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# Assessment of factors that confound MRI and neuropathological correlation of human postmortem brain tissue

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**Abstract** In spite of considerable technical advance in MRI techniques, the optical resolution of these methods are still limited. Consequently, the delineation of cytoarchitectonic fields based on probabilistic maps and brain volume changes, as well as small-scale changes seen in MRI scans need to be verified by neuronanatomical/neuropathological diagnostic tools. To attend the current interdisciplinary needs of the scientific community, brain banks have to broaden their scope in order to provide high quality tissue suitable for neuroimaging- neuropathology/anatomy correlation studies. The Brain Bank of the Brazilian Aging Brain Research Group (BBBABSG) of the University of Sao Paulo Medical School (USPMS) collaborates with researchers interested in neuroimaging-neuropathological correlation studies providing brains submitted to postmortem MRI in-situ. In this paper we describe and discuss the parameters established by the BBBABSG to select and to handle brains for fine-scale neuroimaging-neuropathological correlation studies, and to exclude inappropriate/unsuitable autopsy brains. We tried to assess the impact of the postmortem time and storage of the corpse on the quality of the MRI scans and to establish fixation protocols that are the most appropriate to these correlation studies. After investigation of a total of 36 brains, postmortem interval and low body temperature proved to be the main factors determining the quality of routine MRI protocols. Perfusion fixation of the brains after autopsy by

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mannitol 20% followed by formalin 20% was the best method for preserving the original brain shape and volume, and for allowing further routine and immunohistochemical staining. Taken to together, these parameters offer a methodological progress in screening and processing of human postmortem tissue in order to guarantee high quality material for unbiased correlation studies and to avoid expenditures by post-imaging analyses and histological processing of brain tissue.

**Keywords** Brain · Banking · Postmortem · MRI · Neuropathology · Confounder

**Abbreviations**

| | |
|---|---|
| AAF | Acetic acid-alcohol-formaldehyde |
| BBBABSG | Brain Bank of the Brazilian Aging Brain Research Group |
| CSF | Cerebrospinal fluid |
| DTI | Diffusion tensor imaging |
| FLAIR | Fluid-attenuated inversion recovery |
| GFAP | Glial fibrillary acidic protein |
| MR | Magnetic resonance |
| MRI | Magnetic resonance imaging |
| PET | Positron emission tomography |
| PMI | Postmortem interval |
| 3D | Tridimensional |
| USMS | University of Sao Paulo Medical School |

## Introduction

Brain tissue provided by specialized human brain banks is used in biochemical, immunohistochemical, autoradiographic, and molecular studies. Previous studies were mainly focused on the elucidation of the neurochemical composition, metabolism, transmitters and receptor distribution of normal brains, and on the changes of these parameters during development, aging and neurodegenerative diseases (Hulette 2003; Ravid et al. 1992; Schmitt et al. 2007). With the advance of neuroimaging methods, several of the aforementioned aspects of the human brain can be assessed in-vivo by structural and functional MRI as well as by PET studies. Imaging studies are likely to result in a scrutinized analysis of human brain tissue, but the findings should be neuropathologically/anatomically verified prior to be used in clinical practice. To attend these novel needs of the scientific community, structured brain banks should broader their scope to provide high quality tissue suitable for neuroimaging- neuropathology/anatomy correlation studies.

Since 2005, the Brain Bank of the Brazilian Aging Brain Research Group (BBBABSG) of the University of Sao Paulo Medical School (USPMS) collaborates with researchers interested in neuroimaging-neuropathological correlation studies including dementias, white matter hyperintensities and epilepsy. As a particularity, the BBBABSG is linked to the MRI section of the USPMS, so the brains can be scanned postmortem in-situ within a short postmortem interval. The advantages of postmortem MRI in-situ is discussed elsewhere, but briefly it permits both to correct the invariable deformations induced by fixation, such as unpredictable swelling, shrinkage, and brain deformation (Challa et al. 2002; Kretschmann et al. 1982; Uylings et al. 1986), and to acquire a signal very similar to the signal acquired in-vivo.

