

Effect of Dexamethasone in the Development of O1 or O4 Immunoreactive Oligodendrocyte of the Neonatal Rat

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Abstract : White matter disease (WMD) study, which underlies the subsequent progress of cerebral palsy as well as cognitive impairment of premature and/or low birth weight infants, has been focused either on the hypoxia-ischemia damage or cytokine-induced brain damage related with maternal or fetal inflammation. Also, dexamethasone (DEXA) may increase the risk of neuropsychological problem including adverse cognitive and behavioral outcome in preterm infants. Thus, we hypothesized that perinatal DEXA would damage and trigger the death of developing oligodendrocytes (OL) progenitors, and subsequently disturb myelination. In this study, DEXA was administered to neonatal rats for 3 consecutive days subcutaneously between postnatal day 1 (P1) and P3. By using immunofluorescent staining of stage specific OL progenitor markers such as O4 and O1, the morphological changes of OL progenitors were examined and the apoptosis of OL progenitors were visualized by TUNEL staining. Results depicted that relative number of O1 immunoreactive (IR) cells were less to that of O4 IR cells. Multipolar O1 IR cells with short dendritic processes were observed in both control and DEXA group at P3. In the total O1 immunoreactive cells, the relative percentages of apoptosis cells were calculated at P3 as 8.7% in control, 23.0% in DEXA group. The relative percentages of apoptosis in the total O4 immunoreactive cells were measured at P3 as 3.0% in control and 13.5% in DEXA group.

OL progenitors' apoptosis may contribute to the overall reduction of immature OLs in cerebral white matter. Therefore, specific stages of OL maturation could clinically be an important factor in determining the susceptibility to DEXA. To elucidate the disease mechanism of the white matter disease, further investigation may be needed whether OL progenitors' decrease by the DEXA administration affects to the myelin formation as developmental stages.

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Key words : White matter disease (WMD), Dexamethasone (DEXA), Oligodendrocytes (OL), O1, O4

Introduction

In most preterm infants, white matter disease (WMD)

is one of leading causes of adverse problems such as motor and psychiatric disorders (Krägeloh-Mann et al. 1995, Whitaker et al. 1997). The majority study on WMD, which underlie the subsequent progress of cerebral palsy as well as cognitive impairment in survivors of premature (Back et al. 2001) and/or low birth weight infants (Saigal et al. 2000), was focused on the hypoxia-ischemia (HI) damage or cytokine-induced brain dama-

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ge related with maternal or fetal inflammation (Kumral et al. 2007), since these were well known leading causes in the pathogenesis of periventricular leukomalacia (PVL). In order to investigate the underlying mechanisms behind white matter lesions, a number of animal models of WMD have been developed based on administration of either microbes/bacterial products (Debillon et al. 2000, Follett et al. 2000, Eklind et al. 2001) or the induction of HI (Uehara et al. 1999, Follett et al. 2000, Back et al. 2002). The neonatal rat brain injured by the HI can increase the pyknotic oligodendrocyte (OL) progenitors in ischemic cerebral hemisphere and trigger the death of late OL progenitor via an apoptotic pathway which is similar to that of neonatal neuron (Back et al. 2002). However, birth asphyxia in the neonate occupies only a small portion of diversified factors known to cause cerebral palsy (CP) (Hagberg et al. 2002).

Postnatal systemic corticosteroid treatment, which has been basically used to prevent or treat bronchopulmonary dysplasia (BPD), exert beneficial effects on the neonatal lung, such as improvement in gas exchange, lung mechanics, and time until extubation and decrease the duration of ventilator dependency (Committee on Fetus and Newborn 2002, Rajadurai and Tan 2003, Grier and Halliday 2005). However, in addition to the aforementioned causes of WMD, randomized clinical trials of early postnatal steroid therapy for the prevention of BPD have raised the risks of CP, and increased the rate of subnormal cognitive function (Wilson et al. 2006; Baud and Sola 2007). In particular, dexamethasone may eventually increase the risk of neuropsychological problem including adverse cognitive and behavioral outcome in very low birth weight and/or preterm infants (Hauser et al. 2008, Lajic et al. 2008). Studies using 3-D magnetic resonance imaging (MRI) to quantify at term cerebral tissue volume also suggested that postnatal systemic dexamethasone (DEXA) treatment for neonatal chronic lung disease impaired brain growth,

