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A Double Burial from Shahr-I Sokhta Necropolis (Iran). Bioarcheological Investigations and Non-Invasive Biotechnological Studies on Fragments of Human Remains

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Abstract

The archaeological site of Shahr-i Sokhta, in the Sistan region of south-eastern Iran, is noted for the exceptional preservation of human remains documented in its necropolis. This report describes the results of a non-destructive multidisciplinary investigation into a double burial excavated at Shahr-i Sokhta, preserving two skeletons of subadults. The first part of this study provides an archaeotanatological and bioarchaeological description of the burial. Next, we detail the results of the biotechnological techniques applied to bone fragments pertaining to the femurs of the buried children. Following a non-destructive process for studying these samples, we employ a micromolecular technique based on morphological observation using optical, scanning and transmission electron microscopy. This molecular analysis of the bone fragments proves the presence of organic compounds such as tubulin. Through these investigations, we demonstrate that it is possible, utilizing only a few grams of sample, to obtain useful details for scientific research based on non-destructive interdisciplinary investigations.

Keywords

Bioarchaeology, Microanalysis, Human Tubulin, Non-Destructive Analysis, Osteocyte

1. Introduction

Shahr-i Sokhta is located in the Sistan region about 57 km from Zabol. The hot and dry climate, salinity, and gravelly morphology of the site ensure the preservation of organic remains. While today the excavation area is characterized by a desert climate, analysis of the subsoil has reconstructed evidence for some forms of agriculture in the past (Costantini et al., 2007; Sajjadi, 2007). Biotechnological techniques, meanwhile, have provided further and useful information on the archaeological settlement (Biscione, 2010; Milanesi et al., 2016; Milanesi et al., 2019). The methods described in this paper respond to the need to safeguard the subsoil and the precious materials it contains, a goal that has been underserved by the destructive excavation techniques normally employed. The idea of using non-destructive and protective techniques arose from an observation during the 2007 excavation campaign at Shahr-i Sokhta that a series of skeletons that had been recently uncovered incurred rapid damage after being exposed to temperature and brightness. The two child skeletons, which are the object of this study, for example, began to degrade noticeably within a few hours of their removal from the soil. Small bone fragments were secured, and the skeletons were documented photographically. After a few years, we can now demonstrate that even this limited sample of data can provide essential information.

The structural complexity of mineralized tissues is fundamental for understanding the diagenesis of human bones. For instance, osteocytes, often preserved in excavated human remains, can be considered the most numerous lenticular shaped cells in the bone, and are trapped in the calcified matrix within bone cavities. These cells convert mechanical stimuli into internal biochemical signals by means of special cellular components or proteins such as tubulin (Haridy et al., 2021), which act as mechano-sensors (Qin et al., 2020). Microtubules are the main cytoskeletal filaments of eukaryotic cells and participate in the relationship between cell shape, cell division and intracellular transport. The structural genes coding tubulin subunits, namely the α-tubulin and ß-tubulin genes, have received increasing attention in recent decades (Jayaswal et al., 2019), especially for their use as markers to determine the polymorphism of housekeeping genes. In this context, the cytoskeletal apparatus contributes both to the configuration of the cell body of osteocytes and to the peculiar protrusion of primary cilium cells, which is configured as an important sensor coordinating bone homeostasis (Hoey et al., 2012). Environmental factors such as soil pH, soil hydrology and temperature influence the preservation of skeletal tissues. In particular microbial degradation, loss of organics, and mineral changes influence

DNA degradation in mineralized tissues, and multiple diagenetic pathways may act simultaneously on the post-mortem interactions of archaeological skeletal materials (Kendall et al., 2018). The amount of water (waterlogged level) can inhibit diagenetic processes and microbial interaction (Nielsen-Marsh et al., 2000; Lai et al., 2018). Research has highlighted the key role of bacterial communities in the loss of endogenous DNA in some archaeological samples (Rollo et al., 2002). On the other hand, some samples obtained after colonization by bacteria retain their characteristics when subject to fast dehydration, a process responsible for better tissue preservation and useful for amplification and sequencing by PCR (Zaremba-Niedźwiedzka & Andersson, 2013).

This study demonstrates that non-destructive investigations of small quantities of samples can provide a useful basis for archaeotanatological and bioarchaeological studies. In particular, with just a few fragments of a human biological sample, it is possible to conduct micromolecular studies aimed at investigating the morphological and chemical content of cellular residues and the potential evidence of tubulin in archaeological bone tissue.

2. Archaeological Contextualization

Shahr-i Sokhta is a large archaeological site measuring 150 ha (Sajjadi et al., 2003) and located in Iran near the borders with Pakistan and Afghanistan. Several years of archaeological investigations have uncovered a funerary area extending over 20 ha. So far, 1200 tombs have been documented in this area (Biscione et al., 1977; Sajjadi et al., 2008). The 2007 excavation campaign revealed a well-preserved burial (number 8116, square NFH) consisting of two subadult skeletons accompanied by two jars and a bowl (**Figure 1**). The recent analysis of

Figure 1. Samples taken from the compact bones of the children's femur (Circle-point; left = SX and right = DX) from the grave 8116 of the NFH square at Shahr-i Sokhta.

Ascalone (2022) has revised the chronological sequence of Shahr-i Sokhta, including the original dating of the burial to 2200 BCE proposed by Salvatori and Tosi (2005).

