Platelet Microparticles Induce Angiogenesis and Neurogenesis after Cerebral Ischemia

Yael Hayon[1], Olga Dashevsky[2], Ela Shai[2], Alex Brill[2], David Varon[2,*] and Ronen R. Leker[1,*]

[1]*Department of Neurology, Peritz and Chantal Scheinberg Cerebrovascular Research Laboratory, and* [2]*Department of Hematology, Coagulation Unit Hadassah University Hospital*

Abstract: Activated platelets shed microparticles, which contain a variety of growth factors central to angiogenesis and neurogenesis. The aim of this study was to explore whether platelet derived microparticles (PMP) can boost endogenous neural stem cells dependent repair mechanisms following stroke in a rat model.

To examine the effects of PMP therapy in-vivo, we delivered PMP or vehicle via a biodegradable polymer to the brain surface after permanent middle cerebral artery occlusion (PMCAO) in rats.

Rats were tested with the neurological severity score and infarct volumes were measured at 90 days post-ischemia. Immunohistochemistry was used to determine the fate of newborn cells and to count blood vessels in the ischemic brain.

The results show that PMP led to a dose dependent increase in cell proliferation, neurogenesis and angiogenesis at the infarct boundary zone and significantly improved behavioral deficits.

Keywords: Angiogenesis, Cerebral ischemia, Microparticles, Neurogenesis, Platelets, Stroke

INTRODUCTION

Stroke is a leading cause of chronic disability and a leading cause of mortality and therapies aimed at reducing this morbidity are currently limited. Stimulation of endogenous repair mechanisms based on endogenous neural stem cells (eNSC) appears to be a promising novel therapeutic option because these cells can differentiate into neurons and glia and secrete trophic, survival and support factors [1-3].

Platelets store large amounts of trophic factors including vascular endothelial growth factor (VEGF), basic fibroblastic growth factor (FGF2), platelet derived growth factor (PDGF) and brain-derived neurotrophic factor (BDNF) and also contain membrane receptors including VEGFR1, and VEGFR2 among others [4-7]. Upon activation, platelets shed small subcellualr fragments (0.1-1 μm in size) called platelet microparticles (PMP) that contain all these factors [5-6].

We and others have shown that PMP, in addition to their procoagulant and inflammatory effects, promote all stages of angiogenesis *in-vitro* and *in-vivo* and are capable of improving revascularization in a rat myocardial infarction model [6, 8]. We also showed that incubation with PMP lead to increased neural stem cell (NSC) proliferation and survival *in-vitro* and increase the differentiation potential of endogenous NSC to glia and neurons [9].

*Address correspondence to these authors at the Stroke Service and the Peritz and Chantal Cerebrovascular Research Laboratory Hadassah Ein Kerem P. O. Box 12000, Jerusalem 91120, Israel; Tel: 972-2-6776945; Fax: 972-2-6437782; E-mail: leker@hadassah.org.il
Coagulation Unit Hadassah Ein Kerem P. O. Box 12000, Jerusalem 91120, Israel; Tel: 972-2-677-7672; Fax: 972-2-644-9580;
E-mail: dvaron@hadassah.org.il
Both correspondence authors contribute equally

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Thus, activated platelets and the resulting PMP serve as a mini-storage system of multiple growth and pro-survival factors crucial for both NSC and endothelial precursor cells (EPC).

Newborn cells often depend on cues from blood, therefore we hypothesized that augmenting angiogenesis and neurogenesis together *in-vivo* will have a synergistic effect on both systems and would result in better functional outcome following ischemic injury.

Here we show that PMP may promote cell driven angiogenesis and neurogenesis with a significant functional gain in a rat model of cerebral ischemia.

MATERIALS AND METHODS

PMP Preparation

PMP were produced from platelets obtained from healthy volunteers according to institutional Blood Bank regulations and with the approval of the institutional ethics committee. Outdated platelets portions were obtained from Blood Bank and leukocytes were removed using PLX-5A leukocyte-reducing filter (Asahi Kasei Medical Co, Ltd., Tokyo, Japan). Platelets were pelleted at 750 x g in the presence of 5mM citric acid, resuspended in 0.5ml PBS (with Ca, Mg), and thrombin (2 U/ml) was added. After 5 min incubation, platelet aggregates were removed along with thrombin residues and microparticles were isolated by supernatant centrifugation at 100000x g for 1h at 4°C. PMP were identified by flow cytometry using CD41 antibodies. Total protein amount in the obtained PMP was determined using the Bradford method.

In this study MP were separated by centrifugation at 100,000 g and thus the resulted pellet, which is referred to as PMP, contains both PMP and exosomes.

