ORIGINAL ARTICLE

Comparison of β -Amyloid Plaque Labeling Methods: Antibody Staining, Gallyas Silver Staining, and Thioflavin-S Staining

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Key words: β -amyloid plaques; Alzheimer's disease; antibody staining; Gallyas silver; thioflavin-S

Objective To evaluate senile plaque formation and compare the sensitivity of three different β -amyloid (A β) labeling methods (antibody staining, Gallyas silver staining, and thioflavin-S staining) to detect A β deposition.

Methods APPswe/PSEN1dE9 transgenic mice (APP/PS1) of different ages were used to examine spatiotemporal changes in A β plaque deposition. Antibody staining, Gallyas silver staining, and thioflavin-S staining were used to detect A β plaque deposition in the same brain region of adjacent slices from model mice, and the results were compared.

Results With aging, A β plaques first appeared in the cortex and then the deposition increased throughout the whole brain. Significantly greater plaque deposition was detected by 6E10 antibody than that analyzed with Gallyas silver staining or thioflavin-S staining ($P < 0.05$). Plaque deposition did not show significant difference between the APP/PS1 mice brains assayed with Gallyas silver staining and ones with thioflavin-S staining ($P = 0.0033$).

Conclusions The APP/PS1 mouse model of Alzheimer's disease could mimick the progress of A β plaques occurred in patients with Alzheimer's disease. Antibody detection of A β deposition may be more sensitive than chemical staining methods.

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ALZHEIMER'S disease (AD) is the most common neurodegenerative disorder and a major cause of dementia.¹ Its pathology shows β -amyloid (A β) deposition, tau hyperphosphorylation, brain atrophy, and neuronal death.² Evidence has been shown that A β is generated from neurotoxic amyloid oligomers and fibers. A β is derived from cleavage of amyloid precursor protein (APP) by beta secretase and gamma secretase, and spreads among the brain and biofluids such as blood and cerebrospinal fluid.³

Over the years, many pathological methods have been used for detection of A β or neurofibrillary tangles (NFT), such as Congo red, silver, immunohistochemical, and thioflavin-S staining.⁴⁻⁶ Indeed, several comparative studies on different staining methods have been performed,⁷⁻⁸ which focused on staining morphology of A β -plaques, time-cost issues, or experimental repeatability. To our knowledge, no study reported on sensitivity of the various staining methods. In the present study, we investigated spatiotemporal changes in A β plaque deposition in the brains of APP/presenilin 1 (PS1) mice at different ages. Accordingly, we compared the sensitivity of three different staining methods including antibody, thioflavin-S, and Gallyas silver staining to assess A β plaques. Further, we improved the experimental methods to minimize nonspecific reactions or fluorescence quenching, building on previous research.⁹

MATERIALS AND METHODS

Animals

Different ages of B6C3-Tg (APPswe, PSEN1dE9) 85Dbo/Mmjax mice (34829-JAX, Jackson Laboratory, Bar Harbor, ME, USA), also known as APP/PS1 mice, were individually kept in cages with littermates and free access to water and food under 12-hour light/dark schedules.

All animal works were supported by the Experimental Animal Center of Peking Union Medical College, and in accordance with the institutional guidelines of the Beijing Administration Office of Laboratory Animals. All efforts were made to minimize animal suffering and reduce the number of animals used.

Anatomical processing

In total, 54 male APP/PS1 mice (aged from 2-12 months) were randomly divided into three groups: immunohistochemistry, modified Gallyas silver, and thioflavin-S staining groups respectively. After the mice were anesthetized, the brains were fixed by 4%

paraformaldehyde perfusion through the heart. Paraffin-embedded blocks were prepared by sequential dehydration in graded ethanol before embedding and serial sectioning to a thickness of 4 μ m. Sections were mounted on precoated glass slides.

Pathological staining

Dewaxed brain sections were rehydrated in distilled water and placed in a stainless-steel pressure cooker containing antigen retrieval solution. After 3 minutes of heating under pressure, sections were placed in a room-temperature water bath for 30 minutes, then washed twice in phosphate buffered saline (PBS) for 5 minutes each.

For immunohistochemical staining, 6E10 antibody (1:500; Biolegend, San Diego, CA, USA) was used because of its good specificity and sensitivity. The epitope lies within amino acids 3-8 of beta amyloid (EFRHDS). This antibody reacts to amino acids 1-17 of A β and detects abnormally processed isoforms. After incubation with 6E10 antibody at 4°C overnight, sections were treated with SP Rabbit & Mouse HRP Kit (CwBio, Beijing, China), according to the manufacturer's protocol. Counterstaining was performed with hematoxylin (Golden Bridge Biotechnology Co., Ltd., Beijing, China) for the appropriate time. After each incubation step, sections were washed three times with tris-buffered saline with Tween 20 (TBST) for 5 minutes each.

For modified Gallyas Silver staining, deparaffinized slides were pre-incubated with 0.25% potassium permanganate solution for 5 minutes and 1% oxalic acid for 1 minute, followed by incubation in lanthanum nitrate-sodium acetate solution.¹⁰ For induction of nucleation sites, sections were placed into an alkaline silver iodide solution for 30 minutes at 37°C. Next, sections were incubated in a physical developer solution for 15 minutes, rinsed in gold chloride solution, and then fixed in 1% thiosulfate solution. After each incubation step, sections were washed in distilled water for 5 minutes.

