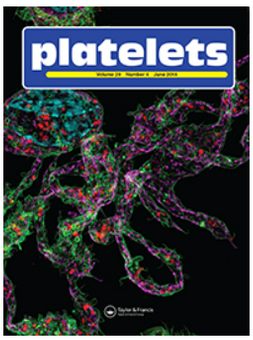

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SYSTEMATIC REVIEW

Efficacy of platelet concentrates in bone healing: A systematic review on animal studies – Part B: Large-size animal models

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Abstract

In the presence of large bone defects, delayed bone union, or nonunion and fractures, bone reconstruction may be necessary. Different strategies have been employed to enhance bone healing among which the use of autologous platelet concentrates (APCs). Due to the high content of platelets and platelet-derived bioactive molecules (e.g., growth factors, antimicrobial peptides), they are promising candidates to enhance bone healing. However, both preclinical and clinical studies produced contrasting results, mainly due to a high heterogeneity in study design, objectives, techniques adopted, and outcomes assessed. The aim of the present systematic review was to evaluate the efficacy of APCs in animal models of bone regeneration, considering the possible factors that might affect the outcome. An electronic search was performed on MEDLINE and Scopus databases. Comparative animal studies with a minimum follow up of 2 weeks, at least five subjects per group and using APCs for regeneration of bone defects were included. Articles underwent risk of bias assessment and quality evaluation. Fifty studies performed on six animal species (rat, rabbit, dog, sheep, goat, mini-pig) were included. The present part of the review considers studies performed on small ruminants, dogs, and mini-pigs (14 articles). The majority of the studies were considered at low risk of bias. In general, APCs' adjunct positively affected bone regeneration. Animal species, platelet and growth factors concentration, type of bone defect and of platelet concentrate used seemed to influence their efficacy in bone healing. However, sound conclusions were not drawn since too few studies for each large-size animal model were included. In addition, characterization of APCs' content was performed only in a few studies. Further studies with a standardized protocol including characterization of the final products will provide useful information for translating the results to clinical application of APCs in bone surgery.

Keywords

Animal models, bone regeneration, platelet concentrate, platelet-rich plasma, platelets

History

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Introduction

In the last 20 years, autologous platelet concentrates (APCs) have been widely used for tissue regeneration in several fields of medicine, such as orthopedics, sports medicine, oral and maxillofacial surgery, dentistry, ophthalmology, and plastic and cardiac surgery [1]. Among APCs, platelet-rich plasma (PRP) is the most known. A second-generation APC called platelet-rich fibrin (PRF) was also developed and used in oral, maxillofacial, and plastic surgery [2]. PRP is defined as an autologous derivative of anticoagulated whole blood (WB) with a supra physiological concentration of platelets, and generally is used in gel or liquid form [3]. PRF differs from PRP under many aspects. First, the PRF preparation protocol is very simple, avoiding the use of anticoagulant. Therefore, platelet activation and fibrinogen polymerization occur without blood manipulation, during centrifugation, instead of being induced using specific chemical activators at the time of *in situ* application. In the PRF protocol, clotting is favored by the contact with silica-coated or glass tubes that initiates the clumping of the red blood cells, resulting in separation of the blood component, and in a few minutes activates most platelets, which release coagulation factors. Therefore, during centrifugation the polymerization of fibrinogen into fibrin occurs differently and faster than PRP [4]. The physiological architecture of PRF, which can be used in clot or membrane form, consists of a mature and dense fibrin network in which almost 100% of platelets and about 50% of leukocytes of the blood sample are collected [5]. The fibrin clot in the PRF is more condensed than the PRP gel, allowing a slow and prolonged release of the content (including the growth factors (GFs) entrapped in the PRF clot) [6,7]. *In vitro* studies showed that PRF releases gradually GFs and cytokines for at least 10 days, whereas PRP gel displayed a different kinetics, releasing most of the GFs and bioactive molecules immediately after activation and up to 6–8 h after its application [6–9]. However, it has been reported that, after such intense release of the granules' content, also PRP may continuously release GFs at a slow rate, up to several days, with a kinetics dependent on the GF type [6–8]. A study that investigated