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Table 1. Antibodies Used for Characterization of Cells

Primary Antibodies				
R&D	1:200	Neuronal progenitors marker		Rabbit anti Sox2
Chemicon	1:200	Neuronal stem cells marker		Rabbit anti Nestin
Santa Cruz	1:100	Neuroblast marker		Goat anti DCX
Chemicon	1:200	Blood vessels marker		Rabbit anti VWF
Serotec	1:200	Endothelial cells marker		Mouse anti RECA1
Chemicon	1:200	Neuronal marker		Mouse anti NeuN
Dako	1:200	Astrocytes marker		Rabbit anti GFAP
Chemicon	1:200	Oligodendroglial marker		Rabbit anti GalC
Serotec	1:100	Activated microglial marker		Rat anti CD68 (ED1)
BD biosciences	1:200	Newborn cells marker		Rat anti BrdU
Secondary Antibodies:				
Invitrogen	1:200	Alexa Fluor 488	IgG	Donkey anti goat
Invitrogen	1:200	Alexa Fluor 555	IgG	Donkey anti mouse
Invitrogen	1:200	Alexa Fluor 555	IgG	Donkey anti rabbit
Invitrogen	1:200	Alexa Fluor 488	IgG	Goat anti rat
Invitrogen	1:200	Alexa Fluor 555	IgG	Goat anti rat

The exosomes are smaller vesicles within the platelets which are released during platelet activation. Thus from now on we refer to PMP as a mix of these two structure populations.

Animals

The study was conducted according to the Institutional Animal Care and Use Committee guidelines in compliance with National Institutes of Health guidelines. Adult spontaneously hypertensive rats (SHR; 13 weeks old; n=6/group) were used for these experiments and treated with PMP or vehicle and sham operated rats (n=3) served as further controls.

Focal Ischemia

SHR underwent permanent distal middle cerebral artery occlusion (PMCAO) resulting in fronto-parietal cortical injury that typically involves 19-24% of hemispheric volume [2]. Briefly, animals were anesthetized with inhalation isoflurane and placed in a stereotaxic head holder. The skull was exposed and the middle cerebral artery was exposed via a craniotomy and then electrocoagulated and cut. Immediately following ischemic onset the brain surface was covered with a commercially available biodegradable polymer (5X5 mm gelfoam - Pharmacia Upjohn pharmaceuticals) containing vehicle or different concentrations of PMP attained upon saturating the gelfoam for 5 minutes in solutions containing different concentrations of PMP (10μg/ml or 100μg/ml). By topical application of PMP we aimed to generate their potential regenerative effect, while avoiding their well known pro-thrombotic effect when circulating in blood vessels. The temporalis muscle and skin were sutured and the animals were given carprofen to control post procedure pain. Sham operated animals (n=3) were anesthetized, their scalps were incised, but ischemia was not induced.

BrdU injections

Animals received intraperitoneally (IP) injection of the tracer Bromodeoxyuridine (BrdU, 50mg/kg q12 hours for 7 days beginning on day 1 post ischemia) to detect proliferating cells. BrdU is incorporated into the newly synthesized DNA of replicating cells during the S phase.

Disability Evaluation

Animals were weighed and examined on days 1, 3, 5, 7, 10, 14, 21, 28, 35, 42, 49, 63 and 90 after stroke with a standardized neurological disability scale [2]. The disability scale consists of 10 items evaluating motor paralysis, walking ability, agility and limb placement. A rat with maximal disability scores 10 points and rats with no disability score 0.

Injury Size

Brain slices cut at 200 micrometers apart were obtained from all animals in the study. Sections were stained with Giemsa and photographed on line with an image acquisition system. Estimation of the lesioned area was performed with SigmaScan-Pro image-analysis software (SPSS inc. Richmond CA). The volume of injured tissue was derived by subtracting the volume of the injured hemisphere from that of the normal hemisphere and dividing the result by the volume of the normal hemisphere. Results are presented as % of the normal hemisphere [2].

