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RESEARCH ARTICLE

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Ultrastructural preservation of tissues and their reaction to the infection with trichinella in the El Plomo mummy

Muscle fiber ultrastructure and trichinosis/mummy of the Cerro El Plomo

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Abstract

The El Plomo mummy was a pre-Columbian Incan child who was found mummified in the Andes Mountains above an altitude of 17,700 feet. In the environment, natural mummification occurred due to low temperatures and strong winds. Dating measurements (relative dating) by experts from the National Museum of Natural History of Chile established that the mummified body corresponds the Inca period (1,450 to 1,500 AD). In 2003, the body was transferred to the University of Chile Medical School for exhaustive medical examination. Tissue samples from the right quadriceps muscle were extracted and fixed in glutaraldehyde and postfixed in osmium tetroxide to obtain ultrathin sections to be observed by transmission electron microscope. Images were recorded on photographic paper, digitalized and analyzed by experts on morphology. Results showed a preservation of cell boundaries in striated muscle cells, but specific subcellular organelles or contractile sarcomeric units (actin and myosin) were unable to be recognized. However, the classical ultrastructural morphology of the polypeptide collagen type I was preserved intact both in primary and secondary organization. Therefore, we concluded that the process of natural mummification by freezing and strong winds is capable of damaging the ultrastructure of muscle cells and preserving collagen type I intact.

KEYWORDS

El Plomo mummy, striated muscle, collagen, trichinella

1 | INTRODUCTION

Mummification by natural influences results in so-called natural mummies, whereas mummification induced by active (human) intervention results in so-called artificial mummies. Many ancient cultures used burial rites -which to some degree involved both natural and artificial mummification methods. Since they are so uniquely well-preserved, mummies may give many insights into mortuary practices and burial rites (Lynnerup, 2007).

A mummy is the preserved body of a human or an animal being. Any dead body that still has skin on it is considered a mummy. When the Arabs invaded Egypt in the seventh century AD, they believed that the dark resin coating on the mummies was bitumen or asphalt, and so they began referring to the mummies as "mummiya" or Arabic for bitumen. It is from the word "mummiya" that the modern day word "mummy" has evolved (Australian Museum, 2009).

Mummies are found in many places of the world. Some of the world's best-known mummies were created accidentally, when a body's final resting place happened to prevent the natural process of decay. But many cultures around the world have sought to mummify their dead on purpose. Arid desert winds and blazing hot sand occasionally dried corpses out quickly enough to mummify them. Initially, mummification was so expensive that it was a privilege enjoyed only by the Pharaoh and few nobles. The ancient Egyptians believed that the dead lived on in the next world, and that their bodies had to be preserved forever as they were in life. They believed that the body

would serve a person in the next world and therefore spent much effort in developing methods of embalming. Mummies were made naturally or by embalming, which is any process that people use to help preserve a dead body. Some bodies became mummies because there were favorable natural conditions when they died (Mahajan & Amin, 2011).

It is possible to investigate ancient mummified remains through histological tools such as immunohistochemistry because of its ability not only to detect proteins but also to isolate their location to specific tissues and thereby improve confidence that the results are genuine; it can detect proteins, found their precise location into a specific tissue and thereby improve the collected data (Wick, Kalischnig, Maurer, Mayerl, & Muller, 2001). A mouse model of Egyptian mummification has been used to demonstrate that the survival of proteins, judged by the retention of immunohistochemical staining, varies markedly. Some survive the process well, whereas others become barely detectable despite the morphology of the tissue being excellently preserved. Previous results show that protein preservation is multifactorial, with tissue type and degradation, and the properties of the proteins itself having significant effects. Proteins forming large, multisubunit complexes such as collagen IV appear to be more resistant to degradation than those that do not. However, modern modeling studies cannot replicate the full extent of degradative processes and taphonomic changes experienced by real mummies (Metcalfe & Freemont, 2011).

This study addresses tissue preservation and paleopathological aspects in the El Plomo mummy in Santiago de Chile, Chile. The aim of this research is to analyze the structural tissue organization of striated muscle and connective tissue, associated with the infection with *Trichinella* cysts described in the El Plomo mummy (Rodriguez et al., 2011).