3. Methods and Material

3.1. Archaeothanatology and Bioarchaeology

The archaeothanatological approach of this study follows the approach of Duday (2009) and Harris and Tayles (2012). Determinations of the sex and age of the skeletons are made according to Byers (2022) and Buikstra and Ubelaker (1994). Estimations of stature, meanwhile, are calculated according to Sjøvold (1990).

3.2. Sampling

The two fossil skeletons were found in grave 8116 (square NFH) of the large necropolis located at 30˚39'63"N; 61˚23'59.9"E on a plateau about 55 km SW of Zabol in SE Iran (**Figure 2** circle-point). Excavation of the skeletons began with the removal of a first surface layer, followed by a second layer of agglomerated gravel preserving the samples in an anaerobic environment. To prevent contamination of the remains, the excavation team donned sterile gloves and masks, removing the samples within a few minutes of their unearthing. Two fossilized

Figure 2. The archaeological site of Shahr-i Sokhta, Sistan, Iran (30˚39'N; 61˚24'E) lies on a Plio-Pleistocene plateau lies about 55 Km SW of Zabol in SE Iran (Circle-point).

femur fragments measuring a few centimetres were acquired (**Figure 1** circle-point). These samples were quickly deposited in a sterile Falcon capsule and stored under aseptic conditions at room temperature.

3.3. Transmission Electron Microscopy

Next, the bones were prepared for ultrastructural observations. Fragments were fixated in 3% glutaraldehyde and 1% osmium tetroxide for 30 minutes, then gradually dehydrated at room temperature in anhydrous ethanol mixed with decreasing amounts of water (40%, 60%, 80%, and 100%) infiltrated with Spurr epoxy resin, then cured at 70˚C for 7 hours in an oven. Thin sections of the polymerized samples were cut using an LKB III Ultrotome microtome with a diamond blade and examined using a Zeiss Axiophot 400 optical microscope. The ultra-thin sections were collected on copper grids, stained for 3 minutes in 2% uranyl acetate and 2% lead citrate, and subsequently observed using a Philips Morgagni 268D transmission electron microscope (TEM).

3.4. Scanning Electron Microscopy and X-Ray Microanalysis

A sample of bone fragments was coated with graphite (Edwards Scancoat S150A) and observed under a scanning electron microscope (Philips XL20). To determine the chemical elements, the instrument was equipped with an X-ray microanalysis probe, which was used at an acceleration voltage of 20 kV. The concentration of chemical elements on the bones was determined with an error of about 1%. The mean concentrations and standard deviations of each element were measured at five different points in the samples. The X-ray beam was 1 μm wide and penetrated to a depth of 2 μm.

3.5. DNA Isolation from Fossil Human Skeletons and PCR Conditions

70 mg of bone samples from each skeleton were used to extract DNA. DNA was isolated by use of a commercial kit, the GeneElute Mammalian mini prep kit (Sigma Aldrich). Minor modifications were made to minimise the presence of PCR inhibitors that are often present in fossil bones (Rohland & Hofreiter, 2007). The samples were homogenised in a small ceramic mortar 5 cm in diameter. Total DNA was resuspended in 30 microliters of elution buffer provided by the kit. All steps were performed at room temperature (about 20˚C), thereby reducing further degradation of the ancient DNA. The PCR amplification was performed in two-sequential steps under a sterile biohazard laminar flow. The first PCR reaction consisted of a total volume of 12.5 μl containing: 2.5 μl DNA, 0.25 mM of dNTPs, 0.25 μM of each primer, 1× Green GoTaq Reaction buffer containing 1.5 mM MgCl2, 0.1 U of Taq DNA Polymerase (Promega). The first PCR amplification step was performed according to the following cycle scheme: 95˚C for 5 minutes, 39 cycles at: (94˚C for 30 seconds, 60˚C for 30 seconds, and 72˚C for 1 minute), 72˚C for 10 minutes. Primers were synthesized to amplify

small sections of the highly conserved ß-TUB genes. The Forward primer sequence is: AGGGATAGAACGGCTTGAATGG, while the Reverse is: TAGGAGTGCTGAGTTTGAGAGTGC. A second PCR step using the same conditions was conducted using 5 microliters of the amplicon obtained after the first amplification step and increasing the PCR mixture proportionally to a final volume of 50 microliters. The PCR products were checked with standard 1% agarose gel in TAE buffer. The DNA on gel was stained with FluoroVue (Smobio) dye in agreement with the manufacturer's instructions.

4. Results and Discussion

4.1. Archaeothanatology

The double burial consisted of an inhumation grave holding two children (**Figure 1**).

The body (A) observed on the right of **Figure 3** was probably deposited first, followed by the body on the left (B). This depositional hypothesis is suggested by the right femur of A, broken about mid-diaphysis, possibly indicating that it was superimposed on the right tibia of B. Due to the limitations of the existing photographic documentation, however, it cannot be excluded that the femur passed under the tibia. On the basis of the close anatomical resemblance of the two subjects, it can be hypothesized that the inhumations and therefore the deaths of both subjects were contemporary, with the deceased deposited and covered immediately. The absence of earth between the skeletons further strengthens this hypothesis. Both skeletons lie on their side (right side A and left side B) with the upper limbs hyperflexed (171˚A, 168˚B), the hands folded in front of the face, and the lower limbs flexed (50˚A, 23˚B). Previous excavation campaigns in Shahr-i Sokhta have documented the remains of clothing and reed mats or baskets