Immunohistochemistry

Rats were perfusion fixed, brains were frozen-sectioned and double or triple stained for immunohistochemical evaluation using fate specific antibodies (Supplementary Table **1**, n=6/group). Cells were counted in regions of interest including the subventricular zone (SVZ) and peri-lesioned cortex in coronal brain slices (sliced at 12 microns)

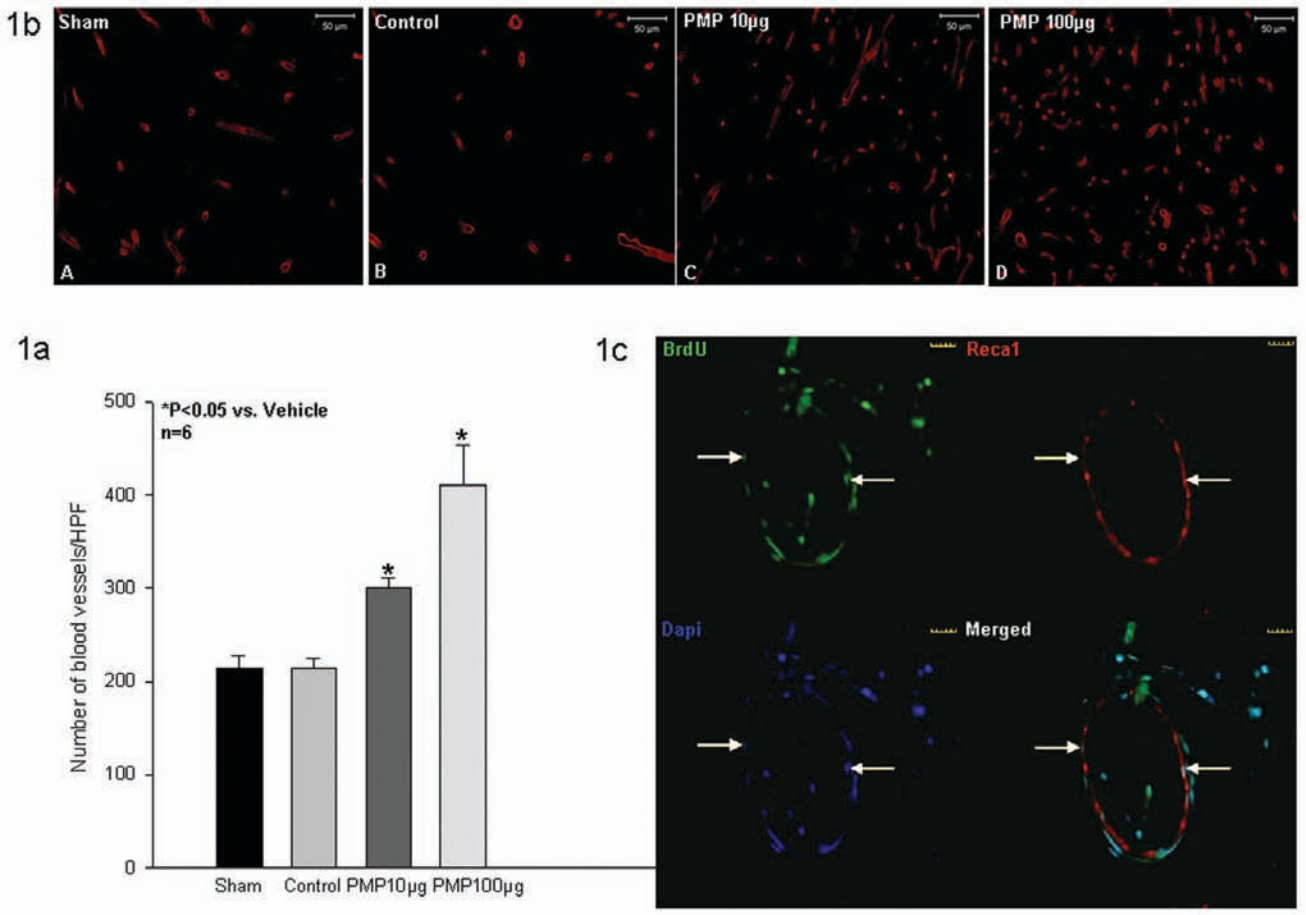

Fig. (1). PMP increase angiogenesis in the ischemic hemisphere
Immunohistochemistry was evaluated at the infarct border. Blood vessel density was counted at the peri-infarct area (n=6/group). Fig. (**1a**) is a bar graph showing blood vessel density in the tissue adjacent to the infarct. Fig. (**1b**) shows low power photomicrograph (x100) from the infracted hemisphere of sham operated animals (**A**), vehicle treated (**B**) and PMP treated (**C**, **D**) stained with an antibody to RECA1. Note that blood vessel density is much higher in the PMP treated animals. Fig. (**1c**) shows co-localization of BrdU (green) and the endothelial marker RECA1 (red) in vessel walls of a PMP-treated animal. Nuclei are counterstained with DAPI (blue).
HPF = high power field, Bar graphs 50µ
Data are represented as mean ± SEM, *p<0.05.

100 microns apart from Bregma +2.2 to Bregma -1.3 as previously described [2].

