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Human brains found in a fire-affected 4000-years old Bronze Age tumulus layer rich in soil alkalines and boron in Kutahya, Western Anatolia

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ABSTRACT

Undecomposed human bodies and organs always attracted interest in terms of understanding biological tissue stability and immortality. Amongst these, cases of natural mummification found in glaciers, bog sediments and deserts caused even more attention. In 2010, an archeological excavation of a Bronze Age layer in a tumulus near the Western Anatolia city Kütahya revealed fire affected regions with burnt human skeletons and charred wooden objects. Inside of the cracked skulls, undecomposed brains were discernible. To analyze the burial taphonomy of the rare phenomenon of brain preservation, we analyzed brains, bone, teeth and surrounding soils elements using Inductively Coupled Plasma-Mass Spectrometer (ICP-MS). Adipocere formation or saponification of postmortem tissue fat requires high levels of alkalinity and especially potassium. Indeed, ICP-MS analysis of the brain, teeth and bone and also of the surrounding soil revealed high levels of potassium, magnesium, aluminum and boron, which are compatible with the famous role of Kütahya in tile production with its soil containing high

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level of alkalines and tile-glazing boron. Fatty acid chromatography revealed simultaneous saturation of fats and protection of fragile unsaturated fatty acids consistent with soil-presence of both pro-oxidant and anti-oxidant trace metals. Computerized tomography revealed protection of diencephalic, metencephalic and occipital tissue in one of the best-preserved specimens. Boron was previously found as an intentional preservative of Tutankhamen and Deir el Bahari mummies. Here, in natural soil with its insect-repellant, anti-bacterial and fire-resistance qualities it may be a factor to preserve heat-affected brains as almost bioporcellain specimens.

% Ö z e t

Çürümemiş insan vücutları ve organları her zaman biyolojik doku stabilitesini ve ölümsüzlüğü anlamak isteyen araştırmacılar için dikkat çekici olmuştur. Bunların arasında buzullarda, turba bataklık sedimentlerinde ve çöllerde rastlanan doğal mumyalaşma daha da dikkat çekici olmaktadır. 2010 yılında Batı Anadolu şehri Kütahya'nın yanında bulunan Seyitömer höyüğünde orta Bronz çağı katmanı kazılırken, kısmen yanmış insan iskeletler ve tahta parçalarının yanında çatlamış kafatasları içerisinde çürümemiş beyinlere rastlandı. Gömülme koşullarının anlaşılması için, hem beyinlerin, hem kemikler, dişler ve etraftaki toprağın elementleri Inductively Coupled Plasma – Mass Spectrometer (ICP-MS) tekniği ile analiz edildi. Ölü mumu olarak da bilinen adipocere formasyonu ya da saponifikasyon post-mortem yağ dokusunun yüksek miktarda alkalin özellikle potasyumla muamelesini gerektirmektedir. Gerçektende, ICP-MS analizleri beyin, diş ve kemik dokusu ile içlerinden çıktıkları toprağın içerisinde yüksek miktarda potasyum, magnezyum, alüminyum ve boron tesbit etti. Bu da Kütahya'nın çini üretimindeki bilinen rolü ve toprağındaki alkalenler ve çini parlatmada kullanılan bor varlığı ile örtüşüyordu. Yağ asidi kromatografisi aynı anda hem yağ asitlerinin satürasyonuna hem de frajil doymamış yağ asitlerinin korunmasına işaret ediyordu. Bu da toprakta hem oksidan hem de anti-oksidan elementlerin dengeli varlığına bağlandı. Bilgisayarlı tomografi diensefalik, metensefalik ve oksipital korteksin en iyi korunmuş örnekte mevcut olduğunu gösterdi. Daha önce yapılan çalışmalar Tutankamon ve Deyr El Bahari mumyalarında borun kasıtlı bir koruyucu olarak kullanıldığını göstermişti, böcek uzaklaştırıcı, anti-bakteriyel ve ısı-rezistansı sağlayıcı etkileri ile ısıya maruz kalmış beyinlerin adeta biyo-porselene dönüşmesinde de bor etkin olmuş olabilir.

Introduction

Human undecomposed corpses and organs thousands of years old are extremely unique and very special cases not only for archeological anthropologists but also for life scientists to illuminate new associations between different living conditions, dietary habits and human health. Indeed, the famous example of Ötzi from Austria-Italy border, a 5000-years old Neolithic human corpse found in Alps revealed many details about ancient human life (Makristathis et al., 2002; Sjøvold, 1998).

In this study, we would like to report ancient human brains found near the Western Anatolian city Kütahya (Fig. 1a) in Seyitömer Höyük (layered tumulus, Fig. 1b). Seyitömer Höyük is situated southwest of the village of Seyitömer and 26 km northwest of Kütahya. The mound, 23 m high and 150 m in diameter, was occupied from the Chalcolithic to the Roman periods and later. The site was

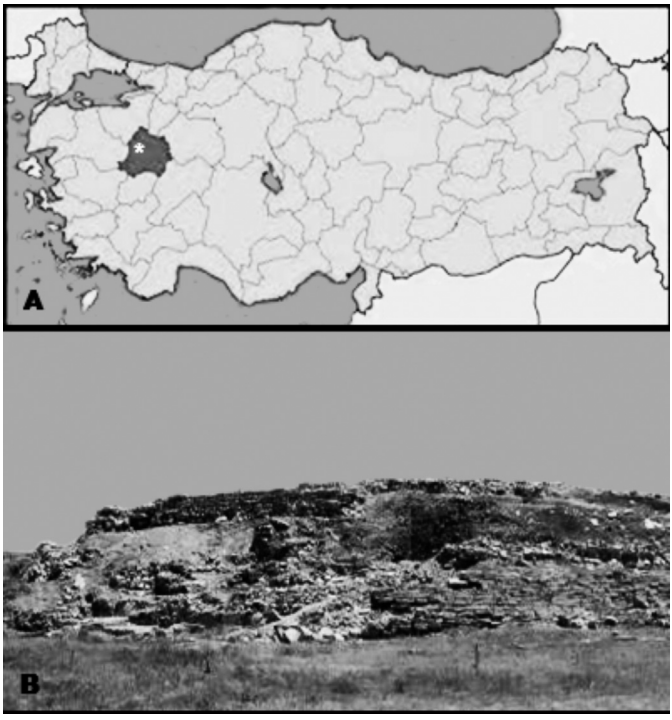

Fig. 1. (a) Location of the city Kütahya in Western Anatolia, Turkey. (b) General view of the Seyitömer Höyük.

investigated in 1989 by a team from Eskişehir Museum and in the 1990s by Afyon Museum. The mound was situated within the area of the Seyitömer Lignite Company, which intends to mine coal beneath it, and thus excavations were revived in 2006 as a five-year salvage project led by A. Nejat Bilgen of Dumlupınar University.