2 | MATERIAL AND METHODS

2.1 | Incan child's body

The Cerro El Plomo mummy belongs to the body of an Incan child from 8 to 9 years old. According to studies of funerary clothing carried out by specialists of the National Museum of Natural History of Santiago de Chile, this mummy reached the region of the Mapocho possibly circa 1,500 AD, year which coincides, according to history, with the expansion of the Incan Kingdom.

The site of its finding is a cave built of stones about 5,420 m (17,000 feet, 35°05′56.90″ S and 70°64′33.10″O) high; this area usually remains covered with snow throughout the year, with extreme low temperatures and strong winds. At the time of its discovery, leather moccasins, furnishings and offerings made of fine silver were found standing out between two llamas and a statuette of a carefully dressed woman, among others (Cabezas, 1986). The child was naturally frozen (mummified) and remained so until 1954.

On February 1, 1954, two local muleteers who knew the rural roads, caves, and cliffs among the Andean mountains between Argentina and Chile, found in the mummified child in a sector known as "Pirca de los Indios" ("Wall of the Indians," in Spanish). After finding on the ground some Indian utensils and gold ornaments, the muleteers dug in the ice and an Indian child buried in a natural ice chamber appeared a few meters below the surface. He was taken to a cave at 4,000 meters high and hid there. Negotiations for its sale with the Museum of Natural History lasted over a month and then was taken down to the town of Puente Alto (stay of 1 month, 33° 36'56.50''S and 70°34'10.95'' W, and 700 m high). Currently, the body is protected under optimal conditions in a proper acclimatized chamber to ensure the preservation of the frozen body. The case remains in the basement of the dependencies of the National Museum of Natural History in Santiago de Chile.

2.2 | Transmission electron microscopy (TEM)

In September 2003, the mummified and lyophilized body of the child from Cerro El Plomo was moved from the National Museum of Natural History of Santiago of Chile to the Imaging Unit at the Teaching Hospital of the University of Chile Medical School with the purpose of applying modern technology to extract more information from the child's body and to contextualize its origin and the location where it was found in 1954.

A rounded sample from the right quadriceps muscle was collected with a 0.3 cm diameter steel trocar resulting in a sample of 0.25 cm depth, and immediately immersed in a freshly prepared cold (4°C) solution of 1% glutaraldehyde in phosphate buffer at pH 7.2 and an osmolarity of 320 mOsm for a period of 12 h. Then the sample was postfixed in 1% OsO_4 in the same pH and osmolarity conditions. According to routine protocols, the sample was dehydrated in ethanol at increasing concentrations and then embedded in epoxy resin LX-112 (Ladd Research Inc., Burlington). Semi-thin sections were obtained (1 micron in thickness) and stained with toluidine blue 1%, to achieve the location of the lesion. The parasite *Trichinella* spp was diagnosed by optical microscopy (Rodriguez et al., 2011). The ultrathin sections (100 nm) were obtained in an ultramicrotome Porter-Blum MT2-B equipped with a diamond blade. Stain with uranyl acetate and lead citrate for gray enhancement was used. The observation was made in transmission electron microscopes models Hitachi H-500 and H-7100, with respective accelerating voltages of 100 and 75 kV.

Digital electron micrographs were recorded with a MegaView III CCD-SIS using iTEM software (Olympus Soft Imaging System GmbH, Münster, UK). Similar conditions for normal and pathological muscle were employed (Popescu et al., 2011).

The observations were made at different magnifications, clearly identifying the muscle fibers, remnants of the parasite, and the ultrastructure of collagen fibers (from 7,000× to 30,000×). Scanned images (JPGE format, 300 dpi) were imported, included and described as results in this study.

3 | RESULTS

3.1 | Ultrastructure of skeletal muscle fibers

As shown in Figure 1A–D, striated muscle fibers are seen in longitudinal and parallel disposition. The process of mummification by freezing

