Forensic Sci Med Pathol (2015) 11:395–404
DOI 10.1007/s12024-015-9691-7

ORIGINAL ARTICLE

Optical clearing of the dura mater using glycerol: a reversible process to aid the post-mortem investigation of infant head injury

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Accepted: 20 June 2015 / Published online: 1 July 2015
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Abstract
Purpose In cases of suspected abusive head trauma, a thorough and systematic study of the cranium and its contents is essential, preferably using the best available methods for observing the brain and its coverings. Building upon recent developments in skull bone removal techniques in infant autopsies, we have assessed the use of two optical clearing agents (OCAs), glycerol and mannitol, on pediatric dura mater in an attempt to increase the transparency of this tissue and thereby enhance the post-mortem assessment of infant head injuries, particularly subdural hematomas.
Methods Extracorporeal testing revealed glycerol to be the more effective OCA. Therefore, in situ investigations were commenced using glycerol during 33 pediatric post-mortem examinations.
Results An increase in the transparency of the dura was observed in 32 of the 33 cases, within 1 min of application of the OCA. In a 2 year old with cerebral palsy, only partial optical clearance of the dura was seen, most likely due to a significantly atrophic brain, prominent gelatinous leptomeninges, and abnormally thickened dura. This technique allowed for detection of minimal amounts of subdural bleeding over the convexities, before dissection of the dura, avoiding post-mortem blood spillage from artifactually disrupted bridging veins. Optical clearing of the dura aided in the evaluation of patterns of subdural hemorrhage in three cases of non-accidental head injury, three cases of peri-natal head injury and one case of overlaying, apparently resulting in minor crush injury to the head.
Conclusions We have demonstrated that glycerol is an effective and easy-to-use OCA to effect the readily reversible optical clearing of human infant calvarial dura at autopsy.

Keywords Post-mortem · Infant · Head injury · Dura mater · Glycerol · Optical clearing

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Introduction

The dura mater is a typical fibrous connective tissue [1], being relatively thick and dense [2]. It consists of an outer endosteal layer and an inner meningeal layer. The composition and increasing thickness of the dura varies with age, making this membrane difficult to see through when observing the brain with the naked eye during a post-mortem examination. In a relatively recent study, the median infant dural thickness by age was found to be 485 μm at 0–90 days, 663 μm at 91–180 days, 607 μm at 181–270 days, and 469 μm at 271–365 days [3]. There is a predominance of collagen fibers (over 90 % of the dura's thickness [4]), especially in the endosteal layer, which are highly corrugated, and layered on top of each other [5]. The collagen fibers are arranged in parallel bundles with differing orientations, varying from highly aligned to apparently random and arranged in lamellae [6].

Light-based imaging systems, such as optical coherence tomography (OCT), fluorescence microscopy, and confocal

imaging, require light to penetrate through biological tissue. However, these tissues highly scatter light, decreasing the effective depth of imaging [7]. Optical clearing agents (OCAs) are used to aid such imaging systems by decreasing the amount of scattered source light, increasing tissue transparency, and increasing the depth of imaging.

We postulated that the application of an OCA could be used to increase the transparency of the dura mater during post-mortem examination of head injuries, aiding in the assessment of areas of bleeding associated with this membrane. We present the translation of a method often used for increasing imaging depth of tissues (i.e., the use of glycerol as an OCA), to aid in the visualization of the brain surface and pathologies associated with head injury through the intact infant dura in 33 pediatric autopsies.

Materials and methods

Case selection

Cases were identified as part of a regional pediatric/perinatal autopsy service at Leicester Royal Infirmary between September 2013 and May 2015. For all cases, tissue that was retained at autopsy for research purposes had appropriate parental consent.

Extracorporeal testing of optical clearing of the dura mater

Extracorporeal testing of optical clearance using glycerol was undertaken on samples of dura mater from three pediatric autopsies (a 6 week old male, a 9 week old male, and a 3 day old male) and one 36 week old stillborn fetus. An additional extracorporeal experiment was also performed on the dura from a 7 week old female to compare mannitol and glycerol (100 %). As part of the post-mortem examination of the brain and meninges, the dura mater was removed from the cadavers using standard autopsy technique by a specialist consultant pediatric pathologist. Samples of parietal dura approximately 6 cm × 6 cm in size were immersed in 100 % v/v glycerol or 50 % v/v glycerol/distilled water for 10 min. Digital photographs of the same tissue samples were taken against a patterned background adapted from a USAF 1951 resolution test target for evaluation of optical clearing using a Canon EOS 500D camera with an attached Canon EFS 17-85MM lens set to automatic focus and exposure, before and after immersion in glycerol. Within the patterned background, three horizontal and three vertical bars constitute an element block and there are six element blocks in a group. There are four groups in total (number −2 to 1). As the group and element number increases, the size of the bars and spaces between the bars decreases (Fig. 1). Photographs were dimensionally normalized in Photoshop Elements (version 10) to ensure that the target pattern backgrounds were reproduced to the same scale. Eight independent observers with optometrically corrected vision were then asked to assess each photograph (at a size of 17.5 cm × 20.5 cm) to determine the smallest group and element number that they could visually resolve by separation of the vertical and horizontal bars of the element blocks viewed through the dural tissue. Photographs were viewed from a distance of 60 cm on an Apple Mac Pro 2 × 3.06 GHz 6-core Intel Xeon with an Apple LED cinema display LCD monitor with a 27″ screen and a resolution of 2560 × 1440. The element blocks were assigned numbers on a linear scale from 1 to 24, with one representing the smallest element block, to allow arithmetic means and standard deviations (SD) to be calculated for each photograph. Image J image analysis software (National Institutes of Health, USA) was used to determine the pixel dimensions of the spacing's of the element bars in order to calculate the percentage change in size of element blocks the participants could resolve before and after immersion of the dura in glycerol.