For thioflavin-S staining, modifications were made to minimize background staining based on numerous studies.¹¹⁻¹² Like silver staining, sections were first pre-incubated with potassium permanganate solution and oxalic acid, then placed in 3% sodium borohydride solution for 5 minutes. To enhance the contrast between thioflavin-S and A β plaques, sections were incubated with 0.5% thioflavin-S dissolved in 50% ethanol for 30 minutes. This was followed by differentiation in two changes of 80% ethanol for 10 seconds each, and then incubation in 5 $\times$ PBS solution at 4°C for 1 hour.

Measurement of A β plaque and statistical analysis

After pathological staining, the number of A β plaques in the cortex and hippocampus was counted. Binary images were measured using ImageJ software (National Institutes of Health). As shown in Figs. 1-3, area occupied by A β plaques in the cortex and hippocampus was counted for each staining method for data analysis. To establish an accurate comparison of selectivity and sensitivity, adjacent brain sections were selected for A β plaques staining with each of the methods. As showed in Fig. 4, three areas of 1 mm $\times$ 1 mm in each of three selected slices were analyzed. All photographs were captured with an inverted fluorescence microscope (DMI4000B; Leica, Wetzlar, Germany).

Prism software 7.0 (GraphPad Software Inc., La Jolla, CA, USA) was used to calculate statistical significance. One-way analysis of variance (ANOVA) followed by Tukey's or Dunnett's tests was used for multiple comparisons. Data are presented as mean $\pm$ SEM. *P* val-

ues <0.05 were considered statistically significant.

RESULTS

To examine A β plaque distribution at different ages of APP/PS1 mice, 6 different time points were chosen: 2, 4, 6, 8, 10, and 12 months ($n=9$ in each group), which represent most of a mouse's lifespan.

First, 6E10 antibody immunohistochemistry assays were performed (Fig. 1A). Senile plaques first appeared in the cortex and hippocampus at 4 months, and then spread throughout the entire brain. Noticeably, A β deposition increased more rapidly with age in the cortex than the hippocampus. Quantification of area occupied by A β plaques in the cortex [Fig. 1B, $F(5, 30)=41.35$, $P<0.0001$] and hippocampus [Fig. 1C, $F(5, 30)=28.7$, $P<0.0001$] showed aggravated plaque formation with increasing age.

Gallyas silver staining is a classical argyrophil

Figure 1. Distribution of β -amyloid plaque in the cortex and hippocampus of APP/PS1 mice. Senile plaque formation in 2, 4, 6, 8, 10, and 12-month-old APP/PS1 mouse brain tissues detected by 6E10 immunohistochemistry staining (A). Scale bar in A: 200 μ m. Quantification of area occupied by A β plaques in the cortex (B) and hippocampus (C) at different ages ($n=3$ for each group). Error bars represent mean $\pm$ SEM. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ compared with 2-month mice, based on one-way ANOVA followed by Dunnett's test.

protein assay that is broadly used in neuropathy. We used a modified method that extends the incubation time of alkaline silver iodide solution, thereby increasing the staining effect. Senile plaques were detected at the earliest in 4-month-old brain (Fig. 2A), consistent with 6E10 immunohistochemistry results. As expected, fewer plaques were detected by Gallyas silver staining, particularly in aged mice. Likewise, senile plaques increased in the cortex [Fig. 2B, $F(5, 30)=99.79$, $P<0.0001$] and the hippocampus [Fig. 2C, $F(5, 30)=26.5$, $P<0.0001$] with age.

Next, we examined A β plaques in the brain of APP/PS1 mice with thioflavin-S staining. A considerable number of plaques observed by 6E10 immunostaining and Gallyas silver staining were also detected by thio-

flavin-S staining (Fig. 3A). It is worth noting that many small background fluorescent dots were also detected, which could easily lead to a false positive result. Thus, to objectively evaluate thioflavin-S staining, we excluded this noisy signal using the in-built particle measure function of ImageJ. Similarly, thioflavin-S staining positive results improved in the cortex [Fig. 3B, $F(5, 30)=76.36$, $P<0.0001$] and hippocampus [Fig. 3C, $F(5, 30)=17.94$, $P<0.0001$] with age.

Subsequently, we compared staining results among 6E10 immunostaining, Gallyas silver staining, and thioflavin-S staining in contiguous sections of one mouse. For each slice, three 1 mm² areas were randomly selected, which corresponded to the other two staining methods (Fig. 4A-C). 6E10 immunostaining

Figure 2. Distribution of β -amyloid plaque in the cortex and hippocampus of APP/PS1 mice. Senile plaque formation in 2, 4, 6, 8, 10, and 12-month-old APP/PS1 mouse brain tissues assayed by modified Gallyas silver staining (A). Scale bar in A: 200 μ m. Quantification of area occupied by A β plaques in the cortex (B) and hippocampus (C) at different ages ($n = 3$ for each group). Error bars represent mean $\pm$ SEM. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ compared with 2-month mice, based on one-way ANOVA followed by Dunnett's test.

