A comprehensive study of dura mater biomineralization: morphological, crystallographic, and immunohistochemical aspects

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Introduction

Biomineralization is a process of formation of biominerals widespread among living organisms. Pathological biomineralization refers to the deposition of calcium compounds outside the tissues of the skeleton and teeth [12]. Biomineralization of the dura mater is observed in 12.5 % of the population, mainly in men with an average age of 53 years. Moreover, in more than 75 % of cases, biomineralization of the vascular plexus is simultaneously observed [25]. Most often, calcifications are localized in the sickle of the brain (26.8 %), the petrocline ligament (13.2 %) and the tent of the cerebellum (6.8 %) [11]. In children, biomineralization of the dura mater is extremely rare (less than 1 % among patients examined by
The phenomenon of calcification is also characteristic of tumours of the dura mater - meningiomas. They usually have an expansive nature of growth and are considered histologically benign [14]. Calcifications are found in 20-25 % of meningiomas, but since they are often found in meninges, the problem of differential diagnosis between relatively "normal" tissue of meninges and calcified meningiomas arises [1, 16].

There is no unequivocal opinion about the mechanism and significance of meningioma calcification. Perhaps it is related to the process of tumour cell degeneration and serves as a barrier against the further spread of the tumour [8].

After neurosurgical interventions for the removal of tumours, traumatic brain damage or other reasons, it is necessary to reconstruct the dura mater to minimize the risks of complications: fistulas, infections, brain keels, scars and adhesions [4, 13].

There is data on autografts, for example, temporal fascia. This method has advantages: ease of material processing, price and good biocompatibility. However, the autograft cannot be used when the size of the defect is significant; this method requires an additional operation and creates a risk of complications at the removal site [2, 6].

The use of homografts today is not a good option due to the risks of prion infection transmission [9, 21].

A promising solution is using synthetic materials, including nanomaterials, to create an artificial dura mater. Obstacles can be insufficient biocompatibility or neurotoxicity, so these materials require detailed study before possible use [5, 17, 19, 23].

Therefore, the study of the morphological features of the biomineralization of the dura mater can create a basis for the development of new models of artificial dura mater and can also contribute to improving the diagnosis of tumours of the meninges and the central nervous system. This work aims to study the morphological features of the dura mater with signs of biomineralization.

Materials and methods
Protocol of the Ethics Commission
This study was approved by the Commission on compliance with bioethics in conducting experimental and clinical research at the Academic and Research Medical Institute of Sumy State University (protocol № 2/12 dated December 8, 2022). All studies used the Declaration of Helsinki (6th edition, revised 2008, Seoul) and the Universal Declaration of Bioethics and Human Rights (2006).

Sample collection
The research was conducted on tissues obtained during autopsies in the pathology department of the Sumy Regional Clinical Hospital (Sumy, Ukraine). 30 samples of dura mater with signs of biomineralization (group I) and 30 samples without these signs (group II) were examined. Group I contained 15 samples from female patients and 15 from male patients. Group II included 14 samples from female patients and 16 samples from male patients. To detect pathological biomineralization, the tissue of the dura mater was examined using histological, histochemical, and immunohistochemical methods and scanning electron microscopy with energy-dispersive X-ray spectroscopy.

Histological examination (hematoxylin-eosin staining)
We removed dura mater samples from the falx cerebri area and fixed them in a 10% buffered formalin solution (CAS № 50-00-0) for 24 hours. After that, we cut out strips measuring 2.0 x 0.5 x 0.1 cm, dehydrated and embedded in paraffin in the ATM-4M carousel-type apparatus (Ukraine). Paraffin blocks were cut at a thickness of 7 microns using a Shandon Finesse 325 rotary microscope (Thermo Scientific, Waltham, MA, USA). After that, the paraffin sections were stained with hematoxylin-eosin. All photos were taken using a Zeiss Primo Star microscope with a Zeiss Axiocam ERc 5s camera and "Zen 2.0" software (Carl Zeiss, Jena, Germany).

Based on the results of the histological examination, we divided the dura mater samples into two groups (30 samples each): with signs of biomineralization (group I) and without them (group II).