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the overall amount of released GFs and the induction of cell migration *in vitro* up to 28 days showed that PRF may offer significant advantages over PRP [9]. Secondly, the volume of plasma used to prepare the clot is different in the two APCs. In PRF, fibrinogen in the plasma is not concentrated by blood centrifugation. During PRPs' preparation, the plasma column is separated in different fractions, while in PRF there is no fractioning and the total amount of plasma available to forming the final clot is higher than in PRP. Minor drawbacks of PRF respect to PRP may be noted. Since anticoagulant is not used, blood drawing and PRF preparation must be done intrasurgically soon before the application, as opposite to PRP, for which the blood can be drawn from patients before starting the surgical procedure and then activated when needed, during surgery. Since PRP can be used in liquid form, it may also be applied by infiltration in the tissues, while this is not feasible with PRF. Table I summarizes the main properties and biological activities of APCs and the main benefits produced, which support their use in a number of clinical applications, though there is still no clear evidence that the biological response to a given type of APC is clearly different than others in specific interventions. The rationale of the use of PRP and PRF in hard and soft tissue regeneration is the high content of GFs and bioactive molecules, released by activated platelets, which are claimed to enhance and speed up the healing process. Indeed, platelets are involved in wound healing and inflammation and have the capability to induce a number of biological activities, among which osteoclast-like cells formation from bone marrow cells, as demonstrated in a murine study [10]. Platelet-GFs contribute at different levels to angiogenesis, macrophages recruitment, chemotaxis of keratinocytes, fibroblasts, and mitogenic activity [1]. In particular, some platelet-GFs are strongly involved in bone regeneration: b-fibroblast growth factor (bFGF) induces preosteoblast proliferation and differentiation; insulin-like growth factor (IGF-1) stimulates expression of alkaline phosphatase, osteopontin, and osteocalcin in bone marrow stromal cells; and platelet-derived growth factor (PDGF) may exert positive effect on bone regeneration through its mitogenic activity and synergy with transforming growth factor- beta1 (TGF-1). The latter is the most important GF released by platelets during bone healing and contributes to proliferation of fibroblasts, marrow stem cells, pre-osteoblasts and osteoclasts activity. In addition, vascular endothelial growth factor (VEGF) enhances angiogenesis, which is fundamental in bone regeneration [11]. Also, other molecules and properties of APCs such as antimicrobial, analgesic, and anti-inflammatory activities may be useful for bone tissue regeneration [1,3,12]. In addition, it was reported that platelets might activate peripheral blood mononuclear cells, which released insulin-like-10 (IL-10), an anti-inflammatory cytokine involved in tissue regeneration [13]. Despite these positive features in a number of processes involved in tissue regeneration, efficacy of APCs in bone healing is still debated [1,11,14,15]. *In vitro* studies reported a dose-dependent effect of PRP in osteoblasts and fibroblasts differentiation, with the best results obtained with a moderate platelet concentration compared to a high one [1,11]. In contrast, results obtained from *in vivo* and clinical studies are controversial and the different platelet concentration of PRP used, different protocols, as well as type of bone defect and different animal species have been claimed as possibly responsible for this variability [11,14,16–18]. A high heterogeneity was found in clinical studies, which used APCs for bone reconstruction: PRP used as an adjunct to bone grafts was reported to have a beneficial effect in the treatment of intrabony defects, while it seemed apparently useless in improving bone formation in sinus lift procedures [15,19]. In contrast, a recent systematic review reported that PRF in combination with bone grafts may enhance graft maturation [20]. PRF is currently mostly used in oral surgery procedures, compared to PRP, whose use also extends to other fields of surgery [20]. However, a comparison of clinical studies is often difficult due to the variable protocols, bone grafts combination, platelet concentration, and methods in assessing bone regeneration [18–20]. In addition, there is a lack of randomized controlled clinical trials on the efficacy of APCs in bone injuries [19–22]. So, the aim of this systematic review was to summarize the preclinical evidence of the APCs' efficacy used alone or in combination with bone grafts in animal studies. Possible factors that might influence APCs' outcome and the possible translation of results in clinical practice were also evaluated. In the present review, only studies performed on large-size animals (small ruminants, dogs, mini-pigs) were analyzed.

Table I. Main biological activities and clinical indications of platelet concentrates.

