ORIGINAL ARTICLE

Mobilization of circulating progenitor cells following brain injury in premature neonates could be indicative of an endogenous repair process. A pilot study

Efstathiou N[1], Soubasi V[1], Koliakos G[2], Kyriazis G[3], Zafeiriou DI[4], Slavakis A[5], Kantziou K[1], Pozotou T[6], Chatzizisi O[3], Drosou-Agakidou V[1]

[1]1st Neonatal Department and NICU, Hippokration General Hospital, Aristotle University of Thessaloniki
[2]Biochemistry Department, Medical School, Aristotle University of Thessaloniki
[3]Immunology Laboratory, Pulmonology Department, Papanikolaou General Hospital, Aristotle University of Thessaloniki
[4]1st Paediatric Department, Hippokration General Hospital, Aristotle University of Thessaloniki
[5]Biochemistry Department, Hippokration General Hospital
[6]Medical school, Aristotle University of Thessaloniki
Thessaloniki, Greece

Abstract

Background: Preclinical data and adult studies have showed an endogenous regeneration process following brain damage that involves mobilization of progenitor cells. This process is not well described in preterm neonates. The present study aims to investigate the mobilization of Circulating Progenitor Cells (CPCs) and their relation to biomarkers of brain injury in preterm neonates.

Methods: This is a prospective cohort study of preterm infants with gestational age (GA) < 34 weeks. Serial cranial ultrasounds scans were performed in all neonates. Brain injury was defined by the presence of intraventricular hemorrhage grade III/IV, cystic periventricular leukomalacia or infarct. Peripheral blood samples were collected from all neonates on days(d) 1, 3, 9, 18 and 45 of life for the measurement of levels of CPCs [early and late Endothelial Progenitor Cells (EPCs), Haematopoietic Stem Cells (HSCs) and Very Small Embryonic-Like Stem Cells (VSELs)], Neuron-Specific Enolase (NSE), S100b, Erythropoietin (EPO) and Stromal Cell-Derived Factor-1 (SDF-1) .

Results: Ten out of the 23 preterm infants included in the study developed brain injury; the remaining thirteen infants served as controls. In the brain injury group a significant increase of HSCs (d9, d45), early EPCs (d3, d9, d18) and late EPCs (d1, d3, d9, d18, d45) was observed compared to controls. VSELs on d45 were significantly higher in controls. S100b on d1, EPO on d1, SDF-1 on d3 and NSE on d18 were significantly increased in the brain injury group. Moreover, CPCs were significantly related to S100b, NSE, EPO and SDF-1 levels at multiple time points.

Conclusions: The observed pattern of CPCs mobilization and its association with biomarkers following brain injury in preterm neonates indicate the existence of an endogenous brain regeneration process. Enhancement of this process with exogenous progenitor cell transplantation might be a powerful therapeutic strategy to restore brain damage and improve the neurodevelopmental outcome in premature infants. Hippokratia 2015; 19 (2):141-147.

Keywords: Preterm, neonates, brain injury, progenitor cells

Corresponding Author: Nikolaos Efstathiou, 61 Olynthou St, 54351 Thessaloniki, Greece, tel: +306936119294, e-mail: nikos.efstathiou@gmail.com

Introduction

Progenitor cells are pluripotent cells of primitive origin involved in organogenesis during the embryonic period. They have the ability of self-renewal and multilineage differentiation. Specifically, if found in a new environment or stimulated by appropriate growth factors, they may alter their direction of differentiation (pluripotency). In extrauterine life they are found in various tissues in a "resting" state, both locally in the target organ (in particular the subventricular zone and hippocampus in the brain) and distantly (mainly in the bone marrow). These cells are mobilized following tissue damage and migrate to the site of the injury in a chemotactic way, participating in an endogenous regeneration process[1–3]. Chemotactic factors are secreted locally and attached to the surface receptors of progenitor cells, promoting migration of the latter to the injured area. Although the mechanism of action of these Circulating Progenitor Cells (CPCs) is not fully elucidated, it is believed that key mechanisms involve immunomodulatory effects, neuroprotection, neovascularization, and neurogenesis[4–6]. The Central Nervous System (CNS) injury occurring during the perinatal period and the cerebral palsy that often follows cannot be treated effectively with current therapeutic modalities and therefore regenerative medicine is a very promising strategy in this area. Both endogenous Neural Stem Cells of the brain (NSC) and peripherally administrated progenitor cells have been shown to promote regeneration in different experimental neonatal brain injury models. However, the results

vary depending on the type of cells involved, the route of administration and especially the time of administration[5,7,8]. Therefore, it is important to clarify the above factors before bedside application of CPCs[6].