Histochemical study by the von Kossa method
Staining of dura mater samples by the von Kossa method began with deparaffinization (twice for 5 min in a solution of xylene (CAS № 95-47-6)) and dehydration (twice for 5 min in a solution of 96 % ethanol (CAS № 64-17-5), 10 min in a solution of 70 % ethanol (CAS № 64-17-5)) to distilled water. Then, the samples were placed in a beaker with a 5 % solution of silver nitrate (CAS № 7761-88-8) under intense illumination (in front of a 60-watt lamp, having previously wrapped the beaker in foil) for 1 hour. To remove silver nitrate residues, the samples were washed three times with distilled water and placed in a sodium thiosulfate solution (CAS № 10102-17-7) for 5 min. The samples were washed with tap and distilled water. The samples were then stained in a 0.1 % solution of nuclear fast red (CAS № 6409-77-4) for 5 min. The samples were washed with tap and distilled water. Dehydration, clarification, and synthetic coating were then performed.

Histochemical study with alizarin red S (Dahl-McGee modification)
Staining of dura mater samples with alizarin red S began with deparaffinization (twice for 5 min in a solution of xylene (CAS № 95-47-6)) and dehydration (twice for 5 min in a solution of 96 % ethanol (CAS № 64-17-5), 10 min in a solution of 70 % ethanol (CAS № 64-17-5)) to distilled water. Then, the samples were stained for 2 min with a solution of alizarin red S. To prepare the solution, 0.5 g of alizarin red S (CAS № 130-22-3) was dissolved in 45 ml of distilled water, and the pH was adjusted to 6.3-6.5 for 10 % NH4OH solution (CAS № 1336-21-6). The samples were then
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rinsed with distilled water for 5-10 sec and treated with acidified ethanol (CAS № 7647-01-0) for 15 sec. Dehydration, clarification, and synthetic coating were then performed.

**Histochemical study by the Van Gieson method**

Staining of dura mater samples by the Van Gieson method began with deparaffinization (twice for 5 min in a solution of xylene (CAS № 95-47-6)) and dehydration (twice for 5 min in a solution of 96 % ethanol (CAS № 64-17-5), 10 min in a solution of 70 % ethanol (CAS № 64-17-5)) to distilled water. Then Weigert's hematoxylin (50 drops of Weigert's solution I per 25 drops of Weigert's solution II) was dripped onto the samples. Weigert's solution I is a mixture of 1 g of hematoxylin (CAS № 517-28-2) and 100 ml of 96 % ethanol (CAS № 64-17-5). Weigert solution II is a mixture of 4 ml of ferric chloride (CAS № 7705-08-0), 1 ml of concentrated HCl (CAS № 7647-01-0), and 95 ml of distilled water. Then the samples were washed in 2 portions of water and treated with a solution of acidified ethanol (CAS № 7647-01-0) until the background became clear. Then a solution of picrofuchsin (a mixture of 10 ml of a saturated aqueous solution of picric acid (CAS № 88-89-1) with 1 ml of a 1 % aqueous solution of acid fuchsin (CAS № 3244-88-0)) was dripped onto the samples. Dehydration, clarification, and synthetic coating were then performed.

**Histochemical study by PAS reaction method**

The PAS method started with the preparation of Schiff's reagent. For this, 1 g of basic fuchsin (CAS № 569-61-9) was added to 200 ml of boiling distilled water and boiled for 5 min with constant stirring. Then the solution could cool to 60-70 °C and filtered through a paper filter. Then the solution could cool to 50 °C. 20 ml of 1 N hydrochloric acid (mixture of 16.4 ml of concentrated HCl (CAS № 7647-01-0) and 3.6 ml of distilled water) was added and allowed to cool to 20-25 °C. Then 1 g of sodium bisulfite (CAS № 7631-90-5) was added. The solution is stored for the first three days in a dark place at room temperature, then in the refrigerator.

Staining of dura mater samples by the PAS reaction method began with deparaffinization (twice for 5 min in a solution of xylene (CAS № 95-47-6)) and dehydration (twice for 5 min in a solution of 96 % ethanol (CAS № 64-17-5), 10 min in a solution of 70 % ethanol (CAS № 64-17-5)) to distilled water.

Then the dura mater samples were dewaxed to distilled water. The samples were then treated with a 1 % solution of periodic acid (CAS № 10450-60-9) for 10 min at room temperature in the dark. The samples were washed in tap water (5 min) and distilled water (2 min). Then cold Schiff's reagent was dripped onto the samples for up to 10 min at room temperature and under light illumination until they turned pink. The samples were washed in tap water (several times until the water turned red) and distilled water (2 min). After that, the samples were stained with a hematoxylin solution (CAS № 517-28-2) for 4 minutes. The samples were washed twice in tap water. Dehydration, clarification, and synthetic coating were then performed.

