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ARE ROUTINE METHODS GOOD ENOUGH TO STAIN SENILE PLAQUES AND NEUROFIBRILLARY TANGLES IN DIFFERENT BRAIN REGIONS OF DEMENTED PATIENTS?

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SUMMARY

Introduction: *Numerous clinical cases have been reported showing the clinical picture of dementia but not meeting the neuropathological criteria for Alzheimer's dementia (AD). Different methods used to stain senile plaques (SPs) and neurofibrillary tangles (NFTs) might account for this discrepancy.*

Subjects and methods: *Here, brains of 11 patients with dementia were examined. Cryosections and paraffin sections from 6 different brain regions (frontal medial, temporal medial and occipital gyrus, hippocampus, superior parietal lobe and cerebellum) of all cases were stained with Bielschowsky, Campbell, Gallyas and Congo red stains each.*

Results: *The study shows that the Bielschowsky silver stain is insufficient for detecting SPs and NFTs, whereas two other methods proved to be more accurate. SPs were found in similar frequency in all brain regions examined (exception: cerebellum). The highest amount was shown with Campbell silver stain in paraffin sections. In Congo red only 25 percent of these SPs were stained, which is probably due to a great number of them not containing any amyloid. NFTs were found almost exclusively in the hippocampus. The highest number was detected with Gallyas silver stain in cryosections.*

Conclusion: *These results may suggest that Campbell stain for SPs and Gallyas stain for NFTs should be the methods routinely used.*

Key words: *Alzheimer's disease – neuropathology - silver staining - senile plaque - neurofibrillary tangle*

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INTRODUCTION

Senile plaques (SPs) and neurofibrillary tangles (NFTs) are regarded as the main neuropathological changes in Alzheimer's disease (AD). They mainly occur in the parietal temporal neocortex and the hippocampus, but also in subcortical areas and in the cerebellum. SPs and NFTs can be detected by a number of silver stainings such as Gallyas (1971) Bielschowsky (1911) or Campbell (1987), with immunological methods using antibodies against Aß4 (Davies et al. 1988) and Tau-protein (Grundke-Iqbal et al. 1984) as well as by amyloid specific Congo red stain. Usually, post mortem histopathological proof of SPs and NFTs can confirm a clinically based presumption of AD (Jellinger & Bancher 1998).

But a growing number of cases has been reported which either showed the clinical picture of AD but did not meet the neuropathological criteria (Brunnström & Englund 2009, Brayne et al. 2009) or, on the other hand, showed clinically non demented patients with amounts of SPs and NFTs sufficient for AD diagnosis (Schmitt et al. 2000, Gay et al. 2008). This raises the question of whether Bielschowsky and Congo red stains, the most widely used methods for showing these histopathological changes, can be seen as suitable and sufficient for routine diagnostics. Therefore, these staining methods were compared to those established by Gallyas and Campbell for their ability to stain senile plaques and neurofibrillary tangles in different brain regions of demented patients.

SUBJECTS AND METHODS

For this study 11 cases were examined. Inclusion criteria were age of 80 years and over and the clinical picture of dementia. All cases examined were in-patients in Max Bürger Hospital, Berlin. Patients´ ages ranged from 82 to 101 years with a mean of 90. Only one patient was male, 10 were female. The diagnosis of dementia was based on DSM-IV and could be confirmed by the patients´ charts. Dementia had lasted for 2-7 years (mean: 3.8 years) and was staged as severe with all 11 patients. For one of the patients, duration of illness could not be estimated due to lack of information on patient history.

The autopsy was carried out in the department of pathology in Klinikum Westend, Berlin. Further preparation of dissected brains was done in the department of neuropathology of Klinikum Steglitz Berlin. The brain weight varied by between 950g and 1280g, with an average of 1119.1g. The brains went through autolysis for 10 to 24 hours (mean: 15.5 hrs).

After dissection the brains were fixed in 4% formalin for 14 days. Blocks of tissue were then taken from the left hemisphere and processed in the department of geriatric psychiatry. For examination, blocks were taken from the medial frontal gyrus, the medial temporal gyrus, the hippocampus, the anterior part of the occipital gyrus, the frontal part of the superior parietal lobe and from the posterior part of the left cerebellar hemisphere. 6mm sections were cut from these blocks and embedded in paraffin, the rest was used for cryosectioning. Paraffin sections were cut at 7µm, the cryosections at 70µm.