Figure 3. Distribution of β -amyloid plaque in the cortex and hippocampus of APP/PS1 mice. Senile plaque formation in 2, 4, 6, 8, 10, and 12-month-old APP/PS1 mouse brain tissue tested by modified thioflavin-S staining (A). Scale bar in A: 200 μ m. Quantification of area occupied by A β plaques in the cortex (B) and hippocampus (C) at different ages ($n=3$ for each group). Error bars represent mean $\pm$ SEM. * $P<0.05$, *** $P<0.001$ compared with two-month mice, based on one-way ANOVA followed by Dunnett's test.

identified more positive plaques than Gallyas silver and thioflavin-S staining ($P<0.05$). Moreover, there were no statistically significant differences between the latter two methods [Fig. 4D, $F(2, 15)=8.559$, $P=0.0033$]. Antibody detection of A β was more sensitive than Gallyas silver and thioflavin-S staining.

DISCUSSION

Many pathological staining methods have been developed to describe pathological lesions related to neurological disorders. Although these methods have been used in research and clinical applications for decades, very few studies have compared the various methods. In this study, we investigated the spatiotemporal distribution of A β plaques in APP/PS1 transgenic

mice and improved the staining quality by adjusting the procedure. We found that immunohistochemical staining is the most sensitive method for labeling A β plaques, compared with Gallyas silver staining and thioflavin-S staining.

Immunohistochemistry is an effective method to selectively detect proteins in tissues based on antibody antigen reaction, which is widely used in pathophysiology. Thioflavin-S or thioflavin-T can bind to the cross-beta-sheet structure of A β that is found in neuritic plaques, NFTs, neuropil threads, and vascular amyloid.¹³ NFTs and A β plaques can be distinguished by fluorescence microscopy based on their shape. Studies have shown that thioflavin-T has higher permeability capacity through the blood-brain barrier than thioflavin-S.¹⁴ Several attempts have been made to improve

Figure 4. Comparative analysis of β -amyloid plaques stained by different methods. Senile plaques in adjacent slices of the same APP/PS1 mouse stained by 6E10 immunohistochemistry (A), modified Gallyas silver staining (B), and modified thioflavin-S staining (C). Quantification of plaque density in the same region (D, $n=9$ for each group). Error bars represent mean $\pm$ SEM. * $P<0.05$, ** $P<0.01$ based on one-way ANOVA followed by Tukey's test. n.s.: not significant. Scale bar in A: 200 μ m.

the sensitivity and reduce non-specific staining.¹⁵⁻¹⁶ We extended the incubation time with a low concentration of thioflavin-S (0.5% in 50% ethanol) and high concentration of PBS to prevent fluorescence quenching. These improvements effectively reduced the influence of background staining (Fig. 3A). Several neuronal structures can be identified with different silver stains by using various solvent solutions or silver salts based on its argyrophil character.

Over the past two decades, studies have compared staining results of NFTs by various methods. In dog brain, previous studies have noted similar plaque morphology between immunohistochemistry and various silver stains.¹⁷ While another study in AD patient brain suggested that Campbell and Gallyas stain are superior to Bielschowsky or Congo red stain.¹⁸ However, to date, there has been little discussion on comparisons within the same slice. Consecutive sections from the same mouse will obtain more reliable results (Fig. 4A-C). Further, no mature tangles around plaques have been observed in APP/PS1 mice, which avoids NFT disturbance.¹⁹ Thus, in the present study, we have gone some way towards

enhancing our understanding of amyloid plaque staining.

Conflicts of interest statement

The authors declare that they have no competing interests.

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检测淀粉样沉淀蛋白的三种染色方法比较： 抗体染色、Gallyas 银染和硫磺素 S 染色

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关键词: 淀粉样沉淀; 阿尔茨海默病; 抗体染色; Gallyas 银染; 硫磺素 S 染色

目的 探究阿尔茨海默病小鼠模型的淀粉样沉淀的形成过程。比较抗体染色, Gallyas 银染和硫磺素 S 染色 3 种染色技术检测淀粉样沉淀的效果。

方法 检测不同月龄的 APPswe/PS1dE9 转基因小鼠 (APP/PS1) 大脑淀粉样沉积的时空变化。对小鼠同一脑区的临近切片行抗体染色, Gallyas 银染和硫磺素 S 染色, 并对比其染色效果。

结果 随着模型鼠年龄的增大, 淀粉样沉积首先出现在的大脑皮层, 随后扩散至整个大脑。与 Gallyas 银染和硫磺素 S 染色比较, 6E10 抗体染色能检测到更多的淀粉样斑块沉积 ($P < 0.05$)。Gallyas 银染和硫磺素 S 染色的检测结果之间没有显著的统计学意义 ($P = 0.0033$)。

结论 APP/PS1 转基因小鼠模型能够模拟阿尔茨海默病患者脑内的淀粉样沉积过程。抗体染色得到的结果相较于其它两种检测方法更为可靠。