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Preservation of cell structures in a medieval infant brain: A paleohistological, paleogenetic, radiological and physico-chemical study

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ABSTRACT

Cerebral tissues from archaeological human remains are extremely rare findings. Hereby, we report a multidisciplinary study of a unique case of a left cerebral hemisphere from a 13th century AD child, found in north-western France. The cerebral tissue–reduced by ca. 80% of its original weight–had been fixed in formalin since its discovery. However, it fully retained its gross anatomical characteristics such as sulci, and gyri; the frontal, temporal and occipital lobe as well as grey and white matter could be readily recognised. Neuronal remains near the hippocampus area and Nissl bodies from the motor cortex area were observed (Nissl, Klüver–Barrera staining). Also, computed tomography (CT) and magnetic resonance imaging (T1, proton density, ultra short echo time sequences) were feasible. They produced high quality morpho-diagnostic images. Both histological and radiological examinations could not confirm the pathologist's previously suggested diagnosis of cerebral haemorrhage as the cause of death. Reproducible cloned mtDNA sequences were recovered from the skeleton but not from the brain itself. This was most likely due to the combined effect of formaldehyde driven DNA–DNA and/or DNA–protein cross-linking, plus hydrolytic fragmentation of the DNA. The chemical profile of the brain tissue, from gas-chromatography/mass-spectroscopy analysis, suggested adipocerous formation as the main aetiology of the mummification process. The hereby presented child brain is a unique paleo-case of well-preserved neuronal cellular tissue, which is a conditio sine qua non for any subsequent study addressing wider perspectives in neuroscience research, such as the evolution of brain morphology and pathology.

Introduction

Preserved cerebral tissues from archaeological human remains are extremely rare findings; usually soft tissue decomposition and taphonomic conditions preclude the preservation of such organs. Naturally mummified human remains usually do not have well-preserved brains due to the rapid autolysis that occurs within the brain in the immediate post-mortem period (Gerszten and Martífinez, 1995) and because in anthropogenic mummification (e.g. ancient Egyptian mummies), the cerebral tissue was often removed as part of the embalming process (Peck, 1980).

Thus, very few preserved cerebral tissues have been reported in the literature (Table 1). Reports of such specimens dealt with naturally mummified remains from ancient Egypt (Elliot-Smith, 1902; Karlik et al., 2007), northern Chile (Gerszten and Martífinez, 1995), prehistoric Florida (Doran et al., 1986), Korea (Kim et al., 2008) and medieval Denmark (Tkocz et al., 1979). Forensic cases have also been reported (Eklektos et al., 2006; Radanov et al., 1992). Due to the scarcity of the material and the lack of modern techniques, almost no multidisciplinary studies have been undertaken for the examination of such unique findings.

The present study reports macroscopic, microscopic, radiologic, biomolecular and physico-chemical observations of a medieval brain found in north-western France. The aim of this study was to examine the macro- and micromorphological characters of the preserved tissue, to test a previous diagnosis of cerebral haemorrhage and to compare its preservation to other cases of preserved cerebral tissue. Ancient DNA analysis was performed for assessing the potential for DNA retrieval from the formalin-fixed brain sample, while bone samples from the individual were used as a control to check for DNA preservation in the specimen in general. In addition, physico-chemical examinations with gas chromatography-mass spectroscopy (GC-MS)

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Table 1
Summarised data on preserved, historical brain tissues reported in the literature.

Sample type	*N*	Age/sex	Provenience	Dating	Analysis performed					Findings and mummification factor	Reference
					Macroscopic	Histological	Radiological (CT, MRI)	Biomolecular	Physico-chemical		
Skeleton	5	1 adult F[a], 3 adult M[b] and 1 subadult	Windover pond, Florida, USA	7790–8290 BP		x		x		Presence of cellular structures, e.g. neurons and intact DNA; NM[c]	Doran et al., 1986; Pääbo et al., 1998; Royal and Clark, 1960
Mummy	1	adult M	Ötztal Alps, Italy	5200 BP		x				Identification of myelin sheaths; NM	Hess et al., 1998
Mummy	1	16 years old M	Egypt	3250 BP	x	x	x		x	Good macroscopic condition, no cellural evidence; NM due to adipocere	Elliot-Smith, 1902; Karlik et al., 2007; Lewin and Harwood-Nash, 1977
Mummy	15	8 F and 7 M adults	Desert of Northern Chile	3000 BP–1500 AD	x	x				Good macroscopic condition, identification of myelin, pathological findings, no neurons recognised; NM	Gerszten and Martífinez, 1995
Bog body	1	adult M	Cumberland, UK	Bronze or Iron Age	x					NM	Powers, 1960
Skeleton	1	Adult (?)	Droitwich, Worcestershire, UK	Romamo-British					x	NM due to adipocere	Oakley, 1960
Skeleton	56	–	Svendborg, Denmark	1236–1540 AD	x	x		x	x	Presence of neuronal and glial processes (?), presence of Nissl substance in very few cases; NM due to adipocere	Tkocz et al., 1979
Skeleton	1	1.5 year old child	Quimper, France	1250–1275 AD	x	x	x	x	x	Very good macroscopic condition, identification of neurons and Nissl substance, no biomolecular evidence; NM due to adipocere	Papageorgopoulou et al., 2010 (present study)
Mummy	1	Child	Yangju, Korea	15th century AD	x	x	x			Shrunken brain remnant with heavy calcification-like substances over its surface; NM	Kim et al., 2006a,b; Shin et al., 2003a,b
Mummy	1	adult F	Yongin, Korea	15th–16th century AD	x	x	x (no MRI)	x		Good macroscopic condition, identification of myelin sheaths, no cellural and biomolecular evidence; NM	Kim et al., 2008

[a] F = female.
[b] M = male.
[c] NM = natural mummification.

were performed in order to characterise its chemical composition and mode of preservation.