principally affecting cerebral cortex (Murphy et al. 2001). Moreover, experimental studies have demonstrated deleterious effects on central nervous system development attributable to steroid administration (Ahlbom et al. 2000, Baud 2004, Newnham and Jobe 2009). Supporting several reports indicated that exposure to excess systemic corticosteroid may cause developmental fetal brain damage and lead to some long term sequelae, which would be catastrophes in life, since the developing brain is particularly sensitive to systemic corticosteroid treatment (Matthews and Challis 1997, Ahlbom et al. 2000, Baud 2004, Newnham and Jobe 2009).

Steroid treatment during critical periods of brain development may impair myelination and brain cell division, resulting in long-term behavioral effect (Weichsel 1977). Single doses of DEXA given to rats on postnatal day 4 or 7 are associated with subsequent behavioral disturbances, reduction in cerebellar weight and impairments in spatial learning and motor coordination (Benesová and Pavlík 1989). *In vivo* administration of glucocorticoids to infant animals decreased the myelin formation (Gumbinas et al. 1973). DEXA administered intraperitoneally to 3-day-old rats for 7 consecutive days suppressed the expression of genes related to glial functions, especially myelination (Tsuneishi 1991). However, the exact pathophysiology as to how postnatal DEXA affects the myelin synthesis is not clearly known.

Thus, we hypothesized that postnatal DEXA would injure and trigger the death of developing OL progenitors via apoptotic pathway, and subsequently impair myelination. To test this hypothesis, we administered DEXA subcutaneously to neonatal rats for 3 consecutive days between postnatal day 1 (P1) and P3, and observed the morphological changes of developing OL progenitors and subsequent hypomyelination, using immunofluorescent staining against surface markers of variable developing stages of OL progenitors such

as O4 and O1, and we observed the death of OL progenitors via apoptotic process by double-labeling staining of TUNEL and O4/O1.

Materials and Methods

1. Animal preparation and postnatal treatment

This study was performed in accordance with the institutional guidelines which are in compliance with current international laws and policies (NIH Guide for the Care and Use of Laboratory Animals, NIH publication No. 80-23, 1985, revised in 1996) and were approved by 'Dankook University Institutional Animal Care and Use Committee' (DUIAC), which adheres to the guidelines issued by the Institution of Laboratory of Animal Resources (ILAR). Timed-pregnant Sprague-Dawley rats (Cheil Co. Korea) were purchased and housed individually in breeding cages and allowed food and water *ad libitum*. Pups from rats were pooled at postnatal day 1 (P1), and then randomly redistributed as the control (n=10) and DEXA groups (n=10). The DEXA group received subcutaneous injections of DEXA (1.0 mg/kg body weight) at the dorsum of the neck daily morning for three consecutive days (P1 ~ P3) respectively. The control group received equal amount and frequency of subcutaneous injections of normal saline. 8 hours after injecting DEXA, P3 pups were decapitated under anesthesia. The brains were removed after perfusion with 4% paraformaldehyde and stored in the same fixative for 1 day. The brains were sequentially incubated in the 10, 20 and 30% sucrose solution at 4°C, after which they were stored at -80°C until they were used.