In a further extracorporeal experiment, similar sized samples of cadaveric parietal dura were immersed in three different concentrations of mannitol (80, 160, 320 g/L) as a comparison to a sample of dura from the same subject immersed in 100 % glycerol.

In situ optical clearing of the dura mater

Following successful results of the extracorporeal testing, the use of glycerol (100 %) as an OCA was adopted routinely into local pediatric autopsy practice for the

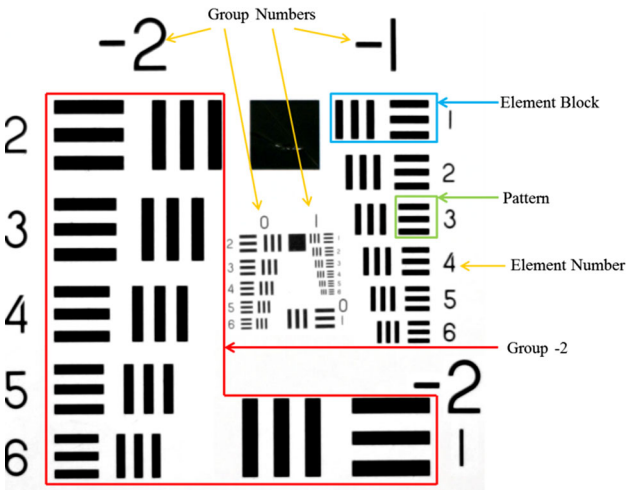

Fig. 1 The group and element numbers on a USAF 1951 resolution test target

evaluation of subdural brain pathologies. Glycerol was applied to the dura mater in situ as part of the post-mortem examination of the brain in 33 neonatal, infant, and early childhood autopsies. Included were 20 males and 13 females, with an age range from 1 day to 3 years and a median age of 14 weeks. There were three cases of non-accidental head injury (NAHI), and three cases of perinatal head injury. Initially, opportunistically selected cases were used to evaluate the effects of the glycerol on the dura. Later in the series, cases were selected where the application of glycerol was considered to enhance the viewing of specific brain pathologies.

The infant calvarial bones were removed using a method discussed in an earlier publication [8] which leaves the dura mater intact. The dura was then rinsed with tap water to remove surface blood and bone debris. The surface was blotted dry with a towel. Glycerol (100 %) was then painted onto the surface of the dura using a soft brush. Depending on the thickness of the dura in each case, 1–3 applications of glycerol were applied to the dura over a period of 1–2 min.

Histology

Samples of dura from the extracorporeal experiments that were untreated and treated with 100 % glycerol were processed for routine histology in a CPA accredited hospital histopathology laboratory by standard methods. Standard Hematoxylin and Eosin (H&E) sections were analyzed by a consultant pediatric pathologist using an Olympus BX45 clinical microscope equipped with planar objectives. Masson's trichrome, Perls' stain (for iron), Glial fibrillary acidic protein (GFAP), CD68, and beta amyloid precursor protein (β-APP) staining were also undertaken. Digital micrographs were taken using Jenoptik ProgRes CapturePro v 2.8.8 software.

Results

Extracorporeal optical clearing

No difference could be detected with the naked eye when comparing the transparency of the dura before and after the tissue was treated with the three different concentrations of mannitol. In comparison, an obvious change in a parallel sample of dura immersed in glycerol was associated with an increase in the transparency and accompanying shrinkage and stiffening of the tissue. A very noticeable increase in the transparency of the dural tissue was seen during the extracorporeal experiments with both 50 and 100 % glycerol (Fig. 2a–d). Against the USAF 1951 resolution test target pattern, the mean number on the linear scale seen by the independent observers for the section of dura before immersion in 100 % glycerol was 11.50 with a SD of 2.20, corresponding approximately to element 1 in group 0. After immersion the mean number on the linear scale was 6.75 with a SD of 0.89, corresponding approximately to element 6 in group 0. The mean number on the linear scale seen by the independent observers for the section of dura before immersion in 50 % glycerol was 16.13 with a SD of 2.30, corresponding approximately to element 3 in group -1. After immersion, the mean number on the linear scale was 7.00 with a SD of 1.93, approximately corresponding to element 6 in group 0. The increase in transparency for the dural tissue before and after immersion in 100 and 50 % glycerol reduced the size of the element block the observers could resolve by 5 and 9 element blocks respectively. Using the pixel data (Table 1), this reduction in resolvable target size was calculated to be a percentage decrease of 60.67 and 73.59 % for the 100 and 50 % glycerol experiments respectively.