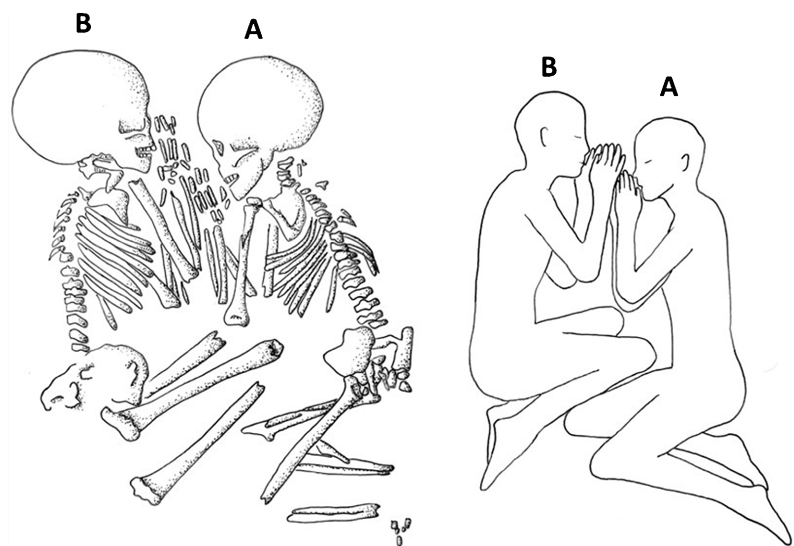

Figure 3. Plan of the double inhumation of children (left) and reconstruction of arrangement of the deceased in the grave (right). A shows the body buried as first and B as second.

together with lace beads found near the cervical spine (Piperno & Salvatori, 2007; Sajjadi et al., 2003), thus suggesting that it was customary to dress the deceased at the time of burial. Organic materials such as mats and clothes are in some cases shown to have dissolved before the soft tissues of the subjects they adorned (Harris & Tayles, 2012), even if traces remain. In the tomb of the two children, however, no traces of clothing were found, and it is plausible that the individuals were buried without clothing. Their high level of preservation is due to the surrounding sediment matrix consisting of very fine-grained soil which implemented a progressive replacement of the soft tissue.

4.2. Bioarchaeology

Judging from the photos taken with metric scales, the teeth conserved in the dental socket of the superior and lower dental arcades suggest an age at death between 6 ± 24 months for both individuals. Namely, the maximum length of the long bones of subject A, with a maximum length of the left humerus of 197 mm, corresponds to an age of 8 - 10. Subject B, with a maximum femoral length of mm 244, was aged 6 - 9 years old (Stloukal & Hanakosa, 1978). No evidence of pathological modifications was observed on the bone remains of children.

4.3. Micromolecular Morphology

The bones contain fibres providing elasticity and minerals that ensure compactness. The main proteins and minerals were deposited by the secretory activity of osteocytes. These latter cells are abundant in bone and serve as the main regulators of bone homeostasis through calcium modulation. They also act as main responses to external mechanical stimuli that induce signalling pathways involved in osteocytes (Qin et al., 2020). The TEM study performed on the samples showed different ultrastructures. The left skeletal bone appeared well preserved with numerous compact fibres (**Figure 4(A)**). In contrast, the right skeleton showed numerous fibres with a less dense structure (**Figure 4(C)**). Morphological analysis carried out via scanning electron microscopy (SEM) of the left skeleton showed a well-preserved structure with the presence of some *canalicula* (**Figure 4(B)**), while the right skeleton appeared to have deteriorated and poorly preserved (**Figure 4(D)**). Human bone is only one of several vertebrate collagenous tissues that are strengthened and hardened *in vivo* by the precipitation of poorly soluble inorganic minerals. Human femur bone has approximately 26 wt% organic matter, 9 wt% - 10 wt% water and 64 wt% mineral fractions (Zioupos et al., 2000). The chemical composition of the fossilized bones was evaluated by SEM-EDAX microanalysis (**Figure 5**). Phosphorus (P) is an essential element of the mineralized bone component and homeostasis appears to be controlled by osteocytes which play an active role in bone mineralization and phosphate regulation (Feng et al., 2009). Phosphorus (P) was detected in both samples but was more abundant in the left skeleton. Calcium (Ca), which plays an essential role in bone firmness (Bonjour, 2013), was contained in similar quantities in the two samples.