It is very hopeful that long-term clinical experience with the use of human umbilical cord blood-derived stem cells (HUCBC) in hematologic diseases supports their safety[5]. In addition, the first sporadic clinical trials -using autologous HUCBC in neonates with hypoxic-ischemic encephalopathy (HIE) and in older children with cerebral palsy- indicate the feasibility of the procedure[6,9-11].

In the accessible literature, there are no clinical studies focusing on the CPCs kinetics in neonates with brain damage. The present study was designed to investigate the possible mobilization of progenitor cells in prematurity-associated cerebral injury.

Study Population and Methods

Study design and population

This is a single-center prospective cohort study in preterm infants with a gestational age of less than 34 weeks who were admitted within the first 24 hours of life to our tertiary neonatal intensive care unit. Exclusion criteria included intrauterine growth retardation, major congenital or metabolic disorders, congenital coagulopathies and twins with major abnormalities in their sibling.

Peripheral blood samples were collected from all neonates on days (d) 1, 3, 9, 18 and 45 of life, for the measurement of levels of CPCs, Neuron-Specific Enolase (NSE), S100b, Erythropoietin (EPO) and Stromal Cell-Derived Factor-1 (SDF-1). Cranial ultrasounds scans (CUS) were performed routinely within 24 hours of birth, on day 3 and then on a weekly basis till discharge in all preterm infants. The cerebral injury was defined by the development of intraventricular hemorrhage (IVH) grade III or IV, cystic periventricular leukomalacia (PVL) or infarct. Data on demographic, perinatal characteristics and prematurity-associated complications, were collected for all the patients.

The primary aim was the association of CPCs' kinetics with the presence of brain insult and their relationship with chemotactic factors. This study was approved by the ethical committee of Aristotle University of Thessaloniki, and written informed consent was obtained from all parents.

Flow Cytometry

Blood samples were collected in ethylenediaminetetraacetic acid (EDTA) containing tubes, and -soon after collection- incubated with monoclonal antibodies [PE-antiCD184(CXCR4), FITCH-antiCD34, PECy5-antiCD45 (BD Biosciences, San Jose, CA, USA) and APC-antiCD133 (Miltenyi Biotec, Bergisch Gladbach, Germany)]. Red blood cells were removed by employing lysing buffer (BD Pharm Lyse; BD Biosciences, San Jose, CA, USA). Subsequently, samples were washed and analyzed with a BD FACSCalibur instrument (BD FACSDiva Software, version 6, BD Biosciences, San Jose, CA, USA). 100,000 events were acquired. Levels of each cell population were expressed as a percentage of the total events.

CPCs of interest were populations enriched in Haematopoietic Progenitor Cells (HSCs: $CD34^+/CD184^+/CD45^+$), Very Small Embryonic-Like Stem Cells (VSELs: $CD34^+/CD184^+/CD45^-$), and Endothelial Progenitor Cells (EPCs). Early and late EPCs were defined as $CD34^+/CD45^{dim/-}/CD184^+/CD133^+$ and $CD34^+/CD45^{dim/-}/CD184^+/CD133^-$ respectively. A detailed description of gating strategy is displayed in Figures 1 and 2.

Biomarkers

Biomarkers were measured in serum or plasma samples (stored at -80° C) using commercially available assays according to the manufacturer's instructions. Biomarkers of brain injury S100b and NSE were assessed in the serum using immunochemiluminometric assay (Liaison, DiaSorin-SpA, Saluggia, Italy). Serum levels of EPO were measured using immunochemiluminometric assay (Immulite 2000XPi, Siemens, Llanberis, United Kingdom), and plasma levels of SDF-1 were assessed using ELISA (Quantikine ELISA, R & D Systems, Minneapolis, USA).