During Seyitömer Höyük excavations, an early stage of the middle Bronze Age layer (4c layer) was encountered (Fig. 2). Signs of a likely fire disaster and partly burnt human skeletons were encountered

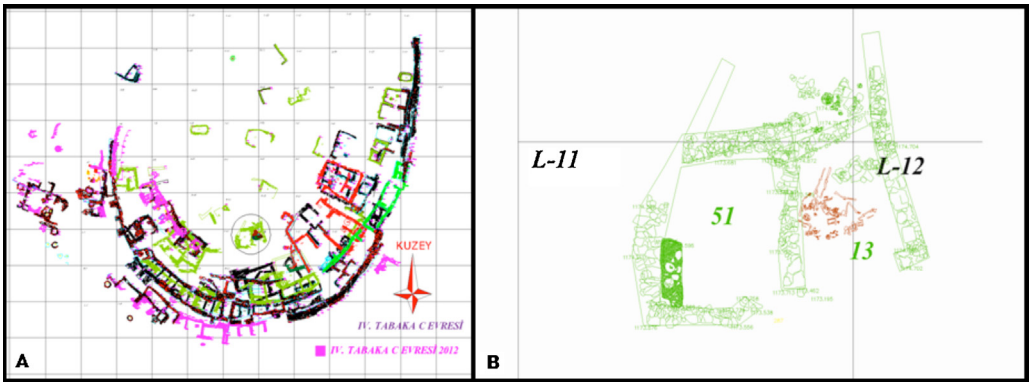

Fig. 2. (a) Schematic drawing of the Middle Bronze Age layer, where six partly carbonified human skeletons were found. (b) Close-up view of the encircled area in (a), where the skeletons were placed.

near burnt wooden debris. In some of these skeletons, four cracked skull bones were surprisingly containing relatively well-preserved human brains. Among these four human brains, the best preserved, intact specimen was kept for museum exhibition and histological studies were performed on the two of partly fragmented brains.

A fault line crossing the same layer may indicate a possible earthquake causing rapid burial and fire exposure of the corpses. Human brains are among the fastest decomposing tissues after death by liquefaction necrosis. Thus, these findings were quite rare even for archeologists frequently encountering human skeletons. To enlighten the underlying conditions of these rare findings, we aimed to employ a multi-disciplinary archeometric approach to define the burial taphonomy and natural mummification of Seyitömer brains. To achieve our goals, we first made morphological descriptions of the best preserved specimen with naked eye and computerized tomography (CT) and also investigated the specimen's microscopical features. Then we analyzed fatty acids using gas chromatography and elemental contents using Inductively Coupled Plasma-Mass Spectrometer (ICP-MS).

Materials and methods

Dating of the samples

Dating of the samples from the 4c layer was performed first by optically stimulated luminescence (OSL, instrument DTU model Risø TL/OSL-DA-20, performed by Sevgi Altınok under supervision of Erhan Altunel, Professor of Osman Gazi University, Department of Geology). The samples were dated to 1900–2000 BCE. Classical archeological dating evaluating pottery of the same layer, where brains were found, confirmed the results of OSL.

Histo-pathological analysis

Carbonized tissue samples consisting of brain tissue were highly fragile. Since almost all histological procedures are normally performed in water containing tissues, three different tissue processing methods were undertaken to overcome processing difficulties of water-free ancient tissues. Techniques were as follows:

Formic acid treatment/decalcification-method: Carbonified brain materials taken from already-degraded brain samples were soaked and shaked in formic acid for 90 min such that samples segregated into tiny particles. Then, these tiny pieces were blotted and transported into cassettes, washed with tap water for 1 h and then rinsed for overnight. Routine tissue processing and paraffin embedding (TP/PE) methods were employed, which followed staining with hematoxylin-eosin (H&E) and toluidine blue (TB).

Cryostat sectioning: The tissues were directly sectioned via a cryostat apparatus superseded by routine TP/PE and H&E/TB staining.

Reprocessing method: Appropriate procedures were followed both for formalin-paraffin processing and cryostat sections. These comprised exposure of brain specimens to 96% ethanol for 120 min, 70% ethanol for 90 min and 50% ethanol for 60 min. Then, pieces were rinsed with tap water and kept overnight. After completion of these steps, all samples were processed by routine formalin-paraffin procedures and then sectioned with cryostat.

Computerized tomography analysis

Serial sections of 0.75 mm thickness of brain parenchyma in accordance with compatible anatomic positions were realized using 16 consecutive CT apparatus (Siemens Sensation 16) by applying 120 kV 320 effective mAs parameters. 0.75 mm section thickness reconstructions were acquired by standard algorithm. These data were used for production of multiplanar reconstructions with 3 orthogonal plane images.

Inductively Coupled Plasma-Mass Spectrometer (ICP-MS) analysis of chemical elements

Elements were analyzed not only in the mummified brain tissue, also in the skull bone and teeth of the corresponding corpse as well as in the barley seeds found in the same layer and surrounding soil. By this, it was aimed to obtain a comprehensive picture of the environment, which could affect the body chemistry with biogenesis and diagenesis.

Initial washing procedure

For elemental sampling, different specimen elemental contents except the brain were analyzed both as crude samples and after a specific washing procedure to eliminate the external contamination. Washing procedure was not applied to brain samples as the brain tissues absorbed the washing chemicals and water and did not dry completely. Samples (tooth, bone, seed and soil) were gently washed with de-ionized water (Millipore Direct Q3, Billerica, MA, USA), dried at 40 °C and subsequently treated with ultrasonic bath in diethyl ether (analytical grade, Sigma–Aldrich, Steinheim, Germany) for 60 min, then again dried at 40 °C. They were then treated with ultrasonic bath in formic acid (≥98% purity, Merck, Darmstadt, Germany) for 5 min and washed with de-ionized water 3 times and dried at 40 °C for the last step of washing procedure.

Sample preparation procedure for microwave acid digestion

The processed teeth and bone weights were between 0.37 and 0.44 grams and digested in 20 ml nitric acid (65%, Suprapure, Merck, Darmstadt, Germany). For seeds and soil, 10 ml nitric acid (65%) was used for digestion, whereas for brain samples a mixture of 3 ml deionized water and 7 ml nitric acid was used. Four teeth, four brains, two seeds and one soil sample were analyzed, each of these samples were run for 3 times and the averaged results were listed in the results table. The acid digestion protocol was completed using a microwave (CEM, Mars5, Matthews, NC, USA).

Microwave acid digestion

The acid digestion processes for bone, brain, teeth, seed and soil samples were performed with a heating program under 100% power and with ramp times of 25 min for bone and teeth specimens, 10 min for seeds and soil and for 20 min for brain specimens, respectively. For bone and tooth 1172 kPa, for soil and seed 1379 and 1724 kPa and for brain 1172 kPa pressures were employed under temperatures of 210 °C, 170 °C, 200 °C and 210 °C, respectively. Under maximal pressure and heat, brain specimens were held for 5 min and all other specimens were held for 10 min.

