A quantitative study on changes of the myelinated fibers in the cerebral cortex of cortical dysplasia rats

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Abstract
An animal model of cortical dysplasia was established through X-ray irradiation induced subcortical heterotopic nodules in rats. Transmission electron microscopy detection of the ultrastructure and the stereology examination showed that there was a significant decrease in cerebral white matter and hippocampal volume, the total volume, volume density, length density and total length of the myelinated fibers in the white matter of cortical dysplasia rats. Subcortical heterotopic nodules of the hippocampal CA1 region and synaptic number density in the CA3 region were reduced by 33.97% and 45.55%, respectively, in CD rats compared with normal rats. Our experimental findings indicate that erosed subcortical heterotopic nodules, decreased total length of myelinated nerve fibers and demyelination directly lead to a reduction of white matter volume.

Key Words: cortical dysplasia; heterotopic nodules; white matter; myelinated fibers; medullary sheath; stereology

INTRODUCTION
Human cerebral cortex dysplasia (CD), a neuronal migration disorder and malformation of cortical development, often causes clinical seizures, developmental delays, partial neurological disorders and mental retardation. CD is a major cause of intractable epilepsy[1]. Although CD has been reported in a number of clinical behavioral studies, and electroencephalogram, radiological and pathological research[2], and our research group has also investigated the pathogenesis of CD[3-4], the fundamental mechanism leading to epilepsy still remains unclear. In addition, stereological studies, which are very important to elucidate the associated mechanism[5] are scarcely reported. Therefore, we aimed to further clarify the reason for the reduction of subcortical white matter volume and the changes in synaptic numbers in heterotopic nodules in the CA1 region of CD rats. This experiment was the first demonstration of the quantitative analysis on the numerical density of the synapse in subcortical white matter, the total volume, total length, length density and volume density of myelinated nerve fibers in the cerebral cortex of CD rats using the combination of a new stereological method[6] and electron microscopy.

RESULTS
Quantitative analysis of the experimental animals
Sixteen pregnant Sprague-Dawley rats at day 17 of pregnancy were equally and randomly divided into a normal group (normal feeding) and a CD group (CD rats were established via X-ray irradiation). Neonatal rats at 56 days after birth were utilized in this experiment.

White matter and hippocampal volume reduction in CD rats
The stereological detection showed that the hippocampus and white matter volume was reduced by 33.97% and 45.55%, respectively, in CD rats compared with normal rats ($P < 0.05$; Table 1).

| Group               | Hippocampus (mm$^3$) | CE (%) | White matter (mm$^3$) | CE (%) |
|---------------------|----------------------|--------|------------------------|--------|
| Normal              | 81.51±7.50           | 6.8    | 74.49±7.56             | 2.9    |
| Cortical dysplasia  | 53.92±6.93$^*$        | 7.2    | 40.56±5.74             | 5.4    |

CE: Stereological sampling error. Data are expressed as mean ± SD, with eight rats in each group. $^*P < 0.05$, vs. normal group (two-sample t-test).

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**Length density and total length of myelinated nerve fibers reduced in CD rats**
The length density of myelinated nerve fibers in cerebral white matter of CD rats was 64% lower than normal rats ($P < 0.05$) and the total length of myelinated nerve fibers was reduced 80% compared with normal rats ($P < 0.05$; Figure 1, Table 2).

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![Ultrastructure of myelinated nerve fibers in cerebral white matter of rats (transmission electron microscopy, × 6 000).](image1)

(A) In cortical dysplasia rats, the myelinated nerve fibers show apparent stratification, collapse, disruption, and disordered arrangement.

(B) In normal rats, the myelinated nerve fibers are normal with plasma membrane rings of regular heights and dense structure.

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**Volume density and total volume of myelinated nerve fibers reduced in CD rats**
The volume density of myelinated nerve fibers in cerebral white matter of CD rats was decreased 11% compared with normal rats, but the difference was not statistically significant ($P > 0.05$). The total volume of myelinated nerve fibers in cerebral white matter of CD rats significantly decreased 52% compared with normal rats ($P < 0.05$; Table 3).

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**Synaptic number density in CA1 heterotopic nodules and CA3 region reduced in CD rats**
The synaptic number density in subcortical heterotopic nodules in the CA1 and CA3 regions of the hippocampus were reduced by 26% and 28% respectively in CD rats compared with normal rats, with no significant difference between the two groups ($P > 0.05$; Table 4).

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**DISCUSSION**

Preliminary studies using of myelin staining and electron microscopy observed the demyelination of myelinated nerve fibers in white matter, showing myelin disintegration, degeneration and loss of axons in CD rats\(^2\). This experiment was the first to apply novel unbiased stereological methods to further quantify the number of myelinated nerve fibers in cerebral white matter of CD rats.

Results of this study showed that the total length of myelinated nerve fibers was shortened. However, this alteration was not solely caused by the demyelination of myelinated nerve fibers, it may also be caused by axon fracture and oligodendrocytes\(^6\). The demyelination of only myelinated nerve fibers in CD rats cannot fully explain the reduction in white matter volume, but we did not investigate the total length of non-myelinated nerve fiber in rat cerebral cortex, and no related studies are...
found, so the total length of non-myelinated nerve fibers may be increased, decreased or normal. Strong evidence indicates that the cerebral changes in CD rats include the presence of myelinated nerve fiber demyelination and axonal loss, which was consistent with our previous findings. The reduction in oligodendrocytes should be investigated in further research.