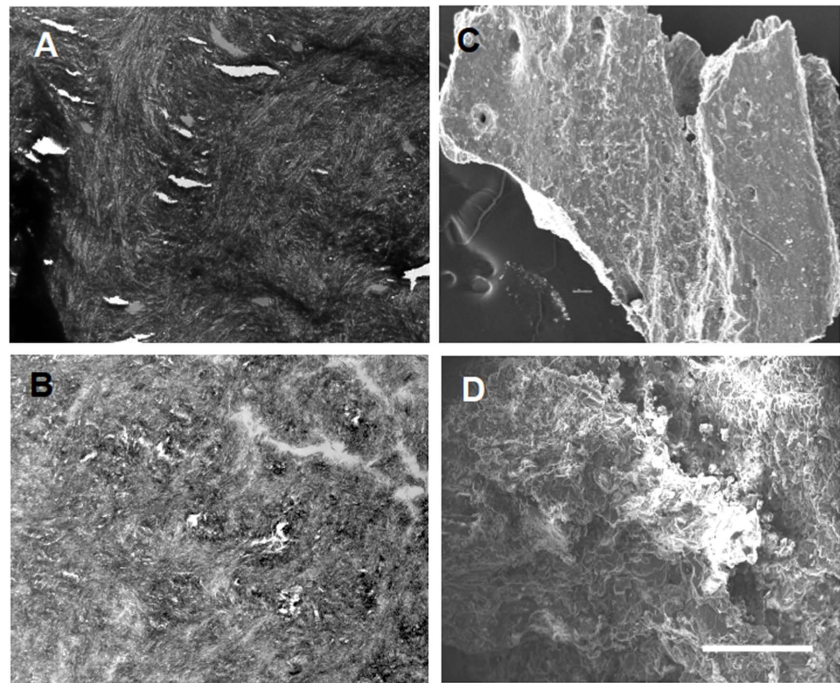

Figure 4. TEM morphology observation of samples a well preserved archaeological left skeleton with numerous details (A) and the right skeleton showing a large quantity of fibres although less electrondense (B); Bar = 5 µm. SEM morphological observation of the archaeological left skeleton with well preserved osteocytes (C) and the archaeological right skeleton deteriorated and poorly preserved (D); Bar = 500 µm.

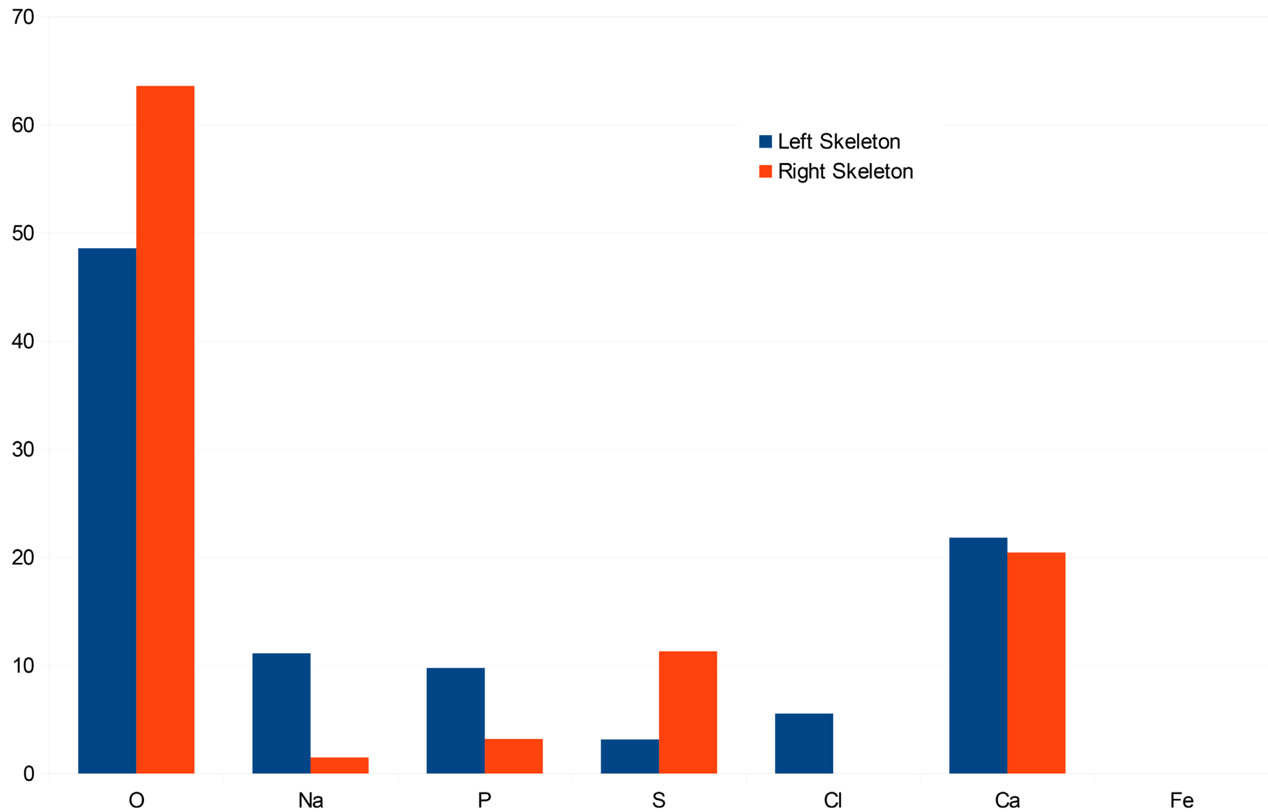

Figure 5. Microanalysis shows the chemical composition of the minerals on the surface level of the femurs fragment.

In the necropolis of Shahr-i Sokhta, after bones found under aerobic conditions are unearthed, the skeletons disintegrate and disappear after a few days, confirming the crucial role of deteriogenic oxygen (O), (Surabian, 2012). This compound is contained in greater quantities in the right sample, which displays a deficiency of Sodium (Na), Phosphorus (P), the absence of Chlorine (Cl) and an abundance of sulphur (S). In the left skeleton, oxygen was detected in a smaller quantity, while we observed the presence of Na, P and Cl and a deficiency of S. This could confirm the quality of the left skeleton's preservation.