ICP-MS analysis

ICP-MS (Thermo Scientific X Series II, Bremen, Germany) with an RF power of 1300 W was employed. Argon flow rates were 13.2 L/min for plasma gas, 0.78 L/min for auxiliary gas and 0.87 L/min for nebulizer gas, respectively. The dwell time was 10 ms and each sample was run as 3 replicates as described. The spray chamber temperature was 3 °C. The cones were Ni (sample and skimmer) and sample uptake was continued for 60 s.

Calibration standards

For calibration standards (High-Purity Standards, Charleston), indium (Absolute standards, Inc., Hamden, CT, USA) was used as an internal standard with calibration points set for blank, 5, 10, 25, 50, 100 and 250 ng/mL. Correlation coefficients were $r^2 \geq 0.999$.

Fatty acid chromatography in brain specimens

Accurately weighted portion of brain sample was homogenized in cold 154 mM NaCl. Total lipids and added internal standard (100 μg nonadecanoic acid in choloroform, Sigma Chemical Co, St Louis) were extracted with chloroform/methanol (2:1) containing 0.005% butylated hydroxytoluene. The chloroform phase was removed and evaporated to dryness under a stream of nitrogen. Total lipids were saponified with 2% KOH in methanol and the fatty acids (FA) methylated with 14% BF_3 (boron

trifluoride) in methanol. The resulting FA methyl esters (FAMEs) were extracted with hexane and analyzed by capillary gas chromatography (Perkin-Elmer 8420 Capillary Gas Chromatography (GC), Gouda, The Netherlands. Column: 50× 0.25 mm WCOT fused silica, CP-sil 88; flame-ionization detector temperature 300 °C; oven temperature program from 150 to 230 °C at 2 °C min^{-1}; carrier gas N_2). The mass spectra of FAME from representative samples were obtained using a Hewlett-Packard (HP) 6890 capillary GC interfaced with a HP mass selective detector and controlled by a HP Chem Station (Column: 25 mm × 0.25 mm ID, QC2xBPx70; detector temperature 280 °C; oven temperature program from 100 °C to 290 °C at 3 °C min^{-1}; carrier gas helium). FAMEs were identified by their retention time and compared to those of authentic standards (Sigma Chemical Co, St Louis), and by GC-Mass Spectrometry. The detector response factors were determined by injecting equal amounts of FAs and internal standard methyl esters on to the column. Their amounts were estimated by calculating the corresponding areas of FA and internal standard.

Results

Anatomic description of the best preserved Seyitömer brain

Macroscopic findings

Brain weighed 65grams and its dimensions were measured as 210 mm × 120 mm × 60 mm. Naked eye examination revealed disturbed bilateral frontal and frontotemporal surface anatomic structures. Multiplanar photographic pictures were taken for further detailed neuroanatomic analyses. Gross examination revealed that the brain base including left cerebellar hemisphere, brain stem (pons and medulla oblongata), and bilateral temporal lobes were relatively well preserved (Fig. 4a–c). Bilateral frontal base structures including gyrus rectus, orbital sulci, orbital gyri, olfactory sulci were destroyed (Fig. 3). Structures anterior to the line drawn through temporal poles barely showed their original anatomic details (Fig. 3). When compared with human postmortem brain, there was a striking decrease by volume and weight (5%) due to environmental conditions and time. This was explained by vaporization of water content of neural tissue. Other than distorted and/or destroyed frontal and frontotemporal neuroanatomical structures, diencephalic, posterior fossa (cerebellum) and metencephalic (pons and medulla oblongata) landmarks were compatible with the modern human brain.

Fig. 3. Superior view of an unearthed brain with distorted fronto-temporal structures.

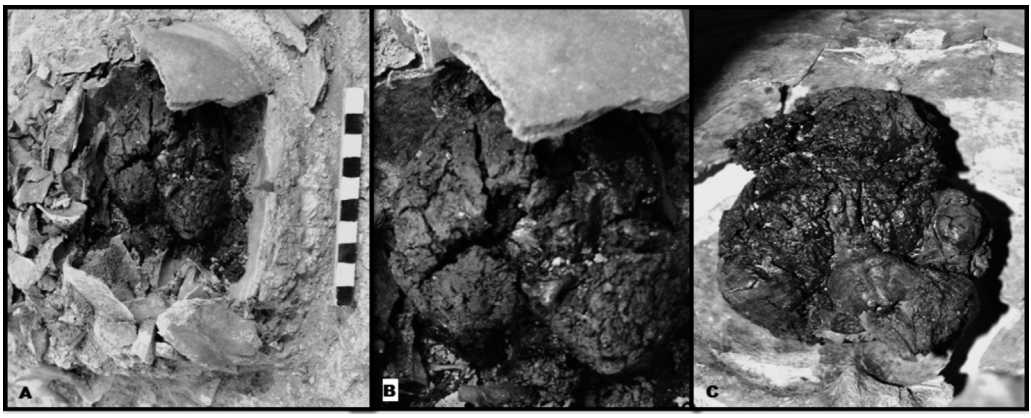

Fig. 4. (a and b) In situ view of a protected Seyitömer brain with well-preserved basal brain structures. (c) A similar view of the brain basal structures, when the surrounding skull was removed.

Radiological anatomical findings

Almost all anatomical structures of medulla oblongata, pons, middle cerebellar peduncle, mesensephalon, fourth ventricle and occipital cortical structures were relatively well preserved. Brain parenchyma was detected in approximately 500–1000 window gap with an average of 450 HU densities. CT technology revealed structures of diencephalic, metencephalic and occipital tissues similar to those of modern human relevant structures other than remarkable total shrinkage of ancient brain. Fig. 5a and c shows modern human brain, whereas Fig. 5b, d and e shows Seyitömer brain sample at similar planes.

Microscopical findings

Brain tissue analyses did not reveal any identifiable histologic landmarks in these techniques. In cryostat sections, followed by routine TP/PE and H&E/TB staining, there were eosinophically hypostained lacunar structures and the thin linear basophilic threads (Fig. 6a; 40× magnification and Fig. 6b; 200× magnification), which may be either calcium deposits or fungi and which were discussed below. Histological investigations did not provide any protected neural tissue patterns. Just some basophilic structures were found among the brown fragmented tissue debris, which could be either fungi or calcium deposits as discussed below.

Elemental findings in Seyitömer brain

All results of the elemental analysis are presented in Table 1.

Potassium, magnesium, aluminum and manganese. Potassium, magnesium and aluminum were strikingly abundant in soil. Potassium level in modern human skeleton was found to be less than 600 μg/g, and in Roman skeleton samples it was between 280 and 470 μg/g. Potassium level in Seyitömer bones was 670 μg/g. Brain tissue also contained considerable amount of potassium likely with both biogenesis and diagenesis.

Magnesium was absorbed by teeth, yet brain tissue also contained very high amounts of magnesium (5253 μg/g dry tissue). When magnesium levels of bone were compared with the modern bone, a strikingly higher level in Seyitömer bone was obvious (In late Roman skeletal examples average magnesium levels of 0.3 μg/g were found, which was less than 0.6% in modern skeletal tissue) (Zapata et al., 2006).