In this paper we present and discuss the parameters established by the BBBABSG to select and to handle brains for neuroimaging-neuropathological correlation studies and to exclude inappropriate/unsuitable autopsy brains. In our experience, short postmortem time is essential for a good gray/white matter contrast in postmortem MRIs. Special attention will be given to avoid or to minimize fixation-induced brain swelling and distortion in order to facilitate fine-scale MRI-neuropathology correlations and to optimize immunohistochemistry and stereological protocols.

## Material and methods

The BBBABSG was created in 2003 by a multidisciplinary group of the University of Sao Paulo Medical School aiming to provide high quality material to a research network unraveling multiple aspects of aging brain processes and related neurodegenerative diseases (Grinberg et al. 2007).

The protocols have been approved by the local ethics committees and a written informed consent form was obtained for every case through relatives of the deceased.

The following questions were addressed:

(a) How the postmortem delay influences the signal acquired on postmortem MRI in-situ?

(b) How storage of the corpses prior to the MRI affects the quality of the signal acquired?
(c) Whether fixation by immersion or by perfusion yields better preservation of brain shape and tissue quality
(d) Which fixative is the most suitable for neuroimaging/neuropathology correlation: formalin 10%, formalin 20%, acetic acid-alcohol-formaldehyde (AAF) or alcohol 70%?

To find out how the postmortem delay and the storage of the corpse affect the signal acquired on postmortem MRI in-situ 12 brains were scanned. Additional 24 brains were subjected to different fixation protocols without being scanned for testing fixation method and fixative effects on brain size, volume and tissue preservation (Table 1).

In the 36 cases, the brains were taken from deceased subjects older than 50 years with no cognitive decline. The subject's clinical and functional statuses were assessed through a reliable informant. This protocol includes a series of semi-structured scales and questionnaires that cover major functional abilities and are validated for assessment with an informant. Additionally, information was collected on demographic characteristics, medication, medical history, cardiovascular risk factors, and family history of dementia. Postmortem interval (PMI) was recorded and CSF pH measured. CSF pH lower than 6.0 was an exclusion criterion in accordance with the BBBABSG's protocol. The full autopsy was performed by the pathologist in charge for this study. For further information see (Grinberg et al. 2007).

Prior to autopsy, the first 12 brain were submitted to MRI sequences within less than 16 h PMI using a 1.5 Tesla GE Scanner at the Department of Radiology, University Sao Paulo, equipped with 33 mT/m gradients and echo planar capability. After autopsy the brains were fixed in-toto by immersion (20 l/brain) or by perfusion (5 l/brain + post-fixation by immersion) using four fixative solutions (Fig. 1). As part of the perfusion protocols the blood was washed out from the vascular system prior to the fixation using either NaCl 0.9% or 20% mannitol + heparin.

**Table 1** Casuistics ($N = 36$)

| Postmortem MRI in-situ (mean age; F:M) | No MRI (mean age; F:M) |
|---|---|
| 12 (65 years; 4:8) | 24 (63.6 years; 8:16) |

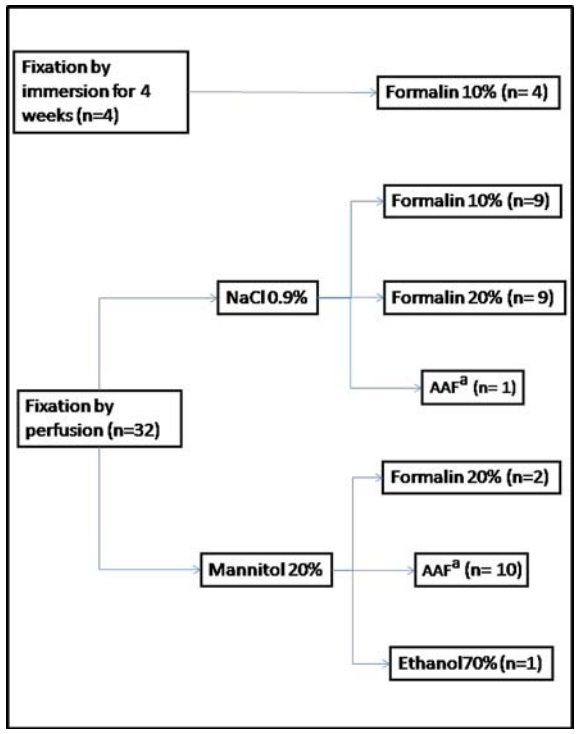


**Fig. 1** Fixation protocols used in this study. ([a] = 80 parts of 80% ethanol, 15 parts of formaldehyde and 5 parts of acetic acid. NaCl = sodium chloride. For post-fixation, the same substances used for initial fixation were adopted)