Materials and methods

The left cerebral hemisphere was found inside the skull of an 18-month-old infant; age determination was made based on dental eruption (Tavernier, 1994) and the length of the long bones (Scheuer and Black, 2000). The skeletonised body of the infant (T. 69 US 38-81) dating from the 13th century (1250-1275 AD, dendrochronology) was exhumed from a burial site in Quimper-Bretagne, France (Dietrich et al., 2005). The city of Quimper, with an average height of 50 and 60 m above sea level, is characterised by the Atlantic tides and the confluence of three rivers (Odet, Steir and Jet), which join in the middle of Quimper's centre, not far from the site where the infant's burial was discovered. The presence of a salt and fresh watery, acidic, clay soil environment is believed to be responsible for the excellent state of preservation of this infants' burial (Le Bihan and Villard, 2005).

The body of the child was wrapped in a leather envelope and deposited into a wooden coffin; a pillow was placed under its head. The macroscopic observation of the skull showed the presence of a multifragmentary, non-depressed, mainly horizontal circular fracture involving the frontal and both parietal bones without signs of remodelling. The fracture line formed a nearly complete circular fracture of the cranial vault and did not show any signs of healing (Dietrich et al., 2005). There was no evidence to indicate whether the fracture was peri- or post-mortem.

The specimen has been preserved since its initial recovery in 1998 (Dietrich et al., 2005) in 1% formalin solution. Standard histological analysis of the brain cortex was performed: one sample (3 mm × 3 mm × 2 mm) from the area identified as precentral gyrus (motor area), and a second sample (2 mm × 3 mm × 3 mm) from the area identified as hippocampus and parahippocampal gyrus was performed. The samples were rehydrated in a Ruffer solution (Ruffer, 1921), fixed in 4% formalin and immersed in multiple baths of progressively more concentrated ethanol to dehydrate the tissue; xylene was used as a clearing agent. The tissues were embedded in paraffin wax and sectioned into ultra-thin sections (3–4 μm) using a rotation microtome (Microm HM 325, Adamas Instrumenten, Rhenen, Netherlands). Routine hematoxylin–eosin and Giemsa staining were performed as well as, Nissl (cresyl-violet), Luxol Fast Blue and Klüver–Barrera staining for the detection of neural remnants. Gram, Brown–Brenn, periodic acid-Schiff (PAS) and Warthin–Starry silver stains were performed for detection of micro-organisms and hemosiderin stain (Berliner blue) for positive identification of hemosiderin deposits. The sections were examined under a widefield microscope (Zeiss Axiophot, Carl Zeiss AG, Feldbach, Switzerland).

DNA survival was assessed from both a small sample of the formalin-fixed brain, plus one control, unfixed, bone fragment each from the rib and vertebra, obtained from the skeleton. All pre-extraction/decontamination, extraction and PCR set-up steps were performed in a dedicated ancient DNA laboratory (University of Copenhagen), which is isolated from the modern/post-PCR labs, making it amenable to the analysis of low copy number ancient samples. The lab area is under positive air pressure and incoming air is subject to HEPA filters, which ensure that the air in circulation is clean. In addition, all equipment (e.g. pipettes) and work surfaces are cleaned regularly with bleach and subject to UV treatment to get rid of exogenous DNA. All lab users are required to wear full body suits, facemasks and gloves prior to entering the aDNA lab, and in this particular case gloves were changed often to keep cross-contamination to a minimum.

The DNA extraction from the brain sample was performed using the heat-alkali method proposed by Shi et al (Gilbert et al., 2007; Shi et al., 2004). Specifically, 100 μl of 0.1 M sodium hydroxide (NaOH) was added to the sample and placed on a heating block at 100 °C for 20 min. The mixture was cooled, neutralized using an equimolar solution of hydrochloric acid (HCl) and then purified using the Qiagen DNeasy Blood and Tissue kit (Qiagen, Inc., Valencia, CA). The purified DNA was eluted with 100 μl AE buffer (10 mM Tris–HCl, 0.5 mM EDTA). The DNA extractions from the bone samples utilised a bleach pre-treatment of the powdered material to remove any external sources of contaminant human DNA (Malmström et al., 2005), followed by digestion and purification of DNA using a modified version of the protocol outlined by Yang and colleagues (Yang et al., 1998). The quality of the extracted DNA was assessed using PCR, which targeted a 69 bp region (excluding primers) of the mitochondrial Hypervariable 1 region, using primers that PCR amplify between nucleotide positions 16249 and 16317 of the Cambridge Reference Sequence (Anderson et al., 1981). Reactions were performed in 25 μl volumes, using Platinum Taq Hifidelity (Invitrogen, Carlsbad, CA), which included 2 μl of template DNA, 200 nM of each primer, 2 mM of $MgSO_4$, 1× PCR buffer, 200 mM of dNTPs, 0.4 mg/ml of BSA and reconstituted with water up to the total volume of 25 μl. PCR conditions were as follows: initial denaturing at 94 °C for 4 min, 42 cycles of 15–s denaturing at 94 °C, 20 s annealing at 56 °C, 30-s extension at 72 °C and a final extension at 72 °C. PCR amplified DNA was visualised on 2% agarose gels. Amplified products were cloned using the Topo TA cloning system (Invitrogen) and 8 cloned amplicons were sequenced for each PCR by the commercial Macrogen facility (Macrogen, Seoul, South Korea).

