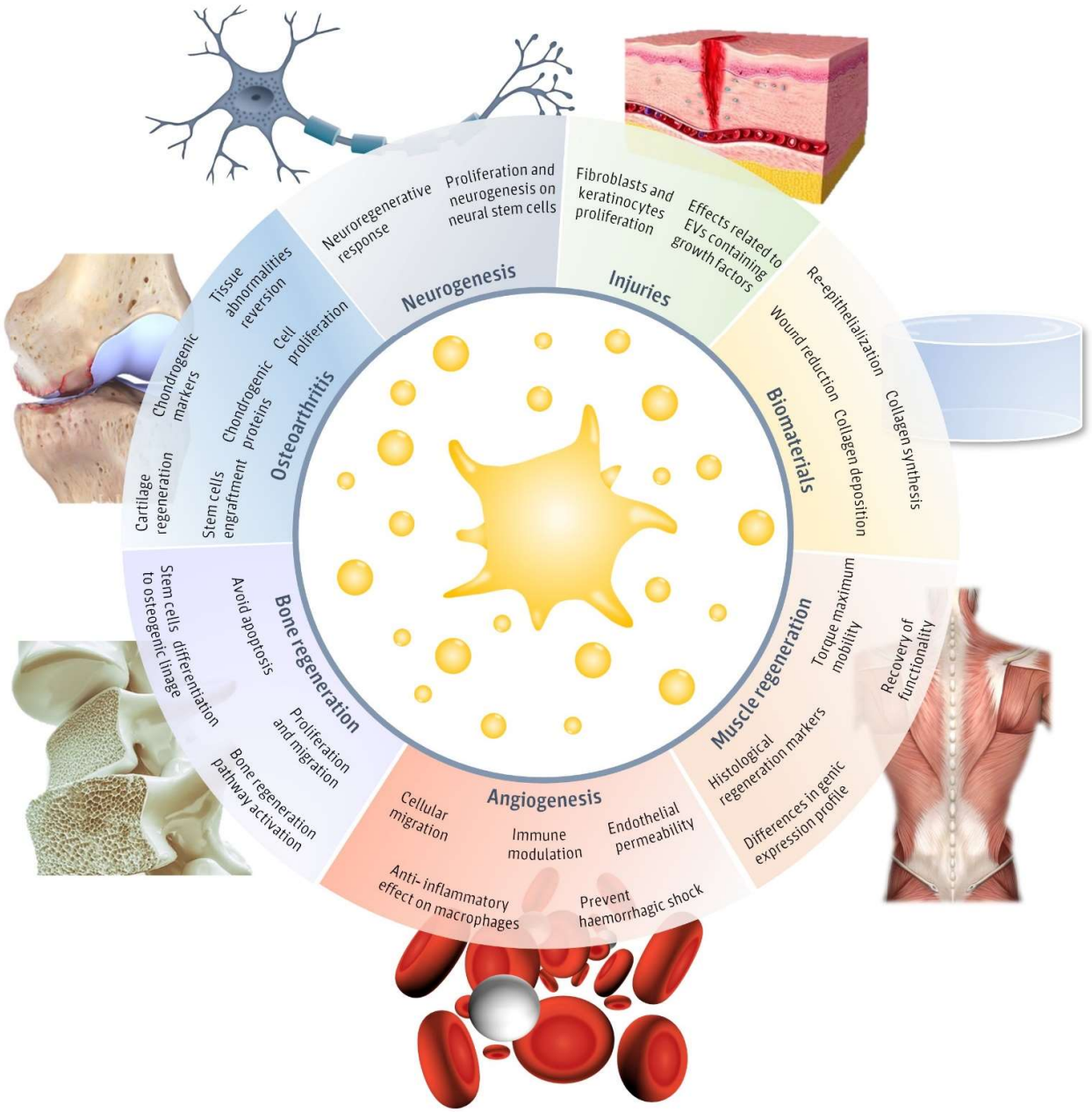

Figure 1. Regenerative applications of platelet derived extracellular vesicles (pEVs). Main regenerative effects reported for pEVs in regenerative fields, including musculoskeletal regeneration, neural regeneration, wound healing, angiogenesis and blood coagulation. This figure was created using freepik images.