Aluminum levels in Seyitömer bones were higher than the modern bone, which were also high in barley seeds. While aluminum level in the modern human skeleton was found to be less than 20 μg/g, it was 94 μg/g in Seyitömer sample. In Roman skeletal samples aluminum level was 200 μg/g on average, which varies between around 50 and 1300 μg/g (Zapata et al., 2006). Brain tissue contained

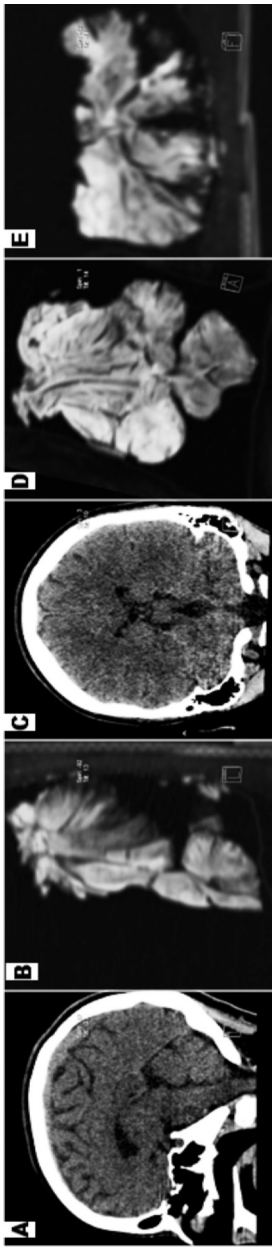

Fig. 5. (b, d, and e) Computerized tomography revealed morphology of the Seyitömer brain in comparison to modern human brains (a and c) at similar planes.

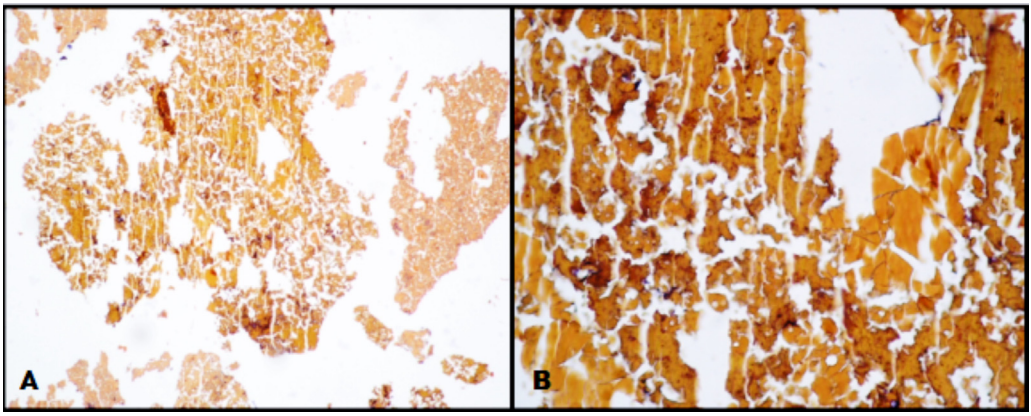

Fig. 6. In cryostat sections, followed by routine TP/PE and H&E/TB staining, there were eosinophilic-hypostained tissue debris and the thin linear basophilic threads (a; 40× magnification and b, 200× magnification).

even higher levels of aluminum (265 μg/g dry tissue), which biogenesis contributing more according to our view.

Manganese was also high in soil, teeth and more profoundly bone tissue had accumulated manganese. Normal manganese level in modern skeleton was less than 10 μg/g; which is 155 μg/g in the Seyitömer sample, higher than average manganese levels around 20–25 μg in Roman skeleton samples (Zapata et al., 2006). Interestingly, archeological artifacts from Japan also revealed higher manganese levels around 380, 120 and 300 μg/g from Jomon-, Yayoi- and Kofun skeletons of 3000, 2300 and 1700 years old age, respectively (Hisanaga et al., 1989). Japanese researchers commented on a great effect of soil contamination for the increased bone manganese levels. Brain uptake was less than teeth but still higher than the modern brain (16.7 μg/g dry tissue in Seyitömer sample versus about 0.3 μg/g wet weight of the modern brain tissues) (García et al., 2001).

Nonetheless, we should admit that elemental analyses in human brains are performed still in a limited number of samples and thus, relevant other species brains may also be taken into consideration, whether the detected amounts are normal. Modern rat brain manganese level is approximately

Table 1
Elemental content of Seyitömer brain and its correspondent teeth and bone tissues and of the surrounding soil as assessed by ICP-MS.

Element [μg/g dry tissue]	Soil		Seed (Barley)		Teeth	Bone	Brain
	Washed	Unwashed	Washed	Unwashed	Standard	Standard	Unwashed
Potassium	2627	7431	781	26,813	1049	671	1842
Magnesium	7777	18,187	13,564	1060	2803	1277	5253
Aluminum	11,763	18,872	677	1903	7.9	94	265
Manganese	168	662	74	75	25	155	16.7
Boron	15	60	41	825	26	25	49
Zinc	86	170	142	959	160	129	106
Copper	23	36	45	120	4.4	7.8	21.1
Molybdenum	7	14	1.2	23	5.5	10.5	77
Iodine	2	4	7	13	0.8	1.9	8.3
Selenium	0	0	2.3	0	0	0	4.8
Tin	0	0.48	0	0.47	0	0	3.2
Strontium	0	0.5	225	17	111	90	34
Barium	212	403	58	0	23	39	16.6
Lead	627	884	3.6	17	4	41.5	10.5
Nickel	71	106	129	236	7	32.7	9.8
Arsenic	19	31	2.4	0	2.8	3.2	1.8
Uranium	0.7	1.3	1.3	1.3	0.88	2.52	0.48

between 1.3 and 2.2 μg/g (Saito and Saito, 1996), which may indicate that accumulated amounts may be within the normal range.

Boron. Boron was present in soil, teeth and bone tissue and barley seeds contained significant amount of boron. Interestingly, boron was also in the brain and its uptake was at similar amounts of teeth and bone, which may have occurred with both diagenesis and biogenesis. Its significance was ascertained in the Discussion section.

Zinc and copper. Zinc and copper values in Seyitömer brain were comparable to the modern brain. Zinc value in the brain was approximately about 60% of the soil and higher than the modern brain (106 μg/g versus approximately 20 μg/g wet weight of modern brain tissues) (García et al., 2001). To have a further idea, if fresh rat brain tissue levels are analyzed using ICP-MS, zinc levels are found approximately between 30 and 76 μg/g (Saito and Saito, 1996). Zinc value of the bone (129 μg/g) was similar to those found in two different late Roman skeletal examples (approximate average values between 130 and 200 μg/g) and at the range of normal skeletal examples (less than 200 μg/g) (Zapata et al., 2006). We concluded that tissue levels of zinc represented biogenesis events in Seyitömer samples.