Biological activity	Clinical benefits
Enhancement of soft tissue healing	Faster closure of surgical wounds Faster epithelization and maturation of skin and mucosa Healing of chronic ulcers Improvement of healing of tendon, ligament, muscle injury
Enhancement of hard tissue healing	More predictable healing of bone defects Higher quality and quantity of newly formed bone Improved osseointegration of implants
Mechanical and cohesive properties; pro-coagulation activity	Improvement of bone graft handling and reduction of granules dispersion Control of intraoperative bleeding Control of intrasurgical complications like maxillary sinus membrane perforation
Control of inflammatory cytokines and modulation of microvessel permeability	Reduction of pain, swelling, and other postoperative symptoms, which improves patients' quality of life, satisfaction, and treatment acceptance
Antimicrobial activity	Control of postoperative infection

Methods

A systematic electronic research was performed on MEDLINE and Scopus using the following terms: platelet-rich-plasma; platelet-rich-fibrin; plasma-rich-in-growth-factors; platelet concentrate; PRP; PRF; PRGF; bone regeneration; bone healing; bone repair, with the limitation to animal studies. Such terms were combined using Boolean operators AND, OR. The last electronic search was performed on May 2016. Additional manual research was performed on reference lists of reviews focused on APCs and bone healing as well as those of the selected articles.

Inclusion criteria

Inclusion criteria were divided into primary and secondary. Studies that did not meet these criteria were excluded. Primary inclusion criteria were the following: articles in English language, animal studies, articles published on peer-reviewed journals, bone lesions (no soft tissue and osteochondral lesions), use of PRP or PRF alone or in association with bone grafts (no multiple compound mixtures), presence of negative controls, comparative studies, follow-up $\geq$ 2 weeks, animals/samples per group $\geq$ 5, and full-text availability. Studies on implant osseointegration and ectopic bone formation were excluded. Secondary inclusion criteria regarded the protocol and features of the APCs used and included platelet counts of PRP and/or GFs dosage reported, use

of allogeneic PRP or PRF, and details of the protocol used to produce PRP or PRF reported.

Articles selection

First, title and abstract of the articles found through the electronic search were screened in order to assess the fulfillment of primary inclusion criteria. When the title and abstract did not provide adequate information to make a decision, the full text of publications was obtained and screened (first phase of selection). Only studies that meet the primary inclusion criteria were included in the second phase of selection. The latter phase was based on the assessment of the secondary inclusion criteria previously described. Articles that performed an inadequate platelet counts (e.g., provided indication only of the fold increase of platelets in PRP compared to WB) and that did not meet the other secondary inclusion criteria were excluded.

Risk of bias assessment

The risk of bias of the included studies was assessed, according to the Animals in Research: Reporting In Vivo Experiments guidelines [23]. The following criteria were adopted: ethical statement, experimental animals, experimental procedures, sample size calculation, randomization of groups, blinding of the evaluator, statistical analysis, financial conflict of interest, number of animals analyzed, and translation of the results in the discussion. The indication of the platelet baseline in WB was also added to these criteria. All criteria were evaluated as adequate (score = 1), unclear or incomplete (0.5), and inadequate (0). Among the details regarding the animals used, indication of the age was considered more important than sex and weight. Studies were then classified at high (total score = 0–4), medium (>4–7), or low risk of bias (>7–11).

Results

Initially, 476 articles were found by electronic search and 8 more articles were added by hand-searching on articles' and reviews' references (see the flowchart in Figure 1). After screening of titles and abstract, a total of 358 articles were excluded because they did not satisfy the inclusion criteria. In the first phase of selection, the full text of 126 articles was analyzed and 51 articles were excluded for the following reasons: 47 articles did not have an adequate sample size (at least five animals/samples per group); 2 articles did not clearly report the number of animals employed; 1 article did not have the minimum follow-up requested and another evaluated the effect of PRP on implant osseointegration. In the second phase of selection, a total of 25 articles were excluded: 21 articles were excluded for inadequate or lacking PRP characterization; 3 articles were excluded because they employed human PRP in animal models of bone healing [24–26]; 1 article did not report in detail the procedure to produce PRP [27]. Finally, a total of 50 articles were included: in the present review, only those performing on large-size animals (small ruminants, dogs, minipigs) are examined [28–41]. Seven included articles were performed on small ruminant model of bone healing [28–34], three on canine model [35–37], and four on porcine one [38–41]. The main information of the included studies is reported in Table II. It must be noted that all the included studies used PRP and only one of them additionally tested PRF. They are ordered according to the animal species and to the platelet concentration of PRP. In addition, Table III indicates the characterization of PRP for each study. The lack of PRP characterization (except for platelet counts) in half of the included studies is noteworthy. When performed, PRP characterization was different between studies.