4.4. Micromolecular DNA Study

The DNA study was performed on the femurs in an attempt to understand the nomadism or sedentarism of the settlement's inhabitants. During the excavation, local authorities permitted the acquisition of only a small sample quantity. However, once placed in aerobic conditions, the skeletons quickly became deteriorated and volatilized. Despite the small available sample size, it was decided to prepare a test for investigating the presence of tubulin in the bones. In human subjects, the temporal bone is known to have a high degree of mineralisation, and this is likely due to high levels of protein and endogenous DNA that can survive in archaeological specimens compared to non-petrous skeletal elements (Gamba et al., 2014; Jørkov et al., 2009). Like other archaeological bone fragments, our samples taken relating to human femurs showed the presence of biomolecules such as DNA. Agarose gel analysis of the PCR products revealed amplicons of 140 bp after the second round of amplification (**Figure 6**). DNA sequencing results were obtained only for PCR products derived from the left

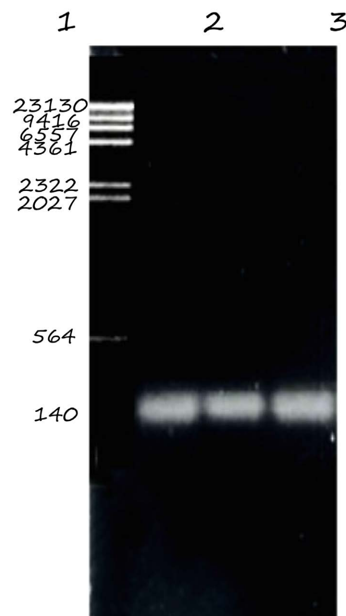

Figure 6. Analysis by agarose gel of 140 bp PCR products obtained from human skeletons. From left: 1) Lambda/Hind III [250 ng]; 2) Human archaeological skeleton on the right; 3) Human archaeological skeleton on the left.

skeleton. We found low amounts of nucleic acids in the right skeleton. Osteocytes found in archaeological bones may contain a significant trace of nucleic acids and the α-tubulin and ß-tubulin genes are considered the main cytoskeletal markers of eukaryotic cells, especially for the purpose of determining the presence of particular housekeeping genes and assessing the changes occurring in DNA traits with which they are associated. CLUSTAL W direct comparison of the left skeletal PCR products (**Figure 7**) targeting the ß gene-TUB revealed a significant correspondence with the 3'-prime end of the *Homo sapiens* tubulin beta 4B class IVb (TUBB4B), mRNA (Jayaswal et al., 2019). In particular, amplicons detected the 3-prime end of the untranslated mRNA which was enriched with several untranslated sequences. Conserved non-coding elements (CNEs) show an extraordinary degree of conservation between species and are distributed in a non-random way on chromosomes which tend to cluster near genes with regulatory roles in cell development. Vertebrate genomes contain thousands

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CLUSTAL O(1.2.4) multiple sequence alignment


NM_006088.6      AGCACTCTGCGCGCCCGCTCTTCTGCTGCTGTTTGTCTACTTCCTCCTGCTTCCCCGCCG   60
SK               ------------------------------------------------------------   0

NM_006088.6      CCGCCGCCGCCATCATGAGGGAAATCGTGCACTTGCAGGCCGGGCAGTGCGGCAACCAAA   120
SK               ------------------------------------------------------------   0

NM_006088.6      TCGGCGCCAAGTTTTGGGAGGTGATCAGCGATGAGCACGGCATCGACCCCACGGGCACCT   180
SK               ------------------------------------------------------------   0

NM_006088.6      ACCACGGGGACAGCGACCTGCAGCTGGAACGCATCAACGTGTACTACAATGAGGCCACCG   240
SK               ------------------------------------------------------------   0

NM_006088.6      GCGGCAAGTACGTGCCCCGCGCCGTGCTCGTGGATCTGGAGCCCGGCACCATGGACTCCG   300
SK               ------------------------------------------------------------   0

NM_006088.6      TGCGCTCGGGGCCCTTCGGGCAGATCTTCCGGCCGGACAACTTCGTTTTCGGTCAGAGTG   360
SK               ------------------------------------------------------------   0

NM_006088.6      GTGCTGGGAACAACTGGGCCAAGGGGCACTACACAGAAGGCGCGGAGCTGGTGGACTCGG   420
SK               ------------------------------------------------------------   0

NM_006088.6      TGCTGGATGTTGTGAGAAAGGAGGCTGAGAGCTGTGACTGCCTGCAGGGTTTCCAGCTGA   480
SK               ------------------------------------------------------------   0

NM_006088.6      CCCACTCCCTGGGTGGGGGGACTGGGTCTGGGATGGGTACCCTCCTCATCAGCAAGATCC   540
SK               ------------------------------------------------------------   0

NM_006088.6      GGGAGGAGTACCCAGACAGGATCATGAACACGTTTAGTGTGGTGCCTTCGCCCAAAGTGT   600
SK               ------------------------------------------------------------   0

NM_006088.6      CAGACACAGTGGTGGAGCCCTACAACGCCACCCTCTCAGTCCACCAGCTCGTAGAAAACA   660
SK               ------------------------------------------------------------   0

NM_006088.6      CAGACGAGACCTACTGCATTGATAACGAAGCTCTCTACGACATTTGCTTCAGAACCCTAA   720
SK               ------------------------------------------------------------   0