Copper was accumulated interestingly more in the brain than bone and teeth. Copper levels both in the Seyitömer brain (21.1 μg/g) and bone (7.8 μg/g) were found to be at comparable levels to the modern brain and bone, respectively. Normal copper level of the modern human brain is 6 μg/g of its wet weight (García et al., 2001) and normal copper level of the modern rat brain is approximately between 8 and 19 μg/g (Saito and Saito, 1996). Normal copper level in the modern human skeleton should be less than 30 μg/g, whereas two late Roman examples revealed levels around 12–14 μg/g (Zapata et al., 2006). When teeth copper level of 4.4 μg/g is considered, it is exactly between 4 and 5 μg/g teeth copper levels found in samples from 3700 to 5000 years old skeletons excavated at Early Bronze Age and Neolithic sites in Poland (Gleń-Haduch et al., 1997). We propose that the tissue copper levels in Seyitömer samples represent mostly biogenetic events.

Molybdenum and iodine. Molybdenum was scarce in soil and teeth and bone uptakes were at the level of soil. Seyitömer brain has concentrated molybdenum, which levels were 30–50% of the modern brain (μg per mg of "dry brain tissue" levels are compared). Modern rat brain molybdenum level is between 90 and 190 μg/g (Saito and Saito, 1996). Iodine was scarce in soil, Seyitömer brain accumulated 3-times more iodine than the bone tissue. Biogenesis events may have determined tissue levels of molybdenum and iodine.

Selenium and tin (stannum). Selenium was not present in soil, bone or teeth. Interestingly, Seyitömer brain contained selenium that was also present in seeds, a likely food source. Most strikingly, tin was not present neither in the soil, seeds, teeth nor bone but Seyitömer brain was positive for tin.

Strontium, barium and lead. Strontium levels in Seyitömer bone were comparable to the modern bone. In Roman skeletons, high levels of strontium were encountered between 500 and 2000 ug/g, yet modern bone strontium was less than 200 μg/g (Zapata et al., 2006). Yet, when brain accumulation of strontium was concerned, 34 μg/g was significantly higher than the approximate levels between 0.01 and 0.02 μg/g wet brain tissue in the modern human brains (Rahil-Khazen et al., 2002). Here, we assumed that brain strontium level was more a result of biogenesis considering also high strontium levels in barley seeds.

Barium level of 39 μg/g in the Seyitömer skeleton was compatible with less than 50 μg/g levels in modern skeletons. In Roman skeletons average levels were around 130–150 μg/g varying in a wide range between 10 and 850 μg/g's (Zapata et al., 2006). Seyitömer brain did not have strong uptake for the barium, despite its relatively high level in the soil.

Seyitömer's brain lead level was 10.5 μg/g versus a mean 0.3–0.4 μg level of the modern brain (García et al., 2001). Seyitömer skeleton's lead level of 42 μg/g was compatible with the modern human skeleton level, which was less than 100 μg/g (Zapata et al., 2006). Seyitömer's teeth level of 4 μg/g was exactly between the 4 and 5 μg/g teeth levels found in samples from 3700 to 5000-years old skeletons

Table 2
Fatty acid contents of Seyitömer brain in comparison to modern brain and in-watery conditions mummified sample (refer to the French Mummified Table in the main text).

Fatty acid (%)	In water mummified brain	Modern brain tissue	Seyitömer brain
Gingkolic acid [C13:0]	1.4	n.d.	n.d.
Myristic acid [C14:0]	11.6	0.4	6.4
Pentadecanoic acid [C15:0]	6.9	0.2	n.d.
Palmitic acid [C16:0]	44.1	23.3	30.6
Palmitoleic acid [C16:1, n−7]	n.d.		1.9
Heptadecanoic acid [C17:0]	3.1	0.3	n.d.
Stearic acid [C18:0]	8.0	27.5	31.2
10OH-Stearic acid [C18:0 10OH]	6.0	n.d.	n.d.
Vaccenic acid, cis [C18:1, n−7]	n.d.		7.6
Oleic acid [C18:1, n−9]	19.1	45.8	3.5
Linoleic acid, cis [C18:2, n−6]	n.d.		3.1
Linolenic acid [C18:3, n−3]	n.d.		2.2
Octatetradecanoic Acid [C18:4, n−3]	n.d.		3.2
Eicosenoic acid, cis-11[C20:1, n−9]	n.d.	2.6	2.7
Arachidonic acid [C20:4, n−6]	n.d.		1.91
Eicosapentanoic Acid [C20:5, n−3]	n.d.		1.5
Nervonic acid [C24:1, n−9]	n.d.		3.3

n.d. – not determined.

excavated at the Early Bronze Age and Neolithic sites in Poland (Gleń-Haduch et al., 1997). We thought again that the brain lead levels reflected biogenesis.

Nickel and arsenic. Nickel was present in the soil, teeth and more markedly bone tissue concentrated nickel. Seyitömer brain levels were significantly higher, when compared with the levels of the modern brain (9.8 μg/g versus about 0.1 μg/g in the modern brain) (García et al., 2001). Seyitömer bone's nickel level of 33 μg/g was also high considering the approximate 9 μg/g nickel level in modern Japanese skeletons. Interestingly, archeological skeletons from Japan revealed nickel levels around 20 μg/g from Jomon-, Yayoi- and Kofun skeletons of 3000, 2300 and 1700 years old age, respectively; which were closer to Seyitömer sample (Hisanaga et al., 1989).

Arsenic was present in soil, which was concentrated in the teeth and bone about 3 μg/g; a level of 1.8 μg/g existed in the Seyitömer brain, which was higher than the level of the modern brain tissue (less than 0.05 μg/g of the wet weight) (García et al., 2001).

Fatty acid findings

All comparative results of fatty acids are shown in Table 2.

Gas chromatography (GC) analysis revealed a reverse ratio of saturated to unsaturated fatty acids (FA). There were some fragmented brain pieces in a couple of skulls, which colors and tissue properties were the same as the one completely protected brain with intact and macroscopically distinctive anatomy. GC analysis was performed in fragmented brain pieces. Brain tissue specific FA nervonic acid was still present (3.3%) despite being one of the unsaturated fatty acids.

All data were compared first with the findings of the Vienna Group (at the Clinical Microbiology and Hygiene Institute, University of Vienna) (Makristathis et al., 2002). These findings were obtained from corpses naturally mummified at very high altitudes, glaciers, glacier lakes and deserts (tissues dated to around 50, 500, 1000, 2500 and 5000 years, respectively) (Makristathis et al., 2002). Secondly, the data were compared with a recent study findings of the Swiss Mummy Project in which a unique case of a left cerebral hemisphere from a 13th century child, found in north-western France was analyzed (Papageorgopoulou et al., 2010). The corpse's discovery was near to a confluence point of 3 rivers, rich in fresh watery acidic, clay soil.