Statistical Analysis

Data are presented as mean (± standard deviation) or median (range) depending on normality. Differences in the values of the quantitative parameters between groups were evaluated by the t-test (independent or paired) or the Mann-Whitney test and the Wilcoxon Signed Rank, and correlations between quantitative parameters were assessed using Pearson's correlation or Spearman's rank correlation, as appropriate. For a comparison of related samples the Friedman test was used. Data analysis was performed by using SPSS statistical package (SPSS statistics, IBM Corporation, version 20, Chicago, IL, USA) and $p < 0.05$ was considered as statistically significant.

Results

Twenty-three infants with gestational age of 29.8 ± 3.11 weeks and birth weight of 1503 ± 528 gr fulfilled the criteria and were included in the study. Ten infants developed brain injury [IVH Grade 3 (n = 6) or Grade 4 (n = 2), PVL (n = 1) or infarction (n = 1)] and comprised the brain injury group. Thirteen infants who did not develop apparent brain damage served as controls. Perinatal characteristics and clinical outcomes of the studied neonates are presented in Table 1.

One hundred and four blood samples were collected for determination of CPCs and biomarkers. Data were excluded from the analysis when sepsis (n=2) or necrotizing enterocolitis (NEC) (n=5) occurred within one week prior to sampling, to obviate possible major confounding factors.

Circulating Progenitor Cells

Cell populations enriched in HSCs were higher in the brain injury group compared to controls, with significant differences on the 9th and 45th day of life ($p < 0.05$). EPCs were also higher in the brain injury group, with differences being significant on the 3rd, 9th, and 18th days of life for early EPCs ($p < 0.05$) and on all time points for