Sections of dural tissue immersed in 50 and 100 % glycerol resolved to the same element block, However, the tissue immersed in 50 % glycerol started at a larger sized element block, resulting in a larger percentage decrease for the size of bars that the participants could resolve before and after immersion in glycerol.

The 100 % glycerol appeared subjectively to dehydrate the dural tissue more than the 50 %, which would be expected owing to the absence of water molecules in the 100 % glycerol.

In situ optical clearing with glycerol

As optical clearing is mediated at least in part, by dehydration of the tissue, 100 % glycerol was chosen for in situ autopsy practice as it was expected that the fluid in the underlying brain tissue would re-hydrate the treated dura, thereby reducing the effect of clearing. An increase in optical clearance of the dura could be seen with the naked eye within the first minute of application of glycerol during in situ use in all but one subject. In nearly all cases, application of glycerol resulted in the blood vessels within and below the dura mater and on the surface of the brain becoming more readily apparent (Fig. 3a, b). In situ application of 100 % glycerol did not appear to dehydrate the dural tissue to the same degree when compared to that apparent in the dura mater in the extracorporeal experiments, most likely due to continued hydration of the dura from the underlying brain tissue. With prolonged application (>10 min or so) some adherence of the dura to the brain tissue was observed. We observed that the increase in optical clarity and the accompanying dehydration of the dural tissue, which causes a slight shrinkage and decrease in the flexibility of this membrane, can be completely and almost instantaneously reversed with the application of

