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Natural preservation of tissues not so bad after all

Maria Jeziorska[1], Patricia Lambert-Zazulak[2], Otto Appenzeller[3], Rosalie David[2], Sonia E. Guillién[4]

[1] Laboratory Medicine Academic Group and [2]KNH Centre for Biomedical Egyptology, University of Manchester, Manchester M13 9PT, UK;
[3] New Mexico Health Enhancement and Marathon Clinics Research Foundation Albuquerque New Mexico 87108 USA;
[4] Centro Mallqui, Lima, Peru

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Abstract

We compared the preservation of antigens of various naturally mummified human tissues from the Atacama Desert, Southern Peru with matching samples from Egyptian mummies obtained from the Manchester Museum Mummified Tissue Bank. We studied intervertebral discs and joints, various soft tissues, major blood vessels, brain meninges and skin. Immunolocalisation of structural proteins, intracellular intermediate filaments and neural markers varied from sample to sample and required cumbersome and lengthy procedures but was generally more successful in naturally mummified tissues from the Atacama Desert. Our findings confirmed our experimental data which showed that when using the same antigen retrieval methods, the investigated proteins from naturally desiccated human tissues retained their antigenicity better than those which were in contact with natron and especially those which were also treated with resins

Introduction

A pathologist assessing tissue sections expects that tissue was processed according to widely accepted, routinely used methods. To avoid autolysis and putrefaction, a biopsy should be immediately immersed in fixative (e.g. in the most commonly used 10% buffered formalin for no longer than 24 hours), washed in buffer, dehydrated in graded alcohols and processed through clearing agent to paraffin wax. Any serious deviation from this routine causes changes in the tissue affecting its microscopical appearance and 'stainability'. This later becomes apparent when using histochemical and immunohistochemical methods. In rare instances when the tissue is damaged during processing, e.g. over-fixed or dried out – its assessment becomes difficult or impossible. Probably the most important reason for that is no point of reference for the observations. It is also well known that immunohistochemistry methods are notoriously difficult to apply to tissue sections obtained from very old paraffin blocks (e.g.30-40 years old).
The initial desiccation during mummification stops autolysis and putrefaction after a certain amount of initial damage has already occurred. However, the undisputable damaging factor is the antiquity of the material. Mummified human remains have their unique and long history which influences the antigenicity of their component tissues. The inevitable changes brought about by time alone exceed by far the changes found in contemporary forensic investigation. Additional factors influencing preservation of the tissue are substances used for mummification and impurities they may contain, plant extracts used for ritual washing, oils and resins, changes in humidity (especially after unwrapping of the mummy, damage caused by bacteria, fungi, insects and animals (Fulcheri 1995; Aufderheide 1998 and 2002)
The aim of this study was to compare preservation of antigens of various naturally mummified human tissues from Atacama Desert, Southern Peru with matching samples from Egyptian mummies obtained from Manchester Mummified Tissue Bank.

Material and methods

Material from Egyptian mummies came from the Manchester Museum Mummified Tissue Bank (MMMTB). This material consisted of endoscopic biopsies, tissue fragments from canopic jars, skin samples, fragments of auricular cartilage and intervertebral discs (materials donated to the MMMTB by the Leicestershire Museum and Art Gallery; Oriental Museum, Durham; University of Colorado, Boulder and American Museum of Natural History). Matching tissue samples from naturally mummified human remains were donated for this study by Sonia Guillién from mummies found on Western coast of South America (Atacama Desert, Southern Peru).

We focused on localisation of neural, vascular and matrix components using contemporary tissues as a positive control.

Histochemistry

Haematoxylin and Eosin (H&E), Miller stain for elastic fibres, Picrosirius red, Masson's Trichrome, Toluidine blue, PAS Haematoxylin, Gram stain and Grocott's Methenamine Silver (GMS) were used.

Immunohistochemistry

A panel of monoclonal and polyclonal antibodies to the pan-neural marker PGP9.5 (a ubiqiutin present in abundance in central and peripheral nerve system), S-100 protein (present in Schwann cells), substance P, collagens type I, II, IV and VI, laminin, elastin, α-smooth muscle cell actin (α- SMC actin), CD68 (macrophage marker) and von Willebrand factor (endothelial cell marker) was used. Different antigen retrieval methods (and combinations of methods) have been used to disclose antigens in tissue (enzymatic pre-digestion with trypsin, pepsin, proteinase K and a panel of proteases, microwave and water-bath pre-treatment with citrate buffer pH 6.0 and 9.0).
Some of the mummified tissues were heavily contaminated by fungi and bacteria (as disclosed by PAS, GMS and Gram stains). We have used a protease inhibitor cocktail (Roche Diagnostics Co, IN) to avoid any possibility of further degradation of the tissue during rehydration process in case of viability of the microorganisms.