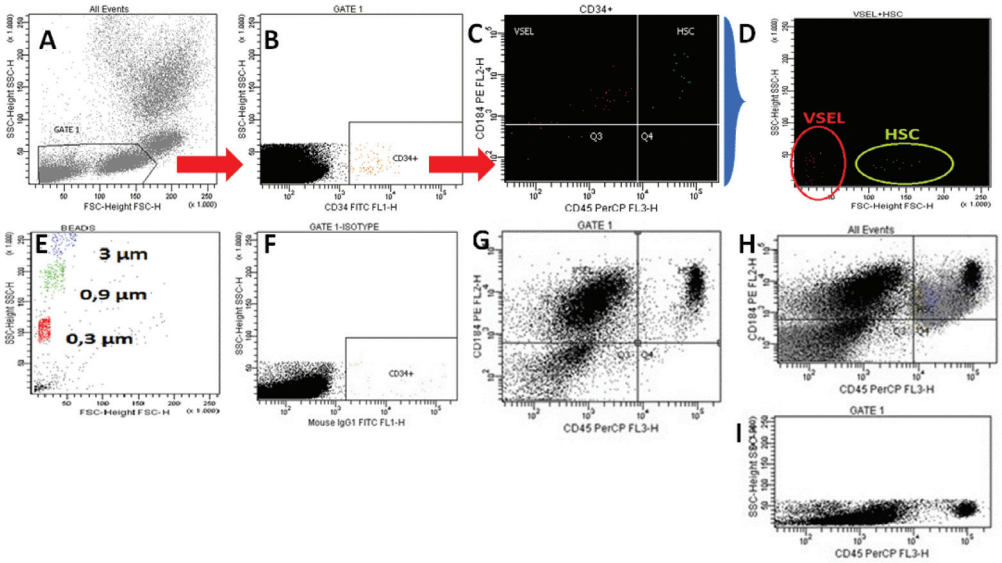

Figure 1: Gating strategy for Very Small Embryonic-Like Stem Cells (VSELs) and Haematopoietic Stem Cells (HCSs): **Panel A:** Peripheral blood total nucleated cells (TNCs) are visualized by dot plot based on FSC/SCC signals. The Gate 1 is set on the lymphocyte region, extended to the left towards small events, in order to allow high enrichment in VSELs. The threshold set on FSC was set very low and size-predefined beads were used to define a sorting region containing small objects (panel E). **Panel B:** Cells from Gate 1 are further analyzed for CD34 antigen expression, and $CD34^+$ events are subsequently analyzed for CD184 and CD45 antigen expression on panel C. **Panel C:** Two populations of $CD184(CXCR4)^+$ cells are distinguished based on CD45 expression. The upper left quadrant represent non-haematopoietic stem cells $CD34^+/CD184^+/CD45^-$ cells, enriched in VSELs, while the upper right quadrant represent $CD34^+/CD184^+/CD45^+$ cells , enriched in HSCs. **Panel D:** Cell populations from panel C are pictured in the SSC/FSC dot plot, where their position and size are clearly delineated (see also in correspondence panel E). Specific cell size (estimated using size-predefined beads) as well as constellation of antigen expression characterizes and separates Very Small Embryonic-Like Stem Cells enriched population from debris, circulating cell-derived microparticles or other cell populations[12,13]. The position of the gates is based on the negative isotype control values (panel **F** and Figure 2/panel G), but also designed to sort cells based on cell population distribution (panels **G**, **H**, **I**) and subjective judgment. Representative data are shown.

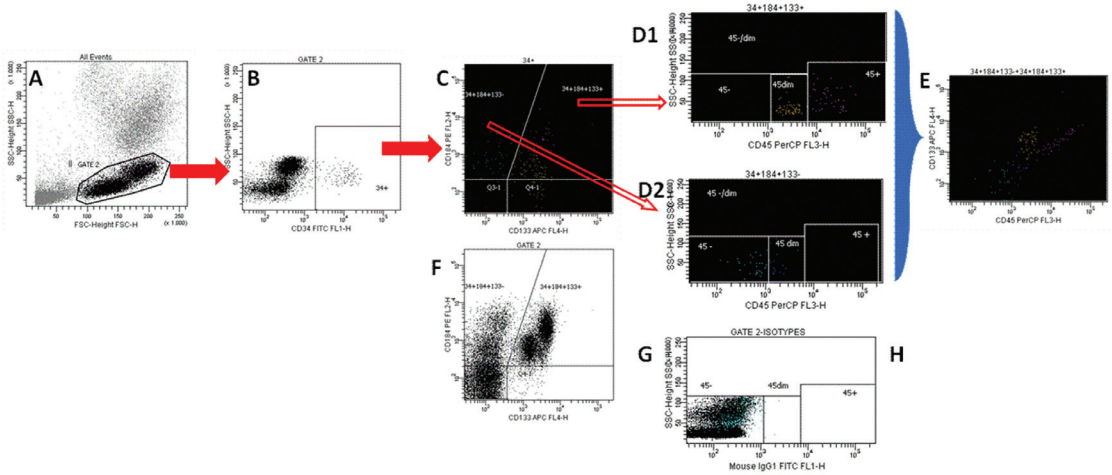

Figure 2: Gating strategy for Endothelial Progenitor Cells (EPCs): **Panel A:** TNCs are visualized by dot plot based on FSC/SCC signals. The Gate 2 is set on the mononuclear region (MNCs). **Panel B:** Cells from Gate 2 are likewise further analyzed for CD34 antigen expression, and $CD34^+$ events are subsequently analyzed for CD184 and CD133 antigen expression on panel C. **Panel C:** Two populations of $CD184(CXCR4)^+$ cells are distinguished based on CD133 antigen expression. The upper right quadrant represent $CD34^+/CD184^+/CD133^+$ cells, while the upper left quadrant represent $CD34^+/CD184^+/CD133^-$ cells. Each subpopulation is further analyzed for CD45 antigen expression in panels D1 and D2 respectively. **Panel D1 & D2:** Populations are subdivided according to CD45 antigen expression. Early $(CD34^+/CD45^{dim/-}/CD184^+/CD133^+)$ and late $(CD34^+/CD45^{dim/-}/CD184^+/CD133^-)$ EPCs are displayed. **Panel E:** All EPCs subpopulation are visualized in a CD133/CD45 dot plot, where their antigen phenotype is more clearly delineated. The position of the gates is based on the negative isotype control values (panel **G** and Figure 1/panel F), but also designed to sort cells based on cell population distribution (panels **F**, **H**) and subjective judgment. Representative data are shown.