2. Tissue preparation Immunofluorescent staining of O4, O1 and TUNEL staining

Immunohistochemical staining was performed with

cryostat sections (18 µm thick) of the brains as described before (Kim et al. 2007). In brief, the coronal sections were incubated with 1 : 100 diluted monoclonal O4 (Chemicon, CA, USA), and 1 : 100 diluted monoclonal O1 (Chemicon, CA, USA), respectively at 4°C overnight. After 3 washings in PBS for 10 min, the sections were incubated with 1 : 200 diluted secondary antibodies (Alexa Fluor 555 anti-mouse IgG, Molecular Probes, Invitrogen) at room temperature for 1.5 h. The stained tissue sections were washed in PBS and processed for non-isotopic terminal deoxynucleotidyl transferase-mediated digoxigenin-dUTP nick end-labeling (TUNEL) staining [TUNEL Apoptosis Detection Kit (DNA Fragmentation/Fluorescence Staining, Upstate Co, USA), where 37 U terminal deoxynucleotidyl transferase (37 U TDT; catalog no. 17-141b, Millipore, Billerica, MA), 1 pmol biotin dUTP (catalog no. 17-141d, Millipore), terminal deoxynucleotidyl transferase buffer, and bovine serum albumin (BSA; 1 mg/mL) were contained] to determine the cell-type specific apoptosis in the white matter. Tissue sections were incubated with proteinase K (20 mg/mL, Sigma, St Louis, MO) at room temperature for 15 min and then washed extensively 3 times with Tris-buffered saline (TBS; 10 mM Tris-HCl, pH 7.4, and 150 mM NaCl) for 5 min each wash. The sections were exposed to equilibration buffer containing terminal deoxynucleotidyl transferase buffer (TDT, Invitrogen, USA) and BSA (1 mg/mL) for 30 min. Each slide was then incubated for 3 h, at 37°C, with a solution containing 37 U TDT, 1 pmol digoxigenin-11-dUTP, TDT buffer and BSA (1 mg/mL). Slides were washed in TBS buffer and processed with anti-digoxigenin antibody. The sections were observed under an Axioscope microscope (Carl Zeiss, Germany) with a fluorescence attachment.

3. Quantification of immunofluorescent cells

In order to quantitatively evaluate the immunofluo-

rescent staining data cell counting was performed in the corpus callosum (white matter). Randomized counting ($200\times$ magnification, one visual field= 0.784 mm^2) of immunofluorescent cells were done by selecting three sections per single animal. Three visual fields from each section were counted and calculated as an average number per visual field. Average cell number per sections was recalculated and average cell number per one animal was summed up. Results were expressed as mean \pm standard errors of the mean (SEM), DEXA and control group's brains were compared at each time point using *Student's t-test*, with $P < 0.05$ considered statistically significant.

Results

Using immunofluorescent staining and TUNEL assay, we investigated the cellular distribution of late OL progenitor (Pre OL, O4) and immature OL (O1), and apoptotic profile of O1 and O4 immunoreactive (IR) cells in the selected white matter regions of P3 rat pups' brain after injection of DEXA. In both control and DEXA group, O1 and O4 IRs were observed at P3, though the relative number O1 IR cell was much smaller than that of O4 IR cell (Figs. 1, 2 and 3). The relative ratio of O1 to O4 IR cell number in the control group, were 0.52 at P3. And the relative ratio of O1 to O4 IR cell number in the DEXA group, were 0.82 at P3.

1. O1 immunoreactive cells in the white matter

The distribution of O1 IR cells in the white matter was similarly uniform in both groups at P3 (Fig. 1A and D). In both control and DEXA group, a number of O1 IR cell was observed in the immature OL though the relative number of O1 IR cells in DEXA group was much smaller than that of the control group (Fig. 1A and D and Fig. 3). The O1 IR cells were mostly observed as asymmetric simple multipolar with short

dendritic processes at P3 (Fig. 1G and H). Compared with mean O1 IR cell number in control group at P3, the relative percentage of O1 IR cell numbers in the DEXA group were calculated as 52.9% (Fig. 3, $P < 0.05$).

