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Accepted Manuscript (Uncorrected Proof)

Title: Short Communication: The Effect of Different Concentrations of Methylprednisolone on Survival, Proliferation and Migration of Neural Stem/Progenitor Cells

Running title: Effects of Methylprednisolone on Neural Stem/Progenitor Cells

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Please cite this article as:

DOI: http://dx.doi.org/10.32598/bcn.2021.35.4
Abstract

Introduction: To address the question whether combination of methylprednisolone (MP) as an anti-inflammatory drug used in neurodegenerative diseases and neural stem/progenitor cells (NS/PCs) is safe, the present study was designed.

Methods: Embryonic rat NS/PCs were exposed to different concentrations of MP and survival by MTT assay, proliferation by analyzing the number and diameter of neurospheres and the migration of the cells by neurosphere assay were evaluated.

Results: The viability of NS/PCs reduced following exposure to 10, 15 and 20 µg/ml of MP. In addition, although the number of neurospheres didn’t change, exposure to different concentrations of MP resulted in formation of smaller neurospheres. Despite these undesirable effects, the highest concentration of MP (20 µg/ml) increased the migration capacity of the NS/PCs.

Conclusion: The combination of MP and NS/PCs is not recommended due to the adverse effects of MP on survival and proliferation of NS/PCs.

Keywords: Methylprednisolone, Neural stem cells, proliferation, survival, migration
Introduction

Inflammation is one of the major events contributes to pathology of neurodegenerative diseases such as spinal cord injury (SCI), Alzheimer’s disease, Parkinson’s disease, multiple sclerosis and epilepsy (Gao & Hong, 2008). On the other word, immune system activation seen in neurodegenerative disease has both photogenic and supportive roles (Amor, Puentes, Baker, & Van Der Valk, 2010). Hence, management of inflammation is very important issue in treatment of neurodegenerative diseases.

One of the main drugs administered for management of inflammation following neurodegenerative disorders is methylprednisolone (MP). For instance, intravenous injection of high concentration of MP (30 mg/kg of body weight in the first time followed by 4.5 mg/kg each hour for 23 hours) is used during the first hours after SCI to reduce the secondary injury (Bracken et al., 1990). In addition, using this drug as a promising approach for treatment of Alzheimer’s disease (Alisky, 2008), Parkinson’s disease (Sato, Asoh, Metoki, & Satoh, 2003), multiple sclerosis (Saidha, Mok, Butler, Fanning, & Harrington, 2010), stroke (Altamentova et al., 2020) and epilepsy (Almaabdi et al., 2014) has been reported. Although some neurological improvements have been reported following administration of MP, the systemic administration of high dose of the drug shows important side effects including wound infection, pneumonia, gastrointestinal bleeding and myopathy (Gerndt et al., 1997; Qian et al., 2005). Thus, changing the route of administration from systemic to local might be a promising approach for decreasing these adverse effects of MP.

On the other hand, the treatment of neurodegenerative diseases has not be restricted to pharmacotherapy and new approaches like the application of neural stem/progenitor cells (NS/PCs)
are in progress (Ronaghi, Erceg, Moreno-Manzano, & Stojkovic, 2010; Russo, 2020). Several basic investigations indicated that transplanted NS/PCs survive, migrate and differentiate into neurons, astrocytes and oligodendrocytes (Aligholi et al., 2016; Cummings et al., 2005). In addition, NS/PCs release trophic factors such as nerve growth factor and brain derived neurotrophic factor which can be helpful for neuroregeneration (Lu, Jones, Snyder, & Tuszynski, 2003). Moreover, functional recovery has been reported after transplantation of NS/PCs to the damaged tissue of the spinal cord (Sankavaram et al., 2019). Despite these outstanding properties of NS/PCs, the transplantation of them is required to be combined with other agents to modulate their behavior (Garbossa et al., 2019).

Based on the above statements, combination of NS/PCs and MP has been considered. In this sense, what is needed to be clear is the effect of MP on the behavior of NS/PCs. One study reported that administration of MP after brain ischemia increased survival and migration of NS/PCs (hong Jing, ping Hou, feng Song, & Yin, 2012). Using MP in a brain injury model showed that this drug supported oligodendrocytes but didn’t influence the survival of neurons (Lee et al., 2008). In addition, the proliferation of NS/PCs in a SCI model decreased by MP (Obermair, Schroter, & Thallmair, 2008). In an in-vitro study, a reduction in proliferation of spinal cord-derived NS/PCs after exposure of MP was reported (Wang et al., 2014). Due to these discrepancies, the present study evaluated the effects of different concentrations of MP on survival, proliferation and migration of rat embryonic NS/PCs using neurosphere assay.
Materials and methods

All methods were performed in accordance with the institutional guidelines of Shiraz University of Medical Sciences for animal care and use.