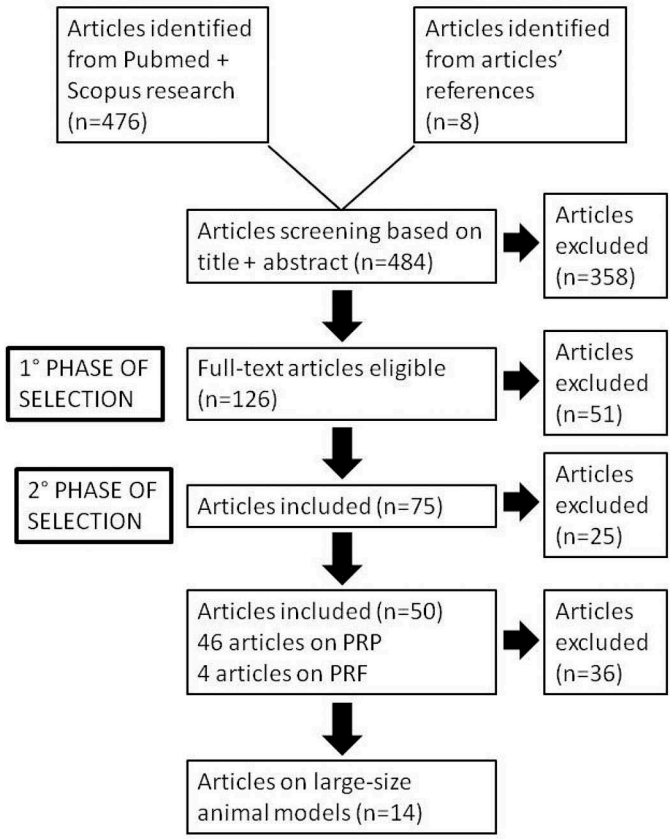

Figure 1. Flowchart of the article selection procedure.

Leukocyte concentration was measured only in two studies. In contrast, five studies reported the dosage of GFs in PRP, particularly TGF-1 and PDGF (Table III). Most of these studies were performed on porcine models of bone regeneration.

Results of the bias assessment are reported in Table IV. Half of the included studies (7/14) presented a low risk of bias. However, only one study reported the sample size calculation. Seven out of 14 studies did not perform the platelet count in WB.

Discussion

Techniques for enhancing bone regeneration are necessary in the presence of large bone defects, nonunion, and delayed bone union or healing of fractures [42]. Graft biomaterials, recombinant osteoinductive proteins [11], cell-based therapy [43], and APCs are used in both preclinical and clinical studies to accelerate bone healing. APCs contain higher platelet and GFs concentrations than patient's WB. The high level of GFs (particularly TGF-1 and PDGF) released by activated platelets constitutes the main reason of using APC for tissue regeneration. In soft tissue healing, PRP has shown interesting results in case of diabetic lower-limb ulcers and other skin and mucosal lesions [1,44]. In contrast, its efficacy in bone healing is controversial [11,16,19]. Variable methods of preparation and the different features of PRP used have been claimed as the principal factors responsible for the contrasting results observed [11,14,16]. PRF was introduced in 2001 and presented easy preparation, lack of anticoagulants, and additional activators, allowing a more physiological fibrin clot formation than PRP. PRF membranes were used in combination with bone grafts to enhance bone regeneration. *In vitro*, PRF showed a slower and longer-lasting release of GFs [6–9], which permitted more durable proliferation and differentiation of rat osteoblasts than PRP [2]. The aim of the present review was to

Table II. Characteristics of the included studies using autologous platelet concentrates for bone regeneration.