## Neuroimaging-neuropathology correlation studies

Briefly, the brains were embedded in celloidin, cut in thick serial sections (440 μm) and subjected to a computer-assisted 3D reconstruction. For more details, see (Teipel et al. 2005; Heinsen et al. 2000, 2004).

Tissue preservation was evaluated by visual inspection and histological analyses. Fixed brains exhibiting a reddish or dark green discoloration in their central parts or pronounced tissue softness were discarded. Histological criteria for discarding brains were absence of staining of Nissl substance in larger neurons as well as absence of nucleoli. All brains were subjected to routine and immunohistochemistral staining for testing the effect of the fixative. The differences between final and initial measurements for each fixation protocol were compared by Kruskal-Wallis tests.

## Results

### The impact of postmortem interval and temperature in the signal acquisition by in-situ scanning

PMI ranged from 4 to 16 h (Table 2). Postmortem signal was very similar to the one expected in-vivo (Fig. 2). However, in general when the PMI was longer than 10 h and/or the corpse was stored at 4°C prior the in-situ scan, CSF nulling was absent in the FLAIR acquisition, an increased signal sharply delineated the pial and ependymal interfaces and artifacts in T2-weighted images as well as artifacts on DTI acquisitions were observed (Fig. 3).

In case 1, we obtained postmortem MRI scans in-situ and after formalin fixation of the brain of a 78-year-old man who had been cognitively normal before death. MRI of the fixed brain showed a lower sensitivity to some signal changes as compared with in-situ MRI. For instance, gray matter was hyperintense in relation to the white matter on the short TR sequence acquired on fixed brains, while the opposite was observed on in-situ scans. CSF was hypointense in relation to the gray matter and approximately isointense in relation to the white matter on the long TR/ short TE sequence in-situ, while the fixative was hyperintense in relation to the brain parenchyma on fixed brains. Moreover, due to fixation deformations, the coordinates of the small dimension signal changes were different on in-situ and on fixed brain images. Finally, diffusion weighted MRI of the fixed brains yielded no sufficient signal.

### Effects of fixation method and fixatives on brain deformation and tissue preservation

Immersion fixation had a clear-cut gradient effect on the brains even after prolonged fixation (> 3 months). The more superficial tissue was well fixed, while the results on deep brain structures were unpredictable. On visual inspection, both thalamus and basal ganglia exhibited a reddish discoloration that proved to be autolytic changes after microscopic investigation. For this reason, immersion fixation was discontinued in favor of perfusion fixation that provided a more rapid and uniform penetration of the fixing agent into all parts of the brain. When the blood was washed out by instillation of NaCl 0.9% solution, brain tissue was swelling up to 45% during subsequent formalin perfusion fixation. This swelling was only minimally reversed during the post-fixation. In addition, gray/white matter transition became blurred.

Therefore, NaCl 0.9% was replaced by 250 ml of mannitol 20%. The mannitol protocol resulted in much less alteration of the brain volume (Table 3).

Concerning the fixatives, formalin 10% was not efficient in fixing the deep structures even by perfusion. The brain fixed by perfusion of ethanol 70% did not harden and kept a reddish coloration even 2 months after fixation.

Both perfusion of formalin 20% and AAF resulted in a very good fixation. AAF caused much less alteration of the brain volume (Table 3) and yielded excellent neuron and glial staining using routine histological as well as immunohistochemical methods. However AAF obviously dissolved parts of the myelin sheath causing excessive accumulation of Buscaino bodies. On other hand, perfusion of formalin 20% resulted in an initial swelling reverted after 1 month.