Culture of NS/PCs

The ganglionic eminence of 13.5 day old rat embryo was harvested using stereomicroscope. After mechanical cutting by surgical knife, the specimens were dissociated by using 0.05% Trypsin/EDTA (Invitrogen, USA) for 5 min at 37°C. Then, soybean trypsin inhibitor was added (Sigma, USA). After centrifugation and discarding the supernatant, the single cells were plated in Dulbecco’s modified Eagle’s medium/F12 (Invitrogen, USA) containing 1% N2 supplement (Invitrogen, USA), 2% B27 supplement (Invitrogen, USA), 1% penicillin/streptomycin, 1% glutamax (Invitrogen, USA), 20 ng/ml epidermal growth factor (EGF; Miltenybiotech, Germany) and incubated at 37°C and 5% CO2. During the next days, NS/PCs proliferated as free-floating clusters (neurospheres). When the diameter of the spheres became about 200 µm, subculturing was done and the cells were re-plated into fresh growth medium. Following the second passage, the obtained NS/PCs were used for the study.

Study design

The cells obtained from the second passage (3×10⁴ cells per well) were cultured in 96-well plates and used for investigation. A range of 0.25 µg/ml to 1000 µg/ml of MP was used in MP-based previous in-vitro studies (Kuppermann, Zacharias, & Kenney, 2014; Mealey, Chen, & Schanz, 1971; Wang et al., 2014). In the present investigation, we selected concentrations of 0, 5, 10, 15 or 20 µg/ml of MP (Sigma, USA) due to the toxicity of this drug in higher concentrations for NS/PCs (according to our
Cell viability, proliferation and migration of NS/PCs were evaluated using the following methods (4 well/group).

**Cell viability assessment**

The NS/PCs were exposed to different concentrations of MP for 7 days. Then, MTT assay was done to evaluate cell viability. Briefly, the cells were incubated by MTT solution for 4 hours, then the reaction was ceased by Dimethyl sulfoxide (DMSO). Absorbance was measured by an ELISA microplate reader at 570 nm.

**Proliferation assay**

Two parameters including the number and the diameter of neurospheres were considered as proliferation index. The NS/PCs were cultured in 96-well plates contained the neurosphere medium (3 well per group). The number of neurospheres in each well was calculated on days 3, 5 and 7 under an inverted microscope (Optika, Italy). Moreover, the diameter of neurospheres was measured in 5 photos taken from corners and center of each well by using Infinity software on days 3, 5 and 7. The average diameter of two diagonals perpendicular to each other was reported as the neurosphere diameter.

**Cell migration assay**

To evaluate the migration of the cells, the neurospheres were cultured in Poly-L-Ornithine - treated plates, then the migration was monitored by taking photos on days 1, 3, 5 and 7. The average distance passed by the three cells located in the farthest distance to the margin of the neurosphere was measured by Infinity software as an index of cell migration.
Statistical analysis

Data are presented as mean ± standard deviation (SD) of the mean. Normal distribution of data was tested prior to statistical analysis. One-way analysis of variance (ANOVA) was used if data were distributed normally and for nonparametric data, the Kruskal-Wallis ANOVA test (KWT) was performed. LSD was used as post hoc test. P<0.05 was considered statistically significant.

Results

Primary culture

The isolated ganglionic eminence was cultured in serum-free medium. As indicated in figure 1, the NS/PCs were proliferated as free-flouting neurospheres. One week after primary culturing, the diameter of the neurospheres was more than 100 micrometers, thus, passaging was done. The single cells obtained from the second passage were used for the rest of the study.

The effect of MP on viability of the NS/PCs

The results of MTT assay at day 7 post-treatment showed that following exposure to 10, 15 and 20 μg/ml of MP, the survival of the NS/PCs decreased significantly compared to that of the group without any drug exposure (p<0.05). Whereas, the cell viability didn’t change by 5 μg/ml of MP (figure 2).

The effect of MP on proliferation of the NS/PCs

The number of neurospheres produced from the single cells after 3 days did not significantly differ between the MP-treated groups and the non-treated group. Although, the number of neurospheres significantly decreased in the 15μg/ml of MP group compared to that of the 5 and 10 μg/ml of MP
At days 5 and 7 post-treatment of the NS/PCs with MP, there was no significant difference in the number of neurospheres among the groups.