NM_006088.6      AGCTGACCACGCCCACCTATGGTGACCTGAACCACCTGGTGTCTGCTACCATGAGTGGGG   780
SK               ------------------------------------------------------------   0

NM_006088.6      TCACCACCTGCCTGCGCTTCCCAGGCCAGCTCAATGCTGACCTGCGGAAGCTGGCTGTGA   840
SK               ------------------------------------------------------------   0
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NM_006088.6       ACATGGTCCCGTTTCCCCGGCTGCACTTCTTCATGCCCGGCTTTGCCCCACTGACCAGCC   900
SK                ------------------------------------------------------------   0

NM_006088.6       GGGGCAGCCAGCAGTACCGGGCGCTGACCGTGCCCGAGCTCACCCAGCAGATGTTTGATG   960
SK                ------------------------------------------------------------   0

NM_006088.6       CCAAGAACATGATGGCTGCCTGCGACCCCCGCCATGGCCGCTACCTGACGGTTGCCGCCG   1020
SK                ------------------------------------------------------------   0

NM_006088.6       TGTTCAGGGGCCGCATGTCCATGAAGGAGGTGGATGAGCAAATGCTTAATGTCCAAAACA   1080
SK                ------------------------------------------------------------   0

NM_006088.6       AAAACAGCAGCTATTTTGTTGAGTGGATCCCCAACAATGTGAAAACGGCTGTCTGTGACA   1140
SK                ------------------------------------------------------------   0

NM_006088.6       TCCCACCTCGGGGGCTAAAAATGTCCGCCACCTTCATTGGCAACAGCACGGCCATCCAGG   1200
SK                ------------------------------------------------------------   0

NM_006088.6       AGCTGTTCAAGCGCATCTCCGAGCAGTTCACGGCCATGTTCCGGCGCAAGGCCTTCCTGC   1260
SK                ------------------------------------------------------------   0

NM_006088.6       ACTGGTACACGGGCGAGGGCATGGACGAGATGGAGTTCACCGAGGCCGAGAGCAACATGA   1320
SK                ------------------------------------------------------------   0

NM_006088.6       ATGACCTGGTGTCCGAGTACCAGCAGTACCAGGATGCCACAGCCGAGGAGGAGGGCGAGT   1380
SK                ----------------------------------------------------AAGGGGGT   9
                                                                     * ** * **

NM_006088.6       TCGAGGAGGAGGCTGAGGAGGAGGTGGCCTAGAGCCTTCAGTCACTGGGGAAAGCAGGGA   1440
SK                TTGGGCGGGC-------------ATATGTTAGTAGGGTGTATGAGCCAGTATACAACCGG   56
                  * * *  **               *    ***     *   * *    * * *  *  *

NM_006088.6       AGCAGTGTGAACTCTTTATTCACTCCCAGCCTGTCCTGTGGCCTGTCCCACTGTGTGCAC   1500
SK                ACCGGTGATAGCTACATAGAGCCATAA---AGGCCTTGTGATCTTTG----------CTA   103
                  * * ***  * **   **    *         * * ****  ** *           *

NM_006088.6       TTGCTGTTTTCCCTGTCCACATCCATGCTGTACAGACACCACCATTAAAGCATTTTCATA   1560
SK                ATTTTTTATGCACTCTCAAACTCAGCCCTCCTAAAAAA----------------------   141
                   *  * * * * ** ** * **    **     * * *

NM_006088.6       GTG    1563
SK                ---    141
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Figure 7. Clustal W of the sequence of the PCR product obtained from the left archaeological skeleton aligned with the *Homo sapiens* tubulin beta 4B class IVb (TUBB4B), mRNA Beta-TUB. A significant match was found at the 3'-prime end of the sequence, as shown by the asterisks.

of conserved non-coding elements that often function as tissue-specific enhancers (Alison et al., 2011). The selection acting on the enhancer sequences is mainly responsible for the retention of these regions, which may be associated with a physicochemical stability of the CNE motifs among the taxa (Hezroni et al., 2017). Finally, some factors, including DNA quality and amplification efficiency, could interfere and affect the accuracy and reliability of the results obtained. Therefore, genes such as tubulin that constantly maintain their gene expression and, therefore, their basic functions should guarantee the accuracy of the experimental results obtained (Arya et al., 2017; Nagy et al., 2017). Furthermore, the development of a protocol that works on ancient biological material to obtain information on the conservation of DNA sequences during evolution may be suggested, helping to formulate hypotheses of a phylogenetic nature (Shi et al., 2016).

5. Conclusion

In archaeological excavation, greater attention should be paid to the conservation of organic finds, which are quickly destroyed by oxidation once uncovered. In this study, we have attempted to unite interdisciplinary approaches aimed at safeguarding these precious finds. Through an archaeothanatological and bioarchaeological investigation of the remains and a micromolecular morphology and DNA study of the few fragments of bone tissue collected, we have offered a few hypotheses. Archaeothanatology investigations suggest that the body on the right was placed first due to the overlap of the lower limbs. Furthermore, the children were buried without clothing as they lay on the ground wrapped in very fine powdered inorganic material, stabilizing the bone remains. The teeth preserved in the sockets of the upper and lower dental arches and the length of the long bones suggest an age of about 6.5 years for subject A and about 7 years for subject B. No evidence of pathological changes was observed. Electron microscopy and microanalysis of the bone fragments confirmed the poor preservation state of the right skeleton and, conversely, the good state of conservation of the left skeleton. The latter showed numerous ultrastructural details, including fibres and chemical elements such as Ca, Na, P, Cl and S essential for bone homeostasis. Furthermore, in line with its better state of preservation, the deteriogenic element O was found in less quantity for the left skeleton. Finally, the results of the molecular analysis confirm the presence of amplifiable tubulin in the remains of the left skeleton. The discovery of the *homo sapiens* beta tubulin gene fragment could be useful in the future for further phylogenetic studies. This preliminary interdisciplinary investigation confirms that even a fragment of fossil bone may contain useful information and in-depth studies of small samples can reveal details about the mystery of past life in the future.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