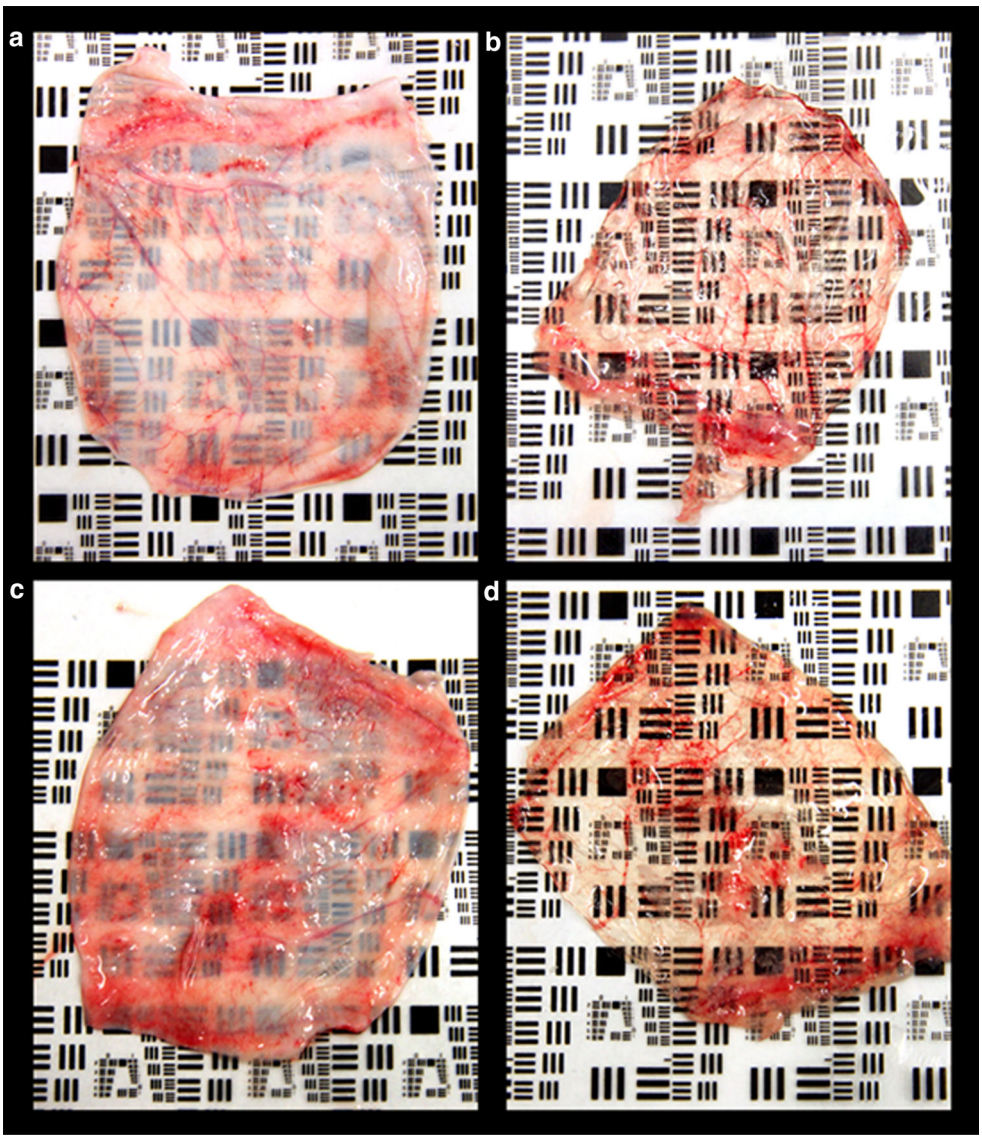

Fig. 2 Samples of parietal dura on a USAF 1951 resolution test target background. **a** Before immersion in 100 % v/v glycerol. **b** After immersion in 100 % v/v glycerol. **c** Before immersion in 50 % v/v glycerol. **d** After immersion in 50 % v/v glycerol

Table 1 Mean element blocks the independent observers could resolve for dural tissue before and after immersion in 50 and 100 % glycerol, and the width of the spaces between the bars of the element blocks, G-glycerol, EB-element block

Tissue sample	Mean EB (element, group)	Mean width of the spaces between the bars of the EB (pixels)
A—Before immersion in 100 % G	1, 0	20.95
B—After immersion in 100 % G	6, 0	8.24
C—Before immersion in 50 % G	3, −1	31.20
D—After immersion in 50 % G	6, 0	8.24

aqueous fluids (including tap water, 0.9 % saline and 10 % formalin) to the tissue (Fig. 4a–c). The clearing effect reliably lasted long enough to allow acquisition of a systematic series of macroscopic photographs of all surfaces of the dura.

In a single case of a 2 year old male severely affected by cerebral palsy, where there was significant atrophy of the underlying brain tissue and prominent gelatinous leptomeninges with thickening of the dura, a partial clearing effect was produced. This partial effect is most likely due

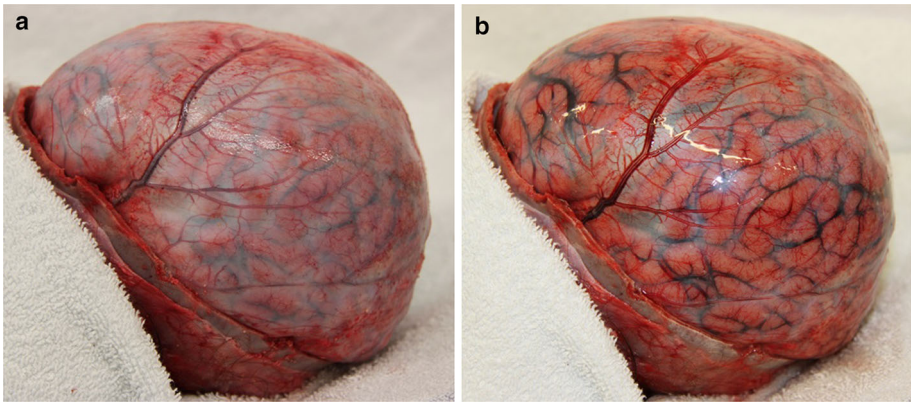

Fig. 3 In situ dural clearing with glycerol on a 13 month old female. **a** Before application of glycerol. **b** After application of glycerol

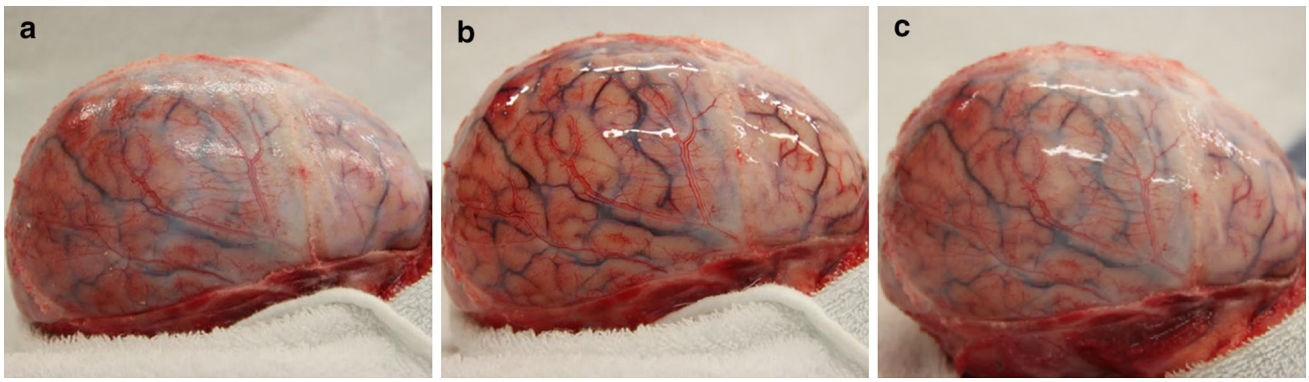

Fig. 4 In situ clearing on a 9 week old male. **a** Before application of glycerol. **b** After application of glycerol. **c** After reversal of clearing with water

to the fluid rich nature of the leptomeninges in this particular circumstance.