Overall, this experiment found that the total length of myelinated nerve fibers was significantly shortened and the total volume was significantly decreased, indicating that the loss of myelin is the main factor in myelinated nerve fibers, while axon fracture and loss play a minor role.

The reduction of synaptic number density in CD rats was calculated in two-dimensional plane, rather than three-dimensional continuous slices under electron microscopy. Further studies are needed to obtain concrete data of synaptic volume.

Under normal circumstances, axons from bilateral cerebral cortex pyramidal cells assemble to form the corpus callosum, which is responsible for the exchange of information between two hemispheres. Corpus callosum neurons overexpress inward rectifying potassium channels and these channels can reduce electrical activity of the corpus callosum neurons. The specific regional projections of the corpus callosum fibers changed significantly in CD rats. Dense projection fibers were decreased at the S₁/S₂ junction, while axons that should terminate in layers II and III were shown to cross through layers II and III and terminate in the superficial layer [7]. However, white matter volume was reduced in CD rats. The corpus callosum fibers projecting at the S₁/S₂ junction were significantly decreased, and the corpus callosum fibers disappeared in the entire sensory cortex including S₁ region and S₁/S₂ junction[7]. This may be due to the decreased electrical activity of neurons and the blocking of synaptic transmission.

To confirm the above speculations, further studies are required to conclusively determine the number and length of the corpus callosum fibers and non-myelinated nerve fibers, to observe the oligodendrocytes that are associated with myelin formation and the microglia cells that play a role in phagocytosis in the nervous system, and to verify the role of demyelination in the loss of myelinated nerve fibers. Moreover, great efforts are needed to detect the volume density of synapses in a three-dimensional plane, and to verify the "bridge" role in neural networks using neurophysiological techniques.

In summary, experimental findings indicate that subcortical heterotopic nodules of erosion, total length of myelinated nerve fibers and demyelination directly lead to reduced white matter volume.

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**MATERIALS AND METHODS**

**Design**
A randomized, controlled, animal experiment.

**Time and setting**
Experiments were performed from January 2007 to December 2010 in the Electron Microscope Room, Chongqing Medical University, China.

**Materials**
Sixteen healthy, pathogen-free pregnant Sprague-Dawley rats at day 17 of pregnancy, aged 8–12 weeks and weighing 250–370 g, were provided by the Experimental Animal Center, Institute of Field Surgery, Daping Hospital, the Third Military Medical University of Chinese PLA, China (license No. SCXK (Yu) 20020003). The experimental disposal of animals complied with the Guidance Suggestions for the Care and Use of Laboratory Animals, issued by the Ministry of Science and Technology of China[8].

**Methods**

**Establishment of CD models**
The CD rat models were established as previously described[9-10]. In brief, eight healthy pregnant rats were exposed to a radiation box (20 cm long, 12.5 cm wide, 11 cm high; Elekta Limited, Stockholm, Sweden), in an ELEKTA 8 MTV linear accelerator (Elekta Limited) at the irradiation dose of 150 cGy and dose rate of 100 MU/min, for an average exposure time of 1.4 minutes. After the X-ray irradiation, the pregnant rats were housed in cages, until their delivery. Neonatal rats were weaned at 21 days after birth and given free access to food and water until 56 days after birth. Successful models were confirmed by pathological observation[3-4].

**Preparation of brain tissues**
The rats were anesthetized 58 days after birth with 3.5% chloral hydrate via intraperitoneal injection. Animals were perfused through the heart with normal saline until the cleaning solution was clarified, then fixed with 2% paraformaldehyde in phosphate-buffered saline and 2.5% glutaraldehyde (20 mL/min, pH 7.4, 4°C). The injection was terminated when the eyeballs were pale, liver became pale, muscle twitched, tail upturned, limb and neck stiffness was present. The brain tissue was removed, and the olfactory bulb, partial frontal lobe and cerebellum were excised. The brain was divided into left and right cerebral hemispheres along the median sagittal plane, and embedded in 6% agar. Brain tissue was sliced into 1-mm-thick continuous sections and 12–16 sections were obtained from each cerebral hemisphere. A square grid (0.39 mm²) was randomly overlaid on each section and grid points falling on each area of interest were counted in each slice to obtain white matter and hippocampus volumes (Figure 2A). The subcortical heterotopic nodules in the CA1 region and the pyramidal cell layer in the CA3 region were observed under an electron microscope in 3–4 slices from one cerebral hemisphere in each rat.