Magnetic resonance imaging (MRI) and computer tomography (CT) were performed. MRI images were obtained using a Siemens Avanto TIM 1.5 Tesla scanner with an 8 channel wrist coil. Sagittal T1, an axial PD (proton density) as well as axial and coronar UTE (ultra-short-echo time) sequences were performed (Imaging protocol: FOV 96 cm^2 and 100 cm^2, slice thickness 0.75 and 0.78 mm, TE 0.07 ms, TR 3.60 and 3.66 ms, flip angle 30°) similar to earlier successful experience using UTE for high quality MRI in dry mummified tissue (Rühli et al., 2007). CT-images were captured using a Philips Brilliance 40 multislice scanner (FOV 250 cm^2; 120 kV, 200 mAs, pitch 0.474, collimation 0.625 mm, high resolution mode). All radiological examinations were performed at the University Clinic Balgrist, Zurich, Switzerland. CT-based surface reconstruction (SL: 0.9 mm; 124 slices) was made using Amira® software 4.1., ZUSE Institute Berlin, Germany.

Gas chromatography-mass spectrometry analysis was completed using a 65 mg tissue sample taken from the cerebral cortex of the brain. Cerebral tissue from a fresh anatomical specimen (Institute of Anatomy, University of Zurich, Institute of Anatomy, University of Zurich, part of Body Donation program) was analysed for comparative purposes. The extraction of the brain tissues was performed according to the method of Makristathis et al. (2002). In brief, the tissue was first washed with water, the internal standard (C16:0-d31) was added and then the lipids were saponified using 7.5 M sodium hydroxide and methanol (1 + 1, v/v). Thereafter, methylation of the fatty acids was performed using 6 M aqueous hydrochloric acid and methanol (5.4 + 4.6, v/v). The fatty acid methyl esters were extracted into n-hexane and t-butylethylether (1 + 1, v/v). The organic extract was cleaned up by adding 0.3 M sodium hydroxide. The cleaned organic phase was then injected into the GC-MS (MD 800, Thermo Scientific, San Jose, USA).

Results

Macroscopic examination

Macroscopic examination of the left cerebral hemisphere showed excellent preservation of the main brain structures. The typical pattern of the lateral surface of the brain with sulci (e.g. sulcus

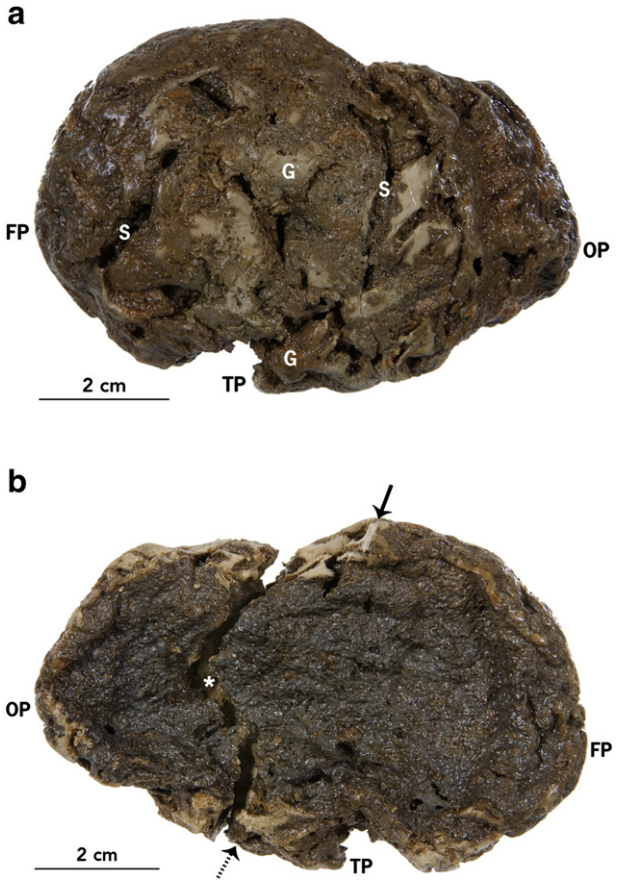

Fig. 1. (a) Lateral aspect of left cerebral hemisphere. The overall anatomical structure is well preserved and structures such as sulci (S) and gyri (G) are clearly identifiable (FP: frontal pole, TP: temporal pole, OP: occipital pole); (b) Medial aspect of left cerebral hemisphere. The medial surface of the tissue is covered by a hard greyish deposit, probably due to weathering (FP: frontal pole, TP: temporal pole, OP: occipital pole, *: post-mortem fracture); black arrows indicate the region of sample extraction for histology.

centralis) and gyri was observed. The frontal, temporal and occipital lobe retained their original shape and could be readily recognised (Figs. 1a, b); the cerebellum and the brain stem were not preserved. The medial side of the left hemisphere showed an unusual concavity without signs of deep cerebral grey matter. No commissural structures are detectable. The ventricular system is not visible by macroscopic inspection (Fig. 1b). Lateral and medial surfaces of the cerebral hemisphere were dark brown in colour with a rough texture that still enclosed remnants of pia mater on the outer surface. Scratching on the surface revealed greyish brain tissue, which macroscopically could not be separated into grey or white matter. The texture of the brain was harder and compacter when compared against fresh or formalin fixated cerebral tissue. The weight of the left hemisphere was 50 grams and it measured 8.8 cm in length and 5.5 cm in width.