In three cases of NAHI (a 2 month old male, a 4 month old male, and a 7 month old female) with bleeding below the dural membrane, 100 % glycerol was used to assess the extent of areas of hemorrhage within and underneath the dura mater (Fig. 5a–f). With the resulting increased transparency of the calvarial dura mater, extensive hemorrhage underneath the membrane could be seen more clearly.

In three cases of peri-natal head injury and one case of confessed overlaying, the latter having apparently led to minor crush injury, glycerol was used to determine whether subdural hemorrhage was present and to increase visualization of obvious patterns of bleeding (Fig. 6a–l). In one case in the series (a 4 week old male), clearing of the dura enabled observation of a focus of extremely thin bleeding (best characterized as a smear of blood), over the occipital convexity (Fig. 6b), which was revealed to be subdural hemorrhage upon subsequent reflection of the dura (Fig. 6c).

No macroscopic areas of intradural hemorrhage were noted in the calvarial dura (i.e., over the convexities of the brain or near the superior sagittal sinus) before or after application of glycerol in any of the cases in this study.

Histology

Histological assessment indicated that there was no significant difference between the appearances of the tissue samples by routine light microscopy when comparing untreated dura mater with samples which had previously been immersed in 100 % glycerol (Fig. 7a, b). There appeared to be only very subtle differences in the appearance of some of the collagen bundles between dura treated and untreated with glycerol (Fig. 7c, d). However, some variation in the appearance of dura taken from various sites is to be expected. Dural tissue treated with glycerol also showed a normal Perls' reaction for iron, and glycerol treatment of the dura did not compromise the interpretation of any of the various immunohistochemical stains undertaken on selected samples of the underlying cerebral convexities.

Discussion

Variation in refractive indices means that light travels at different speeds and angles through biological tissues [9], inducing light scattering. The components of dural tissue,