### Immunohistochemistral studies

Small tissue segments were cut out from parallel unstained serial sections, paraffin embedded, cut at 10–12 μm thickness, deparaffinized, pretreated with citrated pH 6.0 in a steamer for 30 min and immunostained with the following antibodies: AT8, GFAP, CD68, neurofilament and myelin basic protein. Both perfusion fixation with AAF and formalin 20% yielded excellent neuron and glial staining using routine histological, as well as immunohistochemical methods (Fig. 4). However in the former, the Buscaino bodies interfered with the results of the myelin staining. Although these bodies can be removed, myelin integrity is important for some correlation studies.

### Future brain banking aspects resulting from our protocol

In this protocol only each second section of an average of 330 serial sections is gallocyanin stained. Therefore, an average of 165 serial sections is still available for further studies requiring well-defined brain regions of cases characterized by a comprehensive clinical assessment and MRI studies.

**Table 2** Demographics, MRI and fixation protocol used in each case

| MRI protocol | Case number | Age (y) | PMI[a] (h) before MRI | Gender | PMI (h) before autopsy | Fixation by | Fixative | Wash-out solution |
|---|---|---|---|---|---|---|---|---|
| In-situ and after fixation | 1 | 78 | 13 | M | 15 | I[b] | 10% formalin | NA |
| In-situ | 2 | 77 | 13 | F | 15 | I[c] | 10% formalin | NA |
| | 3 | 42 | 14 | M | 16 | I | 10% formalin | NA |
| | 4 | 80 | 11 | M | 13 | P | 20% formalin | NaCl 0.9% |
| | 5 | 53 | 7 | M | 9 | P | 20% formalin | NaCl 0.9% |
| | 6 | 58 | 15 | M | 17 | P | 20% formalin | NaCl 0.9% |
| | 7 | 73 | 6 | M | 8 | P | 20% formalin | NaCl 0.9% |
| | 8 | 62 | 7 | M | 9 | P | AAF[d] | NaCl 0.9% |
| | 9 | 62 | 15 | F | 18 | P | AAF | Mannitol 20% |
| | 10 | 71 | 9 | F | 12 | P | AAF | Mannitol 20% |
| | 11 | 61 | | F | | P | AAF | Mannitol 20% |
| | 12 | | 4 | | 7 | P | AAF | Mannitol 20% |
| No MRI | 13 | 81 | NA | F | 17 | P | 10% formalin | NaCl 0.9% |
| | 14 | 58 | NA | M | 14 | P | 20% formalin | NaCl 0.9% |
| | 15 | 57 | NA | M | – | P | 10% formalin | NaCl 0.9% |
| | 16 | 71 | NA | F | 20 | P | 10% formalin | NaCl 0.9% |
| | 17 | 57 | NA | M | – | P | 10% formalin | NaCl 0.9% |
| | 18 | 76 | NA | M | 10 | I | 10% formalin | NaCl 0.9% |
| | 19 | 54 | NA | M | 16 | P | 10% formalin | NaCl 0.9% |
| | 20 | 54 | NA | M | 20 | P | 10% formalin | NaCl 0.9% |
| | 21 | 64 | NA | M | 13 | P | 10% formalin | NaCl 0.9% |
| | 22 | 57 | NA | M | 17 | P | 10% formalin | NaCl 0.9% |
| | 23 | 66 | NA | M | 18 | P | 20% formalin | NaCl 0.9% |
| | 24 | 74 | NA | M | 16 | P | 20% formalin | NaCl 0.9% |
| | 25 | 54 | NA | F | 16 | P | 20% formalin | NaCl 0.9% |
| | 26 | 54 | NA | M | 19 | P | 20% formalin | NaCl 0.9% |
| | 27 | 76 | NA | M | 9 | P | AAF | Mannitol 20% |
| | 28 | 66 | NA | M | 16 | P | AAF | Mannitol 20% |
| | 29 | 87 | NA | F | 12 | P | AAF | Mannitol 20% |
| | 30 | 55 | NA | M | 12 | P | AAF | Mannitol 20% |
| | 31 | 83 | NA | F | 13 | P | AAF | Mannitol 20% |
| | 32 | 54 | NA | M | 13 | P | AAF | Mannitol 20% |
| | 33 | 62 | NA | M | 10 | P | 70% ethanol | Mannitol 20% |
| | 34 | 71 | NA | F | 24 | P | AAF | Mannitol 20% |
| | 35 | 55 | NA | F | 15 | P | Formalin 20% | Mannitol 20% |
| | 36 | 61 | NA | M | 21 | P | Formalin 20% | Mannitol 20% |