One of the main fields in which the applications of pEVs have been studied are injuries. Concretely, it has been reported an increase of fibroblast and keratinocyte migration and proliferation *in vitro*, associated to the wound healing process [17,18]. These effects may be related to the pEVs cargo, which was positive in different growth factors [17]. Even more, the evaluation on a diabetic rat model confirms *in vivo* the wound regenerative effects observed for pEVs [17,19]. In the same direction, more creative experiments sug-

gest that pEVs can be combined with biomaterials or active biomolecules to obtain improved regenerative results. Interestingly, pEVs were combined with a sodium alginate hydrogel in order to achieve a more translational medical product, despite reaching similar properties than using pEVs directly [17]. Another study presented pEVs formulated on a chitosan/silk hydrogel and combined this approach with a plant polysaccharide. This study reports higher collagen synthesis and deposition, wound reduction and re-epithelialization and dermal angiogenesis *in vivo* [19].

Furthermore, in addition to the wound healing properties, two rat model studies suggest that pEVs prevent uncontrolled blood loss and hemorrhagic shock [20–22]. In fact, the pEVs dose-response performed *in vitro* suggests that pEV blood coagulation is dependent on EVs concentration [20], as the International Society of Extracellular Vesicles (ISEV) encourages to test [1]. Even more, pEVs have an effect on endothelial permeability, which mitigates blood loss too [21]. Further studies report that aggregates of thrombin activated pEVs decrease the bleeding time after *in vivo* injuries while decreasing the interleukin concentration too [22]. Moreover, it is important to realize that pEVs are also involved in the inflammatory response. Some studies report that pEVs present an anti-inflammatory effect on macrophages, which decreased the release of cytokines [23]. However, little studies have been performed on evaluating pEVs treatments effects on immune modulation although pEV role is essential for these processes [15].

Another interesting property of pEVs treatments is their angiogenic capability, associated to cellular mobilization and migration. In fact, vasoregeneration and maintenance of arterial integrity after injury have been reported [24–26]. These effects were attributed to pEVs protein cargo and also to some lipid factors [25,26]. Incorporation of pEVs into cells and later phenotypical changes were assessed through *in vitro* studies [24]. Later *in vitro* and *in vivo* experiments confirmed an increase in cell recruitment and adhesion, followed by a regenerative effect [24]. Even more, mice ischemic hearts were analyzed *in vivo* confirming the angiogenic effects of pEVs [26].

In more specific studies, pEVs have also been reported to be involved in the neuroregenerative response. First, *in vitro* studies suggest that pEVs induce proliferation and neurogenesis on neural stem cells, which have been associated to different proteins contained in pEVs [27]. Secondly, i*n vivo* studies show an increase in neural stem cells proliferation and differentiation, in addition to the angiogenic effect. Furthermore, the rat model evaluated improved the neurological functionality after ischemic stroke according to a motor disability test [28]. Overall, it is interesting to notice that the neuroregenerative effects attributed to pEVs follow a dose dependent response, as it has been reported [27,28].

Another field in which pEVs have been evaluated as therapeutical agents is musculoskeletal regeneration. To start, it has been suggested that pEVs may contain a functional miRNA profile that would benefit osteoarthritis regenerative therapies [29]. Chondrocyte cell culture studies have shown that pEVs induce an increase on proliferation and cell migration through the activation of the Wnt/β-catenin pathway [30]. Moreover, pEV treated chondrocytes have shown a decrease in the proinflammatory response and the apoptosis rate induced by inflammation conditions [30,31]. As a functionality test, pEV treatment promoted the expression of chondrogenic markers on patient derived osteoarthritic chondrocytes [31]. Furthermore, these effects observed for pEVs follow a dose dependent response [30]. The functional effects were corroborated in an *in vivo* approach, in which a rabbit model was used. In this study, higher levels of chondrogenic proteins were found for the pEV treated group, while the tissular abnormalities observed in the histological cuts were reversed [30]. Finally, pEVs have also been evaluated in combination with other approaches such as cell therapy. Specifically, pEVs enhance the engraftment of stem cells into articular injured tissue, thus promoting the cartilage regeneration in intra-articular defects [32].