Results

Histochemistry

All histochemical stains were successful (with some adjustments to timing and temperature), although weaker than in contemporary tissues, with the exception of nuclear staining with Haematoxylin. After initial treatment with Celestine blue, Haematoxylin showed nuclei in cartilage, both naturally and natron mummified (Fig. 1). The nuclear preservation in cartilage was comparable to that found in the Tyrolean Ice Man (Hess, 1998). The naturally mummified tissues showed better colour definition for all histochemical stains comparing to natron treated samples as in our experimental mummification of carotid arteries (see "Egyptian versus natural mummification: tracking the differences in loss of tissue antigenicity" in these proceedings).

Immunohistochemistry

Our previous experience with processing of mummified tissue (Jeziorska, 2003) showed that Ruffer's solution (Ruffer, 1921) diminishes the chances of successful immunohistochemical staining. We, therefore, used our modified rehydration and fixation protocol. We decided against the use a fabric softener reported by Turner et al. (1981). We achieved good results in reconstitution of mummified tissue using buffered Tween 20 or Triton X (Sigma-Aldrich Co., St Louis, MO) instead.
Table 1 shows a summary of immunostaining graded semiquantitatively on matching contemporary, naturally, and natron mummified samples and Figures 1-4 show examples

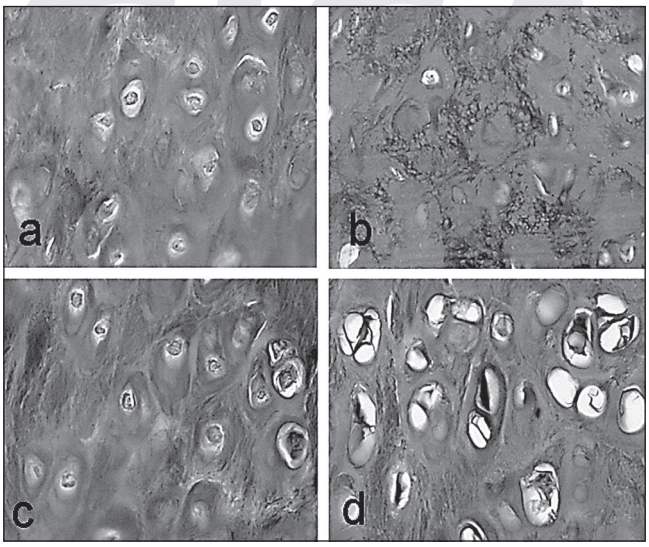

Fig. 1 - Auricular cartilage samples for naturally mummified (a, b) and natron treated (c, d) samples with postitive nuclear staining (H&E).

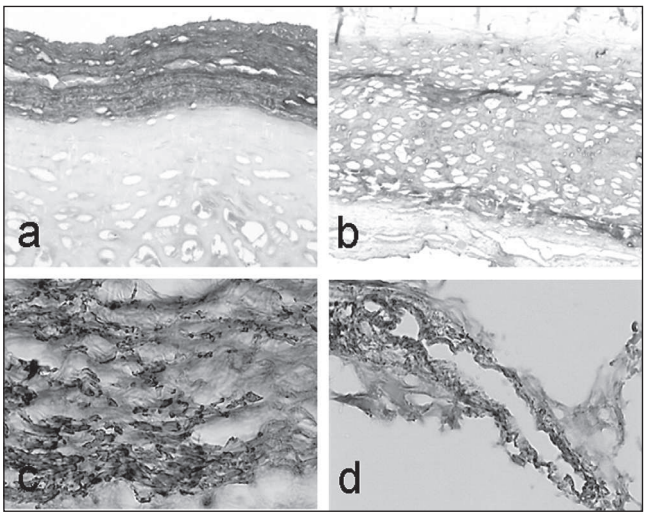

Fig. 2 - Immunohistochemical staining for collagen type I (a) and II (b) in bronchial cartilage, elastin in aorta(c) and collagen type IV in a microvessel (d) in natron mummified tissue.

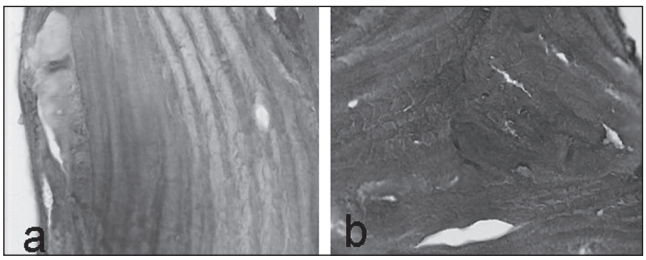

Fig. 3 - Immunolocalisation of collagen type I in natron (a) and naturally (b) mummified bone.