2. O4 immunoreactive cells in the white matter

The distribution of O4 IR cells in the white matter was also similar in both groups at P3 (Fig. 2A and D). In both control and DEXA group, a number of O4 IR cell was observed in the immature OL though the relative number of O4 IR cells in the DEXA group was much smaller than of the control group (Fig. 2A and D and Fig. 3). O4 IRs was observed in the somata as well as on their dendritic processes in both group (Fig. 2G and H). The O4 IR cells mostly appeared to be asymmetric simple multipolar or bipolar morphology with few and short dendritic processes at P3 (Fig. 2G and H). Compared with mean O4 IR cell number in control group at P3, the relative percentage of O4 IR cell numbers in the DEXA group were estimated as 33.7% (Fig. 3, $P < 0.05$).

3. Apoptosis of O1, O4 immunoreactive cells in the white matter

TUNEL assay was performed to investigate and assess the apoptosis of O1 and O4 IR cells by using double immunofluorescent staining. In the total O1 immunoreactive cells, the relative percentages of apoptosis cells were calculated at P3 as 8.7% in control, 10.0% in DEXA group (Figs. 1 and 4). The relative percentages of apoptosis in the total O4 immunoreactive cells were measured at P3 as 3.0% in control and 9.4% in DEXA group (Figs. 2 and 4).