Microscopic and biomolecular examination

Microscopic examination showed remarkable morphological details. The cortex stained more intensively. Grey matter (GM) could be clearly differentiated from white matter (WM), although the six (I–VI) characteristic cellular layers of the grey substance e.g. on the motor cortex could no longer be differentiated. Blood vessels, glia and Nissl bodies could be readily identified (Fig. 2) yet neurons and large pyramidal cells were not visible in the grey matter. However, many observed dark staining structures may have

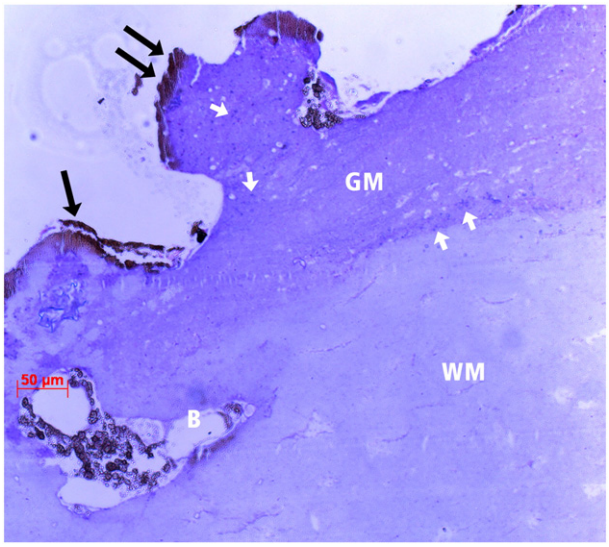

Fig. 2. Micrograph of a Nissl (crystal-violet) stained section of preserved tissue from the motor area (cerebral cortex). Grey (GM) and white (WM) matter is clearly defined, remnants of Nissl bodies (white arrows) and a blood vessel (B) are also observed. Deposits of hemosiderin on the upper surface of the cortex are present (black arrows).

represented nuclei remnants of these cells (Fig. 2). Large neurons were present near the hippocampus area (Fig. 3). The cells had retained their original shape as well as the dendrites, although they were not intensively stained and apparently no nuclei were preserved. However, a structure corresponding to the typical hippocampal organisation, was not identifiable. Hemosiderin deposits were present on the outer surface of the cortex. The positive identification was performed by the use of hemosiderin stain (Berliner blue) (Fig. 4). Due to the loss of clear territorial borders and cortical layering it was not possible to attribute the accumulation of hemosiderin to a specific compartment (Fig. 2). Post-mortem contamination by micro-organisms was also observed (Fig. 5a) on the histological sections from both the motor cortex and the hippocampus area.

Reproducible cloned mtDNA sequences were recovered from the rib DNA extracts, confirming DNA survival in the non-fixed skeletal parts of the body. Unfortunately, the vertebra did not yield any

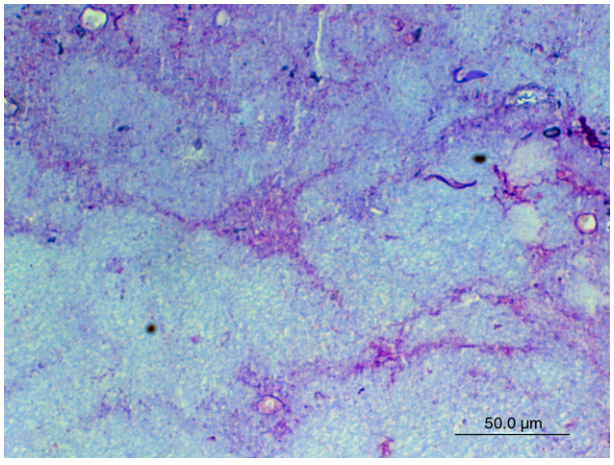

Fig. 3. Micrograph of a Nissl (crystal-violet) stained section of preserved tissue from the hippocampus area. Remnant of a pyramidal cell (neuron) is readily identifiable (arrows).