Table 1: Perinatal characteristics of the 23 preterm infants that were included in the study.

Group:	Brain injury	Control	p
n	10	13	
Gestational Age (wks) (SD)	27.8 (3.4)	31.4 (1.6)	<0.01
Birth Weight (gr) (SD)	1194 (558)	1741 (369)	<0.05
Antenatal steroid - n(%)	3 (30%)	13 (100%)	
Caesarean section -n(%)	6 (60%)	10 (77%)	
Apgar score 1' (median, range)	2.5 (1-5)	8 (3-8)	<0.0005
Apgar score 5' (median, range)	7 (3-9)	9 (6-9)	<0.001
SBE on admission (mmol/L) (SD)	-14.96 (7.5)	-6.29 (2.4)	<0.05
IVH (grade III/IV) - n(%)	8 (80%)	0	
PVL - n(%)	1 (10%)	0	
Infarct- n(%)	1 (10%)	0	
Sepsis- n(%)	1(10%)	1 (7.6%)	
NEC - n(%)	1(10%)	4 (31%)	
Death (n)	7	0	
(day of death: median, range)	(30, 3-57)		

IVH: intraventricular hemorrhage, PVL: periventricular leukomalacia, NEC: necrotizing enterocolitis.

late EPCS (1st, 3rd, 18th, and 45th day ($p<0.05$), 9th day ($p<0.001$)). On the contrary, cell populations enriched in VSELs were significantly higher in controls compared to the brain injury group on the 45th day of life ($p<0.05$). Kinetics of CPCs are shown in Figure 3.

No significant correlations were observed between CPCs and birth weight, gestational age, antenatal steroids or mode of delivery.

Biomarkers of brain injury

Levels of S100b were significantly higher in the brain injury group compared to controls on the 1st day of life [median 2.96 vs 1.54 (mg/l), $p<0.05$], with a trend towards