Discussion

Periventricular leukomalacia (PVL) is a common

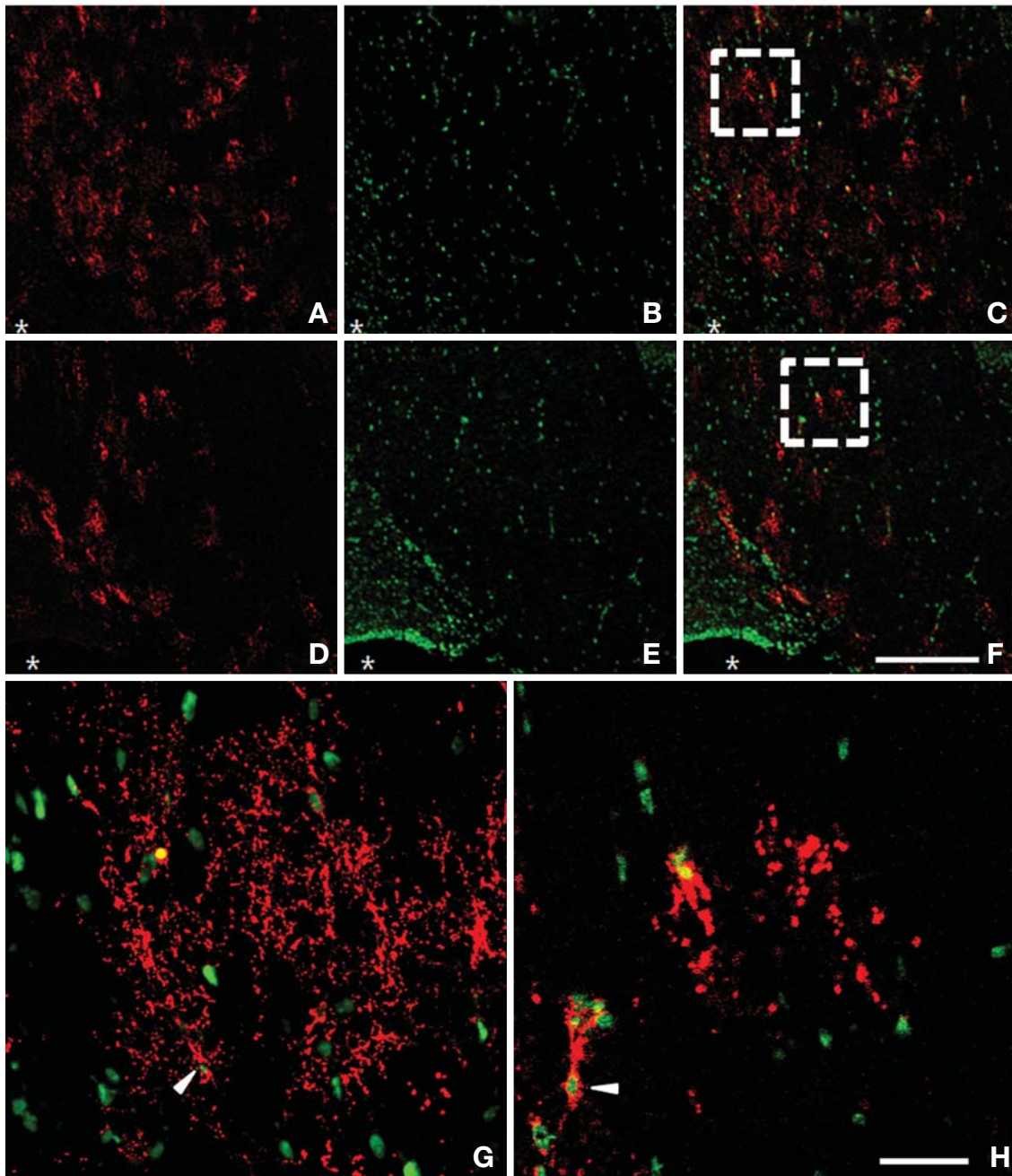


Fig. 1. Distribution of O1 immunoreactive (IR) cells in the white matter of rat pup at P3. Double immunofluorescent labeling for O1 (red) and TUNEL assay (green) in the control (A-C, and G) and DEXA groups (D-F, and H) revealed the apoptosis of O1 IR cells (merge, yellow). O1 IR multipolar cells (arrowheads) with short dendritic processes can be observed (G, H). The dashed-line boxes in image C and G are the regions selected for panels D and H, respectively. (*) indicate ventricle. Scale bars; A-F= 200 μm ; G and H=50 μm .