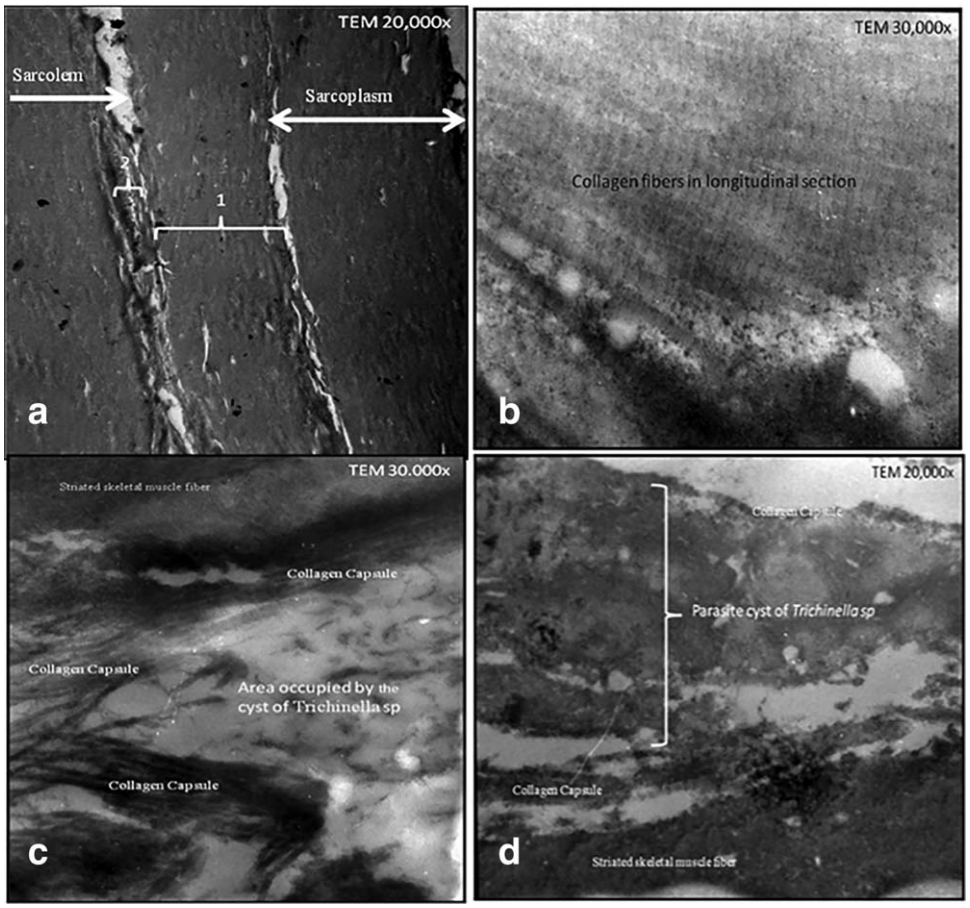

FIGURE 1 **(a)** Micrograph of the ultrastructure of skeletal muscle fibers in the process of mummification by freezing: Mummy of Cerro El Plomo (electronic microscopy transmission, 20,000). **(b)** Micrograph of the ultrastructure of type I collagen fibers in the process of mummification by freezing: Mummy of Cerro El Plomo (eletronic microscopy transmission, 30,000). **(c) and (d)** Micrographs of the ultrastructure of the interaction of skeletal muscle fibers in the presence of Trichinella parasite in the process of mummification by freezing: Mummy of Cerro El Plomo (eletronic microscopy transmission, ×30,000 and ×20,000, respectively)

has provoked a disruption in the normal histological architecture. In a normal tissue, under transmission electron microscopy with 20.000× magnification, the sarcoplasm (cytoplasm) of striated skeletal muscle cell (fiber) normally shows a striated organization (sarcomeres) and subcellular elements -such as many nuclei distributed in the periphery under the plasma membrane, mitochondria, T-tubule system, and others. In Figure 1A, B, D, the sample from El Plomo mummy only shows a homogeneous cytoplasm and no organizational details. Apparently, the process of mummification by freezing altered the structure, survival, and individuality of the elements of the sarcoplasm (Figure 1A).

In the microphotographs obtained with transmission electron microscope, the spaces between the muscle fibers were well defined and contained by the plasma membrane, and were occupied by the endomysium that is formed by basal lamina and dispersed and adjacent reticular collagen fibers, eventually able to penetrate the basal lamina. Commonly, all three layers when seen under light microscopy are called the sarcolemma (plasma membrane).

Similarly, also shown in Figure 1A, the endomysium was observed between muscle cells, consisting of reticular fibers (type III collagen) and the basal lamina (type IV collagen). However, the mummification by freezing only allows topographical identification of these elements.

3.2 | Ultrastructure of type I collagen fibers

Figure 1B shows the collagen fibers of the perimysium from the right quadriceps muscle in longitudinal arrangement. In general, collagen fibers appeared as highly ordered material, with a strictly longitudinal and parallel. Collagen fibers were interspersed all over the sample with a very tight disposition, even making boundaries between fibers almost unnoticeable. Collagen fiber bundles usually measured between 15 and 30 μm, although some of them were finer or thicker; forming these bundles, there were the fibrils–collagen fibers arranged in parallel.