Seyitömer brains' palmitic acid ratio (30.6%) was higher than in a normal brain (23.3%) but lower than in the French mummified brain (FMB) tissue (44.1%) (Papageorgopoulou et al., 2010). Myristic acid (6.4%) was again higher than normal (0.4%), but lower than in the FMB (11.6%). In naturally

mummified corpses, which were mummified with lack of water-contact, the Vienna Group found myristic acid between 2.8% and 8.5%, and Seyitömer brains' myristic acid ratio (6.4%) was within this range.

In normal brains, the ratio of stearic acid found inside the sphingomyelin of the gray matter is 27.5%; in Seyitömer brains its ratio is 32% and in the FMB is 8% only. Please note that stearic acid is a saturated fat and a dominant product of adipocere formation. Interestingly, if stearic acid needs to be produced industrially, animal fat should be treated under high pressure and high temperature in the presence of water in order to hydrolyze tissue triglycerides. Here, if the water of brain tissue would be thought to provide the necessary water for this reaction, this chemical finding is exactly parallel to our archeological scenario, in which it is anticipated that these bodies were trapped due to an earthquake and a consequent fire. The distribution pattern of other fatty acids has also indicated the same scenario, like a chemical signature.

Considering the fatty acids with double bonds, we have conspicuous findings. Unsaturated oleic acid with a single double bond was the highest (19%) in FMB, when compared with a normal brain (15.8%) and with our sample (3.5%). In water immersed pig adipose tissue, adipocere formation was concomitant with increasing oleic acid; this may explain the striking difference between the Seyitömer and the FMB samples (Papageorgopoulou et al., 2010). The FMB samples were protected in watery conditions, whereas our samples may have been protected only by boiling in their own tissue water and in absence of exogenous dampness. Except the oleic acid, the FMB tissue did not reveal existence of any other fatty acid with double bonds. In our sample we found eicosenoic acid (n−9, 1 double bond) at a level of 2.7%, that was almost the same in a normal brain and lacking in FMB. Cis-vaccenic acid (n−7, 1 double bond) was not reported to be present neither in the FMB nor in the Vienna Group samples of body adipocere. The Vienna Group researchers only detected linoleic acid (2 double bond) in one 1000-years old mummified corpse found in Peruvian Ilo Desert with zero humidity and high temperature. In Peruvian mummy this acid was present at a ratio of 1.2%, while in our sample it was found at a ratio of 2.2%. Also in the Seyitömer sample, we found nervonic acid specific for neural tissue (n−9, 1 single bond) which has not been mentioned in the FMB, nor in the Vienna Group samples.

Linolenic, octatetradecanoic, arachidonic and eicosapentanoic acids with corresponding 3, 4, 4 and 5 double bonds have been found in Seyitömer brains at ratios of 2.2%, 3.2%, 1.9% and 1.5%, respectively; which were, again, completely absent in the FMB and the Vienna Group samples.

Seyitömer brains constitute a very unique example, where intense adipocere formation and fatty acid unsaturation (with a typical signature of stearic and palmitic acids) have occurred side-by-side with saving vulnerable and neural tissue specific unsaturated fatty acids. It might be possible that the surrounding soil containing both oxidizing and reducing elements exerted a balanced mixture that allowed two different mainstream chemical reactions to happen simultaneously.

A condition, in which double-bond fatty acids were better protected seemed to be lack of humidity and exposure to high temperature. Surprisingly, glaciers and watery conditions did not provide proper micro-environment to protect fragile tissue lipids.

Discussion

First record of a finding of preserved brain tissue in an archeological research setting is dated back to 1857, where brains were found in preserved corpses in Peru. In the Peruvian conditions, preservation of corpses depended on the absence of rain, dryness of the soil, and the quantity of niter (potassium nitrate) in the soil (Rivero, 1857). Brains were usually found as a loose flattened masses adherent to the intracranial fossae. The color varied from light brown to nearly black; a whitish, wax-like substance was sometimes found in the center of the mass. The actual weight of the brain, in one case, was probably one-twentieth of its original weight. Incas mummified their dead, mostly the bodies of persons of high social rank, although the process of embalming was apparently nothing more than drying by heat (Lamb, 1901). Cholesterol and fatty acids have been recovered unchanged from various organs of Egyptian mummies, first time in 1907 by Schmidt (King et al., 1929). While fresh brain contains the whole of its cholesterol in the free state, it has been found that as much as 99% of cholesterol in ancient Egyptian brain is present as esters in combination with palmitic and stearic acids (King et al., 1929).

Modern examples of mummified brains: reports from 1960s until today

In 1960, archeologists made a report on findings from a shallow limestone cave under water in Sarasota County, Florida (Royal and Clark, 1960). The cave contained archaic bones, including a skull with naturally preserved portions of brain inside. The charred log from one layer lower than the layer with bones, produced a radiocarbon date of 8000 +/-200 years BP. Some white material with the appearances of a brain was noticed inside the skull (Royal and Clark, 1960). The authors hypothesized that a similar situation to the case described by Breder may have occurred, where the soft parts of a fish failed to decompose in aquarium water containing *Pseudomonas eisenbergii Migula* (Breder, 1957). Also in 1960, brain remnants preserved as adipocere were found in British-Roman skulls from second century CE during sewerage excavations. The brain matrix contained high proportions of clay (Oakley, 1960).

In 1965, archeologists discovered a wooden bridge of Celtic origin near the Lake of Neuchatel in Switzerland (Pilleri and Schwab, 1970). Due to a collapse of this bridge in the year 1 BCE several people were buried under the debris. All the intact human skulls contained cerebral remains. Macroscopically, the cerebral tissue had a humid, grayish-white appearance; the texture of the cerebral matter was typical for brain tissue (Pilleri and Schwab, 1970).

In 1979, skulls excavated from a churchyard in Svendborg, Denmark and dating to the Middle Ages, were reported containing intracranial masses (Tkocz et al., 1979). The masses consisted of brain material preserved in the state of adipocere. Abundance of cholesterol – a neutral lipid typical of human brain cells but not of bacteria – was present besides the phospholipids and fatty acids (Tkocz et al., 1979). Since the bodies were exhumed from a soil affected by frequent floods, the authors have proposed that this protection occurred due to dissolving of soil alkaline with water and subsequent saponification (Tkocz et al., 1979).

In a swampy pond of Windower in central Florida in 1984, human corpses were found three meters below the water level inside a red-brown peat zone (Doran et al., 1986). Radiocarbon dates from bone and from peat matrix gave consistent ages in the range of 7800–8300 years before present. In five skulls, material recognizable as preserved brain tissue was present. Gross examination disclosed the external gyral pattern of atrophic human brains (Doran et al., 1986). Transverse slices of the brain material exposed parietal, temporal and occipital lobes with peat filling all fissures. Magnetic resonance (MR) analysis of one adult male brain at sagittal section revealed occipital and frontal pole, lateral ventricles and cingulate gyrus (Doran et al., 1986).