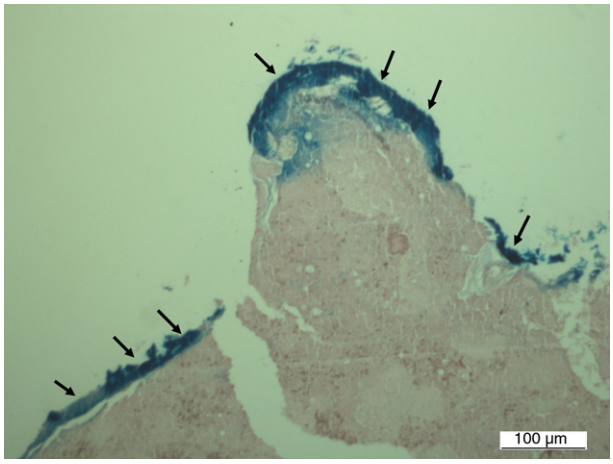

Fig. 4. Micrograph of a hemosiderin (Berliner blue) stained section of preserved tissue from the motor area (cerebral cortex). The remnants of hemosiderin on the upper surface of the cortex are positive identified (black arrows).

amplification products. The sequence recovered from the rib differed by single nucleotide polymorphisms at nucleotide positions 16269 (A-G) and 16298 (T-C) with regards to the Cambridge Reference Sequence. In contrast to the rib, no endogenous DNA could be recovered from the brain.

Radiologic examination

CT and MR imaging verified that the tissue was well preserved, especially the MRI could be successfully performed and provided detail examination of all anatomical features (Figs. 6, 7). Sulci, including sulcus centralis and gyri, corpus callosum, were easily identifiable as well as GM and WM (Fig. 6). No signs of a cerebral haemorrhage (altered signal intensity, structural inhomogeneities) could be identified.

The presence of an almost even distribution of GM in comparison to WM could be detected on CT and MRI images. Nevertheless, the calculation of the exact volumetric distribution of the GM and the WM was not possible due to the mummification and the reduction in weight and volume.

From the methodological point of view, it should be noted that MRI images were successfully obtained from the formalin-fixed ancient brain in the T1 weighted sequence, in the PD sequence as well as in the UTE sequence, respectively.

Physico-chemical examination

The fatty acid composition of the ancient cerebral specimen and the modern brain tissue were evaluated. The main fatty acids detected in the infant and the modern brain were ginkgolic acid (C13:0), myristic acid (C14:0), pentadecanoic acid (C15:0), palmitic acid (C16:0), heptadecanoic acid (C17:0), oleic acid (C18:1), stearic acid (C18:0) and 10-hydroxystearic acid (C18:0 10OH). However, ginkgolic acid and 10-hydroxy stearic acid were only found in the mummified specimen and eicosenoic acid (C20:1) only in the modern specimen (Table 2).

Most of the fatty acids were more abundant in the ancient specimen except for oleic acid and stearic acid, which were more abundant in the modern brain tissue. The composition of the ancient tissue was high in saturated fatty acids and low in unsaturated fatty acids, whereas the modern specimen exhibited almost an equal distribution between unsaturated and saturated fatty acids. In the ancient brain tissue, palmitic acid represented the dominant saturated fatty acid followed by oleic and myristic acid respectively, whereas in the modern tissue stearic acid is more abundant followed by palmitic acid. Myristic acid was present in very low concentrations in the modern specimen. Among the