Article	Animal details (species, age, gender)	Experimental model	Type of APC; biomaterial combined with APC	Follow-up	Outcome assessment	Platelet concentration (10^3/μL) Mean value ± SD	Results
Hernandez-Fernandez et al. [28]	Sheep, 4 months, NS	Distraction osteogenesis femoral diaphysis	PRP; none	40 days	CT, h	590.24	Not significant increase of bone formation with PRP. Only significantly wider femur and increased diaphyseal thickness in the PRP group, compared to ctr.
Mooren et al. [29]	Saanen goat, skeletally mature, female	critical-sized defects in the forehead	PRP; AB + BioOss	1,2,6, 12 weeks	h, hm	800	No significant differences between the groups with and without PRP.
Fennis et al. [30]	Saanen goat, 2.5 years, female	Mandibular defect	PRP; AB	3 and 6 weeks	h, hm	800	Significant differences in fibrous capsulation, vascularization, and bone resorption in PRP group, compared to AB alone group. Significant increase of bone formation with the addition of PRP.
Fennis et al. [31]	Saanen goats 2.5 years, female	Mandibular defect	PRP; AB	3 and 6 weeks	r	800	Significant increase in bone healing with PRP + AB compared to AB alone.
Mooren et al. [32]	Saanen goat, NS, female	Critical-sized defects in the forehead	PRP; AB	1,2,6, 12 weeks	h, hm	800	No significant differences between the PRP+ AB and AB alone.
Scholz et al. [33]	Sheep, 2 years, female	Intervertebral body fusion model	PRP; MCM	12 weeks	r, CT, hm, h	1375	No significant differences between the MCM and PRP + MCM group. Slightly lower mineralized cartilage ratio in MCM group compared with the PRP + MCM one.
Jakse et al. [34]	Sheep, adult, female	Bilateral sinus floor elevation	PRP; AB	4 and 12 weeks	h, hm	3810 ± 2468	More newly formed bone in PRP + AB side, compared with AB side, but not statistically significant.
Carvalho et al. [35]	Mongrel dog, adult, female	Intrabony defects near post-extraction sites	PRP; BG and none	90 days	h, hm	463	No significant effect of PRP addition in bone formation compared to ctr and BG alone.
Hatakeyama et al. [36]	Beagle dog, 1 year, NS	Buccal dehiscence from the alveolar crest to the buccal wall of extraction sockets	PRP, PRF; none	4 and 8 weeks	CT, h and hm	PRP: 556 ± 74 PRF: NS	Significantly higher bone formation in PRF vs. ctr (4 weeks) and vs. PRP (4, 8 weeks). Significantly lower newly formed bone in PRP group compared to the ctr. At h, higher rate of bone maturation in the PRP and PRF groups compared to ctr. but not statistically significant.
Choi et al. [37]	Mongrel dog, adult, NS	Bilateral mandibular defects after extraction of mandibular premolars	PRP; AB	6 weeks	FM, h and hm	1120	At h and hm, significantly more bone formation in AB than in PRP + AB. At FM more delay in graft remodeling with PRP + graft than with graft alone.
Jungbluth et al. [38]	Goettingen mini-pig, 18–30 months, female	Critical size metaphyseal tibial defect	PRP; CPG	6 weeks	r, h and hm	1619.5 ± 406.9	Significantly more bone formation and greater bone resorption of the granules in the PRP group compared to CPG alone.
Li et al. [39]	Danish Landrace pig, NS, NS	Anterior lumbar interbody fusion	PRP; β-TCP	3 months	r, CT and hm	1786.6	All β-TCP/PRP levels with partial fusion, compared to β-TCP levels (four non-fusions). No significant difference between β-TCP and β-TCP+PRP.
Hakimi et al. [40]	Goettingen mini-pigs 18–30 months, female	Critical-sized tibial defect	PRP; AB	6 weeks	*Ex vivo* r, hm and h	2120.3 ± 550.7	Significantly greater new bone area in the PRP + AB group compared to AB alone. At h, more prominent presence of multinucleated giant cells/ macrophages in the PRP + AB group than AB alone.
Schlegel et al. [41]	Pigs, 12 months, female	Critical-sized defects in the forehead	PRP; AB; bovine collagen	2, 4, 12 weeks	r and h	NS	Significantly greater bone formation only at 2 weeks with the addition of PRP to AB. No significant effect on bone formation with PRP added to bovine collagen. No significant differences between the two types of PRP, prepared with two different methods.

NS = not specified; AB = autologous bone; APC = autologous platelet concentrate; MCM = mineralized collagen matrix; BG = bioglass; CPG = calcium phosphate granules; β-TCP = β-tricalcium phosphate; CT = computed tomography; h = histology; hm = histomorphometry; r = radiography; FM = fluorescence microscopy;

Table III. Characterization of the platelet concentrates in the included studies.