In 1992, Bulgarian forensic examiners reported seven mummified brains found among 39 human skeletons in a modern mass grave site in Sofia district; three of the brains were intact (Radanov et al., 1992). The preserved structures strongly resembled human brains, although they were hard in consistency and black in color. Their analysis revealed that these brains contained more manganese, aluminum, silicon and titanium than normal brains, which is compatible with our elementary findings.

In 1995, a series of mummified bodies from coastal deserts of Northern Chile was published. The selected places are among the driest points on earth. People have inhabited this arid coastline for at least 8000 years (Gerszten and Martínez, 1995). No artificial mummification process was practiced by these people in the majority of their burials, allowing for a unique opportunity to examine relatively well-preserved ancient human tissues (Gerszten and Martínez, 1995). In several cases, the dura, falx cerebri, and both cerebral hemispheres were well preserved. The cerebral hemispheres varied in color from light to dark brown and had the consistency of friable, gritty-like material. The cerebellum and brain stem were difficult to identify (Gerszten and Martínez, 1995).

In 2003, three frozen bodies belonging to three children were reported recovered in an archeological excavation carried out at an altitude of 6739 m above sea level on the summit of Mount Llullaillaco, on the border between Argentina and Chile (Previgliano et al., 2003). Children had been sacrificed 500 years ago in times of the Inca Empire. Computer tomography (CT) of the three frozen mummies was performed on a nonhelical scanner. A clear differentiation of white and gray matter in the brain and cerebellum due to the change of fat into adipocere and the deposition of calcium salts were shown on the cranial CT scans. The pons, medulla oblongata, and the spinal cord could also be seen (Previgliano et al., 2003).

In 2008, a unique brain mummification case from Korea was reported (Kim et al., 2008). Among the tombs of the Joseon dynasty (1392–1910), frequent spontaneous mummifications are encountered due to lime in tombs. One observed corpse among these specimens belonged to a female from 15th to 16th centuries. After cutting the skull, it was found that the surface of the brain was very sticky and fragile. The left and right hemispheres as well as the cerebellum and brain stem could be identified. On the surface of the cerebral hemispheres, residual vessels, as well as the gyri and sulci of the brain were identified (Kim et al., 2008). On the medial surface of the brain, a well-preserved thalamus could be discerned. The brain was also observed in axial CT views. Various parts of the brain, including the right and left cerebral hemispheres, the temporal and parietal lobes of the cerebrum, and the cerebellum could be identified (Kim et al., 2008).

The discovery in 2010 of an 18-month old infant brain from 13th century was published with many details (Papageorgopoulou et al., 2010). The infant was discovered in a wooden coffin in Quimper, France, not far from a site where three rivers confluence. Thus, the researchers concluded that the presence of soil salts and watery acidic clay had provided the mummifying conditions (Papageorgopoulou et al., 2010). The frontal, temporal and occipital lobes retained their original shape and could be readily recognized; the cerebellum and the brain stem were not preserved. Lateral and medial surfaces of the cerebral hemisphere were dark brown in color with a rough texture that still enclosed remnants of pia mater on the outer surface (Papageorgopoulou et al., 2010). CT and MR imaging verified that the tissue was well preserved, especially the MRI provided detailed examination of all anatomical features. Sulci, including central sulcus and gyri, corpus callosum, were easily identifiable (Papageorgopoulou et al., 2010).

In 2012, Prats-Munoz and colleagues reported findings of spontaneously mummified brain tissue from several individuals buried in a 15-meter-high entrance carstic cave from the island of Minorca. The individuals dated to approximately 3000 years BP, that corresponded to the late Mediterranean Bronze Age (Prats-Muñoz et al., 2012). The reported brain tissues did not preserve any anatomical detail, yet microscopically the authors found eosinophilic reticular material besides some structures, which they proposed to be brain nuclei and transversing nerve fibers. They claimed that the dry climate of the island, elevated location of the cave which precluded exposure to rain, the acid cave soil and walls rich in sulfates and plaster, all contributed to the protection of nervous tissue in skulls (Prats-Muñoz et al., 2012).

Fungus-like material in mummified brain tissues? When they are really vegetal structures, when just calcium deposits?

Fungus-like vegetal structures were detected in 2100-years old mummified brains found near the Neuchatel Lake (Pilleri and Schwab, 1970). In Svendborg mummified brains, both light and scanning electron microscopy revealed presence of bacterial colonization (Tkocz et al., 1979). Among the South American mummified brains, in several cases, yeast and spores were present within the CNS parenchyma (Gerszten and Martínez, 1995). These organisms had the features of Microsporidia (Gerszten and Martínez, 1995).

In the mummified brain from the South African bushveld, there appears to have been a post-mortem contamination of the brain tissue by organisms with extremely thick cell walls and a granular cytoplasm (Eklektos et al., 2006). These were potentially either fungal spores or cysts produced in the lifecycle of a parasite and were found close (within 100 μm) to the surface of the mummified cerebral cortex (Eklektos et al., 2006). In the 3200-years old Egyptian Nakht brain of spontaneous mummification, structural anomalies were observed in the solochrome cyanin R- and H and E-stained sections (Karlik et al., 2007). The exact identification of these thin linear eosinophilic threads besides the hypostained structures was not determined. Studying their published pictures, these structures resemble quite well those, which other researchers defined as vegetal structures/fungi, but Karlik et al. (2007) interpreted these structures as signs of possible calcification. They concluded that the calcium ions would be released from the tissue because the normal proteins, subcellular structures, and membranes were altered during mummification. In the 13th century mummified brain from Quimper, post-mortem contamination with micro-organisms was observed on the histological sections as fungal hyphae stained with Periodic Acid Schiff stain (Papageorgopoulou et al., 2010).

The presence of bacteria and/or fungi may have some significance for the mummification process. The bacteria and fungi could have either produced putrefaction or, as had been suggested long ago, they could have released antibiotic compounds to prevent other microorganisms from degrading the brain tissue.

Postmortem alkaline environment's role in lipid saponification and preservation of Seyitömer brains: Notter experiments

In 2009, Notter and colleagues investigated early stages of adipocere formation in both pig and human adipose tissue in aqueous environments (Notter et al., 2009). They analyzed subcutaneous adipose tissue from both species after immersion in distilled water for up to six months. Early-stage adipocere formation in pig samples during later months was detected, but not in human samples. In the case of both unsaturated myristic and oleic acids, human adipose tissue had significantly higher mean concentrations of each fatty acid over the entire sampling period compared with pig adipose tissue. The saturated stearic acid, on the other hand, remained significantly higher in pig adipose tissue, in which it rose above basal levels, whereas it declined in the human adipose tissue (Notter et al., 2009). Interestingly from day 0 to day 180, saturated fat palmitate levels declined in human adipose tissue, whereas in pig adipose tissue these levels first decreased and then increased above the starting value (Notter et al., 2009). Again, unsaturated oleic and linoleic acids increased in the human specimen, while their levels significantly decreased in the pig adipose tissue. Fresh pig adipose tissue contained large amounts of potassium – K (>80%) with <10% of the tissue containing sodium – Na. Conversely, fresh human adipose tissue contained more than 75% Na and <20% K tissue (Notter et al., 2009). Another striking difference was more than 11-fold increase of magnesium levels in pig adipocere, besides the slight increase of magnesium in human adipocere tissue (Notter et al., 2009).