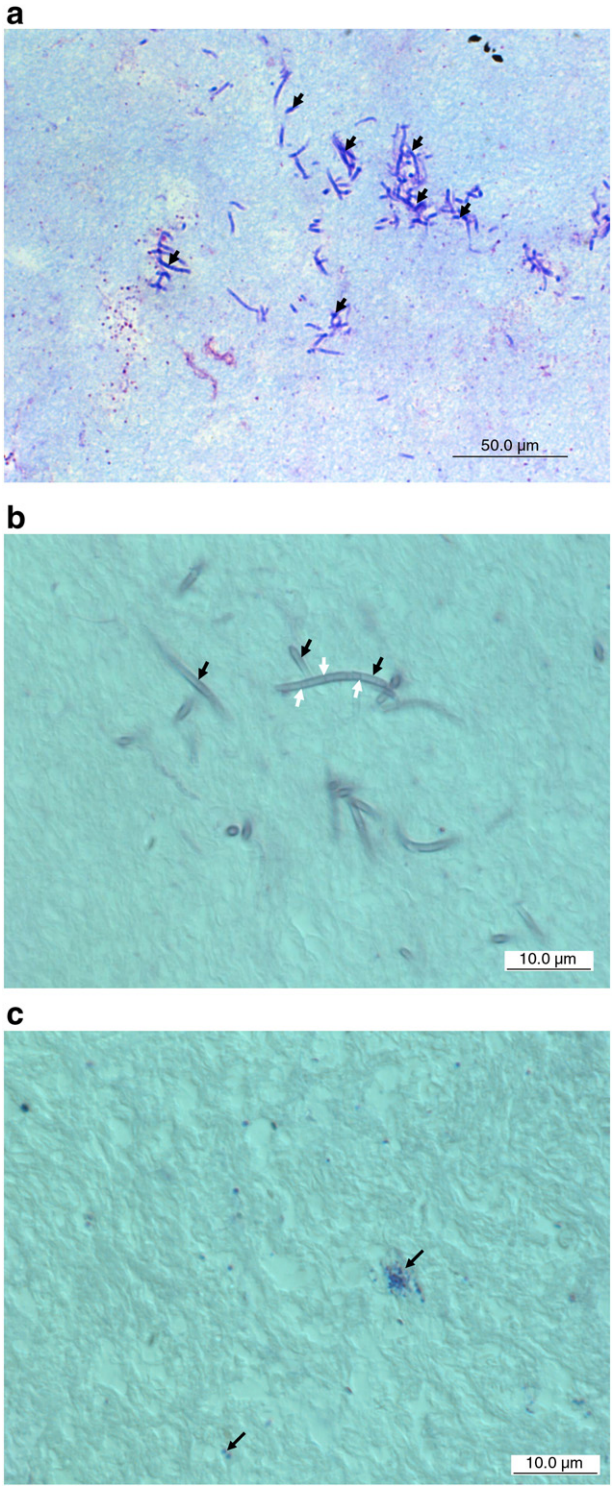

Fig. 5. (a) Micrograph of a Nissl (crystal-violet) stained section of preserved tissue from the hippocampus area. Micro-organisms (black arrows) due to post-mortem contamination are observed. (b) Fungal hyphae stained with periodic acid-Schiff (PAS) stain, the hyphal wall (black arrows) and the septum (white arrows) can been observed. (c) Brown-Brenn gram staining of spores present in the brain tissue; mature spores stain gram-positive (violet) (black arrows).

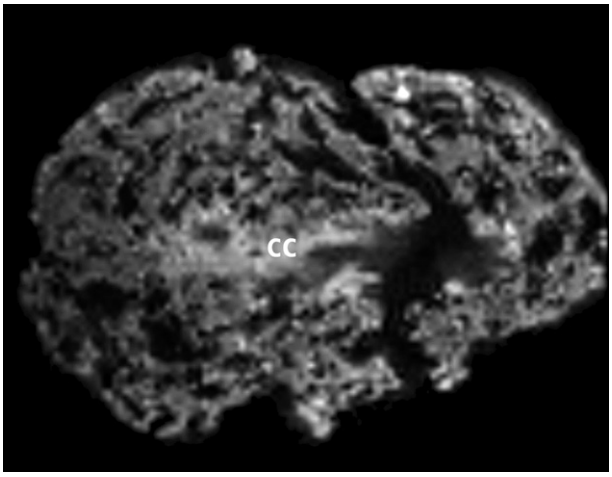

Fig. 6. MRI; anatomical features e.g. sulci, gyri and corpus callosum (CC) are visible.

unsaturated fatty acids, oleic acid was the most abundant in both specimens.

Discussion

Anatomical and biomolecular findings

Cell structures in ancient or modern post-mortem preserved cerebral tissues have not been identified in histological sections until now (Bohnert et al., 1998; Eklektos et al., 2006; Karlik et al., 2007; Kim et al., 2008; Radanov et al., 1992; Tkocz et al., 1979). Usually the basic anatomical features (e.g. lobes) in the preserved cerebral tissues were not well defined (Kim et al., 2008; Lewin and Harwood-Nash, 1977). The left cerebral hemisphere reported here, shows excellent macroscopic detail with good preserved and readily recognisable occipital, frontal and temporal lobes as well as gyri and sulci.

Compared to similar specimens from prehistoric USA (Doran et al., 1986), ancient Egypt (Elliot-Smith, 1902; Karlik et al., 2007; Lewin and Harwood-Nash, 1977), a Romano-British site in Great Britain (Oakley, 1960), medieval Denmark (Tkocz et al., 1979) and modern adipocerous bodies (Bohnert et al., 1998; Eklektos et al., 2006; Radanov et al., 1992), the present specimen is even better preserved. Cell structures like Nissl bodies have been observed in the medieval cerebral tissues from Denmark (Tkocz et al., 1979), prehistoric Florida (Doran et al., 1986) and from South Africa (Eklektos et al., 2006), while in all other cases preservation was poor especially at the cellular level. The neurons remains described by Doran and colleagues (1986) exhibit the same characteristics as in the present case. Unfortunately, they did not provide figures of the neurons that would allow a more exact comparison. Despite the good overall preservation we could not identify a hippocampus formation. We made the greatest possible effort to minimize the destruction of the specimen by restricting the sample sizes. Apart from the missing hippocampus formation, due to the small sample size and the lack of specific anatomical landmarks for macroscopical orientation, the complete loss of the highly exposed medial part of the temporal lobe, which included the hippocampus, might be a plausible reason for the lack of evidence concerning this region.

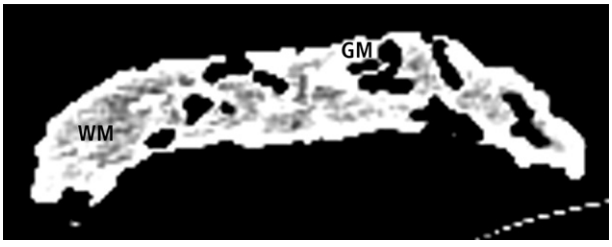

Fig. 7. CT image, dorsal view; grey (GM) and white (WM) matter as well as basic anatomic features are visible.

Table 2
Relative concentrations of the fatty acids in the ancient and modern brain tissue, respectively.

	Ancient brain tissue	Modern brain tissue
C13:0	1.4%	n.d.[a]
C14:0	11.6%	0.4%
C15:0	6.9%	0.2%
C16:0	44.1%	23.3%
C17:0	3.1%	0.3%
C18:1	19.1%	45.8%
C18:0	8.0%	27.5%
C18:0 10OH	6.0%	n.d.[a]
C20:1	n.d.[a]	2.6%

[a] n.d.: not detected.