	Article	Platelet morphology	Leukocytes	TGF-β1	PDGF	VEGF	EGF	IGF-1
Ruminants	Hernandez-Fernandez et al. [28]		X					
	Mooren et al. [29]							
	Fennis et al. [30]							
	Fennis et al. [31]							
	Mooren et al. [32]							
	Scholz et al. [33]							
	Jakse et al. [34]			X	X			
Dog	Carvalho et al. [35]							
	Hatakeyama et al. [36]			X	X			
	Choi et al. [37]							
Mini-pig	Jungbluth et al. [38]			X	X			
	Li et al. [39]		X					
	Hakimi et al. [40]			X	X			
	Schlegel et al. [41]			X	X			X

Table IV. Risk of bias assessment of the included studies.

	Article	Total	Sample size calculation	Randomization	Blinding	WB platelet baseline
Ruminants	Hernandez-Fernandez et al. [28]	6	NA	0	1	0
	Mooren et al. [29]	8	0	0	1	0
	Fennis et al. [30]	6	0	1	0	0
	Fennis et al. [31]	8	0	1	1	0
	Mooren et al. [32]	4	0	0	0	0
	Scholz et al. [33]	7.5	0	1	0	1
	Jakse et al. [34]	8.5	0	0	1	1
Dog	Carvalho et al. [35]	8	0	1	1	0
	Hatakeyama et al. [36]	6.5	0	1	0	1
	Choi et al. [37]	4	0	0	0	1
Mini-pig	Jungbluth et al. [38]	8.5	0	1	1	1
	Li et al. [39]	8	0	1	1	1
	Hakimi et al. [40]	10	1	1	1	1
	Schlegel et al. [41]	6	0	1	0	0

Studies with a total score from 0 to 4 were considered at high risk of bias, from 5 to 7 at medium risk of bias and from 8 to 11 at low risk of bias. The most important parameters are below reported.
NA = not applicable; 0 = inadequate; 1 = adequate; 0.5 = unclear; WB = whole blood.

evaluate the efficacy of PRF and PRP in animal models of bone healing, considering the possible factors that might influence their outcome.

The studies selected for the present review, composed by two parts, were performed on six animal species (rabbit, rat, dog, sheep, goat, mini-pig). Each species presents different features, making interspecies comparison, as well as comparison with bone regeneration in humans, unfeasible. In the present part of the review, only studies using PRP and/or PRF on large-size animal models were considered. These types of animal models are less frequently used than small-size ones as rats and rabbits. Indeed, they were used in the 28% (14/50 articles) of the included studies.

Large-size animal models for bone healing

Animal models play a critical role in many study domains of the biomedical sciences, such as basic sciences, feasibility and bioactivity testing, clinical modeling, and effective prediction (45). The latter is also referred to as "preclinical testing" and is aimed at evaluating the performance and the efficacy of a given method, treatment or adjunctive therapy, simulating the clinical condition on proper animal models. Key criteria for the choice of such models are driven by the comparability of macro- and micro-anatomy, metabolic background, and physiological responsiveness of the tissues under investigation with respect to human ones, as well as of the modalities of therapy application and activity (43). In contrast to small animals like mice, rats, and rabbits, large-size animal models present more similarities with human bone anatomy and/or physiology according to the literature [17,33,35,40,41,45]. Indeed, small ruminants such as sheep and goats are suitable models for testing prosthesis and implants for long bone and joint dimensions similar to humans. In addition, weight-bearing is also very similar [17,46]. Mini-pigs are considered the best animal model of bone regeneration [46]. Their bone structure, anatomy, and bone healing rate are very close to the human ones [17,40]. In dogs, bone composition and structure have several features in common with humans. In the past, canine models were frequently used in dental research [17]. However, their use as animal model raises several ethical problems. Planning of randomized controlled clinical trials on the use of PRP in dogs in the veterinary practice may overcome this problem. Indeed, dogs as humans are affected by several chronic diseases, which are difficult to reproduce in laboratory [47]. Large-size animal models presented other disadvantages compared to small-size animals: high cost, seasonal breeding cycles (sheep), poor availability of biomarkers, long time for bone healing, and availability of appropriate equipment and facilities [17,46]. Interestingly, all the included studies using porcine model showed positive results using PRP in bone healing (Table II). In contrast, the majority of the other studies did not report a significant efficacy of PRP. Since the porcine model is claimed as the best animal model for studying therapies regarding

bone defects regeneration, the results obtained should have a good transferability and one could conclude that PRP may be as well effective for the treatment of bone defects in humans. However, it is noteworthy that all the included studies using PRP in large animal models were performed on healthy animals. One should consider that the etiopathogenesis of diseases leading to bone defects creates a tissue environment, which is quite different from the one present in an experimentally induced bone defect. Indeed, even though PRP has been reported to be more effective in pathological tissues as compared to normal ones [47], the scarce number of articles on spontaneous pathological bone conditions in large-size animal model did not permit to draw definitive conclusions.