Alison, P. L., Kerk, S. Y., Tan, Y. Y., Brenner, S., & Venkatesh, B. (2011). Ancient Vertebrate Conserved Noncoding Elements Have Been Evolving Rapidly in Teleost Fishes. *Molecular Biology and Evolution, 28,* 1205-1215. https://doi.org/10.1093/molbev/msq304

Arya, S. K., Jain, G., Upadhyay, S. K., Sarita, S. H., Dixit, S., & Verma, P. C. (2017). Reference Genes Validation in *Phenacoccus solenopsis* under Various Biotic and Abiotic Stress Conditions. *Science Report, 7,* Article No. 13520.

https://doi.org/10.1038/s41598-017-13925-9

Ascalone, E. (2022). Preliminary Report of 2018-2019 Excavations in Area 33 at Shahr-i Sokhta In E. Ascalone, & S. M. S. Sajjadi (Eds.), *Excavations and Researches at Shahr-i Sokhta 3 (= ERSS 3)* (pp. 145-167). Studies and Publications Institute, Iranian Center for Archaeological Research.

Biscione, R. (2010). Iran: Campagne 2007-2008 a Shahr-i Sokhta (Sistan, Iran Orientale) e nuove ricerche sulla crisi dell'urbanizzazione della fine del III millennio a.C. *AMEA, 52,* 203-289

Biscione, R., Salvatori, S., & Tosi, M. (1977). Shahr-i Sokhta: L'abitato e la sequenza cronologica. In P. Basaglia (Ed.), *La città bruciata del serto salato* (pp. 77-112). Venezia.

Bonjour, J. P. (2013). Calcium and Phosphate: A Duet of Ions Playing for Bone. *Journal of the American College of Nutrition, 30,* 438s-448s.

Buikstra, J. E., & Ubelaker, D. H. (1994). *Standards for Data Collection from Human Skeletal Remains*. Arkansas Archeological Survey.

Byers, S. N. (2022). *Introduction to Forensic Anthropology*. Routledge.

Costantini, L., Costantini-Biagini, L., Delle Donne, M., Sajjadi, M., Strika, S., & Zaruri, M. (2007). Funerary Offerings at Shahr-i Sokhta: Foods and Beverage during the Bronze Age. Archaeological Report 7. In *Proceeding of the 9th Annual Symposium on Iranian Archaeology* (pp. 333-338).

Duday, H. (2009). *The Archaeology of the Dead: Lectures in Archaeothanatology*. Oxbow Books. https://doi.org/10.2307/j.ctt1cd0pkv

Feng, J. Q., Ling, Y., & Schiavi, S. (2009). Do Osteocytes Contribute to Phosphate Homeostasis? *Current Opinion in Nephrology and Hypertension, 18,* 285-291. https://doi.org/10.1097/MNH.0b013e32832c224f

Gamba, C., Jones, E. R., Teasdale, M. D., Mclaughlin, R. L. et al. (2014). Genome Flux and Stasis in a Five Millennium Transect of European Prehistory. *Nature Communication, 5,* 5257. https://doi.org/10.1038/ncomms6257

Haridy, Y., Osenberg, M., Hilger, A., Manke, I., Davesne, D., & Witzmann, F. (2021). Bone Metabolism and Evolutionary Origin of Osteocytes: Novel Application of FIB-SEM Tomography. *Science Advance, 7,* eabb9113. https://doi.org/10.1126/sciadv.abb9113

Harris, N. J., & Tayles, N. (2012). A Hidden Aspects of Mortuary Practices: Archaeothanatology at Ban Non Wat, Thailand. *Journal of Anthropological Archaeology, 31,* 227-239. https://doi.org/10.1016/j.jaa.2012.01.001

Hezroni, H., Ben-Tov, P. R., & Meir, Z. (2017). A Subset of Conserved Mammalian Long Non-Coding RNAs Are Fossils of Ancestral Protein-Coding Genes. *Genome Biology, 18,* 162. https://doi.org/10.1186/s13059-017-1293-0

Hoey, D. A., Chen, J. C., & Jacobs, C. R. (2012). The Primary Cilium as a Novel Extracellular Sensor in Bone. *Frontiers in Endocrinology, 3,* 75. https://doi.org/10.3389/fendo.2012.00075

Jayaswal, P., Shanker, A., & Singh, N. K. (2019). Phylogeny of Actin and Tubulin Gene Homologs in Diverse Eukaryotic Species. *Indian Journal of Genetics and Plant Breeding, 79,* 284-291. https://doi.org/10.31742/IJGPB.79S.1.20

Jørkov, M. L. S., Heinemeier, J., & Lynnerup, N. (2009). The Petrous Bone a New Sampling Site for Identifying Early Dietary Patterns in Stable Isotopic Studies. *American Journal of Physical Anthropology, 138,* 199-209. https://doi.org/10.1002/ajpa.20919

Kendall, C., Eriksen, A. M. H., Kontopoulos, I., Collins, M., & Turner-Walker G. (2018).

