Turk Neurosurg, 2023

Original Investigation

DOI: 10.5137/1019-5149.JTN.41370-22.2

Received: 18.06.2022
Accepted: 18.08.2022

Published Online: 22.02.2023

Histopathologic and Spectrometric Evaluation of Bony Components of Synostotic Suture and Parietal Bone in Children with Sagittal Synostosis

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ABSTRACT

AIM: To understand the characterization of the ossification process both in the synostotic suture and adjacent parietal bone.

MATERIAL and METHODS: The surgical procedure for the 28 patients diagnosed with sagittal synostosis consisted of removing the synostotic bone as a whole, if possible, "Barrel–Stave" relaxation osteotomies, and strip osteotomies to the parietal and temporal bones perpendicular to the synostotic suture. The synostotic (group I) and parietal (group II) bone segments are obtained during osteotomies. Atomic absorption spectrometry was used to determine the amount of calcium in both groups, which is an indicator of ossification. Scanning electron microscopy and immunohistochemistry were employed to assess trabecular bone formation, osteoblastic density, and osteopontin, which is one of the in vivo indicators of new bone formation.

RESULTS: Histopathologically, trabecular bone formation scores did not indicate any significant difference between the groups. However, the osteoblastic density and calcium accumulation in group I were higher than those in group II, and the difference was significant. Osteopontin staining scores in cells showing membranous and cytoplasmic staining with osteopontin antibodies significantly increased in group II.

CONCLUSION: In this study, we found reduced differentiation of osteoblasts despite their increase in number. Moreover, the osteoblastic maturation rate was low in synostotic sutures, bone resorption becomes slower than new bone formation, and the remodeling rate is low in sagittal synostosis.

KEYWORDS: Craniosynostosis, Electron microscopy, Osteoblast, Osteopontin, Sagittal synostosis

ABBREVIATIONS: SEM: Scanning electron microscopy, **AAS:** Atomic absorption spectrometry

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INTRODUCTION

The calvarium aids in brain development by growing in synchronization with the rapidly growing brain tissue throughout the first few years of life. Cranial sutures are composed of undifferentiated mesenchymal tissues between the skull bones, and bone development occurs perpendicular to these structures (i.e., sutures) (15). The suture line that closes prematurely restricts bone growth in the direction perpendicular to the suture line (14), and this results in calvarial and facial deformities, impaired brain development, neurological conditions, increased intracranial pressure, and long-term cognitive and neuropsychological difficulties (27). The pathophysiology of premature suture line ossification is yet to be fully understood, and it is believed to be caused by vascular, mechanical, or genetic elements (9). Although genetic modifications observed in syndromic synostoses promote new bone formation, the cellular processes that cause premature suture closure in sagittal synostosis remain unexplained.

Studies have compared synostotic sutures with each other or with the normal suture, but no study has compared the sagittal synostotic suture with the adjacent parietal bone in sagittal synostosis. Thus, this study aimed to evaluate the association of cell behavior and bone formation with variability in in vivo parameters in sagittal synostosis by comparing the bone structure of the affected suture line and the adjacent parietal bone. While the same mechanisms work in cranium development and suture ossification, different morphologic results occur in adjacent bones during the growth process.

MATERIAL and METHODS

This prospective study included 28 children with pediatric sagittal synostosis who underwent surgery by the craniosynostosis team, which consisted of a leading neurosurgeon and two plastic surgeons at the Department of Neurosurgery. The study obtained ethics committee approval, and the legal representatives of the patients were informed of the preoperative treatment process. In addition to obtaining informed consent for the surgical procedure, a study-specific consent was obtained from the legal representatives, which was prepared based on the type, scope, design, potential risks, rationale, and objective of the study.

The study included patients who did not contraindications for surgery. Patients with syndromic conditions, those with contraindications for surgery, and those from whom family consent was not obtained for the study were excluded. The pathological suture segment (group I) to be resected either as a whole or in a stepwise pattern during the surgical treatment of 28 patients diagnosed with sagittal synostosis, and the parietal bone segment (group II) to be obtained while performing Barrel–Stave osteotomy perpendicular to the prematurely closed suture line in the same session were compared histopathologically and in terms of calcium density (Figures 1-4). By using atomic absorption spectrometry (AAS), calcium levels in the collected bony tissues were determined to indicate calcification, which is a sign of ossification. Scanning electron microscopy (SEM) was employed to demonstrate three dimensionally bone formations in high resolution. Immunohistochemistry was used to assess trabecular bone formation, osteoblastic density, and osteopontin, which is one of the in vivo indicators of bone formation.