Type of bone defect and outcome assessment

Critical-size defects are defined as the smallest wound created intraosseously in a particular bone, which does not spontaneously heal [17,48]. To obtain complete bone healing, use of supplementary biomaterials, cells, and GFs may be necessary [49]. In contrast, non-critical-size defects spontaneously heal and the comparison between different treatments for bone healing may be more difficult [17,48].

The size of bone defect used in preclinical studies influenced the efficacy of PRP in bone healing [42]. Several studies performed on critical-size defects showed good results with PRP [11,49]. In clinical practice, critical-size defects are those that require strategies to regenerate bone [18]. Few included studies were performed on critical-size defects (Table I), but results of PRP in bone healing were controversial. It is important to consider that other factors such as the age of animals used (young vs. adult), location of the defect (e.g., long bone or calvarium), biomaterial used, and time of follow-up were important in assessing the efficacy of therapies for bone healing in animal models [17,42,48]. Several authors reported that the type of biomaterial combined with PRP might strongly influence its efficacy for bone regeneration [18,50,51]. This correlated with the study by Schlegel et al. [41], who reported good results in bone formation by combining PRP with autografts, but not by combining it with bovine collagen. However, a discussion on all possible biomaterials, of natural and synthetic origin, that could be combined with PRP or other APCs is not among the aims of the present review.

The most frequent techniques for bone healing assessment were histology and histomorphometry (Table II), which have a high translational value as well as imaging technologies [17]. Only few included studies performed radiographs or CT and only one study performed other evaluations (e.g., immunohistochemistry) (Table II). It is noteworthy that some clinical studies performed only an imaging assessment to determine bone regeneration [52–55]. Authors' opinion is that both techniques should be performed to evaluate APCs' effect on bone. In addition, a more extensive use of immunohistochemistry or modern technologies (e.g., scintiscan) may provide useful information on the effects of APCs.

Effects of platelet concentration, leukocytes, and GFs on bone healing

For the present review, 21 articles were excluded for an absent or inadequate PRP characterization, which rendered the reproducibility of the results and the evaluation of the effect of PRP itself difficult. Patients as well as animals presented interspecies and intraindividual differences of both platelet counts and GFs content in WB [51], which resulted in PRP with different features [29,32,56]. Plachokowa et al. [51] using CT analysis demonstrated a higher degree of bone union using human PRP + HA/TCP compared to rat and goat PRP combined with the same material. Surprisingly, no data regarding leukocytes and GFs content of different PRPs were available. Platelet concentration was the most frequent parameter measured in PRP (Tables I). It differed among the different animal species and also among the included studies performed on the same species (see Table II). Several authors reported that only certain concentrations of platelets in PRP might be effective in bone formation [51,57,58]. In particular, a relatively low concentration of platelets in PRP seemed to be more effective in bone healing than a high one [1,11]. However, a dose-dependent effect of PRP was not observed in the included studies: we could not find any correlation between platelet concentration and the outcomes (significant or nonsignificant benefit) (Table II). Possible reasons might be the scarce number of included studies and the fact that every animal species might have its own range of the effective platelet concentration, which should be assessed in future preclinical studies on PRP [29,32]. For this reason, the assessment of the platelet concentration in WB of different animal species assumed a critical significance. Unfortunately, most of the included studies did not report such information (Tables III and 4IV).