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In bone regeneration, *in silico* evaluation of pEV miRNA also suggested their potential use for bone repair [33]. These predictions have been supported for some *in vitro* studies, which report that pEVs promote the differentiation of mesenchymal stromal cells into the osteogenic linage [34,35]. It was shown that pEVs can be internalized by stem cells and, after 20 h, they were mainly colocalized in the perinuclear region. Moreover, pEVs induced proliferation and migration of stem cells in a dose dependent manner [35]. Osteodifferentiation effects *in vitro* were determined by Alizarin red staining [35] and the expression of cellular osteogenic markers [34]. The osteogenic effets *in vitro* have been attributed not only to the growth factors pEVs contain, but also to the RNA [35]. In addition, *in vitro* and *in vivo* models of osteonecrosis have been used to test pEV functionality. These models suggest that pEVs can promote proliferation and avoid apoptosis, inducing a bone regeneration effect through the activation of Akt/Bad/Bcl-2 pathway [36]. However, another study performed in pigs had previously reported no significant effects in bone formation, despite having induced angiogenesis in the pEVs treated group [37]. Therefore, it is necessary to perform further experiments, and proper pEVs characterization, to determine their real osteogenic effect.

Finally, pEVs are also associated to muscle regeneration. pEVs induced an increase of histological regeneration markers such as centrally nucleated fibers, after an *in vivo* rat study. Even more, pEVs treated group showed an improved recovery of functionality, associated to the torque maximum mobility [38]. Furthermore, this study compared the gene expression profile of inflammatory, fibrotic and regenerative related markers of pEVs and stem cell derived-EVs. This comparison allowed to see differences on the gene expression despite similar functional regenerative outcomes [38].

3. Platelet derived EVs as therapeutic products

EVs are considered biological products, therefore, isolation and characterization must be reported for their approval as therapeutical agents. For pEVs, low manipulation is needed compared to cell derived EVs, which may ease their clinical use despite still being considered biological medical products [39]. In fact, many factors can alter the nature of EVs, including the isolation methodology, misleading the real effects of pEVs and their clinical translation [40]. Moreover, highly pure pEVs samples are difficult to obtain, as lipoproteins are usually co-isolated with EVs [29,31,41]. For this reason, different methodologies have been tested, such as ultracentrifugation, density gradient centrifugation, size exclusion chromatography, ultrafiltration, or polymer-based precipitation, each of them with their advantages and limitations [42,43]. Overall, it is important to select a methodology that allows to obtain pure functional pEVs through a scalable methodology, compatible with a reproducible and standardized large production [39]. It is important to notice that centrifuge-based methods are still the most common method used in regenerative medicine approaches for pEV obtention (Figure 2).

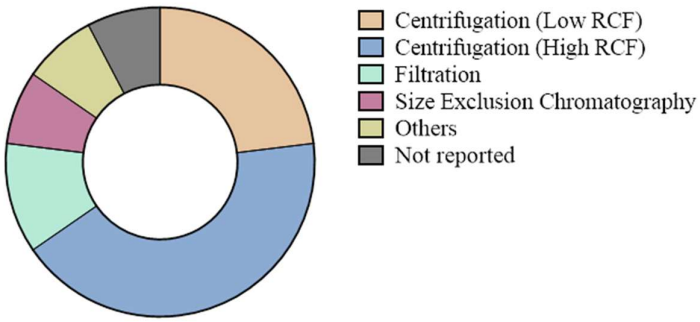

Figure 2. pEVs isolation methods reported for regenerative approaches. Diagram shows the proportion of reports that used centrifugation at low relative centrifugal force (RCF; lower than 80 000 × g), centrifugation at high RCF (higher than 80 000 × g), filtration,

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size exclusion chromatography, other methods or did not report the isolation method used. If two different methods or a combination of methods was used, both groups are represented on the diagram.

Despite the heterogeneity of pEVs, and their dependence on the isolation methods, quality control is necessary. Physicochemical, molecular and functional characteristics must be defined [39]. However, when single EV characterization is limited, therefore bulk analysis is mainly performed for EV samples, as the ISEV recommends [1]. Physical and molecular characterization are reported differently or partially depending on the article (Figure 3). Overall, low RCF centrifugation isolated pEVs present poorer physical and molecular characterization and are usually presented as microparticles instead of pEVs. Thus, "pEVs" is a term that would englobe the heterogenic population that may be obtained through the isolation process [1]. Thereby, it is necessary to reinforce a proper pEV characterization in order to allow their clinical translation [39].