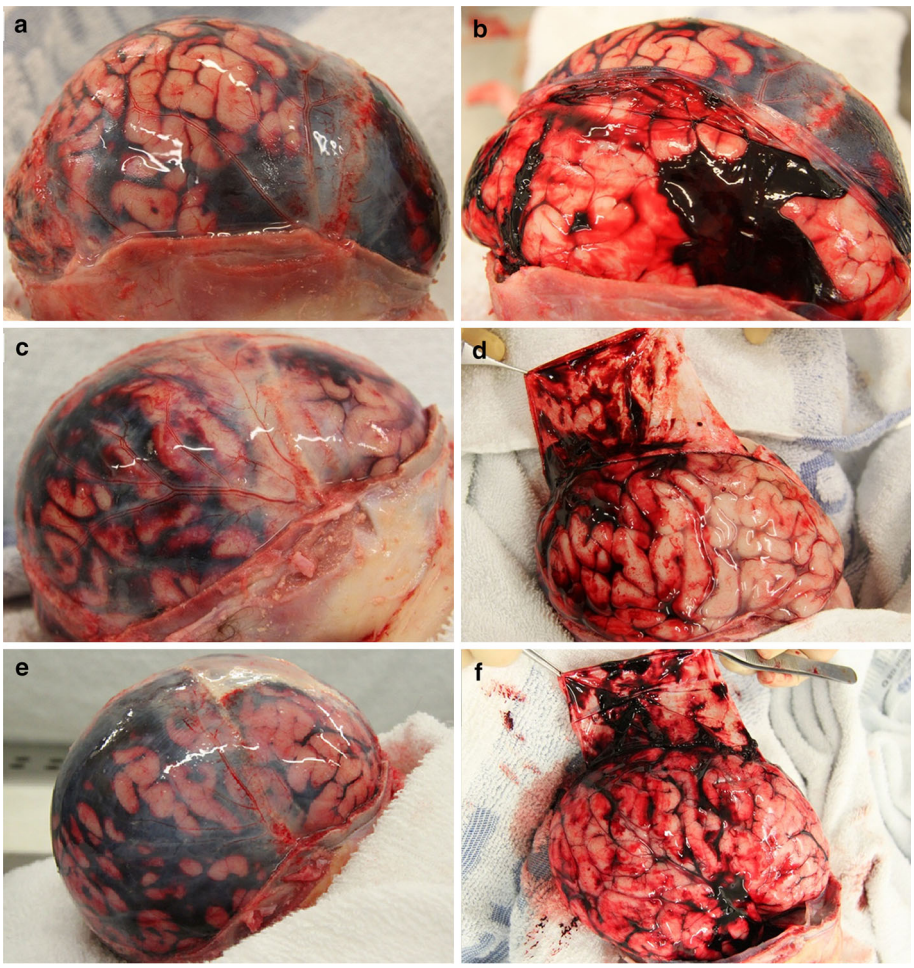

Fig. 5 In situ clearing with glycerol and lifting the dura of three NAHI cases. **a**, **b** 4 month old male, **c**, **d** 2 month old male, and **e**, **f** 7 month old female, all showing bilateral patchy thin subdural hematoma, thickest over the sulci of the brain with extremely thin bleeding over gyral convexities

such as collagen, scatter light to a large degree as they have high refractive indices. The surrounding interstitial fluid and/or cytoplasm have lower refractive indices and therefore scatter light to a smaller degree. Optical clearing agents such as glycerol, with their high refractive indices are hyperosmotic [10] and therefore they diffuse into the tissue they are applied to, reducing variation in refractive indices, in turn reducing the amount of light scattering.

A further effect associated with the process of optical clearing is thought to be dehydration of the tissue as a result of the rate of passive diffusion of the glycerol into the tissue, which is slower than the migration of water out of the tissue because OCAs have high osmolality [11]. The smaller diffusion coefficient of glycerol compared to water results in the glycerol travelling into the tissue and cells at a much slower rate, causing the tissue to shrink [12]. Collagen is soluble in sugar-alcohols [10] and therefore structural modification or dissociation of collagen is also thought to play a role in optical clearing [13]. In this paper we did not observe dissociation of collagen fibers, possibly due to a reversible effect produced by rehydration of the tissue when place in formalin. Rather, if anything, there was slight compaction of some of the collagen bundles in treated dura samples. However, these minimal changes would not be diagnostically significant.

A USAF 1951 resolution test target pattern was adapted for the extracorporeal transparency assessment within this study. Subjective observations by the naked eye against the standardized test target enabled an appropriate comparison of the dural tissue before and after immersion in the OCA. We accept that some variation in observed resolution will occur owing to inter-observer variability; variations in the thickness of the dura; overlaying of the dura on different components of the target pattern; and a variation in the vasculature of the tissue. The latter appear to have resulted in some instances where meningeal vessels have obscured the view through the tissue to the target.

To the best of our knowledge, the use of glycerol to optically clear the human dura mater in situ has not been reported before. There are, however, publications relating to optical clearing of animal dura mater with glycerol [14], in addition to experiments on human dura mater using glucose and mannitol [1, 15–17]. There are numerous studies that have used glycerol on both animal and human skin and sclera [11, 12, 18–23]. The dura mater is comparable to scleral and dermal tissue in that it has a densely