In few studies, leukocytes were reported to diminish PRP efficacy in tissue regeneration due to an increase of inflammatory cytokines production [59]. However, an involvement of leukocytes in antimicrobial activity, as well as soft and hard tissue healing, was reported [60–62]. In the present review, only two studies counted leukocytes in PRP (Table III) and no evaluation of its antimicrobial potential was performed. Also in clinical studies, leukocytes in PRP were evaluated only in few cases [63–65]. Further studies might be necessary to elucidate the role of leukocytes in PRP for bone regeneration. Same conclusions could be obtained regarding optimal GFs levels for bone healing: few preclinical (Table III) and clinical studies [52,54,66–68] measured them. In the included studies performed on mini-pigs, the use of PRPs with different levels of GFs was associated with beneficial results in bone healing (Tables I and III). It was also observed that the effect on bone regeneration of a PRP with a high level of TGF-1 on bone regeneration was not significantly different compared to that of a PRP with a lower level [41]. Since GFs' levels do not depend on platelet counts [14] and several of them (e.g., TGF-1, PDGF, VEGF) contributed to bone healing [11,14,69], it may be important to assess their content in PRP to evaluate the influence of the different GFs' amount in bone regeneration.

An additional problem in assessing PRP effects on bone formation was the variability of protocols adopted for its production in both animal and clinical studies [11,42]. Several authors claimed a standardization of the procedure, which might render more accurate the evaluation of PRP regenerative properties in bone [18,42,63,70].

Bias assessment of the included studies

The majority of the included studies were at low risk of bias (Table III). However, almost all of the included studies did not report a sample size calculation (Table III). It is necessary to ensure that an adequate number of animals is used to achieve a statistical power >80% [17]. An animal study should always comply with the 3R principles (Replacement, Reduction, Refinement) and should report all the details concerning the number of animals used and their characteristics (e.g., species/strain, sex, and age) [23]. It is well known that the age of animals strongly influences the rate of bone healing. It has been suggested to use only sexual mature animals for an experiment on bone healing (except for mice and rats) to avoid the bias of additional bone growth [17,71]. In the present part of the review, the majority of the studies duly reported this information (Table II).

Reporting of the sex of animals used is also important. Some authors suggested to use male animals instead of female ones due to the absence of estrous cycle, which might cause an increase of variability of the results [72].

Use of PRF in animal models of bone healing

In contrast to PRP, few preclinical and clinical studies have been published on the use of PRF for bone regeneration. Only one study included in the present review performed a comparison between PRP and PRF on a canine model of buccal dehiscence after tooth extraction, and PRF produced better outcomes than PRP in bone formation (Table II). However, it is needed to perform more studies, possibly based on the comparison of different APCs, to investigate the *in vivo* regenerative potential of PRF.

Limits of the review

Due to the high heterogeneity of the included studies, a meta-analysis of the results was not performed. In the present review, only studies performed on bone defects and evaluating PRP/PRF effect on bone healing were included. Other types of defects were excluded, such as osteochondral ones, and also studies on implant osseointegration were not considered. Use of APCs might be promising to enhance bone healing in patients affected by bone pathologies or other diseases associated with compromised bone health. However, all the included studies were performed on healthy animals. Therefore, it is difficult to predict how subjects with a compromised bone tissue may respond to treatment involving the delivery of a large amount of platelet GFs. Other properties of APCs (e.g., antimicrobial activity, stimulation of angiogenesis, enhancement of soft tissue healing) might be involved in bone healing, but these aspects were not investigated in the studies performed on large-size animal models.

Conclusion

A very limited number of articles was found per each animal species considered, and most of them only tested PRP. In addition, a strong variability in species, platelet and GFs' amount at baseline, protocol employed, type of bone defect, duration of follow-up, and combination with different biomaterials make it difficult to reach a general consensus on the actual efficacy of platelet concentrates. This adjunctive therapy may be promising to enhance healing in patients with a low bone regeneration potential and those at risk of infections due to the regenerative and antimicrobial potential of platelets. Unfortunately, few studies were performed on animal models of disease or infection. Further studies on such animal models should be performed in order to evaluate the mechanism of APCs in pathologic tissues, rather than in normal ones. This type of model is closer to the clinical condition of patients, who require special strategies for bone regeneration. APCs' characterization is absent or inadequate in the majority of clinical studies and some animal studies. At least the baseline platelet count should be always performed because it correlates to bone healing capability. Also the leukocyte count and the dosage of the main GFs present in APCs (TGF-1, PDGF, VEGF) are suggested. Finally, the role of other platelet-derived-GFs and -molecules (e.g., IGF-1, EGF, bioactive lipids, antimicrobial molecules) in bone regeneration should be investigated for a better understanding of the properties of APCs and their future clinical applications in this field.

Declaration of interest

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