Post-mortem contamination by micro-organisms similar to our case has also been reported by Gerstzen and Martifinez (1995) and Eklektos and colleagues (2006). In the case of Gerstzen and Martifinez (1995) the micro-organisms were identified to be a fungal species such as Microsporidia, in the second case, identification was not possible (Eklektos et al., 2006). Microsporidia may be also present in the paraffin-embedded brain tissue sections of the brain. Gram stain and Brown-Brenn stain have been used to provide reliable results for their identification (Garcia, 2002). Mature spores stain gram-positive (violet) and immature spores stain red. Warthin–Starry stain and PAS stain was also positive for the presence of microsporidia. It should be noted however that these staining methods are not specific enough to prove the presence of microsporidia (Garcia, 2002). The exact identification of the micro-organisms is difficult and multiple diagnostic methods from many body tissues and fluids may be required. Thus no further analysis was possible. We could positive identify only fungi like structures such as mature spores and fungal hyphae and under high magnification (oil immersion) the hyphal wall and the septum (cross-walls along the hyphae).

The weight of the telecephalon (both hemispheres, without midbrain, brain stem and cerebellum) of an 18-month child ranges from 850 to 950 g (Dobbing and Sands, 1973) and the whole brain ranges from 940 to 970 ± 16 g (Dekaban and Sadowsky, 1978). We estimate that the weight of the left cerebral hemisphere had been reduced by about 80%. This reduction is usual in mummified remains (Aturaliya and Lukasewycz, 1999) and has been noticed in all cases of mummified cerebral tissues (e.g. Kim et al., 2008; Radanov et al., 1992; Eklektos et al., 2006). The infant brain was preserved in formalin. A dry conserved brain would be lighter, so direct comparison with other cases of dry conserved brains was not feasible.

The radiological findings have verified the good macroscopic and microscopic picture. Our CT findings match the above mentioned studies in that we also readily observed WM and GM, the sulci, the gyri and the corpus callosum. Until now, MRI imaging of a historic specimen has only by been performed by Karlik et al. (2007), however, our brain revealed a much better image quality due to its formalin-fixation. The acquired images in our case show a better signal intensity probably due to the moist nature of the material.

Both histological and radiological examination could not confirm a previously suggested diagnosis of cerebral haemorrhage. The deposits of hemosiderin on the outer surface of the cortex may indicate that the child had subdural bleeding, due to a skull fracture, but we have no evidence to suggest that this was the cause of death. The presence of hemosiderin suggests that the bleeding occurred at least several days before the death of the infant.

Both MRI and CT were comparable in terms of spatial resolution in the present case. The surprisingly high degree in variety of MR signals in the present case allowed a notable differentiation of main brain compartments such as corpus callosum. However, one may expect a major haemorrhage to be possibly better differentiable by MRI, whereas CT may show probable calcifications particularly well.

Evaluation of brain development, in terms of myelination progress and GM and WM volume changes, permit the study of developmental outcomes to changes in specific anatomic structures (Tzarouchi et al., 2009). Although an exact assessment of the GM and WM was not possible due to the weight and volume shrinkage, it was noted that there was a practically equal proportion of WM to GM. This is normal considering the young age of the individual and the fact that myelination processes are more pronounced during the first 2 years of life. Several studies have suggested that cortical GM and WM volumes represent dynamic changes throughout childhood (Matsuzawa et al., 2001). GM volume has been reported to peak at age 4 and then decreases throughout ones lifetime (Pfefferbaum et al., 1994), while cortical WM volume increases during the first decade of life and remains stable thereafter (Matsuzawa et al., 2001).

Reproducible cloned mtDNA sequences were recovered from the DNA extractions from the rib but not from the brain tissue. This result is consistent with other studies on the quality of DNA extracted from biological tissues that have undergone long-term fixation in formalin, and is likely explained by the combined effect of formaldehyde driven DNA-DNA and/or DNA-protein crosslinking, plus hydrolytic fragmentation of the DNA (Gilbert et al., 2007).

The vertebra did not yield amplification products, possibly due to the poor preservation of nucleic acids in this sample. The sequence recovered from the rib differed by single nucleotide polymorphisms at nucleotide positions 16269 (A-G) and 16298 (T-C) when compared to the Cambridge Reference Sequence. Although this fragment is not long enough to definitively assign a mitochondrial haplogroup to the specimen, it is consistent with a sample of European origin, in particular haplogroup V. The focus of the current study deals solely with the preservation status of the brain and the potential for DNA retrieval from the formalin-fixed brain sample. It might be of future interest to investigate further the child's haplogroup and sex, if preservation allows it, which is however beyond the limits of the present study.

Mummification process

The mummification observed represents a unique case of naturally occurring preservation of human brain tissue in the absence of preservation of other soft tissues. The skeleton, as already described, was found in an area characterised by a fresh-water and salt-water, acidic environment (Le Bihan and Villard, 2005). Since such environments favour adipocere formation (Forbes et al., 2005a,b), physico-chemical analysis was undertaken in order to verify this hypothesis. Adipocere formation is characterised by the hydrolysis of fatty tissue into a mixture of predominantly saturated fatty acids, turning the body fat into a soap-like substance, which has the ability to slow down or inhibit decomposition (Takatori, 1996, 2001).