On the other hand, the diameter of neurospheres dramatically reduced following exposure of the NS/PCs to the different concentrations of MT compared to the non-treated group. This effect could be observed 3, 5 and also 7 days after exposure to MP (P<0.05) except for concentration of 20 μg/ml of MP in day 5 (figure 3, G).

**The effect of MP on migration of the NS/PCs**

As illustrated in figure 4, an increasing trend could be seen in migration capacity of the NS/PCs in the all groups from day 1 to day 7 following exposure to different concentration of MP. In days 1 and 3 after exposure, there was no statistically significance among the groups in migration index. Although, the migration of NS/PCs treated with 20 μg/ml of MP was higher than that of the non-treated group in days 5 and 7 following exposure.

**Discussion**

In the present study, we indicated that high concentrations of MP threatened the survival of NS/PCs and the proliferation capacity of NS/PCs decreased by exposure to different concentrations of MP. However, the highest concentrations of MP (20 μg/ml) enhanced the migration capacity of NS/PCs. As demonstrated in the present study, previous investigations showed the anti-proliferative effect of MP. Wenhao et al. reported that the inhibitory effect of MP on the proliferation of NS/PCs is related to decrease in the expression of hypoxia-inducible factor-1α (HIF-1α) and Hes1(Wang et al., 2014). HIF-1α helps the cells against apoptosis and increases the cell survival (Majmundar, Wong,
Based on the results of another study that evaluated the expression of various genes associated with neurogenesis, the anti-proliferative effect of methylprednisolone is related to up-regulation of ferritin heavy chain 1 (Fth1) and insulin-like growth factor binding protein (IGFBP-3) genes as well as down-regulation of Endothelin receptor type B (EndrB) (S.-Y. Li et al., 2012). Recently, Li et al. reported that MP decreased the survival of fetal neural stem cells. By using an EndrB agonist, they indicated the role of PI3K/Akt Pathway and IncRNA in this adverse effect of MP (S. Li et al., 2020). Our investigation indicated that all studied concentrations of MP decreased proliferation of NS/PCs. Moreover, previous studies indicated that MP inhibited proliferation of the endogenous NS/PCs (Obermair et al., 2008). Accordingly, based on our results and previous investigations, MP inhibits proliferation of both endogenous and exogenous NS/PCs.

The present study demonstrated the improved migration of NS/PCs following exposure to high concentrations of MP as a positive finding. The migration capacity is one of the main factors in NS/PCs transplantation (Arocena & Collinson, 2012) which is reported for MP-treated NS/PCs for the first time in this study. However, further mechanistic investigations in this area are needed. Although, considering the anti-survival and anti-proliferative effects of MP, its effect on migration of NS/PCs may not be a benefit in combination therapy approaches.

In conclusion, MP increased the migration capacity of NS/PCs only in a high concentration, but it reduced survival and proliferation of NS/PCs. These effects of MP on NS/PCs should be considered in future combination therapies including MP+NS/PCs for neurodegenerative diseases.
Acknowledgment

The authors wish to gratefully acknowledge the support of Shiraz University of Medical sciences (grant number 10750-74-01-94).

References


Figures and captions:

**Fig1. Culture of neural stem/progenitor cells (NS/PCs).** The ganglionic eminence was isolated and cultured in a serum-free condition and after seven days the proliferated cells appeared as well shaped and visible neurospheres (A). After the second passage, the neurospheres (B) were dissociated as single cells (C) and were used for the rest of the study.
Fig 2. **Cell viability assay.** The survival of the neural stem/progenitor cells (NS/PCs) was evaluated seven days after exposure to 5, 10, 15 or 20 μg/ml of methylprednisolone (MP) by MTT assay. *: P<0.05 vs the untreated group.
**Fig 3. Cell proliferation assay.** The proliferation capacity of the neural stem/progenitor cells (NS/PCs) was assessed three, five and seven days after exposure to 5, 10, 15 or 20 μg/ml (A-E) of methylprednisolone (MP) as number (F) and diameter (G) of neurospheres. &: P<0.05 vs the 5 and 10 groups. *: P<0.05 vs the other groups. #: P<0.05 vs the other groups except the 20 group.
Fig 4. **Cell migration assay.** The migration of the neural stem/progenitor cells (NS/PCs) as the average distance passed by the three cells located in the farthest distance to the margin of the neurosphere was evaluated in days 1, 3, 5 and 7 post-exposure to 0 (a-d), 5 (e-h), 10 (i-l), 15 (m-p) or 20 (q-t) μg/ml of methylprednisolone (MP). V and W are enlarged views of q and s respectively. 

*: P<0.05 vs the 0 group.