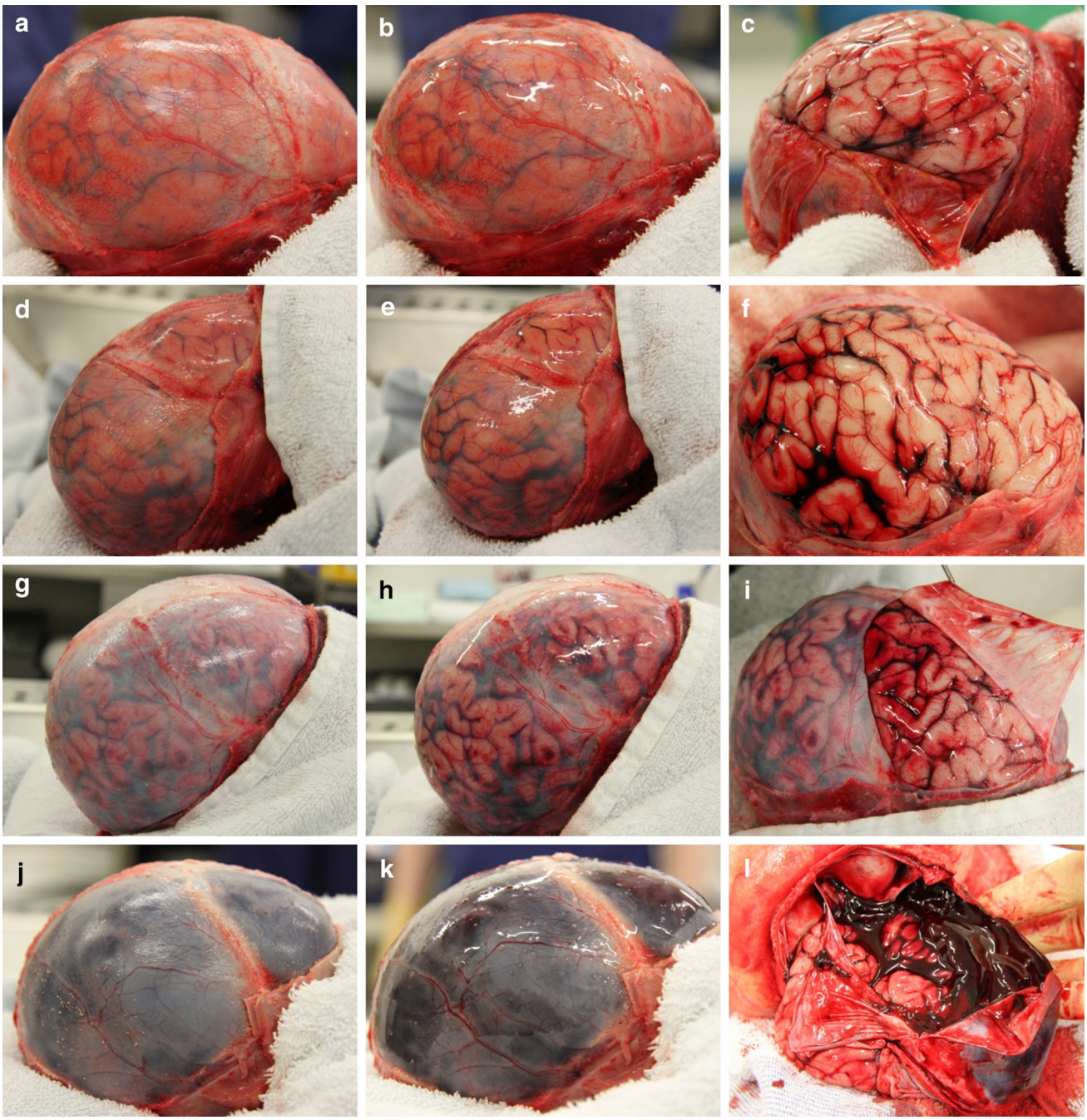

Fig. 6 In situ clearing, before (**a**, **d**, **g**, **j**) and after (**b**, **e**, **h**, **k**) application of glycerol, and lifting of the dura (**c**, **f**, **i**, **l**). **a**–**c** 4 week old male with focal posterior subdural bleeding, revealed on lifted the dura as an extremely thin smear of blood over the occipital convexity. **d**–**f** 1 day old female with peri-natal head injury born by emergency cesarean section following failed suction cup delivery showing a focal thin smear of subdural bleeding below the posterior parietal and occipital dura. **g**–**i** 3.5 month old male with minor crush injury most likely the result of confessed overlay, with very thin subdural bleeding over the right cerebral convexity. **j**–**l** 1 day old female with peri-natal head injury following forceps delivery showing extensive space occupying subdural bleeding extending over the entire convexity

fibrous structure, and, therefore, it has similar optical properties to those tissues [24]. A study using glucose and glycerol on rabbit dura in vitro and in vivo found better results in optical clearance with glycerol compared to glucose [14]. For this reason and owing to the number and wide variety of other studies using glycerol on fibrous tissues, we decided to assess and then apply the use of this particular OCA in the examination of human infant dura mater. We also decided to carry out a comparison of glycerol and mannitol on several pieces of dura from one