[a] Postmortem interval

[b] Immersion fixation

[c] Perfusion fixation

[d] 80 parts of 80% ethanol, 15 parts of formaldehyde and 5 parts of acetic acid

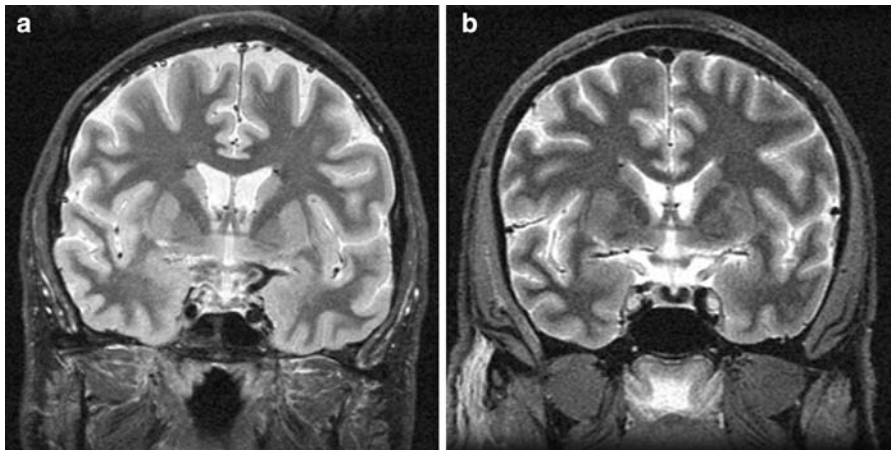


**Fig. 2** Similarity of signal distribution from (**a**) in-vivo and (**b**) postmortem MRI in-situ acquisitions using a proton density weighted spin echo coronal section perpendicular to the posterior-anterior commissure line, the slices are at the level of the anterior commissure plane

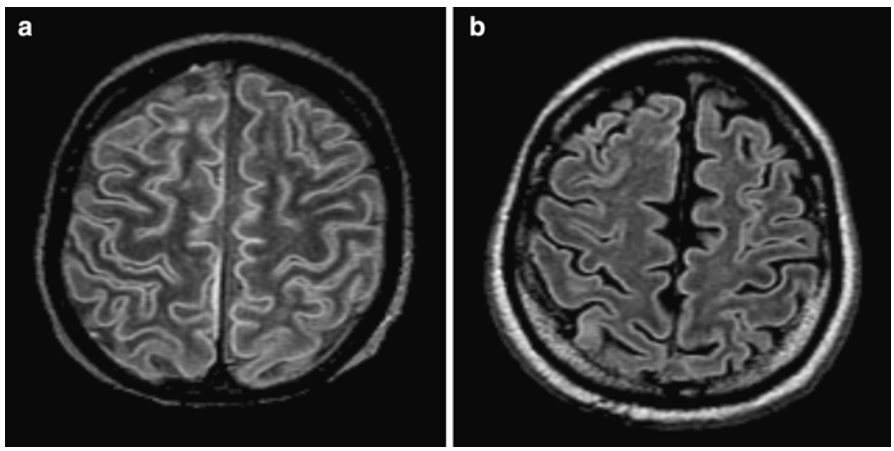


**Fig. 3** FLAIR postmortem MRI acquired in-situ. (**a**) CSF is not suppressed, there is a high signal at the pial surface; (**b**) CSF is fully suppressed, the pial interface is of normal signal