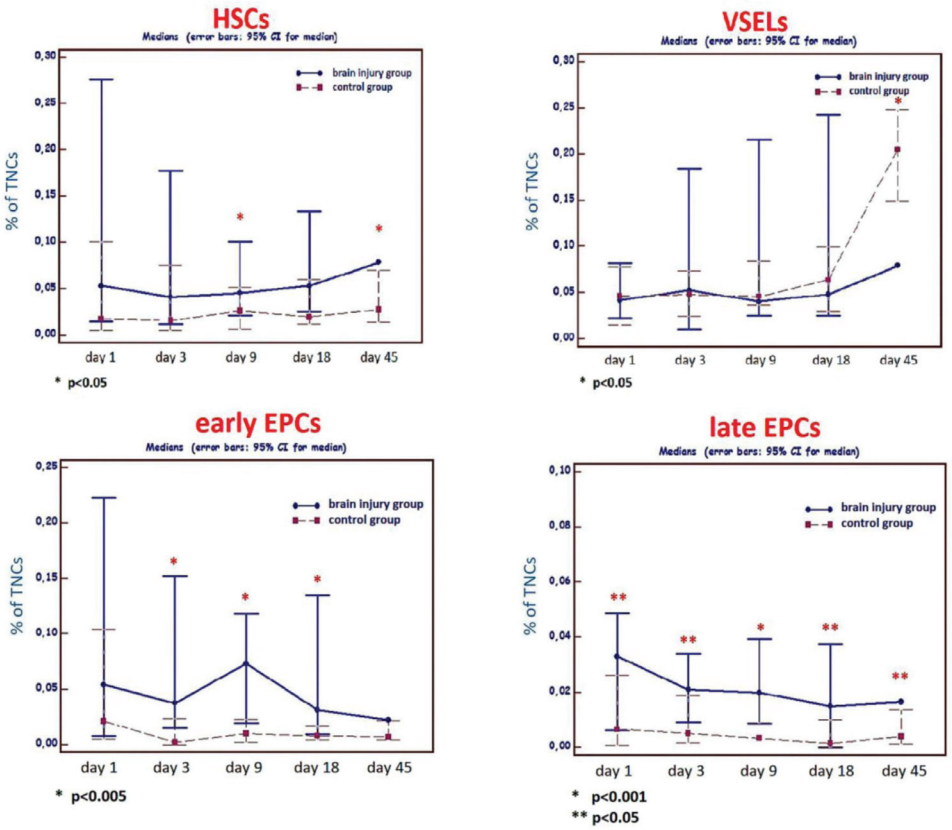

Figure 3: Kinetics of circulating Hematopoietic Stem Cells (HSCs), Very Small Embryonic-Like Stem Cells (VSELs), early and late Endothelial Progenitor Cells (EPCs) from day 1 to day 45 of life in preterms with brain injury and in controls.

decreased levels in the following days. NSE levels were significantly higher in the brain injury group compared to controls on day 18 of life [median 26.2 vs 12.5 (mg/l), p <0.05], while levels in the control group significantly decreased over time (Friedman test, p <0.05) (Figure 4).

Chemotactic factors

Both erythropoietin and SDF-1 were higher in the brain injury group compared to controls early after birth; EPO was higher on days 1 [median 28.0 vs 2.48 (mIU/ml), p <0.05] and 3 of life [median 14.5 vs 8.29 (mIU/ml), p =0.061], whereas SDF-1 was higher on the 3rd day of life [median 648.3 vs 399.9 (pg/ml), p <0.01] (Figure 4).

Correlation of CPCs with biomarkers

CPCs were correlated significantly with biomarkers of brain injury (S100b and NSE) and chemotactic factors (SDF-1 and EPO) measured on the same day of life. Specifically, HSCs were significantly correlated with S100b levels on day 1 (r =-0.44), with NSE levels on days 3, 9 and 18 (r =0.6, r =0.63 and r =0.75 respectively), as well as with SDF-1 levels on day 18 (r =0.6). Likewise, VSELs were significantly correlated with NSE levels on day 3 (r =0.49), with EPO levels on days 3 and 9 (r =0.82 and r =0.78 respectively), and with SDF-1 levels on day 18 (r =0.51). Finally, early and late EPCs were significantly correlated with NSE levels on day 3 (r =0.49 and r =0.51 respectively) and on day 18 (r =0.62 and r =0.57 respectively). Moreover, biomarkers were significantly correlated with CPCs not only on the same day of life, but also with the CPCs of the following days. Multiple positive correlations were found between all subtypes of CPCs and EPO, SDF-1 and NSE levels of the previous days (data not shown).

Correlations were also found between biomarkers of brain injury and chemotactic factors. S100b levels of the 1st day of life were associated with EPO of the same day (r =0.5, p <0.05), as well as with levels of SDF-1 of the 3rd day of life (r =0.52, p <0.05). Borderline correlation was found between NSE levels of the 1st day and SDF-1 levels of the same day of life (r =0.44, p =0.06).

Discussion

This preliminary study shows early mobilization of CPCs in preterm infants with CNS injury that is associated with biomarkers of brain injury and levels of chemotactic factors at serial time points after birth. To date, there is no published data focusing on evolution and correlations of CPCs regarding neonatal brain injury. Moreover, data on the early kinetics of CPCs in preterm infants is scarce.

HSCs apart from their role in hematopoiesis are also involved in the endogenous regeneration process after tissue injury[5] mainly via a paracrine action. In this study, HSCs