Fibrils are observed with a varying diameter (about 9-10 μm) and appeared subdivided by a series of transverse bands under the electron microscope; some were colored in an intensely dark tone while other looked translucent or with lighter shades; these bands were repeated along the fiber in extremely regular intervals (Figure 1B). The distance between the repeating bands (between light and dark bands) within

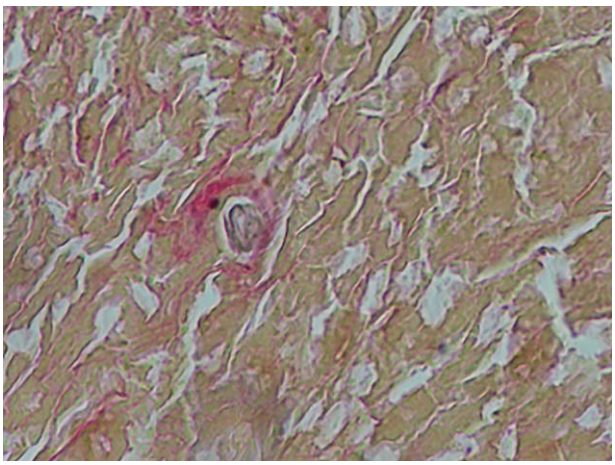

FIGURE 2 Micrographs in the optical microscope that shows deposits of collagen type I (red color) around the cyst of the parasite. Von Kossa's technique [Color figure can be viewed at wileyonlinelibrary.com]

the fibril was always the same: between 60 and 70 nm (typically 64 nm in average). Apparently, the process of mummification by freezing allowed keeping the visual structure of connective tissue and the ultrastructure of collagen type I.

3.3 | Skeletal muscle fibers in the presence of *trichinella* parasite

Histopathologically, the parasite enters the muscle fibers through the endomysium after arriving to the infection site via the blood vessels. The parasite arrives from the interstitium and then enters the muscle cell by breaking the plasma membrane and by dragging in the connective tissue elements. Figures 1C,D and 2 show an area occupied by the parasite bounded centrifugally by variable deposits of collagen, where it is possible to observe its peripheral distribution and cross-striations resulting from the tertiary and quaternary structure organization of the protein.

4 | DISCUSSION

A striated muscle fiber that forms a normal skeletal muscle corresponds to a cylindrical elongated and isodiametric multinucleated structure, with its cytoplasm almost entirely occupied by transverse striations formed by actin and myosin myofibrils organized in the sarcomere, extremely homogeneous distribution and easy to distinguish at low magnification. In the condition of multinucleated cells, the nuclei are organized and distributed in the periphery under the plasma membrane. It is also possible to identify with certainty other subcellular organelles such as mitochondria and glycogen granules.

Similarly, Ramirez, Davila-Vera, and Palacios-Pru (2006) using the transmission electron microscope described the preservation of the structural pattern of striated muscle fibers observed chains of mitochondria interspersed between the myofibrils. Permeabilization with saponin had a direct effect on the contractile apparatus of muscle fibers characterized by disorganization of sarcomeric structures and a significant reduction of the myofilaments.

In the Cerro El Plomo mummy, which was preserved and mummified under natural conditions of low temperatures, presence of snow almost all year round, high wind speed, and altitude hypoxia (5,400 m), it was possible to observe a generally good preservation of striated muscle tissue, which maintains the overall structure of cell boundaries and intercellular space. However, the cytoplasm appeared as a homogeneous mass in structure and staining, without other recognizable and identifiable structures. Apparently, this natural preservation by lyophilization and the elapsed time (since 1,500 AD or so), have triggered the action of cell lysis processes that have affected the physicochemical structure of the cytoplasm, the membranes of subcellular organelles (nuclei, mitochondria, lysosomes, etc.) and sarcomeric striations, leaving a cytoplasm-like colloidal solution liquefaction.

In other studies and with other processes of mummification for shorter times, a common finding in fibers that have undergone preservation is the movement of mitochondria from its normal location into the spaces between the myofibrils in which the contractile component is missing, allowing the assemble of mitochondrial gaps. In muscle cells, non-swollen mitochondrial ghosts were observed, with lysis of their membranes -both on cristae and the outer membrane-, significant reduction of the mitochondrial matrix, disorders in actin myofilaments on sarcomeric I band, with microvesication in the T-system and cisternae of sarcoplasmic reticulum. Importantly, the myosin filaments appeared more resistant and stable.