**Immunohistochemical study with antibodies against osteopontin**

Serial sections of dura mater tissue with a thickness of 3-4 microns, made from prepared histological paraffin blocks, were applied to SuperFrost adhesive slides (Thermo Scientific) and dried at 37 °C for 18 hours. Deparaffinized sections were subjected to unmasking of antigens by the thermal method by heating the sections in a citrate buffer (pH 6.0) at a 95-98 °C. To visualize the results of an immunohistochemical (IGH) study, the detection system “UltraVision Quanto Detection System HRP Polymer” (Thermo Scientific) was used, which involved blocking endogenous peroxidase activity with 3 % hydrogen peroxide, blocking non-specific background staining using “Ultra V Block”, enhancing the reaction “Primary Antibody Amplifier Quanto”. Diaminobenzidine (DAB) was used as a chromogen. Antibodies against osteopontin - OPN (Thermo Fisher Scientific, PAS-34579, dilution 1:300) were used. Active (use of tissue, with a predetermined positive and negative reaction) and passive control of the obtained results were carried out as quality control of the conducted IHC research.

**Scanning electron microscopy (SEM) with energy-dispersive X-ray spectroscopy (EDX)**

Histological sections with a thickness of 10-12 microns were made from paraffin blocks of dura mater tissue, which were placed on a table made of spectrally pure graphite. For maximum attachment of the biological material to the microscope stage and melting of the paraffin, the sections were kept in a thermostat at a temperature of 60 °C for 30 min. To remove paraffin, the samples were covered with xylene three times for 3-4 min, then with 96 % ethanol three times for 5-6 min and rinsed with distilled water. After that, the sample of biological material was additionally grounded with conductive tape wrapped around the stage. The prepared preparations were examined on a scanning microscope SEO-SEM Inspect S50-B (SEO, Sumy , Ukraine) with an energy dispersive spectrometer AZtecOne with an X-MaxN20 detector (Oxford Instruments plc, Abingdon, UK).

**Statistical analysis**

Statistical processing and graphic presentation of the statistical analysis results was performed using GraphPad Prism 8.0. The results of the immunohistochemical study were checked for normal distribution using the Shapiro-Wilk test. In the case of the non-normal distribution of digital indicators, the Mann-Whitney test was used to assess statistical significance. If the data samples had a correct distribution, they were compared using the Student’s parametric t-test. The results were considered statistically significant, with a probability of more than 95 % (p<0.05).

**Results**

A total of 60 samples were examined, including 30 dura mater samples with pathological biomineralization (group I) and 30 control samples without signs of biomineral
deposits (group II). The average age of patients in the group I was 67.00±2.33 years. When studying the distribution of patients by gender in group I, the average age of men was 61.93±3.54 years, and of women 72.07±2.49 years. The average age of patients in the group II was 62.97±3.12 years. When studying the distribution of patients by gender in group II, the average age of men was 57.81±4.45 years, and women - 68.85±3.93 years.

**Histological and histochemical study**

Macroscopically, it was not possible to confirm or deny the presence of biominerals in the dura mater (Fig. 1). Histologically, the tissue of the dura mater, which was removed from the area of the sickle of the brain, represented a dense connective tissue, the inner surface of which was covered with a single-layer flat epithelium. The samples were divided into two groups according to the results of histological examination (hematoxylin-eosin staining).

Group I included 30 samples of the dura mater with signs of biomineralization - small formations of various sizes and irregular shapes (Fig. 2A). These signs were not detected in the control group samples (Fig. 3A).

**Fig. 1.** Dura mater tissue.

**Fig. 2.** Study of the tissue of the dura mater with signs of biomineralization (group I). A - histological examination of the tissue of the dura mater (hematoxylin-eosin staining). B - histochemical staining of dura mater calcifications using the von Kossa method. C - histochemical staining of dura mater calcifications using the alizarin red S method. D - histochemical staining of the dura mater tissue using the Van Gieson method. E - histochemical staining of the dura mater tissue using the PAS reaction. F - immunohistochemical study of dura mater tissue with anti-osteopontin antibodies. Drawings in the insets A-F correspond to the magnified area of this drug. G - SEM of the calcified area of the dura mater. H - EDS spectrum. I - physicochemical study of group I dura mater calcifications using EDS mapping: carbon ions are marked in red, oxygen in green, calcium in blue, and phosphorus in purple.
The result of the examination of the dura mater tissue using the von Koss method was the staining of mineral deposits in brown colour, which confirmed the presence of calcium phosphate compounds in the samples from group I (see Fig. 2B). In the control group, such staining did not occur (see Fig. 3B).