Blood Vessel Density

Slides obtained from homologous areas of rat brains treated with vehicle or PMP were stained with an antibody to blood vessels (RECA1, Table **1**). Images were captured using a camera attached to an Olympus upright fluorescent microscope. Blood vessels were counted in the entire ischemic hemisphere.

Statistical Analysis

Clinical and immunohistochemical evaluations were performed by examiner, blinded to the experimental group. Analysis was performed with the SigmaStat software package (Systat, Richmond CA, USA). Data are presented as Mean ± SE (SEM) as indicated in the legends. Values were compared using one-way analysis of variance (ANOVA) followed by Bonferroni correction for multiple comparisons. P-values < 0.05 were considered significant for all comparisons.

RESULTS

PMP Increase the Number of Blood Vessels in the Affected Hemisphere after Stroke

Blood vessel density in the hemisphere ipsilateral to the stroke was evaluated in all animals (n=6/group) at the infarct border zone using blood vessel and endothelial specific markers including RECA1. The results show that blood vessel density was significantly increased in PMP treated rats (Fig. **1a-b**).

Co-localization of BrdU and endothelial markers served to show that many of these blood vessels were newly formed (Fig. **1c**). Most of the BrdU containing vessels were observed at the area surrounding the infarct. These results suggest that PMP increase angiogenesis at the infarct boundary.

PMP Increase the number of Newborn Cells

Animals (n=6/group) treated with PMP at higher concentrations showed significantly increased numbers of BrdU positive cells. Overall, there was a 2-fold increase in

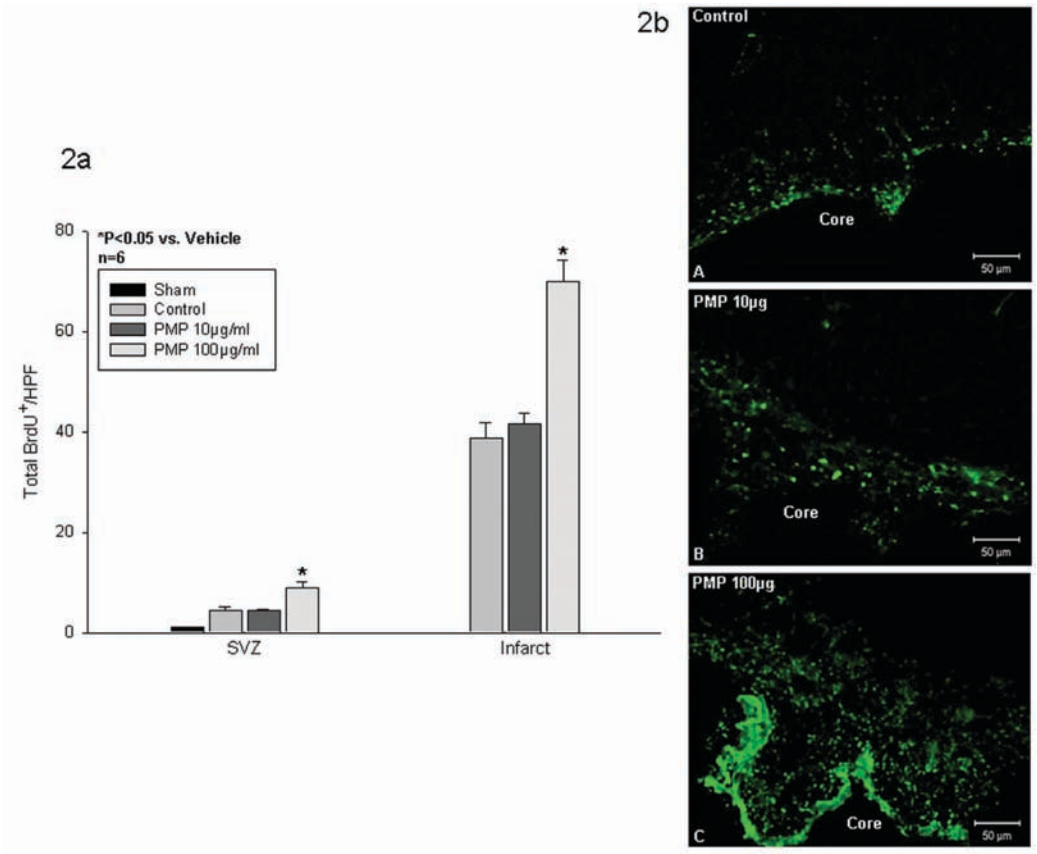

Fig. (2). PMP increase cell proliferation after stroke

The number of proliferating cells ($BrdU^+$) was counted in the SVZ and peri-infarct areas (n=6/group). Fig. (**2a**) is a bar graph showing absolute number of $BrdU^+$ cells in SVZ and peri-ischemic cortex. Fig. (**2b**) is a low power (x100) photomicrograph from the peri-ischemic tissue of vehicle treated (**A**) or PMP treated (**B**, **C**) animals stained with an antibody against BrdU (green). Note that the number of $BrdU^+$ cells is significantly increased in treated animals in a dose dependent manner.