For detection of NFTs and SPs, paraffin and cryosections were stained according to the methods of Campbell, Gallyas or Bielschowsky or with Congo red (after Puchtler et al. 1962). All these staining methods were used for all brain tissues of each single case. Stained sections were examined with an Olympus BH2 microscope using the x125 objective. The size of the visual field was determined by an ocular grid which projected an area of 760x760µm onto the object that was to be examined.

SPs und NFTs were counted and related to an area of 1mm^2 for each visual field of any stained slice. Classic plaques as well as primitive plaques and moss-like network (von Braunmühl 1957) were counted as SPs. Any intra- or extracellular object with a cord-like pattern was counted as NFTs.

The results were statistically calculated (SPSS) with a three factor variance analysis with repeated measurements and the factors „stain“, „slice“ and „brain region“ (MANOVA). Furthermore, Pearson's correlation between histo-pathological variables and co-variables such as age were calculated.

The histo-pathological diagnosis of AD was based on Khachaturian's criteria (Khachaturian 1985), Multi-infarct dementia (MID) was diagnosed by proof of infarcted areas in orientating views of HE and Nissl stains.

RESULTS

The mean numbers of NFTs in five brain regions are shown in table 1. No NFTs were found in cerebellum. With three factor variance analysis we found two highly significant single effects. The Gallyas stain is by far superior to Bielschowsky, Campbell und Congo red stains in marking NFTs. In hippocampus, there are clearly more NFTs than in any other brain region examined. Even there, the Gallyas stain marks even more NFTs than the other stainings such as Bielschowsky (Figure 1). Considering the effects of interaction in variance analysis, using Gallyas stain in cryosections of hippocampus seems advisable for examination of NFTs.

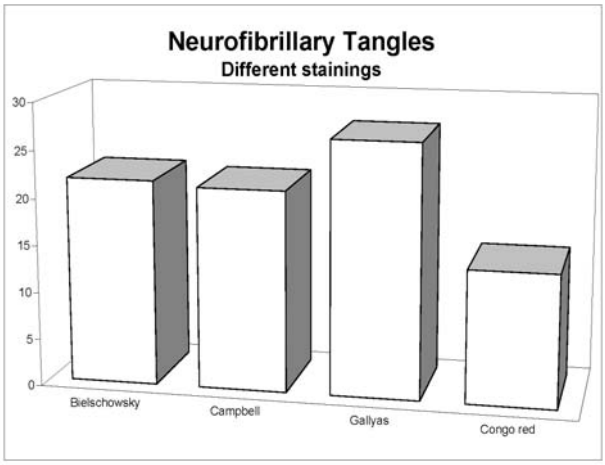

Figure 1. Quantities of NFTs in hippocampus in 4 different stainings. Gallyas stain in cryosections proved to be superior to other methods in staining NFTs

Table 1. Frequency of neurofibrillary tangles (NFTs): Means and standard variances of paraffin (Pa) and cryosections (CS) of the medial frontal (MF) and temporal (MT) gyri, the hippocampus (H), the anterior occipital (AO) and superior parietal lobes (SP) and the cerebellum (CE) of 11 brains, stained with Bielschowsky, Campbell and Gallyas stains and Congo red

		Bielschowsky	Campbell	Gallyas	Congo red
MF	Pa	1.9 (4.9)	1.6 (4.8)	1.9 (5.8)	2.5 (5.6)
	CS	1.6 (4.4)	1.9 (5.3)	1.6 (5.3)	2.6 (6.2)
MT	Pa	0.6 (1.5)	1.8 (5.3)	5.4 (11.6)	2.5 (5.9)
	CS	0.9 (2.4)	2.2 (6.7)	5.5 (12.3)	2.6 (5.6)
H	Pa	21.2 (9.7)	21.0 (9.4)	25.5 (9.7)	15.9 (8.5)
	CS	22.7 (9.9)	22.4 (9.9)	27.5 (9.8)	14.6 (8.5)
AO	Pa	1.6 (3.5)	1.6 (3.9)	2.0 (6.2)	1.5 (2.9)
	CS	1.3 (3.4)	1.9 (4.3)	1.9 (5.8)	1.3 (2.9)
SP	Pa	0.7 (2.4)	1.0 (3.4)	1.5 (4.8)	1.3 (3.1)
	CS	0.9 (2.9)	0.9 (2.9)	1.5 (4.8)	1.0 (2.5)