The formation of adipocere is associated with the conversion of adipose tissue into a grayish-white, wax-like substance, which over time can become an armor-like solid mass. Adipocere is comprised of a mixture of saturated fatty acids that result from the late postmortem changes associated with the decomposition of adipose tissue in the body. The process of adipocere formation is initiated immediately after death by intrinsic lipases that hydrolyze adipose triglycerides to a mixture of free fatty acids (Notter et al., 2009). Given favorable environmental conditions, the unsaturated fatty acids undergo hydrogenation to their saturated form. Hydrogenation of oleic, linoleic, and palmitoleic acids yields stearic and palmitic acids, respectively. Sodium and potassium release during autolysis or elements in the environment such as calcium from soil may react with cleaved fatty acids to form salts of fatty acids. This process is known as hardening and produces an insoluble product, which causes adipocere to have a more brittle quality. The most abundant fatty acid found in adipocere is palmitic acid followed by stearic acid and then myristic acid. In an alkaline environment the process may go on to a formation of water insoluble calcium and magnesium soaps. Thus, the final product of adipocere consists of a mixture of fatty acids with some soaps (Tkocz et al., 1979).

We may assume that elemental milieu of Seyitömer brains led to their resemblance to the pig tissues of Notter experiments rather than to the human tissue. High intrinsic potassium and magnesium concentrations inside the brain combined with diagenesis in the soil may have helped high degree of early saturation of brain tissue lipids into stearic and palmitic acids. Here, it would be proper to emphasize that ancient Egyptians employed natron not only to embalm their dead but also to make faience. Besides the dominance of chloride-, sulphate-, bicarbonate- and carbonate-salts of sodium, the natron mixture also contained magnesium carbonate (1.9%) and alumina (0.7%) (Noble, 1969). Seyitömer is a district of Kütahya known with its production of tiles and ceramics since thousands of years and, as also seen in Seyitömer soil sample, the earth contains everything suitable for the production of ceramic made from aluminum–magnesium-carbonate clays.

Aluminum was blamed to cause Alzheimer disease, yet lack of topographical relationship between sites of aluminum deposition and senile plaques in the Alzheimer's disease brain disproved these claims (Kasa et al., 1995). In brain tissues obtained from cadavers deceased of natural causes, human brain samples of higher age individuals (mean 81 years) exhibited unique deposits of aluminum with

silicone mineralized as chalcedony (Prado Figueroa et al., 2008). Chalcedony has been previously shown as a microcrystalline mineral found in cholinergic nerves of electric fish (*Rajidae*) and it is not known whether it has a functional role in aged brains (Prado Figueroa et al., 2008). If so, it would be tempting to speculate that aluminum was one of the contributing factors preventing degradation of cerebral lipids after death.

Li and colleagues investigated postmortem serum calcium (Ca) and magnesium (Mg) for diagnostic evidence to determine the cause of death (Li et al., 2009). A significant increase in the Mg level occurs in asphyxiation (Li et al., 2009). Hence, elevation of magnesium due to a fire asphyxia in Seyitömer brains is also plausible. The desiccant action of magnesium may have also contributed to both tissue protection and volume reduction of brains.

Manganese is used in the manufacture of ceramics and it is a cofactor of superoxide dismutase. Manganese is involved in desiccation resistance of bacteria, which could prevent protein carbonylation during extremely dry conditions (Fredrickson et al., 2008). Seyitömer brains manganese might be another protective factor during fire vacuum exerted dryness and desiccation.

Old embalming fluids contained arsenic and mercury, which are strongly oxidizing compounds (Piombino-Mascali et al., 2009). Today, formalin/formol has replaced arsenic and mercury's role in coagulating tissue proteins. Very interestingly, the almost-perfect for embalming Salafio solution contained a mixture of strongly oxidizing (formalin, alcohol), neutral (glycerol) and antioxidizing (salicylic acid, zinc) compounds together (Piombino-Mascali et al., 2009).

An ideal medium to protect human tissues decay and simultaneously preserve fine details of their structure may be a well-balanced mixture of oxidizing and deoxidizing compounds. During mummification, strongly oxidizing molecules may provide the first protection against decomposition with protein coagulation and deoxidizing molecules may provide protection of fine molecular details. In Seyitömer soil, there is such a combination of oxidant (such as alkalines, lead, arsenic) and antioxidant elements (copper, zinc), which may have provided a suitable environment for both protection against anatomical decay and preservation of molecules such as brain specific brittle unsaturated fats.

The possible presence of borate in mummification salts used in Pharaonic Egypt was of special interest both historically and biochemically. In two samples, one from Tutankhamen and the second from Deir el-Bahari mummies borate was found, amounting to 2.1 and 3.9 μmol/g, respectively (Kaup et al., 2003). In five of the examined bone fragments from the Junker excavation at Giza similar borate concentrations of 1.2 μmol/g bone were seen (Kaup et al., 2003). The authors have emphasized that the usual borate content of modern autopsy is far below the detection limit, indicating that the elevated borate content in both mummification salt and ancient bone samples was due to an intentional usage for mummification. Boron with insect repellant and fire-retardant activities may have helped mummification of Seyitömer brains.

Another likely reason of Seyitömer brain's elemental content: personal exposures due to mining activities

When we analyzed the distribution of certain elements in teeth, bone and brain, it became obvious that certain elements in the brain exhibited high concentrations despite their low levels in the surrounding soil and in barley seeds as examples of food source. These elements may have accumulated in the brain through biogenesis during the corpses lifetime. These were: tin, copper, lead, arsenic, nickel and selenium. For two elements, selenium and tin, the situation was most puzzling. They were neither present in soil, nor in teeth or bone, but only in the brain. Their presence in the brain was likely due to stronger adherence of the brain proteins to these metals.

Among tin, copper, lead, arsenic, nickel and selenium, at least first four are known to emerge during the Bronze Age mining activities. Bronze is an alloy of tin and copper and arsenic and nickel is present in mine pits of tin (Muhly, 1985). Thus, some of these elements may have accumulated in the corpses due to their occupation and personal differences in the tissues metal ionic concentrations may have influenced post-mortem preservation. Tin deposits in Nothwestern Anatolia, especially in the vicinity of Eskişehir (a city just beside Kütahya) still appear in mineral resource maps published by archeologists (Muhly, 1985). Also an ancient tin mine in the Taurus Mountains of south central

Turkey, which has been dated by radiocarbon and pottery type to the third millennium BCE suggested modern knowledge of tin in Anatolia (Yener et al., 1989).

Conclusion

Seyitömer brain samples found in a Bronze Age Höyük in Western Anatolia contributed further information for new pathways in human tissue taphonomy and provided new impetus for physical anthropologists and archeologists interested in uncovering Anatolian tumuli.

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