**Preparation of electron microscope sections**
Ultrathin sections for transmission electron microscopy were prepared as previously described[11], and an isotropic random slice was obtained with isector method[12].
The field of view for copper mesh was positioned using Nissl staining. Three to four tissue blocks from each sample were embedded and cut into ultrathin sections using a Leica UltracutR ultrathin microtome (Leica, Heidelberg, Germany), to produce one copper mesh. Four pictures of myelinated nerve fibers were photographed in each copper mesh under a JEM-Japan 1011 (HC) transmission electron microscope (Hirachi, Tokyo, Japan). A total of 116 photos (36 in normal rats and 80 in CD rats) at 6000 × magnification were obtained. There were also four photos from hippocampal CA1 or CA3 synapses, a total of 116 photos (36 in normal rats and 80 in CD rats) at 20000 × magnification. According to stereological principles of a photograph on a sample, a total of 348 samples were utilized[6].

Stereological counting of white matter and hippocampal volume

The grid points were randomly superimposed over each brain section under a dissecting microscope (XTL-2400 type, Shanghai Quasi-optical Instrument Co., Ltd., Shanghai, China). The number of measuring points hitting the white matter or hippocampus was recorded. \( \Sigma P_{WM} \) and \( \Sigma P_{H} \), respectively, represent the total number of measuring points in white matter and hippocampus. According to Cavalieri’s formula[13], the volume was calculated as follows:

\[
V_{WM} \text{ or } V_{H} = \frac{1}{M} \frac{\Sigma P_{WM}}{\Sigma A} \text{ or } \frac{\Sigma P_{H}}{\Sigma A}
\]

Where: \( V_{WM} \) is white matter volume, \( V_{H} \) is volume of hippocampus, \( t \) is thickness of the hippocampus, \( a(p) \) is area of each test point, 0.39 mm\(^2\).

Stereological counting of length density and total length of myelinated nerve fibers in white matter

An unbiased counting frame was superimposed under an electron microscope. Myelinated nerve fibers fully positioned within the frame or intersecting with the inclusion line (dotted line) were counted, while fibers intersecting with the exclusion line (solid line) were excluded from the counting. The length density of myelinated nerve fibers \( L_{V} \) was calculated according to the formula[13]:

\[
L_{V} = 2 \times \frac{\Sigma Q}{\Sigma A},
\]

where \( \Sigma Q \) is the total number of myelinated nerve fibers in unbiased counting frames and \( \Sigma A \) is the total area of the unbiased counting frames.

Total length of myelinated nerve fiber = length density × white matter volume.

Stereological counting of volume density and total volume of myelinated nerve fibers in white matter

A counting grid was superimposed onto sections under an electron microscope (Figure 2B). The number of grid points positioned on myelinated nerve fibers \( (P_{WM}) \) and the number of white matter grid points within the entire photo \( (P_{WM}) \) were counted. The volume density \( V_{v} \) of myelinated nerve fibers was calculated according to the formula[13]:

\[
V_{v} = \frac{\Sigma P_{nf}}{\Sigma P_{WM}}
\]

Total volume of myelinated nerve fibers = volume density × white matter volume.

Calculation of synaptic density in subcortical heterotopic nodules in hippocampal CA1 and the pyramidal cell layer of hippocampal CA3

Synapse density refers to the number of synapses per unit area \( (\text{mm}^2) \). An unbiased counting frame was superimposed on a photo and the number of synaptic cross-sections fully positioned within the frame or intersecting with the inclusion line (dotted line). Synaptic density \( N_{x} \) was calculated as the number of synaptic cross-sections per unit area according to the formula:

\[
N_{X} = N_{X} / A_{C},
\]

\( A_{C} \) is area of all unbiased counting frames, \( N_{X} \) is the number of neuron cross-sections. For magnification \( M \),

\[
N_{X} = N_{X} / (A_{C} \times 1/M^{2}) = N_{X} \times M^{2} / A_{C}.
\]

Synapse density counting standard in white matter myelinated nerve fibers, subcortical heterotopic nodules in hippocampal CA1 and pyramidal cell layer in CA3, as well as image acquisition and processing

The widely accepted evidence for recognizing synaptic structure in electron micrographs is as follows[14]: (1) the presence of more than three synaptic vesicles in presynaptic components. (2) identifiable postsynaptic
density in postsynaptic components. However, the presence of synaptic cleft is not essential for identifying synaptic structure. Myelinated nerve fibers and synapses were determined under 6 000–25 000 × magnification, according to the morphological characteristics. Image acquisition was conducted using an electron microscopy camera system. Cells were screened, measured and counted using Image Pro Plus 5.0 image analysis system (Media Cybernetics Corporation, Bethesda, MD, USA).

**Statistical analysis**
Data were analyzed with SPSS 10.0 statistical software (SPSS, Chicago, IL, USA) and expressed as mean ± SD. Group comparison was conducted using two-sample t test. The rank sum test was used for two-group comparison of nonnormal data. A level of $P < 0.05$ was considered statistically significant.

**Author contributions:** Xuntai Ma wrote the manuscript, had full access to data acquisition, integration and analysis, and is the header of the fund. Yong Tang was responsible for the study concept, design and article validation. Yang Lv and Oumei Cheng were responsible for statistical analysis. Yong Yan analyzed data.

**Conflicts of interest:** None declared.

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**Ethical approval:** This pilot complied with the Animal Ethical Requirement of the National or Authority Organization and was given approval from the Animal Ethics Committee of Luzhou Medical College in China.

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