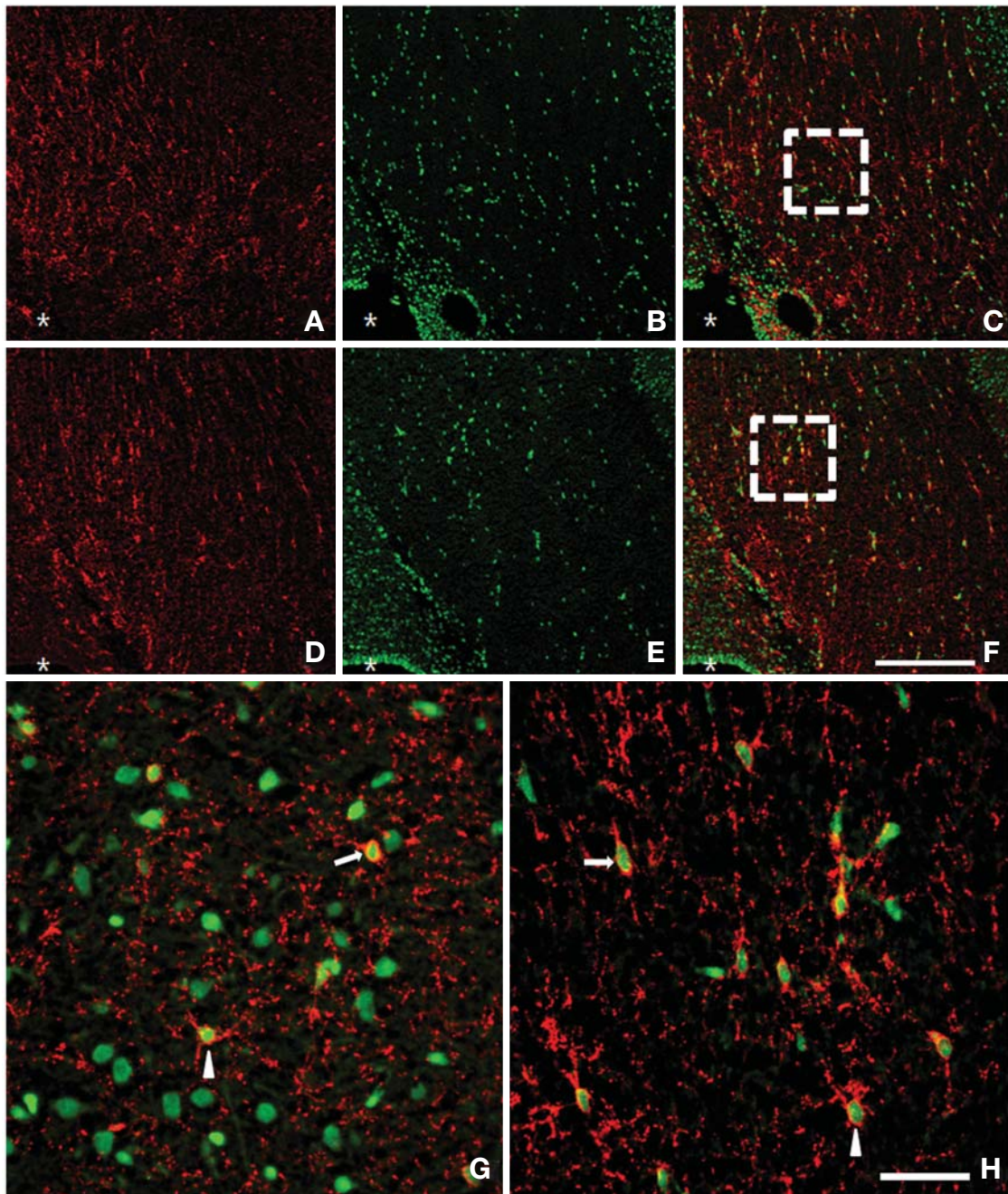


Fig. 2. Distribution of O4 immunoreactive (IR) cells in the white matter of rat pup at P3. Double immunofluorescent labeling for O4 (red) and TUNEL assay (green) in the control (A-C, and G) and DEXA groups (D-F, and H) revealed the apoptosis of O4 IR cells (merge, yellow). O4 IR multipolar (arrowheads) and bipolar cells (arrows) with short dendritic processes can be observed. The dashed-line boxes in image C and G are the regions selected for panels D and H, respectively. (*) indicate ventricle. Scale bars; A-F=200 μ m; G and H=50 μ m

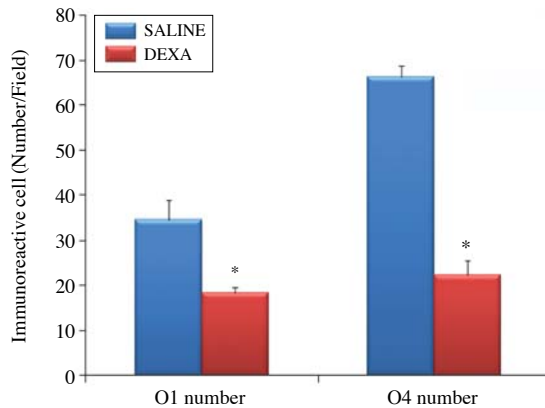


Fig. 3. The numbers of O1, O4 immunoreactive cell at P3 were shown as bar graph at P3. Each bar represents mean \pm SEM of three separate experiments; asterisks (*) indicate statistical significant differences between DEXA and control group at P3 (*, $P < 0.05$).

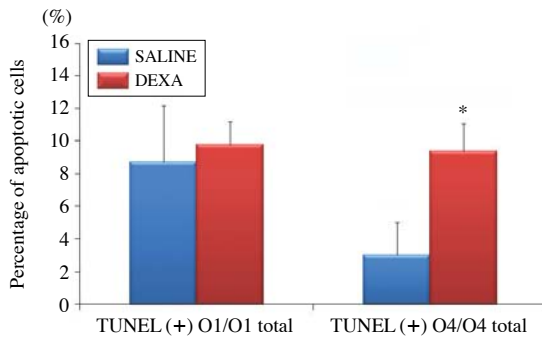


Fig. 4. The percentage of O1, O4 apoptotic cell number to total number of O1, O4 immunoreactive cells at P3 were shown as bar graph. Each bar represents mean \pm SEM of three separate experiments; asterisks (*) indicate statistical significant differences between DEXA and control group (*, $P < 0.05$).