HPF = high power field, Bar graphs 50µ

Data are represented as mean ± SEM, *p<0.05.

the number of $BrdU^+$ cells at the SVZ and at the infarct border zone (Fig. **2a-b**). Importantly, newborn cells accumulated in the cortex surrounding the injured tissue (Fig. **2b**) corroborating previous reports [1-3]. Only a few BrdU positive cells were detected in sham operated animals (n=3), implicating that there was no proliferation and differentiation of those cells. These findings suggest that PMP increase newborn cell proliferation, their survival or both *in-vivo*, which may also promote migration towards the infarct zone and accumulation in this area.

PMP drive Neuronal Differentiation of Newborn Cells

We used immunohistochemistry methods with double and triple staining to evaluate newborn cell fates over time. The absolute numbers of newborn cells that differentiated into neurons (NeuN), astrocytes (GFAP) or oligodendroglia (GalC) were larger in the PMP treated group suggesting that PMP treatment promote differentiation of eNSC (Figs. **3-4**). In contrast to the modest increments in newborn glia, 7% of the $BrdU^+$ cells in the area surrounding the cortical injury expressed neuronal antigens in the group treated with a high concentration of PMP as opposed to 1% of $BrdU/NeuN^+$ cells in vehicle treated animals (Fig. 3a n=6/group), suggesting that PMP actively drive neuronal differentiation *in-vivo*.

A large percentage of newborn cells at the immediate infarct border remained undifferentiated (SOX2 and Nestin positive) in both treated and untreated animals corroborating previous results suggesting that terminal differentiation at this site takes very long time to complete if at all [2, 10].

Nevertheless, the number of these undifferentiated cells was significantly larger in PMP treated animals.

Importantly, almost all of the BrdU/GalC double positive cells were seen in white matter (Fig. **4**) and all BrdU/NeuN colocalizing cells were seen at the area around the immediate infarct border. In contrast, BrdU/GFAP double positive cells were observed both at the SVZ and at the infarct border. These results imply that newborn cell differentiation is influenced by the immediate neighboring tissue and is not entirely cell autonomous. These results also suggest that a secondary stem cell niche at the lesion border forms after stroke in the presence of PMP.

PMP Reduce Neurological Disability in a Dose Dependent Manner

Repeated testing with the neurological disability scale showed that while animals of all groups (n=6) were significantly disabled shortly after injury the deficits consistently improved in a dose dependent manner in animals