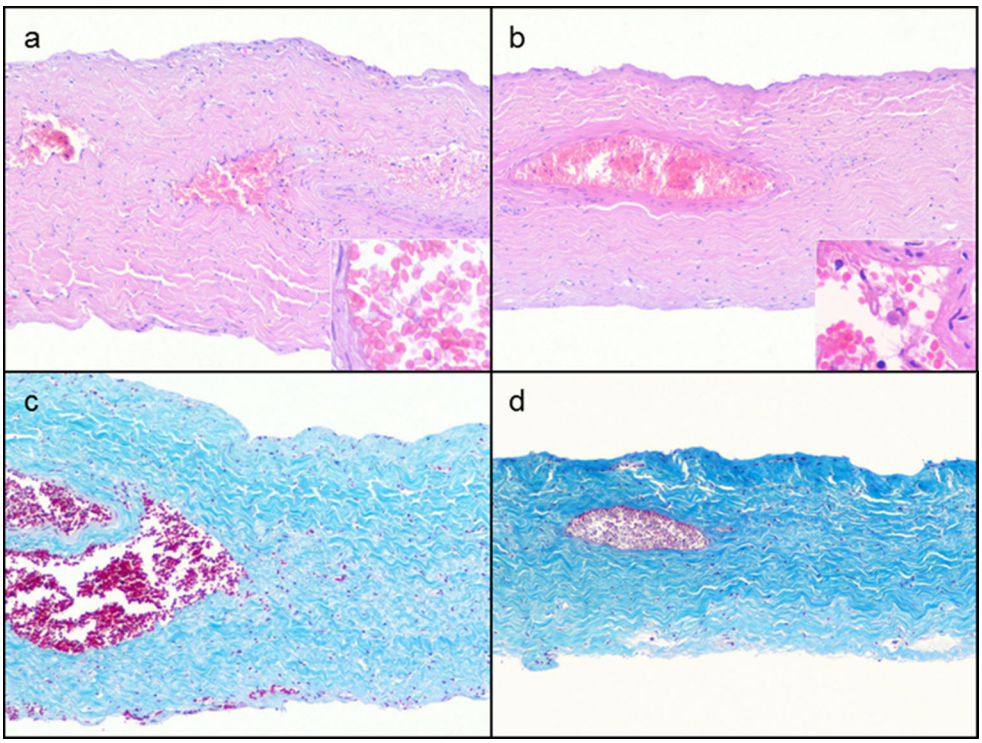

Fig. 7 Digital photomicrographs (×10 objective and ×40 *insets* in **a**, **b**) of parietal dura mater stained with H&E **a** not treated with glycerol, **b** treated with glycerol; and Masson's trichrome, **c** not treated with glycerol, **d** treated with glycerol

case as mannitol is another OCA that has previously been proposed as an agent for clearing of human dura mater in vitro at concentrations of 160 g/L [1, 17]. A possible suggestion for the lack of increased optical clearance observed for the dura immersed in mannitol in our present study is a shift in pH to a more acidic level within the tissue's interstitial fluid caused by the OCA diffusion. A change in pH can result in swelling of a tissue. Swelling of collagen fibers results in an increase in their size and changes in their packing arrangement. These changes increase the scattering of light and therefore a decrease in tissue transparency is observed [16].

Shrinkage of the dura due to dehydration is a consequence of the physicochemical optical clearing process and we have found, along with others [12], that the morphological effects of tissue optical clearance are completely reversible with the application of water or phosphate buffered saline and have no deleterious effect on the subsequent autopsy. However, we would suggest re-hydrating the dura with water before removal of this membrane and continuing with the autopsy assessment of the brain in order to re-establish the flexibility of the dura and restore the ease of its manipulation. We would also recommend that any necessary samples for toxicology be taken prior to the application of glycerol during the autopsy.

No diagnostically significant effect on the histology of the dura could be detected by an experienced pediatric histopathologist when comparing untreated dura samples with those treated with glycerol. Tissues were fixed in a 10 % aqueous solution of formaldehyde which is likely to have resulted in the rehydration of the samples that had previously been immersed in glycerol. One of the suggested effects of optical clearing of tissues is stasis and dilation of micro vessels [22]. No such appearances were noted histologically in the samples of dura in our study. Indeed, glycerol-induced morphological changes in blood vessels, including vascular occlusion have been shown to be reversible upon rehydration of the tissue in in vivo studies on hamster skin [12]. Further, there was no obvious alteration to diagnostically relevant histochemical or immunohistochemical ancillary staining properties of the dura or underling brain tissue following the application of 100 % glycerol to the dura at autopsy.

In the case of a 4 week old male infant and also a 1 day old female, the combination of a subtotal craniectomy approach to calvarial bone removal in combination with optical clearing of the intact dural membrane, allowed detection of a small focus of extremely thin subdural hematoma, best described as a "smear" of minimal bleeding, on the convexity of the occipital lobe. It is almost certain that this finding would have been considered to represent post-mortem artifact if routine autopsy methods had been used to access the brain. Optical clearing with glycerol therefore appears to greatly increase the sensitivity

of detection of bleeding below the dura mater such that all clinically significant subdural hematomas on the cerebral convexities should be assessable using this technique.

We have demonstrated the application of glycerol to the intact infant calvarial dura enables the non-destructive visualization of brain surface blood vessels and also study of the vasculature of the dura itself. One particular advantage is the enhanced ability to directly observe features of head injury including facilitation of the detailed description and photographic documentation of the distribution of subdural hematoma in infant head trauma, free from autopsy-induced artifact. Even the most trivial of convexity subdural hemorrhages can be detected. Although our study cohort only employed this technique in pediatric autopsies, we would expect to see comparable results in older subjects.

Key points

1. Optical clearing agents can increase the transparency of biological tissues in situ, such as the dura.
2. The transparency of the dura can be reversibly increased with glycerol in situ, during post-mortem examination of the dura and the brain surface.
3. Increased transparency of the dura aids in the visualization of certain brain pathologies, including subdural hematoma, before the dura is incised or removed.
4. Diagnostic histological assessment of the dura is not affected by the in situ application of glycerol.
5. This technique allows for the detection of even very small amounts of subdural bleeding.

Acknowledgments This article presents independent research funded by the Home Office Science Secretariat, Science Group. The views expressed in this publication are those of the author(s) and not necessarily those of the Home Office Pathology Unit in Home Office Science. We would like to thank volunteers from the staff of the East Midlands Forensic Pathology Unit who took part in the visual assessment of tissue clearance.

Compliance with Ethical Standards

Conflict of interest None.

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