**Table 3** Weight variation after perfusion, by fixative and wash-out solution

| | Number of brains | Wash-out solution | Weight mean variation before and after perfusion—(final weight-initial weight/initial weight) | Std. deviation | *P* (Kruskal-Wallis test) |
|---|---|---|---|---|---|
| 10% formalin | 9 | NaCl 0.9% | 0.35 | ±0.12 | 0.005* |
| | 0 | Mannitol 20% | – | | |
| 20% formalin | 9 | NaCl 0.9% | 0.25 | ±0.08 | |
| | 2 | Mannitol 20% | 0.09 | ±0.01 | |
| AAF[a] | 1 | NaCl 0.9% | 0.20 | – | |
| | 10 | Mannitol 20% | 0.06 | ±0.04 | |

[a] 80 parts of 80% ethanol, 15 parts of formaldehyde and 5 parts of acetic acid

* $P < 0.05$

## Discussion

Selecting brains for neuroimaging-neuropathological correlation studies is an important step to guarantee unbiased results and avoid waste of time and resources. The signal acquired in postmortem MRI in-situ is much more sensitive for brain changes when the postmortem delay is lower than 10 h and the corpse was not cooled down prior to the scan. The type of fixative and the fixation protocol had a great

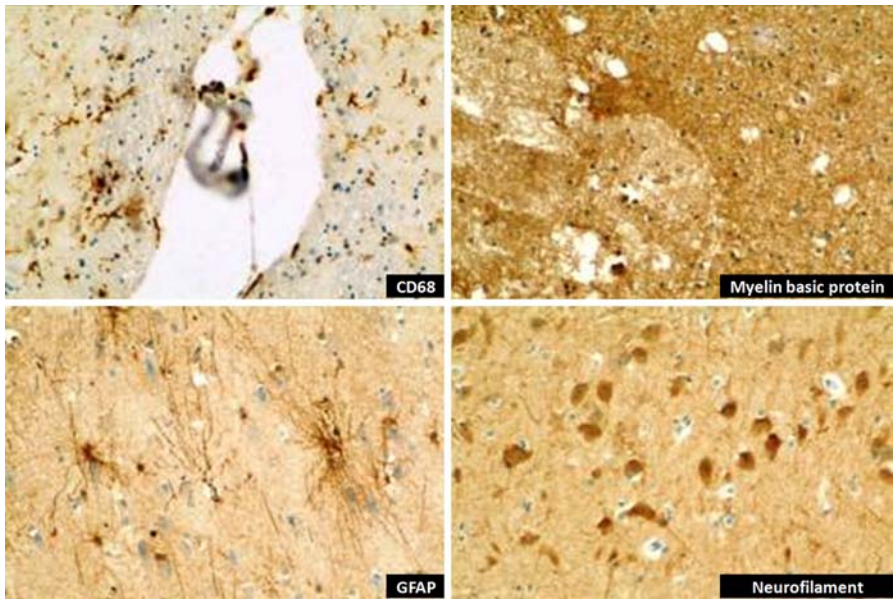


**Fig. 4** Examples of immunohistochemistral results

influence on brain processing and analyses. Perfusion fixation using mannitol 20% followed by formalin 20% proved to be the best method for keeping brain shape and volume more consistent to the original, for preserving the cells and myelin, and for allowing further routine and immunohistochemical staining.

Temperature and water content are major factors determining the intensity of MRI signal. After death, the brain temperature decreases according to a single-exponential function that is influenced by the external temperature (Al-Alousi 2002; Gulyas et al. 2006). This explains why short PMI is important to obtain an postmortem MR signal distribution that is similar to the in-vivo signal. For the same reason, MRI in-situ of corpses stored at 4°C was prone to signal artifacts. Usually, corpses are stored at 4°C before autopsy for delaying autolytic changes, but this procedure is detrimental for the MRI results. Diffusion parameters are also known to be affected by PMI (D'Arceuil and De Crespigny 2007). In our cases, artifacts were detected on DTI when the PMI was over 10 h.

Concerning the water distribution in the tissue, fixation modifies the water content, thus changing the proton density of the tissue, and consequently the intensity of the MRI signal. Moreover, T1 and T2-weighted values are also affected after fixation (Thickman et al. 1983; Fazekas et al. 1993; Grafton et al. 1991; Scarpelli et al. 1994). In our study, variations of signal intensity of fixed brain were considerably reduced compared to the in-situ scan of the same brain (Table 2, case 1).