lesion of periventricular cerebral white matter in the premature infants (Volpe 2000). The principal pathologic feature of PVL is a chronic disturbance of myelination. Myelination is a postnatal event in human brain and early postnatal administration of DEXA to the developing brain may affect this process. The four successive stages of OL development can be defined

by the presence of different type-specific surface antigens (Pfeiffer et al. 1993). In our study, the DEXA was administered daily in the morning for 3 consecutive days (P1 ~ P3). O1 IR cells were characterized by multipolar cells with short dendritic processes (Fig. 1G and H) while the O4 IR cells were morphologically more diverse and represented by simple multipolar or bipolar cell which were less differentiated at P3 (Fig. 2G and H). OL lineage progression in human cerebral white matter between mid-gestation and full term demonstrated similar morphological patterns of immature OLs and Pre OLs (Back et al. 2001). Morphological alterations of O4 IR and O1 IR cells implicated that both pre and immature OLs would go through a reactive response to DEXA insults like other sources of brain injury (Nishiyama et al. 1997, Back et al. 2002).

This study showed that administrations of DEXA in the rat pups induced white matter injury affecting both O4 and O1 IR cells. In brief, at P3, the number of O4 IR cells, assumed as Pre OL, was higher than that of O1 IR cells, assumed as immature OL progenitor cells. Compared to the ratio of O1 to O4 IR cell number in both control and DEXA group, the ratio of DEXA group (0.82) were higher than that of control group (0.52), which could reflect that the larger number O1 IR cells and/or the smaller number of O4 IR cells in the DEXA group at P3. Similar with our data, in the neonatal rats, the same stage of OL progenitors (O4 IR but not O1 IR cells) was vulnerable to HI in immature rats, whereas immature OLs (both O4 and O1 IR cells) are resistant to HI (Back et al. 2002). Furthermore, the white matter susceptibility is related to the presence of OL progenitors (O4 IR but not O1 IR cells) in human (Back et al. 2001). Another studies using these markers have also demonstrated that late OL progenitors are main target cell in PVL (Fern and Möller 2000, Back et al. 2001, Back et al. 2002, Craig et al. 2003), and late OL progenitors *in vitro* are markedly susceptible to free radical-mediated injury than that of matured

OL's (Back et al. 1998, Fern and Möller 2000). The risk for PVL may correlate well with some critical postconceptional age (23~32 weeks) in the human brain development (Back et al. 2002).

The molecular mechanism of apoptosis of pre or immature OLs is presently unclear. According to the previous reports in animal models of WMD by HI injury, caspase-3 would play a critical role in the apoptosis of late OL progenitor (Gu et al. 1999, Back et al. 2002). In the present study, the percentage of O1 and O4 IR apoptotic cells in the number of O1 and O4 IR cells were respectively calculated to evaluate the DEXA effect by TUNEL staining (Fig. 4). The data indicated that the percentage of O4 and O1 IR apoptotic cells was higher than that of control group (Fig. 4), which indicates the susceptibility of DEXA in both O4 and O1 IR cells. In the DEXA group, the percentage of O4 apoptotic cells was little bit smaller than that of O1 apoptotic cells. However, the conclusion of increased susceptibility of O1 IR cells to DEXA in comparison with O4 IR to DEXA cannot be drawn because total number of O4 IR cells as late OL progenitors is much larger than that of O1 IR cells as immature OL progenitors. According to the comparative time schedule for CNS development in mammals (Hagberg et al. 2002), the human preterm (23~36 weeks of gestation) corresponds to postnatal day 3~7 in rat. Furthermore, late OL progenitors are predominant in the white matter and cortex of neonatal rat from P1 to P5 stages (Gard and Pfeiffer 1989).