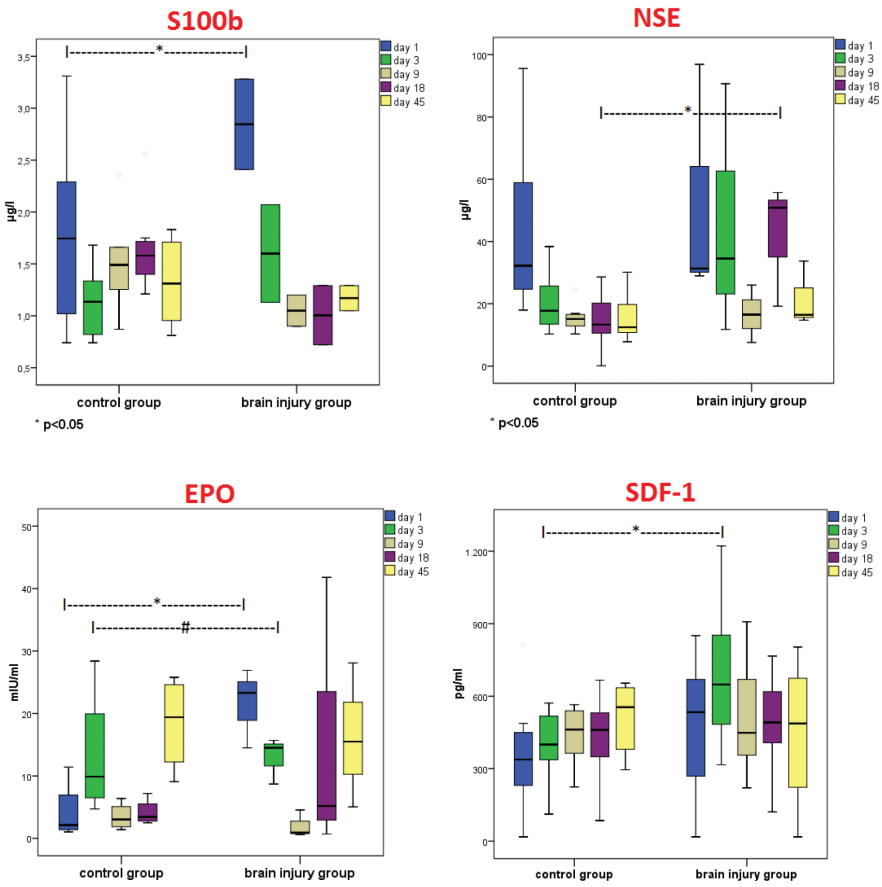

Figure 4: Blood levels of biomarkers S100b, Neuron-Specific Enolase (NSE), Erythropoietin (EPO) and Stromal Cell-Derived Factor-1 (SDF-1) on days 1, 3, 9, 18 and 45 of life in preterms with brain injury and in controls.

were higher in the group of infants with brain injury at all time points with the differences being significant only on days 9 and 45. The failure to demonstrate significant difference at all time points is probably due to the small sample size. So far, only a few published studies -with quite different design- have examined CPCs kinetics, reporting conflicting results. Kotowski et al have found reduced levels of HSCs ($CD184^+/lin^-/CD45^+$) in the umbilical cord blood of premature infants that developed intraventricular hemorrhage in the subsequent days[14]. Borghesi et al have shown correlation of $CD34^+/CD45^+$ progenitor cells (that include the subpopulation of HSCs) with periventricular leukomalacia but not with IVH[15]. Paviotti et al could not demonstrate any correlation of $CD34^+$ progenitor cells on the first day of life with IVH development as well[16].

Endothelial Progenitor Cells were first described in 1997 by Asahara as cells of endothelial cell origin that anchor in bone marrow and migrate in ischemic regions where contribute to neoangiogenesis[17]. Since then their identity has not been determined precisely, and a panel of markers has been used as surrogate markers for cells displaying properties of the putative EPCs[18,19]. Thus, there is great difficulty in comparing different studies. In the present study, the most commonly used markers were adopted, and different gating techniques in flow cytometry were combined to increase accuracy (Figure 2). The infants with brain injury appeared to have significantly higher levels of early EPCs on days 3, 9 and 18 of life and higher levels of late EPCs at all time points compared to controls. The above mobilization of EPCs probably reflects the neoangiogenesis process, in the damaged vasculature of the brain. This neoangiogenesis process is vital both for maintaining the viability of the penumbra and for a possible further regeneration process as well. Safronow et al, also found increased levels of EPCs in infants with IVH[20], whereas Strauss et al found elevated levels of RNA endothelial markers in neonates with increased echogenicity on brain ultrasound and in infants with IVH[21]. However, other studies did not demonstrate such an association[15,16]. A meaningful comparison of the above studies is not possible, given that the putative EPC populations were characterized using different markers.