Staining of the dura mater tissue with alizarin red S confirmed the presence of calcium compounds (orange-red colour) and inorganic iron (purple colour) in the samples of group I (see Fig. 2C). In the samples of group II, we found only iron deposits (see Fig. 3C).

Staining of the dura mater tissue by the Van Gieson method showed the presence of a noticeable amount of collagen fibres (purple-red colour) in the structure of the dura mater of samples of group I (see Fig. 2D) and group II (see Fig. 3D).

Staining of the dura mater with the help of the PAS reaction showed the absence of glycosaminoglycans in the composition of mineral deposits (see Fig. 2E). In the dura mater tissue itself, single PAS-positive cells around vessels were visualized (see Fig. 3E).

**Immunohistochemical study**

Immunohistochemical examination of group I dura mater tissue with anti-osteopontin (OPN) antibodies showed the prominent presence of this protein (see Fig. 2F). The greatest accumulation was observed on the mineral deposits themselves and in the surrounding tissues. An increased level of background chromogen staining (DAB) was also observed. Immunohistochemical examination of the dura mater tissue of group II showed a minimal level of OPN presence in the tissues (see Fig. 3F). The presence of OPN in meningioma tissues of the control group corresponded to 15.08±1.18 cells and it was significantly less than in the studied group 25.29±2.39 cells (p<0.001) (Fig. 4).

**Scanning electron microscopy (SEM) with energy-dispersive X-ray spectroscopy (EDX)**

SEM examination of the dura mater tissue of group I...
Intracranial biominerals are common in non-contrast computed tomography (CT) in children and adults. According to the statistics of CT results, their prevalence varies from 1 % in the young population to 20 % in the elderly. According to the results of autopsies, including microscopic findings during histological examination, biominerals are found in the skull cavity in more than 70 % of patients [10].

In some patients, intracranial biominerals appear as asymptomatic findings on CT; in others, they cause various neurological and cognitive disorders of varying degrees of severity, such as tremors, convulsions, parkinsonism, dementia, psychosis, behavioural disorders, and others [7].

The phenomenon of calcification is characteristic of tumours of the dura mater - meningiomas. They are usually benign neoplasms with an expansive nature of growth. The origin of meningiomas from the dura helps to distinguish them from cancer metastases because the latter is usually intracerebral [18].

But since calcifications are often found in the meninges, the problem of differential diagnosis between relatively "normal" meningeal tissue and calcified meningiomas arises.

In this work, we investigated the dura mater with and without signs of biomineralization using histological, histochemical, and immunohistochemical methods and scanning electron microscopy with energy-dispersive X-ray spectroscopy.

During the histological examination, the samples were divided into two groups based on the presence/absence of signs of biomineralization - small formations of different sizes and irregular shapes. Histochemical methods helped us, to some extent, investigate the composition of these formations. The von Kossa method helped narrow down to calcium phosphate compounds, and alizarin red S staining helped separate iron deposits. The Van Gieson method visualized collagen fibres; the PAS reaction did not reveal glycosaminoglycans in the composition of mineral deposits.

Osteopontin (OPN) is a noncollagenous, sialic acid-rich glycosylated phosphoprotein that regulates osteoblast function during bone formation. In addition, it is also present in various tissues and plays a vital role in inflammation, immune reactions, and bone mineralization [3].

Furthermore, OPN plays an important role in the formation of several human cancers due to the regulation of apoptosis, proliferation, adhesion, migration, invasion, metastasis and angiogenesis. Although staining of OPN has been correlated positively with the development of psammoma bodies in meningiomas, its correlation with clinical recurrence of meningiomas is still largely unknown [15].

In our study, biomineralization is positively correlated with OPN overexpression. We believe a significant amount of OPN is present in connection with remodelling in the calcification process, as this acidic glycoprotein is attracted to hydroxyapatite crystals [22].

SEM data indicate that biominerals in the dura mater are represented by tens to hundreds of formations of various shapes (rounded, oval, and irregular), varying from tens of nanometers to 50 micrometres. According to the data of the element distribution maps and the Ca/P ratio obtained from the spectra (see Figs. 2H, 2I), the mineral of the dura mater deposits is calcium phosphate of apatite composition.

According to the data of the element distribution maps (see Fig. 3G), these data were also confirmed by spectra and element distribution maps (see Fig. 3H, 3I).