Diagenesis of Archaeological Bone and Tooth. *Palaeogeography Palaeoclimatology Palaeoecology, 491,* 21-37. https://doi.org/10.1016/j.palaeo.2017.11.041

Lai, J. et al. (2018). Review of Diagenetic Facies in Tight Sandstones: Diagenesis, Diagenetic Minerals, and Prediction via Well Logs. *Earth-Science Reviews, 185,* 234-258. https://www.sciencedirect.com/science/article/pii/S0012825218300527 https://doi.org/10.1016/j.earscirev.2018.06.009

Milanesi, C., Faleri, C., Cresti, M. et al. (2019). Apple Seeds in an Excavated Roman Amphora Remained Intact for 2000 Years despite Exposure to a Broadly-Degrading Microbial Community. *Journal of Archaeological Science Report, 25,* 472-485. https://doi.org/10.1016/j.jasrep.2019.04.024

Milanesi, C., Scali, M., Vignani, R. et al. (2016). Archaeobotanical Reconstruction of Vegetation and Report of Mummified Apple Seeds Found in the Cellar of a First-Century Roman Villa on Elba Island. *Comptes Rendus Biologie, 339,* 487-497. https://doi.org/10.1016/j.crvi.2016.09.003

Nagy, N. A., Németh, Z., Juhász, E. et al. (2017). Evaluation of Potential Reference Genes for Real-Time qPCR Analysis in a Biparental Beetle, *Lethrus apterus* (Coleoptera: Geotrupidae). *PeerJ, 5,* e4047. https://doi.org/10.7717/peerj.4047

Nielsen-Marsh, C., Gernaey, A., Turner-Walker, G. et al. (2000). The Chemical Degradation of Bone. In M. Cox, & S. Mays (Eds.), *Human Osteology: In Archaeology and Forensic Science* (pp. 439-454). Cambridge University Press.

Piperno, M., & Salvatori, S. (2007). *The Shahr-I Sokta Graveyard (Sistan, Iran): Excavation Campaigns, 1972-1978* (p. 390). Series Report and Memories, New Series, V. 6, IsIAO.

Qin, L., Liu, W., Cao, H., & Xiao, G. (2020). Molecular Mechanosensors in Osteocytes. *Bone Research, 8,* Article No. 23. https://doi.org/10.1038/s41413-020-0099-y

Rohland, N., & Hofreiter, M. (2007). Ancient DNA Extraction from Bones and Teeth. *Nature Protocols, 2,* 1757. https://doi.org/10.1038/nprot.2007.247

Rollo, F., Ubaldi, M., Marota, I. et al. (2002). DNA Diagenesis: Effect of Environment and Time on Human Bone. *Ancient Biomolecules, 4,* 1-7. https://doi.org/10.1080/13586120290018473

Sajjadi, S. M. S. (2007). *Excavation at Shahr-i Sokhta. Graveyard 1977-2000.* ICHTO.

Sajjadi, S. M. S., Canova, M., Costantini, L., & Lorentz, K. O. (2008). Sistan and Baluchistan Project: Short Report on the Tenth Campaign of Excavations at Shahr-i Sokhta. *Iran, 46,* 307-334. https://doi.org/10.1080/05786967.2008.11864751

Sajjadi, S. M. S., Forunzafar, F., Shirazi, R., & Baghestani, S. (2003). Excavations at Sharh-i Soktha. First Preliminary Report on the Excavations of the Graveyard, 1997-2000. *Iran, 41,* 21-97. https://doi.org/10.2307/4300639

Salvatori, S., & Tosi, M. (2005). Shahr-i Sokhta Revised Sequence. In C. Jarrige, & V. Lefevre (Eds.), *South Asian Archaeology 2001* (pp. 281-292). Editions Recherche sur les Civilisations.

Shi, C., Yang, F., Zhu, X., Du, E., Yang, Y., & Wang, S. (2016). Evaluation of Housekeeping Genes for Quantitative Real-Time PCR Analysis of *Bradysia odoriphaga* (Diptera: Sciaridae). *International Journal of Molecular Sciences, 17,* E1034. https://doi.org/10.3390/ijms17071034

Sjøvold, T. (1990). Estimation of Stature from Long Bones Utilizing the Line of Organic Correlation. *Human Evolution, 5,* 431-447. https://doi.org/10.1007/BF02435593

Stloukal, M., & Hanakova, M. H. (1978). Die Lange der Langsknochen altslavischer Bevolkerungen unter besonderer Berucksichtigung von Wachstumsfragen. *Homo, 29,* 53-69.

Surabian, D. (2012). *Preservation of Buried Human Remains in Soil*. Nature Resources Conservaion Service Tolland.

Zaremba-Niedzwiedzka, K., & Andersson, G. E. (2013). No Ancient DNA Damage in Actinobacteria from the Neanderthal Bone. *PLOS ONE, 8,* e62799. https://doi.org/10.1371/journal.pone.0062799

Zioupos, P., Currey, J. D., & Casinos, A. (2000). Exploring the Effects of Hypermineralisation in Bone Tissue by Using an Extreme Biological Example. *Connective Tissue Research, 41,* 229-248. https://doi.org/10.3109/03008200009005292