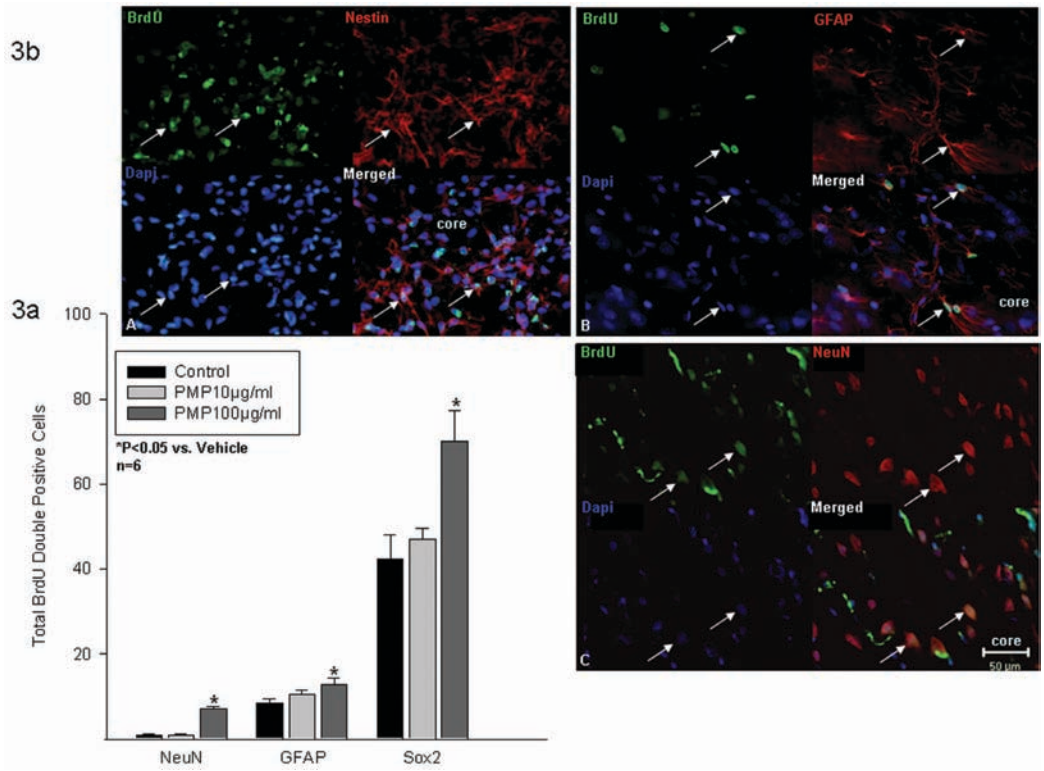

Fig. (3). PMP Drive Neuronal Differentiation

Terminal differentiation of proliferating cells was studied with cell-type specific antibodies (n=6/group). Fig. (**3a**) is a bar graph showing the number of $BrdU^+$ cells that differentiated into glia or neurons or remained undifferentiated and expressed either Nestin or SOX2. Fig. 3b is a series of photomicrographs (x100) taken 90 days after stroke onset from the peri-lesion (panels A, B, C) areas of rats treated with PMP. The images show BrdU (green in all panels) and cell specific antibodies (n=6/group). Markers used include antigens for primitive undifferentiated cells (Nestin, red in A), glia (GFAP, red in B), or neurons (NeuN, red in C). Nuclei were counterstained with DAPI (blue). White arrows point at double positive cells.

Core – infarct core area.

Bar graphs 50µ in all panels

Data are represented as mean ± SEM, *p<0.05.

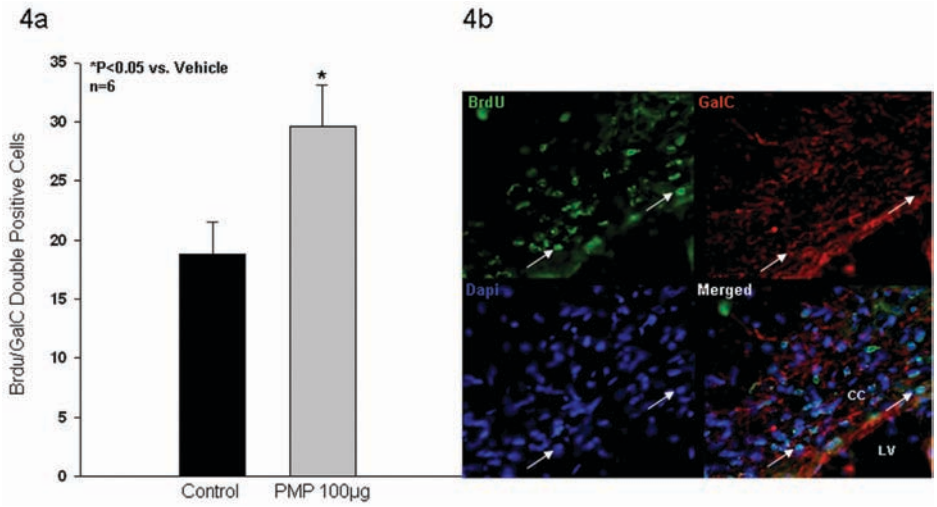

Fig. (4). PMP induce oligodendroglia

Fig. (**4a**) is a bar graph showing absolute number of newborn cells ($BrdU^+$, green) that differentiated into oligodendoglia ($GalC^+$, red). Note that the number was larger in the PMP treated group, suggesting that PMP actively drive cell differentiation into oligodendoglia in white matter. Fig. (**4b**), is a series of photomicrographs (x100) taken 90 days after stroke onset from the corpus callosum of rats treated with PMP.

White arrows point at double positive cells.

CC – corpus callosum, LV – Lateral ventricle.

Data are represented as mean ± SEM, *p<0.05.