The fatty acid profile of the brain tissue matches the changes observed in adipocere formation experiments (Forbes et al., 2005a,b,c), in ancient (Varmuza et al., 2005) and in modern adipocerous cases (Vane and Trick, 2005). In all cases, including the present one, the high concentration of saturated fatty acids and especially the high frequency of stearic acid, 10-hydroxy stearic acid, myristic acid and palmitic acid, were identified as the main constituents characteristic of adipocere (Forbes et al., 2005a,b,c; Makristathis et al., 2002; Takatori, 1996, 2001; Varmuza et al., 2005).

Specifically, the high concentration of 10-hydroxy stearic acid, a substance generated post-mortem and often observed in the adipocere of anthropological and forensic specimens (Varmuza et al., 2005), underlines the process of adipocere as the mummification agent. Palmitic acid, the most predominant fatty acid among the saturated fatty acids in adipocerous cases, was also found in similar high concentrations in this infant brain. Stearic acid is usually the second most abundant fatty acid in adipocere formation, followed by myristic acid (Varmuza et al., 2005). In the present case, however, myristic acid concentration was higher than stearic acid. Similar values have been reported in adipocerous bodies found in mountainous lakes and glaciers (Varmuza et al., 2005).

The pH, temperature and soil type also play an important role in the decomposition of tissues. A damp, warm, anaerobic environment has been suggested to be optimal for the formation of adipocere (Forbes et al., 2005b). A cold, acidic environment, as described in the present case, is sufficient for adipocere formation, but the preventative nature of such an environment decreases bacterial activity and slows down the rate of decomposition and/or conversion to adipocere (Forbes et al., 2005b). Clay soil produces similar results of reduced adipocere formation (Forbes et al., 2005a). This inhibition was obvious in the present case by the lack of a soap-like substance around the skeleton and the excess of adipocere tissue in the brain, which has been seen in similar cases (Bohnert et al., 1998).

Additional remarks concerning the cold, acidic environment and the clay soil type, is that it could induce higher concentrations of 10-hydroxy stearic acid and oleic acid. Yan and colleagues (2001) observed high 10-hydroxyl stearic acid in the early stages of decomposition and Forbes and colleagues (2005b) attribute the high frequency of 10-hydroxy stearic acid from samples buried in cold, acid environment to the retarded rate of decomposition. High concentrations of oleic acid is usually characteristic of non-advanced cases of adipocere and is observed in experimental cases from cold, acidic environments and clay soil types (Forbes et al., 2005a,b). High oleic acid concentrations have also been observed in ancient, e.g. in the Neolithic South Tyrolean Iceman and forensic cases found in glaciers and mountainous environments (Varmuza et al., 2005).

The burial method of the infant (e.g. coffin, body shrouding) should also be taken into account. The body of the infant was wrapped in a leather envelope, which was buried in a wooden coffin. The displacement of the bones in the coffin suggest the lack of an additional shroud or cloth (Langlois and Gallien, 2008). Experiments have shown that coffins retard and clothing enhances adipocere formation (Forbes et al., 2005c). Since there are no reports on the effect of leather on the formation of adipocere and the presence of linen/cotton clothing was not verified, we assume that the presence of the wooden coffin and the leather envelope has not enhanced the formation of adipocere.

Based on this evidence it can be suggested that continuous water immersion in the grave environment may have contributed to the adipocere formation and the preservation of the brain tissue. Water immersion is a standard agent for adipocere formation (Bohnert et al., 1998; Fiedler and Graw, 2003). Recent studies have proved that adipocere can be formed in the absence of a water environment (Forbes et al., 2005a,b; Yan et al., 2001), however, for this specific case, a humid burial environment was verified by the excavators. Other factors, such as the acidic, cold and clay soil environment, as well as the presence of the leather envelope and the coffin did not appear to have any additional effect in the adipocere formation.

The question remains: why was brain tissue, which is the tissue most prone to post-mortem dissolution (Aufderheide, 2003), the only tissue preserved? Hess and colleagues (1998) suggest that the myelin sheaths and the collagen fibres show remarkable resistance to tissue disintegration and therefore may be preserved even up to the final stages of decomposition. This thesis has been adopted also by other researchers (Kim et al., 2008) reporting cases of human brain preservation under unknown conditions.

Similar cases of ancient brain tissue preservation due to adipocere formation have been reported in a site near Moscow, in which two cerebriform fossilized human remains were preserved together with parts of a woolly mammoth (Walter, 1929), in a medieval Danish monastery (Tkocz et al., 1979), in mineral springs in Florida, USA (Doran et al., 1986; Pääbo et al., 1998; Royal and Clark, 1960), in Romano-British skeletal material from Britain (Oakley, 1960), in a Bronze or Iron Age bog body from Britain (Powers, 1960) and in a burial from Sudan (Klohn et al., 1988). Physico-chemical comparison to these specimens was not possible due to lack of available data.

Conclusions

We report a multidisciplinary paleopathological study of a unique case of a very well-preserved medieval infant brain. The cerebral tissue retained its gross anatomical characteristics such as sulci, and gyri. Neuronal remains near the Hippocampus region and Nissl bodies from the cerebral cortex could be identified. Grey and white mater could be readily observed both in the histological sections and the CT and MR imaging. Both the histological and the radiological examination could not confirm the previous suggested diagnosis of a cerebral haemorrhage as the cause of death. Genetic material was not preserved in this case, most likely due to the long duration of formalin fixation of the specimen. The chemical profile of the specimen suggested a reduced formation of adipocere that lead to the excellent preservation of the tissue.

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