The VSELs are very promising pluripotent cells of non-hematopoietic origin, recently discovered in the umbilical cord blood and in the bone marrow, that are involved mainly in organogenesis during embryonic life and in tissue repair after injury[12]. This study presents a different mode of the kinetics of VSELs compared to the other progenitor cell populations. Vivid mobilization was observed only in the control group on the 45th day of life, whereas a trend was obvious since the 18th day. The absence of increased levels in the brain injury group could possibly reflect inhibition of the physiological mobilization during extrauterine life, due to severe brain insult. Similar speculation was stated by Machalinska et al, observing smaller percentages of these cells-lines in infants with severe retinopathy of prematurity[22].

NSE is an isoenzyme involved in glucose metabolism and is found predominantly in neurons while S100b is a protein involved in cellular calcium metabolism and is found primarily in glial cells. Necrosis or apoptosis of these cells leads to the release of these substances into the circulation. They mainly represent diagnostic markers and in a lesser degree prognostic indicators[23,24]. They have been studied extensively in term newborns with perinatal asphyxia, whereas data on levels of these biomarkers are limited in encephalopathy of prematurity. In preterm neonates with asphyxia, Giuseppe et al found significantly elevated levels of both biomarkers[23]. In our study, S100b was significantly increased on the first day of life, probably reflecting the glial death following ischemia. NSE levels in the control group progressively decreased over time, whereas values in the brain injury group varied widely both among subjects and among the different time points. Significantly higher levels in the brain injury group were observed only on day 18 of follow-up. The higher levels in the brain injury group probably reflect prolonged neuronal apoptosis, whereas the observed value fluctuations could be attributed to different severity and duration of brain injury.

Erythropoietin is a chemotactic, pleiotropic growth factor with neuroprotective properties[25,26]. The increased levels found in the group of brain injury on days 1 and 3 of life are consistent with the literature[27] and may reflect a physiological response to the brain damage.

The SDF-1 is a chemotactic factor and a ligand to the surface receptor CXCR4 (CD184) on neural cells and CPCs. It is secreted in the microenvironment of tissue injury, contributing to the chemoattraction of CPCs[28,29]. On day 3 of life the significant increase of SDF-1 in neonates with brain injury probably reflects the above process. Kotowski et al, found no association of this factor with any of the complications of prematurity[14] while Machalinska et al reported increased levels of SDF-1 in premature infants with retinopathy of prematurity[22].

Regarding correlations of CPCs with neonatal characteristics, we did not find any correlation of CPCs with birth weight, gestational age, antenatal steroids and mode of delivery. Existing data is very limited and conflicting[14,15,20,30].

In the present study multiple correlations of CPCs with circulating biomarker levels were found. Multivariate analysis was not possible due to the small sample size. In the brain injury group, the S100b was increased on the 1st day of life, while the SDF-1 and EPO were increased on the 1st and 3rd day of life. CPCs mobilization followed, mainly after the third day. Bui et al found no correlation of EPO with $CD34^+$ cells[30] while Kotowski et al found correlation of SDF-1 levels of the umbilical cord with VSELs of the 14th day of life[14]. The observed correlations and CPCs kinetics in the present study possibly indicate a sequence of events leading to mobilization and migration of the pluripotent cells from bone marrow to the site of the brain lesion[4,28].

In conclusion, results of this study indicate a possible role of CPCs in the pathophysiology of brain damage in premature neonates and in the regeneration process that follows. Their temporal and quantitative correlations

with the occurrence of brain damage and the subsequent secretion of chemotactic factors are indirect indications of an endogenous regenerative process following brain injury that involves pluripotent cells.

We assume that enhancement of this endogenous regeneration process with exogenous progenitor cell administration might be a potential therapeutic strategy. However, future research is necessary to clarify further the optimal basic conditions, such as cell type, timing, and delivery route.

Conflict of interest

Authors certify that there is no conflict of interest.

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