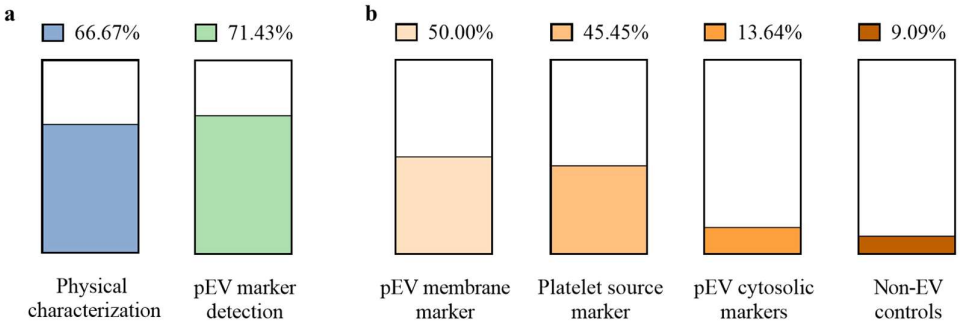

Figure 3. Characterization reported for pEVs used in regenerative applications. (**a**) Percentage of articles which report physical characterization (include nanoparticle tracking analysis, electron microscopy, flow cytometry or dynamic light scattering) and pEV marker detection (include immunolabelling through western blot, flow cytometry or electron microscopy) ; (**b**) Percentage of the different EV markers reported: pEV membrane marker (CD9, CD61, CD63 or CD81), platelet source marker (CD31, CD41 or CD42), pEV cytosolic markers (ALIX, TSG101, HSP90 or HSP101) and non-EV structures (APOA1, APOB100 or calnexin).

In this sense, proper characterization is needed for therapeutical use of pEVs. Average size can be determined through different well stablished techniques, such as electron microscopy, nanoparticle tracking analysis, atomic force microscopy, flow cytometry or resistive pulse sensing [43]. In terms of molecular markers, the most commonly reported molecules for pEVs include surface markers and cytosolic proteins suggested by the ISEV [1]. Therefore, in regenerative therapies, pEVs are mainly reported to present CD9, CD63, CD81 and CD41, among other positive and negative controls (Table 1). Functional effectors of EVs are also analyzed, such as cytokines and growth factors [17,27,35]. Recently, RNA, lipid and metabolite analysis are also performed for EVs characterization [43], and miRNA analysis are emerging as functional indicator for pEVs therapeutical use [29,33]. Finally, pEVs mechanism of action must be addressed through biological assays, since therapeutic activities cannot be determined only by molecular characterization [39].

Table 1. Macromolecule characterization reported for pEVs in therapeutical approaches.

Kind of proteins commonly reported	pEV markers	References
EV membrane markers	CD9	[17,20,21,29–31,34,36,38]
	CD61	[20,23]
	CD63	[17,20,21,30,31,34–36,38,44]
	CD81	[17,20,21,30,36,38]
Platelet source markers	CD31	[21]
	CD41	[20,21,26–28,31,36,45–47]
	CD42	[24]
EV cytosolic markers	ALIX	[29,31]
	HSP90	[20]
	HPS101	[30]
	TSG101	[36]
Non-EVs structures	APOA1	[29,31]
	APOB100	[29,31]
	Calnexin	[36]

Finally, another important consideration for pEVs use as therapeutical products is their storage conditions. More than 56 % of the articles analyzed did not report any specific storage condition. However, frozen storage at -80 ^{0}C was the most common reported way used to determine pEV therapeutical effects. Nevertheless, other articles report a -20 ^{0}C storage [20] or a prior storage of the source, one step before ultracentrifugation is performed [23]. Anyway, it is necessary to perform further evaluation on the storage conditions to compare different approaches and evaluate the stability and functionality over time.

5. Conclusions

In conclusion, pEVs present a huge potential on regenerative medicine therapies. Different approaches have been evaluated, especially in the injury and trauma conditions. Furthermore, restorative effects have been observed in the musculoskeletal and neural environment highlighting their use in healing therapies. However, pEVs have been little studied compared to cell derived EVs, although pEVs translation to clinics seems to be easier. Nevertheless, further characterization report and standardization of requirements should be performed to ease a future clinical use of pEVs.

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