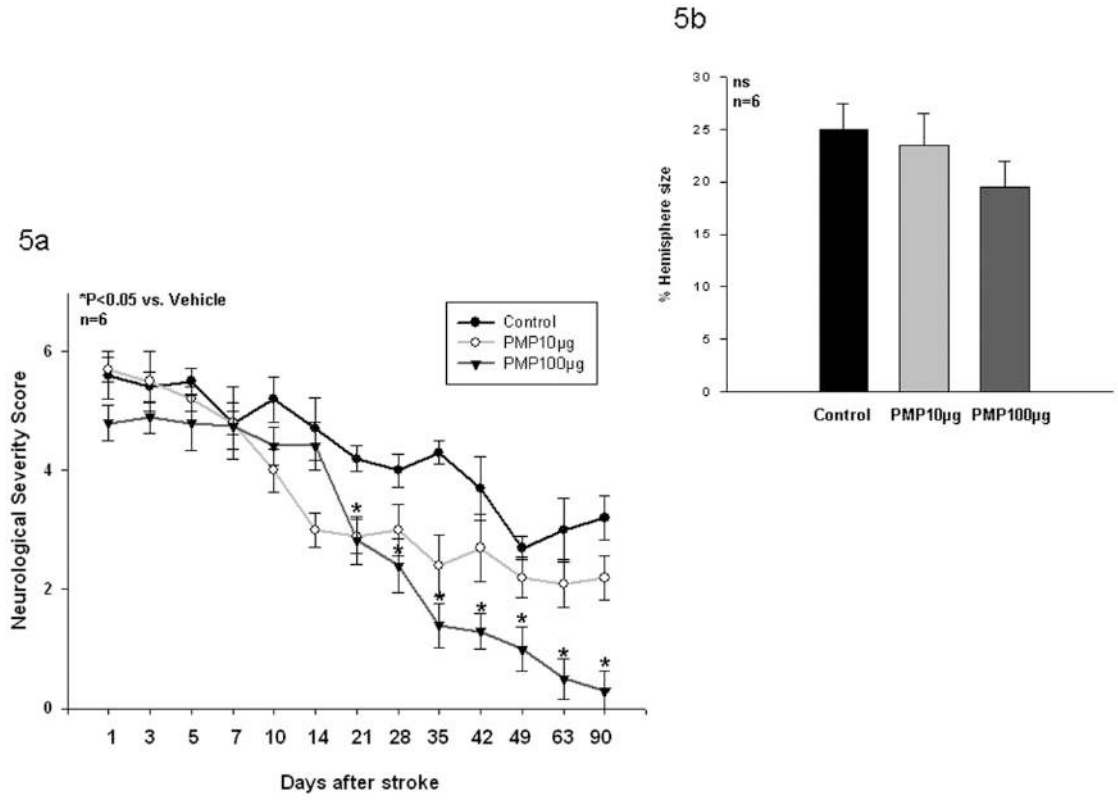

Fig. (5). PMP improve behavioral deficits but do not significantly change infarct size after stroke
Figure 5a shows the clinical deficits at different time points after stroke (n=6/group). Note that treatment with PMP led to dose dependent larger improvements in clinical deficits. Figure 5b is a bar graph showing infarct volumes in the different treatment groups as measured 90 days after stroke.
Data are represented as mean ± SEM, *p<0.05.

treated with PMP compared with those treated with vehicle (Fig. **5a**). In accordance with previous reports behavioral deficits began to improve after 20 days suggesting that this improvement was associated with an increase in neurogenesis [1-3]**.** Animals treated with PMP at high or low concentrations showed injury size that were smaller compared to those of vehicle treated animals although the differences have not reached statistical significance (Fig. **5b**).

DISCUSSION

The current study demonstrates that PMP increase angiogenesis and neurogenesis and improves functional outcome after stroke in a dose dependent manner *in-vivo*.

These results are complimentary to our previous experiments showing that exogenous PMP increase NSC proliferation and survival and increase the differentiation potential of NSC to glia and neurons *in-vitro* [9]. These effects were found to be mediated by the combination of at least FGF2, VEGF and PDGF since blocking the individual effects of these factors, but not those of PF4 which is also contained in PMP, led to partial reversal of the proliferative effects of PMP [9]. Downstream signaling of PMP effects were related to increments in pERK and pAkt which are both implicated in cell survival, angiogenesis and proliferation [9, 11]. Here, we recapitulate these results *in-vivo* and show that when given after cortical ischemia; exogenous PMP can indeed drive proliferation and differentiation of eNSC, leading to improved functional outcome.

These beneficial effects can be linked to better metabolic and functional status of surviving neurons and to an immune modulating effect mediated by stem cells affected by PMP. Although there was no significant change in infract volume, probably because of the duration needed for the growth factors to disengage from the polymer and induce protective effects, we did see a decrease trend in the infarct volume size in animals treated with PMP, that did not reach statistical significance. More direct delivery methods or larger animal groups might result in more significant changes in the lesion volume. The mild neuroprotective effect observed in our experiment may be explained by recent reports that PMP affect the immune system by reprogramming cells such as macrophages or dendritic cells toward a less reactive states leading to an anti inflammatory response [12]. However, the ability of PMP to induce neuroprotection should be further examined.