MANOVA effects: „stain“: F3/30=8.7, p<0.0005, „stain x location“: F15/150=4.9, p<0.0005;
„slice“: F1/10=1.0, p=0.35, „stain x slice“: F3/30=1.8, p=0.16;
„location“: F5/50=37.4, p<0.0005, „slice x location“: F5/50=2.0, p=0.09;
„stain x slice x location“: F15/150=2.0 p=0

For SPs, Campbell stain was clearly superior (Table 2; Figure 2). No differences were found in the amount of SPs between different brain regions except for the cerebellum, where surprisingly only a few SPs (mainly diffuse plaques) could be seen. More SPs could be found in hippocampus, temporal and occipital lobe. Three factor variance analysis showed a significant single effect for paraffin sections, but no interaction effect for Campbell x paraffin slice (Table 2). Strikingly, with Campbell stain more SPs could be found within the same brain region e.g. the hippocampus than with Congo red, indicating numerous non-amyloid plaques while only few SPs really contain amyloid. The same applies to NFTs (Table 1).

Table 2. Frequency of senile plaques (SPs): Means and standard variances of paraffin (Pa) and cryosections (CS) of the medial frontal (MF) and temporal (MT) gyri, the hippocampus (H), the anterior occipital (AO) and superior parietal lobes (SP) and the cerebellum (CE) of 11 brains, stained with Bielschowsky, Campbell and Gallyas stains and Congo red

		Bielschowsky	Campbell	Gallyas	Congo red
MF	Pa	15.9 (8.5)	31.0 (10.3)	15.0 (6.2)	8.9 (6.1)
	CS	14.7 (8.4)	30.4 (10.3)	13.5 (6.1)	8.4 (6.3)
MT	Pa	18.0 (7.9)	32.6 (9.6)	17.8 (6.8)	10.3 (6.4)
	CS	16.7 (8.4)	32.6 (10.8)	16.8 (6.6)	8.3 (6.5)
H	Pa	20.7 (13.0)	32.0 (11.6)	21.1 (9.5)	9.2 (8.6)
	CS	20.7 (13.3)	32.9 (12.1)	21.4 (10.6)	8.7 (8.0)
AO	Pa	17.6 (7.5)	32.2 (7.4)	18.6 (6.0)	6.8 (2.9)
	CS	16.0 (8.7)	31.7 (8.8)	17.6 (6.8)	5.7 (2.4)
SP	Pa	16.0 (10.7)	29.4 (10.9)	15.9 (6.9)	5.1 (3.3)
	CS	15.0 (11.5)	29.0 (11.5)	15.1 (8.1)	4.7 (3.5)
CE	Pa	1.2 (2.3)	2.9 (6.5)	0.4 (1.0)	0.3 (0.7)
	CS	0.9 (2.4)	0	0	0

MANOVA effects: „stain“: F3/30=62.0, p<0.0005, „stain x location“: F15/150=8.5, p<0.0005;
„slice“: F1/10=6.2 p=0.03, „stain x slice“: F3/30=1.0, p=0.4;
„location“: F5/50=19.4, p<0.0005, „slice x location“: F5/50=1.9, p=0.11;
„stain x slice x location“: F15/150=0.4, p=0.9

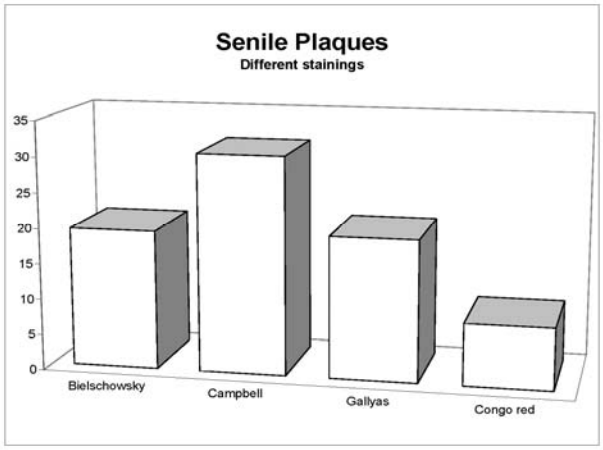

Figure 2. Quantities of SPs in 4 different staining methods (paraffin sections) in hippocampus. For detection of SPs the Campbell stain proved to be highly superior to other methods

DISCUSSION

This study compares the efficiency of the Bielschowsky, Campbell, Gallyas and Congo red staining methods in marking SPs and NFTs. The commonly used Bielschowsky method turned out to be insufficient in marking SPs and NFTs. These were most effectively stained by two different methods. SPs occured with the same frequency in all brain regions examined except for the cerebellum. The highest amount was found with Campbell stain in paraffine sections. Almost only a quarter of these were stained with Congo red, which suggests that a major part of them did not contain any amyloid. NFTs were found almost exclusively in the hippocampus, the highest amount being marked with Gallyas stain in cryosections.