Due to the size of the human brain, autolytic artifacts on deep brain structures after immersion fixation are frequently reported (Adickes et al. 1997; Bodian 1936; Niggeschulze et al. 1977) and were confirmed in our experiments. Therefore, we discontinued immersion fixation. Perfusion fixation yielded a fast and more homogeneous distribution of the fixative within the brain. However, some caveats should be taken into account.

For preventing hardening of the blood in the vessels, blood must be washed out from the vascular system before perfusion fixation. However, when the blood was washed out using NaCl 0.9%, the brain showed a irreversible swelling, especially after formalin perfusion (Table 3). We do not have a clear explanation for this phenomenon; however, previous authors report identical results after whole brain perfusion. Kato found an average of 20% increase of weight in brains perfused with 10% unbuffered formalin. Even after 5 months of fixation the average weight was 14% greater than the original (1939). Frontera reports similar results (1959). Perhaps it can be explained either because isotonic saline induces the movement of extracellular sodium, and chloride ions and water into neurons provoking swelling of the latter (Van Harreveld and Steiner 1970), or by the rapid penetration of formalin in the form of

methylene glycol into the tissue. (Fox et al. 1985). Other studies report less brain swelling after formalin perfusion. The reason may be the use of buffered formalin (Beach et al. 1987) or the mediosagittal severing of the brain prior to fixation (Waldvogel et al. 2006). Our staining and 3D reconstruction protocols require unbuffered fixative and whole brain fixation, respectively. Therefore we did not test these alternatives. An important point is that the fixated specimen can be re-scanned ex-situ and then we can provide better information regarding the fixation effects in the MR signal and volume—at the same time, this would provide a better matching with the histological images.

It has been suggested to replace saline by a substance that is unable to enter the cells in order to prevent swelling of the latter (Cragg 1980). Mannitol, a sorbitol stereoisomer, works as an osmotic diuretic. Its osmolarity and its capacity to open the blood-brain barrier by transitorily shrinking the endothelial cells make mannitol a better substance for washing the blood out. Mannitol-formalin perfusion induced less swelling, but still 15% over the initial weight. AAF perfusion fixation preceded by mannitol rinsing gave the best results with an average swelling of 6% in our cases (Table 3). Using this protocol, brain deformation was minimal and tissue preservation was excellent.

Bodian suggested the combination of alcohol, acetic acid and formaldehyde (AAF) for perfusion fixation of the brain in 1937 (Bodian 1937). Alcoholic fixatives alone often give uneven staining results. Acetic acid may facilitate axonal staining in accordance with its tendency to promote linkages between proteins and reagents. Although, perfusion fixation with AAF resulted in a better brain shape and volume preservation, ideal for 3D reconstruction and for deformity correction, it also caused the formation of Buscaino bodies in the white matter, that interfere with the staining and assessment of this structure.

Another of our concerns was whether routine and immunohistochemistral staining would work properly in our tissue. Excellent results were achieved for AAF and formalin 20% fixation, when pretreatment and blocking of endogenous peroxidase were applied.

MRI is already used by other brain banks after removal of the brain for diagnosing multiple sclerosis plaques, for instance (Bo et al. 2004). At variance of the former procedure, our approach preserves the integrity of the brain and the neuroimaging-neuropathological correlation is far more accurate. In human brain banking, brains are normally severed mediosagittaly. One hemisphere is fixed and the other is frozen. This approach is also possible using our protocol after the MRI in-situ scanning. However, there are still open questions on morphological and pathological asymmetries of human brain that cannot be assessed by the latter technique.

These parameters for selecting brains still have limitations. At variance with experimental animals, human brains are subject to prolonged agonal factors that cannot be controlled. They vary from case to case and they can confound the final result of the study even if the parameters for case selection are respected. However, following the parameters suggested in this study, we are optimistic to provide high quality tissue for the scientific community in order to avoid expenditures by post-imaging analyses and histological processing of brain tissue.

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