Like most other stem cells, eNSC need a tight interaction with surrounding blood vessels in a specialized stem cell niche in order to survive and differentiate [13].

This niche is important not only for supplying the metabolic needs of NSC and provision of paracrine signals but also for their interaction with endothelial progenitor cells (EPC). eNSC and EPC respond to similar stimuli in the same

manner. For example, both cell types proliferate in response to stimulation with VEGF [14-16] or erythropoietin [15, 17] and both migrate according to the same chemokine gradients.

In addition, EPC were shown to interact with resident tissue stem cells affecting the differentiation of both cell types [13].

Our study shows for the first time that angiogenesis and neurogenesis can be simultaneously targeted *in-vivo* with sub-cellular particles.

PMP are shed from platelets upon activation and contain a host of pro-survival and trophic factors including FGF2, VEGF, BDNF, PF4, PDGF and also the receptors necessary for induction of these responses including VEGFR1 [4-7]. In particular, FGF2 is a well known mitogen for NSC [18-20] and VEGF is a mitogen as well as a pro-migration factor for both NSC and EPC [14, 16, 21-25]. Furthermore, BDNF [26] and PDGF are important for cell migration and differentiation [27, 28]. Because of the multitude of trophic factors involved, the *in-vivo* experiment can not answer the question of which factors are responsible for the functional recovery. Hence and because each of these factors activates specific receptors on NSC and EPC it is likely that they all contribute to the overall observed effect as was indeed shown in-vitro [9].

Recent studies also imply involvement of chemokine receptors such as CXCR4 [29] or CCR5 [30] form PMP in affecting different cell types to modulate their regenerative potential.

Another route by which PMP may affect cells, which is not dependent on their growth factors content, is transfer of MicroRNAs (miRNAs) which are small non-coding regulatory molecules that modify gene expression at the post-transcriptional level by binding to target messenger RNAs [31]. miRNAs were found in PMP and are involved in processes such as cell growth, differentiation and tissue remodeling.

Many of the newborn cells at the immediate infarct border remain undifferentiated and express markers of NSC such as nestin and SOX2 or markers for migrating neuroblasts such as doublecortin. These cells are surrounded by blood vessels and the numbers of those vessels were much higher in PMP treated animals. These findings indicate that factors secreted by the PMP including FGF2, VEGF and PDGF may promote migration of newborn cells towards the lesion and form a secondary stem cell niche containing eNSC and newborn blood vessels at this area [32, 33] where cells remain undifferentiated for long periods of time after the initial injury [2, 10].

The relative increase in the number of newborn cells that express neuronal markers in the treated animals was small but significant suggesting that factors within the PMP such as FGF2 and PDGF may actively drive neuronal differentiation [2, 28, 34]. However, the small percentage of newborn neurons does not explain the functional gain observed in animals treated with PMP. Rather, it is likely that the functional gain observed in these animals is related to the ability of the undifferentiated cells to secrete trophic factors [2] and to exert an immunomodulatory effect reducing the anti-neurogenic effects of inflammatory cells in the ischemic hemisphere [35, 36].

Furthermore, the pro-angiogenic effects of PMP may serve to modulate the metabolic activity of neighboring cells possibly having a positive effect on their function.

Cell driven neurogenesis and angiogenesis may be particularly advantageous because of the convenient and inexpensive application of cells or sub-cellular particles that act as mini-storage compartments for a number of pro-survival, pro-migratory and pro-differentiation factors compared with selective application of individual factors.

In this study PMP were administered via a topically-applied biodegradable polymer that is widely used in humans as a hemostatic agent in surgical practice [37, 38]. Although we could not control the rate of discharge of PMP from the gelfoam in these experiments, it is likely that in the future, designer polymers will allow accurate delivery of substances into the immediate peri-lesion area providing the necessary supportive milieu for eNSC and EPC survival and differentiation.

This delivery method was aimed to avoid systemic intravenous administration because of the well known pro-thrombotic effect of PMP while circulating in the blood vessels [4].

This delivery method also appears to be inexpensive and advantageous compared with the use of genetic manipulation using viral vectors to augment growth factor concentrations in the brain. We are also currently exploring different methods of PMP delivery into the brain including direct intra-cerebroventricular injection which may be more widely applied as is the case with bedside placement of intraventricular ommaya reservoirs in cancer patients.

In conclusion, the current study shows that the use of platelet derived sub-cellular particles, is feasible, and leads to combined augmentation of neurogenesis and angiogenesis and results in improved functional gain after stroke.

CONFLICT OF INTEREST

None declared.

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