**Discussion**

Biomineralization occurs in the central nervous system in normal and pathological conditions. Normally, this can be a manifestation of age-related changes. At the same time, it can be a sign of pathology: tumour growth, dystrophy, metabolic disorders, inflammation, intoxication or congenital pathology [20].

Intracranial biominerals are common in non-contrast computed tomography (CT) in children and adults. According to the statistics of CT results, their prevalence varies from 1 % in the young population to 20 % in the elderly. According to the results of autopsies, including microscopic findings during histological examination, biominerals are found in the skull cavity in more than 70 % of patients [10].

In some patients, intracranial biominerals appear in different sizes with a single-layer flat epithelium. Some samples showed probable signs of biomineralization - small formations of various sizes and irregular shapes.
2. Histochemically, the presence of biominerals was confirmed by the von Kossa method (identifying calcium phosphate compounds) and alizarin red S staining (differentiated calcium compounds from iron deposits). Histochemical study of the structure of the dura mater by the Van Gieson method and PAS-reaction of groups I and II showed their similar structure (a noticeable number of collagen fibres, single PAS-positive cells around vessels).

3. Immunohistochemical examination of the dura mater with antibodies against osteopontin revealed a significant difference between the studied and control groups (p<0.001), which confirms the important role of osteopontin in the process of biomineralization in this tissue.

4. Scanning electron microscopy showed that biominerals in the dura mater are represented by tens to hundreds of formations of various shapes (rounded, oval and irregular), the sizes of which vary from tens of nanometers to 50 micrometres. Their composition was analyzed using maps and spectra of energy-dispersive X-ray spectroscopy and most probably corresponded to calcium hydroxyapatite.

References
Біомінералізація - широко розповсюджений серед живих організмів процес утворення біомінералів. В центральній нервовій системі цей феномен зустрічається як в нормі, так і в патології. В нормі це може бути проявом вкрай змін, причому поширеність біомінералів збільшується з віком. В той же час це може бути ознакою патології - пухлинного процесу, порушення метаболізму та інше. Метою даної роботи є вивчення морфологічних особливостей твердої мозкової оболонки з ознаками біомінералізації. В роботі досліджено 30 зразків твердої мозкової оболонки з ознаками біомінералізації (група І) та 30 зразків без таких ознак (група ІІ) отриманих під час розтинів у патологоанатомічному відділенні Сумської обласної клінічної лікарні. Для морфологічної характеристики твердої мозкової оболонки застосували гістологічні методи, також сканувальну електронну мікроскопію. Статистичну обробку результатів імуногістохімічного дослідження проведено в статистичному пакеті GraphPad Prism 8.0 з використанням параметричних і непараметричних методів дослідження. Для розподілу зразків на групи опирались на гістологічний метод - забарвлення гематоксиліном-еозином. Гістологічні методи дослідження проводили за допомогою імуногістохімічного методу. Імуногістохімічна реакція навіяла нейонну ендокрінологічну сітку мінеральних депозитів. Імуногістохімічна характеристика твердої мозкової оболонки з ознаками біомінералізації була визначена за зазначеної методичної діагностики. Загалом сканувальну електронну мікроскопію зображували біомінерали в твердої мозковій оболонці, що відображає унікальну розподіленість окремих мікророзсіченнях. За допомогою сканувальної електронної мікроскопії було зіставлено, що біомінерали в твердої мозковій оболонці представлені дистрибутами-септами утворення різної форми (округлої, овальної та вирізної), розміри яких коливаються від десятків нанометрів до 50 мікрометрів. Їх склад був проаналізовано за допомогою карт та спектрів енергодисперсійної рентгенівської спектроскопії та найбільш імовірно відповідав гідроксиапатиту кальцію. Отже, для твердої мозкової оболонки характерна біомінералізація, що проявляється утворенням мікрокристалічних композитів з гідроксиапатитом. Ключові слова: біомінералізація, гідроксиапатит, кальцієвий біомінералізат, відповідно, біомінералізат, гідроксиапатит, гістологія, гістокімія, сканувальна електронна мікроскопія з енергодисперсійною рентгенівською спектроскопією.

**Author’s contribution**

Denysenko A. P.: research concept and design, receiving data, analysis and interpretation of data, design of the article.

Piddubnyi A. M.: analysis and interpretation of data, critical review, final approval.

Tkachenko I. A.: analysis and interpretation of data, critical review, final approval.

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