The results regarding the different staining methods match those found by Rosenwald et al. (1993). They examined sections of the hippocampi of 35 clinically unspecific brains and compared different silver stains. They, too, found that most SPs could be stained with Campbell's method. For NFTs, the Gallyas stain proved to be superior to Bielschowsky and Campbell stains, a result which has been confirmed by several other groups (Cullen et al. 1996, Uchihara et al. 2000, Uchihara 2007). Uchihara et al. (2000) for example detected up to 80% more NFTs with Gallyas than with Bodian silver stain.

Regarding the revealing of SPs and NFTs, earlier works showed Bielschowsky stain to be the most sensitive method compared to Bodian, Thioflavin-S und Gallyas (Yamaguchi et al. 1988, Wisniewski et al. 1989, Wilcock et al. 1990). Wisniewski and coworkers (Wisniewski et al. 1989) for example were able to stain up to 2.5 times more SPs with Bielschowsky than with Bodian and 1.5 times more than with Thioflavin-S methods. The authors discuss the good outcome for the Bielschowsky method in relation to the high concentration of silver nitrate (20%) used here. Recently, the study of Mavroudis et al. (2010) has used

the Golgi method to visualize the Purkinje cells and their dendritic changes as well as Nissl, Gallyas, Bielschowsky`s, Methenamine Silver staining and Congo red methods, to analyze the morphological changes of the cerebellum in eight brains (four AD cases and four normal controls). Typical neuritic plaques were not seen in the cerebellar cortex, but diffuse plaques were found in the cerebellum in a smaller proportion than in the prefrontal and parietal cortices of the same cases. Interestingly, significant differences among the silver staining methods was not reported. In the study by Yamamoto and Hirano (Yamaomoto & Hirano 1986), 75% more NFTs was stained with Bielschowsky than with Bodian and Thioflavin-S. Yet a comparison to the present results is hardly possible because none of the five studies mentioned included the Campbell staining method. Lamy and coworkers (Lamy et al. 1989) examined sections of the Brodmann area 22 from the temporal lobe of 25 female patients who were over 75 years of age and varied in their clinical impression between healthy and severely demented (Blessed-Score between 0 and 28 points). Comparing seven different staining methods, the authors found that the modified Bielschowsky stain (after Yamamoto & Hirano 1986) marked the highest amount of SPs. Yet it seems important to consider that the authors did not use the Campbell stain. For staining NFTs the Gallyas stain turned out to be the most sensitive, as it did in the present study. But as the difference between the Gallyas and the modified Bielschowsky methods in staining NFTs was not significant, and moreover, the amount of SPs stained with Bielschowsky correlated best with the severity of dementia, the authors prefer the latter for proof of the neuropathological changes specific for AD. Of opposite opinion are Vallet et al. (1992) who compared the four silver staining methods (Bielschowsky, Campbell, Gallyas and Globus) to a modified version of the Thioflavin-S staining and immunohistochemical techniques. While there was no significant difference among the four silver staining methods, all silver staining methods together detected up to 76% less SPs than the modified Thioflavin-S method. Only in staining NFTs did the Gallyas stain prove equal to Thioflavin-S and immunohistochemical methods. Halliday et al. (1994) also found no significant difference between Bielschowsky, Campbell and Gallyas silver stainings in the number of SPs stained, even though the Bielschowsky method showed a higher correspondance to the immunohistochemical methods used. Cullen et al. (Cullen et al. 1996) examined 10µm paraffin sections from the temporal lobe of four AD brains comparing four silver staining (Campbell, Gallyas, Methenamin and Bielschowsky modified after Garvey) with fluorescence methods (Thioflavin-S-, nickel-peroxidase-staining) and immunohistochemical techniques. The authors found no significant difference between the various methods regarding the quantity of SPs stained. In accordance with the present work they confirm that the commonly used Bielschowsky method is inadequate for staining both SPs and NFTs. Moreover it is very susceptible for artefacts when even minimal methodical irregularities occur. The latter might be an explanation for the occasionally contradictory results regarding the amount of SPs and NFTs detected with Bielschowsky. This probably applies as well to the other silver staining methods. With methodical changes of the Bodian silver stain, Kondo and co-workers (Kondo et al. 1993, 1996) could improve the sensitivity for SPs and NFTs. By e.g. the addition of copper and a mean impregnation time of 16-24 hours they were able to detect up to 70% more SPs and NFTs than with the classical version. Similarly, Litchfield and Nagy (Litchfield & Nagy 2001) were able to improve the Bielschowsky staining in sensitivity and specificity by implementing methodical modifications. By changing the incubation temperature to 5°C, they were able to detect up to 80% more SPs compared to Methenamin stain and immunohistochemical methods with antibodies against Tau- and Abeta4-protein.