Nowadays, the neuronal development is dependent on diversified and combination factors (i.e. genetic and epigenetic factors), some of which may affect the susceptibility of the developing brain (Bhutta and Anand 2002). Previous study on the neonatal murine brain from unstressed mothers (Rangon et al. 2007), postnatal administration of exogenous corticosterone failed to find excitotoxic lesions. These results indicated that the level of circulating steroid is not a critical,

but rather, exposure to high level of corticosterone in selective stages of brain development leads to an increased vulnerability to a secondary excitotoxic insult (Rangon et al. 2007). This two-hit hypothesis may be an adaptable theory in the mechanism of CP and WMD associated with prenatal brain injury (Dammann et al. 2004). Consequently, single factors like HI, infectious agents and excitotoxic insults cannot explain the complete mechanism to cover pathophysiology of WMD. Accordingly, we suggest the potential implication of disturbances in white matter development in response to systemic DEXA administration. However, further studies are needed to define and evaluate the molecular signal cascades of apoptosis in late and immature OLs. Also, for better understanding of the biology of oligodendrocyte *in vivo*, mechanisms involved in mature OLs' final outcomes should also be elucidated.

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덱사메타손이 신생 흰쥐의 O1 혹은 O4 면역반응성 회소돌기아교세포의 발달에 미치는 효과

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간추림 : 미숙아 또는 저체중아에서 발생하는 인지장애나 뇌성마비의 원인질환으로 여겨지는 백색질 질병에 관한 연구는 주로 모체나 태아에서 저산소성-허혈성 손상이나 사이토카인에 의해 유도되는 기전에 대한 연구들에만 집중되어 있었다. 일반적으로, 덱사메타손(Dexamethasone)은 인지장애나 행동 장애 등을 포함해 신경정신학적 문제들의 위험을 증가시킨다. 그러므로, 출생전후기에 덱사메타손의 투여는 발달하는 회소돌기아교 전구세포들의 죽음과 손상을 유발해 말이집형성을 방해하게 될 것으로 가정하고 본 실험을 진행하였다. 태생 1일째부터 3일째까지 신생 흰쥐에 덱사메타손을 투여하여 면역형광염색 방법으로 회소돌기아교세포의 발달단계에 따른 표지자자인 O4, O1의 항체를 이용해 염색하였다. 이 염색을 통해 회소돌기아교 전구세포들의 수적, 형태적 변화들을 관찰하였고, TUNEL 분석법을 이용해 회소돌기아교 전구세포들의 세포사멸을 살펴보았다. O1 면역반응성 세포들의 상대적 인 수는 O4에 비해 적었으며, 대조군의 O4에 염색된 세포들은 많은 가지돌기를 가진 못극성으로 DEXA 실험군에 비해 현저한 면역염색성을 관찰할 수 있었다. O1 면역반응성 세포들은 P3에서 짧은 가지돌기를 가진 못극성으로 관찰되었다. 전체 O4 면역반응성 세포에서 세포사가 발생한 상대적 비율은 대조군에서 3%, 덱사메타손의 투여군에서 13.5%였다. 그리고 전체 O1 면역반응성 세포에서 세포사가 발생한 상대적 비율은 대조군에서 8.7%, 덱사메타손의 투여군에서 23.0%로 계산되었다.

따라서, 본 연구를 통해 덱사메타손의 투여에 따른 O1 혹은 O4 면역염색성을 나타내는 회소돌기아교 전구세포의 세포사멸은 회소돌기아교세포 수의 감소를 초래할 것으로 생각되며, 추후 연구에서 발달단계에 따른 말이집을 형성하는 회소돌기아교세포의 감소가 백색질 질병의 병리기전에 어떤 역할을 할 수 있을지에 관한 조사가 백색질 질병의 병리기전을 규명하기 위해 필요할 것이다.

찾아보기 낱말 : 백색질병, 덱사메타손(Dexamethasone), 회소돌기아교세포, O4, O1