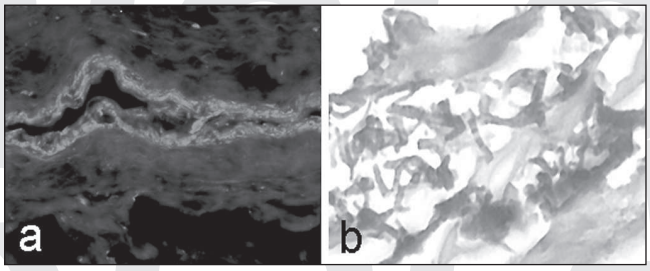

Fig. 4 - The false-positive immuno-fluorescent staining for von Willebrand factor (a; low magnification) on PAS-positive fungal hyphae (b; high magnification) in natron mummified tissue.

	Contemporary	Naturally mummified	Natron mummified
α- SMC actin	++++	+	+++
CD68	++++	+	±
elastin	++++	+++	++
Collagen type I	++++	+++	++
Collagen type II	++++	+++	++
Collagen type IV	++++	++	++
Collagen type VI	++++	+	-
Laminin	++++	±	±
S-100 protein	++++	-	-
PGP9.5	++++	Not tried	+
Substance P	++/+++	Not tried	-
Von Willebrand factor	++++	-	-

Table 1 - Comparison of immunohistochemical staining of matrix, neural and vascular components of contemporary, naturally and natron mummified matching tissue samples.

of immunostaining for different matrix markers in Atacama Desert and Egyptian mummies.
Collagen type I, II, IV, VI and elastin stained better in naturally mummified tissue but α- SMC actin was better in natron treated tissue. CD68 was very weakly positive in naturally mummified tissues and negative in all natron treated samples. S-100 and von Willebrand factor were negative in all samples. We have obtained a false positive result when staining for von Willebrand factor (Fig. 4). A skin sample of natron mummified tissue was incubated with the primary antibody to von Willebrand factor, followed by a secondary antibody conjugated with red fluorescent tag (Texas Red), which showed a substantial amount of positive staining suggestive of longitudinal sections through vessels. In contemporary tissue staining for von Willebrand factor is limited to endothelial cells forming a monolayer on the luminal surface of the vessels. The staining in the sample was relatively thick and parallel to the skin surface with no visible cross-sections of tubular structures resembling vessels. Observation at higher magnification revealed that the staining was of uneven thickness, 'fuzzy' in appearance and the false-positive stainig was found on fungal hyphae disclosed by histochemical PAS stain.

Discussion and conclusions

Obtaining biopsies from mummified tissue The decision to remove tissue from a mummy has to be based on the assumption that it will yield positive results. The biopsies should be small enough and obtained by methods causing the least damage to the mummy (e.g. endoscopic biopsies). At the same time the size of the removed tissue has to allow safe handling and yield enough sections for the performance of the planned experiments.
Processing of mummified tissue for immunohistochemical staining Ruffer's solution (Ruffer, 1921), widely used in rehydration of mummified tissues, due to its high pH gives very good reconstitution of mummified samples due to swelling of the connective tissue. Histochemical stains work well after this type of reconstitution. However, we found that immunohistochemical staining is much weaker or even totally absent after Ruffer's solution. We obtained better results using detergents. We found fixation in 4% buffered paraformaldehyde adequate for our purpose. Our previous experiments on the use of different clearing agents in processing mummified tissue to paraffin blocks resulted in choosing Histoclear (National Diagnostics, Atlanta, GA) for all our samples.
Fungal growth In many samples there was evidence of fungal growth in the form of hyphae penetrating microscopical cracks formed in the tissue by desiccation. While it is possible that this growth was initiated soon after mummification, it is nevertheless less likely in the instance of natron mummified bodies, as the presence of salts composing natron and lack of moisture would preclude or at least hamper growth. In naturally mummified tissue the invasion of micro organisms was likely to occur soon after death. Searching the literature on issues of preservation of mummified remains we found an excellent review of soft tissue taphonomy (Aufderheide, 2002) quoting reports of mistaking fungi for blood cells. The spores and cross-sections through hyphae are deceptively rounded and even the trained pathologist tends to relate observed images to experience gained on contemporary tissues. The issues of modified histochemical stains for micro organisms were extensively discussed by Boano et al (1995).
We have obtained acceptable results in histochemical staining with suboptimally as well as optimally rehydrated mummified tissues, but the latter is essential for successful immunohistochemistry.
It is very difficult if not impossible to compare results of any technique used on tissue of different age and preservation and attribute the differences only to a different mummification process. Nevertheless, it seems to us that naturally mummified human remains displayed surprisingly good antigen preservation as shown by

immunohistochemical staining. Although immunolocalisation of structural proteins, intracellular intermediate filaments and neural markers varied from sample to sample and required cumbersome and lengthy procedures it was generally more successful in naturally mummified tissues obtained from the mummies from the Atacama Desert. Our findings confirmed our experimental data which showed that when using the same antigen retrieval methods, some of the investigated proteins from naturally desiccated human tissues retained their antigenicity better than those which were in contact with natron and especially those which were treated with resins. The presence of fungi interfered in some cases with immunohistochemical staining. We are convinced that immunohistochemistry is a useful method for assessing normal and diseased mummified tissue.

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