In addition to the process of natural preservation by cold and windy weather, presumably the child of Cerro El Plomo should have been in severe caloric restriction and hypoxic stress. In this regard, Ramírez, Martens, and Palacios-Pru (1998) designed an experimental rat model to demonstrate the effects on the myocardium of the simultaneous action of protein caloric restriction and hypoxic stress in a high altitude simulation chamber (3,200 m). In the group with protein caloric restriction, there was a significant decrease in body and heart muscle weight, as well as total protein content; mitochondrial proteins showed a slight increase. These observations correlate with the ultrastructural findings in the same experimental group, which showed fewer contractile myofilaments, undulating configuration of the mitochondrial cristae and increased number of mitochondria. The alterations were more evident in the group subjected to hypoxic stress, with damage to the contractile structures. It is suggested that protein-energy malnutrition accompanied by hypoxia is probably one of the most common factors affecting the occurrence of cardiac disease in the Andean highlands (Ramírez et al., 1998). However, in the normal situation, Sucre, Final, Perez, and Pacheco (1999) described that the ultrastructure analysis of electron microscopy of skeletal muscle in physically active animals showed swollen mitochondria, abundance of lysosomal structures, increased thickness in the basement membrane of intramuscular capillaries and its partial occlusion by the presence of surface infolding into the lumen and macrophages.

Apart from its high tensile strength and elasticity, type I collagen is known for its exceptional durability. In these specimens, the preservation of collagen is facilitated by its sequestration within the bone or by the mineralization of the soft organic tissue (Schweitzer, Wittmeyer, & Horner, 2007). The structure of the tropocollagen triple helix is stabilized by the formation of one interchain hydrogen bond per sequence between the N—H groups of glycines and the C—O groups of prolines on the neighboring chain. These self-assembled fibrils feature a characteristic 67 nm, D-periodic banding pattern (Williams, Gelman, Poppke, & Piez, 1978; Orgel, Irving, Miller, & Wess, 2006). Excellent preservation of collagen has been reported for mummified human tissue (Wick et al, 2001). Optical and scanning electron microscopy investigations have revealed single fibrils and bundles of type I collagen in naturally mummified bodies (Chang et al., 2006).

According to Williams, Edwards, and Barry, (1995) and Hess, Klima, Pfaller, Kunzel, and Gaber (1998), Otzi, the Iceman is a remarkably well-preserved wet mummy and was mummified naturally by a form of freeze-drying. The body was found at 3,200 m above sea level, partly embedded in glacier ice. SEM (scanning) studies of the mummy tissue showed that the collagen fibrils in the skin were structurally preserved. In a research paper by Janko, Zink, Gigler, Heckl, and Stark (2010), morphologically intact type I collagen was identified by topographic analysis from extracted from the mummy. Collagen was arranged as single fibrils in a meshwork or stacked in sheet-like structures, as is characteristic for recent skin collagen. Their results indicated that the type I collagen fibrils were preserved through the ages in the mummified skin of the Iceman. And this observation indicates that some of the collagen was slightly altered. Changes in the mechanical properties of the ancient collagen may have occurred owing to freeze–thaw cycles, irradiation by UV light, or dehydration of the tissue. Freeze–thaw damage can change the molecular structure of collagen, as the crystallization of the ice can disrupt and break the fibrils. Then dehydration and the formation of additional crosslinks appear to be the major factor responsible for alterations of the mechanical properties. Removal of the interstitial water can bring the collagen subfibrils closer together, enabling the formation of additional crosslinks.

5 | CONCLUSIONS

The results presented permit us to conclude that the mummy of Cerro El Plomo, which underwent a process of natural mummification by freezing temperatures, strong cold wind and lyophilization, had an excellent preservation of the ultrastructure of the connective tissue of the endomysium and perimysium, and the reaction of encystment of the parasite (connective capsule). While maintaining skeletal striated muscle cell individuality, the cytoplasm has undergone a process of liquefaction, with the absence of subcellular organelles' individuality. In the parasitic infection, there is conservation of reactive connective capsule of the host, through the observation of the ultrastructural integrity of collagen fibers.

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