In addition to methodical aspects, one explanation for the differences between the staining methods might be that each of them stains different components of SPs and NFTs. As not all the subunits have been formed in every developmental stage, some of the staining methods would not be able to mark all the SPs and NFTs. Congo red for example only stains a low percentage of SPs and NFTs compared to what can be stained with Campbell or Gallyas. Other groups found this for SPs as well as for NFTs (Hansen & Terry 1997, Braak & Braak 1997a, 1997b). Moreover, in the present study Campbell marked by far more SPs and Gallyas more NFTs. This suggests that silver stainings mark a great many SPs and NFTs which do not contain any amyloid. Therefore, after Braak and Braak (Braak & Braak 1997a, 1997b) the Congo red and Thioflavin-S stains are hardly suitable for marking especially extraneuronal neurofibrillar changes, while the silver staining methods of Bodian and Bielschowsky are very susceptible to artefacts and cause misinterpretations of microscope sections by also staining normal fibrillar structures. They recommend Gallyas stain, which has been proved both practical and reliable (Braak et al. 1986, Iqbal et al. 1998).

In summary, comparative studies including our results suggest that each silver staining method has its own “lesion-dependent specificity and within this specificity, its own sensitivity” (Uchihara 2007). For this purpose, stability and reliability of the staining methods are very important for neuropathological examination as well as diagnosis. Unfortunately, all these findings have not become widely accepted, as can be seen in an interview with 104 practising neuropathologists in German-speaking countries (Bancher et al. 1997). More than half of the interviewees (64%) reported using Congo red in AD diagnostics, while only

37% use silver impregnation methods such as Bodian or Bielschowsky and fewer than 10% use the Campbell or Gallyas stain. An interesting point was that 33% of the neuropathologists use immunohistochemical techniques although only 11% believe it will improve their diagnostic judgement. The use of different staining methods which differ in specifity and sensitivity in terms of labelling SPs and NFsT, has been discussed as one possible explanation for the low reliability in neuropathological diagnosis of AD by Dickson (Dickson & Vickers, 2001) with reference to other authors (Hyman 2000, Harding et al. 2000, Jellinger 2009). Interestingly, the interlaboratory comparison study of the BrainNet Europe Consortium,which evaluated the reproducibillity of the assesments of SPs and NFTS and compared the staining between the 15 centers found, that the staining quality and the assesments were most diverse with Bielschowski, followed by Gallyas and immunohistochemical method (Alafuzoff et al. 2006). Furthermore, the same study has shown, that the quantification of SPs was far from to be reliable (Alafuzoff et al. 2008a) and the most uniform staining quality was obtained with the immuno-histochemical methodology (IHC/HPtau) to visualize NFTs and neuropil threats (Alafuzoff et al. 2008b). To improve the reliability of the neuropathological diagnosis of AD, staining methods should be used in a standardized way, as has been previously demanded. As the Bielschowsky stain used commonly in routine diagnostics has proved to be insufficient in marking SPs and NFTs, we suggest the use of Campbell stain for marking SPs and Gallyas stain for marking NFTs.

CONCLUSION

The combination of these silver staining techniques with immunohistochemical methods (Uchichara 2007) and Golgi method (Tsamis et al. 2008) are preferable to improve the correlation between the clinical and neuropathological diagnosis and to contribute to our understanding of dementia diseases.

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