Protection of the preterm brain against inflammatory stress

Impact

The global burden of preterm birth is increasing [1]. Globally, prematurity is the leading cause of death in children under the age of five. Every year, an estimated 15 million babies are born too soon [1,2]. New estimates revealed that preterm births during 2014 reached 8.7% in Europe and was higher in low income countries (13.4%) [2]. With improved perinatal care over the last decades, the survival rates of preterm born infants have improved. Along with improved survival rates, the problem of (long-term) morbidities is emerging. Health care costs increase immensely with lower gestational ages [3,4]. Especially in the first year of an early preterm infant, the average health costs amount to 74000€ [3,4]. Apart from the economic consequences, preterm birth confronts parents and families with psychological and emotional distress. Improving prevention, diagnosis and treatment will decrease those burdens.

In this thesis, I addressed time-dependent changes in the preterm brain in the course of preterm stress, tested therapies (chapter 2&3: cell-based, chapter 4: cell-derived, Discussion Figure 2: small molecular), and identified their potential downstream targets (chapter 2&3: Annexin A1 (ANXA1)). For this purpose, we combined a translational ovine model of perinatal stress (infection/inflammation & HI) with molecular *ex vivo* studies (microglia, chapter 3) and *in vitro* studies (TEER model, chapter 4) to gain mechanistic insights into cell-based therapies. This highlighted ANXA1 as an important mediator of the therapeutic potential of cell-based therapies. We tested the individual therapeutic effect of ANXA1 in a rodent model for neuroprotective and functional improvements (Discussion, Figure 2) and suggest that it has potential to be further explored as biomarker to detect perinatal stress such as HI/antenatal inflammation/infection (Discussion, Figure 1).

Chapter 2 & 3 laid the base for understanding the working mechanisms of early adult stem cell therapy for antenatal inflammation *in utero*. These chapters form the starting point of a larger longitudinal study that closely mirrors the clinical situation (accumulation of perinatal hits). The project design starts with an *in utero* part with the first injurious hit (i.a. LPS) and prenatal stem cell therapy. In the prenatal part, our most important finding is that the combination of antenatal infection/inflammation and stem cell therapy enhances the immune response systemically (lung, spleen, gut) and cerebrally (barrier associated cells and microglia) with a predominant anti-inflammatory profile, though a proinflammatory signature was also detected. These findings highlight the need to focus on the cross-talk between systemic and cerebral response and how stem cells modulate this neuroimmune axis, which is recognized as an important player in brain homeostasis [5]. Combining the finding of the *in utero* part with comprehensive multi-omics analysis of immune cells will give a broader picture of the beneficial effect

of stem cells on multiple organs. Furthermore, it might give insights into epigenetic reprogramming induced by perinatal stress and stem cell treatment which occurs *in utero* and is protracted into adulthood [6-8]. Here, the postnatal parts will be of additive value whereby the consequences of another injurious hit (mechanical ventilation) and a second or first postnatal dose of stem cells will be analyzed. The third part of the longitudinal study includes a long-term follow-up into adulthood, which will give insight into long-term functional improvement of the brain (MRI, gait and maze analysis) in relation to structural/histological examination after stem cell therapy. This study design is unique as it mirrors the clinical situation in a large highly translational animal model. It allows us to test new diagnostic tools, identify biomarkers, optimize functional tests and determine the proper dosing (single vs double dose) and timing of stem cell therapy (prenatal vs postnatal) in the context of antenatal infection and cumulative inflammatory stressors. The long-term study is of high value, especially as long-term studies of the first grown-up survivors of preterm born babies are scarce and small in sample numbers. With the introduction of surfactant in the early 1990s, which has dramatically increased the survival rates, most of the survivors have reached middle age (30s and 40s) and it is known already that survivors have a higher risk for adverse cardiovascular, pulmonary, cognitive and behavioral outcomes that affect life chances and quality of life [9]. Thus, not only the short-term benefits but also potential long-term benefits can be evaluated in this translational study.

As each patient exposes an individual history of risk factors potentially resulting in adverse outcomes, development to improve diagnosis are a premise for optimal treatment. The pathophysiologic mechanisms leading to EoP might be more or less pronounced in HI vs infection exposed infants or underlie different timing windows. This was indicated by analyzing cerebrovascular changes, measured by decrease in ANXA1 expression, after HI or antenatal infection. After HI, ANXA1 loss at brain barriers occurs more acutely (24 hours post hypoxia) and restores at day 3 after HI while in antenatal infection we detected a more prolonged loss of ANXA1, which ranges depending on which brain barrier from 12 hours to four days. Such findings underscore that the individual patient history must be considered to define the optimal window of stem cell. In chapter 4 & the work described in the discussion, we investigated the potential therapeutic role of ANXA1 in HI mediated brain injury. The intellectual property has been patented which offers new scientific and economic opportunities. Importantly, the route of administration and gender appear to be important determinants. Intranasal delivery of ANXA1 offers neuroprotective effects, especially in male mice while intraperitoneal administration seemed to have a more protective effect in female mice. This indicates that sex plays a role in treatment route and therapeutic efficacy. Intraperitoneal

administration was chosen over intravenous administration due to longer half-life in the circulation and technical feasibility in rodent pups [10]. The intravenous administration for ANXA1 could be another approach to target systemic inflammation. Additionally, further research should focus on the sex-differences and whether intrinsic mechanisms (hormones such as estrogens) are responsible for this occurrence and how these intrinsic mechanisms could be exploited for future therapies. The ultimate aim is to optimize the treatment regime (route, dosing, timing) and bring ANXA1 into the clinic. The neuroprotective effect of ANXA1 could be enhanced by cell based-therapies such as MAPCs (MultiStem®Cells) which have regenerative and immunomodulatory properties and are off the shelf products safe to use in clinic. No tissue matching or immune suppression is required for MAPC transplantation. Current clinical phase II/III studies are ongoing for testing the therapeutic potential MAPCs in adults for stroke (TREASURE, NCT02961504; MASTER-2, NCT03545607) and acute respiratory distress syndrome patients (COVID-19, MACOVIA, NCT04367077; ONE-BRIDGE, NCT03807804)[11]. The latter studies yielded promising results in the exploratory study [11,12]. Additionally, those studies received the Fast Track designation and the Regenerative Medicine Advanced Therapy Designation from the FDA. The MASTERS-2 study received positive scientific advice from European Medicines Agency (EMA)[11]. These designations and advices are developed to speed up the patient access to new medicines such as MultiStem® if clinical trials show appropriate safety and therapeutic effectiveness. Consequently, MAPCs can be evaluated as therapeutic intervention in EoP along with standard care such as hypothermia (for HI) or anti-microbial agents (infections).

In conclusion, this thesis contributed to other research in the preclinical testing of cell-based (MSC-EVs, MAPCs) or small molecular (ANXA1) therapeutics that harbor immunomodulatory and regenerative capacities for preterm brain injury. Further studies (preclinical/clinical) will result in commercially available products and/or diagnostic tools that will improve the outcome of babies with preterm brain injury thereby reducing social and economic burden.

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