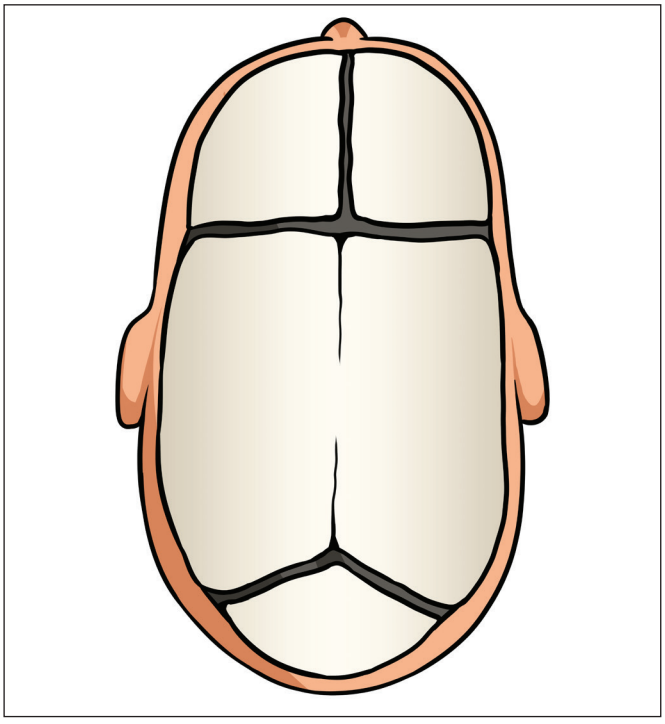

Figure 1: 2D drawing of the cranium and sutures lines.

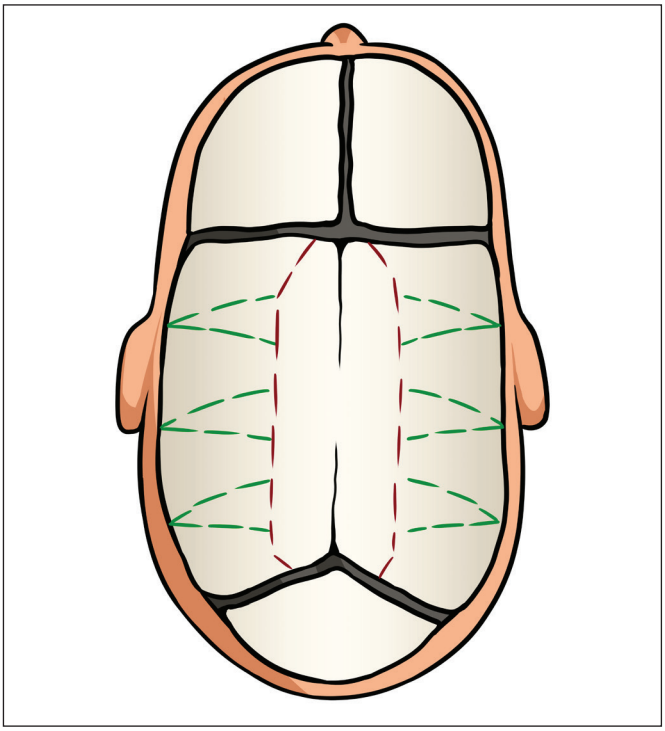

Figure 2: 2D drawing of the surgical planning.

Immunohistochemical Assessment

The samples were fixed in a 10% formaldehyde solution and subsequently decalcified in a 5% ethylenediaminetetraacetic acid solution for immunohistochemistry. After alcohol-xylene follow-up, the decalcified samples were embedded in paraffin blocks. From these paraffin blocks, 4-µm thick sections were cut, and osteopontin (monoclonal antibody, EP106, Epitomics, CA, USA) was applied using hematoxylin and eosin staining and immunohistochemistry. The 4-µm thick sections were transferred to electrostatically positively charged slides and dried at 70°C for 1 h for immunohistochemistry. On a fully automated immunohistochemical stainer, staining with the osteopontin antibody was combined with deparaffinization and antigen retrieval (Ventana BenchMark Ultra, Ventana Medical Systems, Tucson, AZ, USA). A ready-made kit consisting of biotin-free, HRP multimer-based hydrogen peroxide substrate, and 3,3'-diaminobenzidine tetrahydrochloride chromogen suited for the device was used to perform this staining process in completely automatic immunohistochemical equipment. The proportion of the stained cells, rather than the intensity of the stain, was considered in positively stained cells. The rating index was used to determine the proportion of positively stained cells within all surface epithelial cells in each segment. Consequently, the staining rate of <10% was accepted as 0; 11%–30%, 1+; 31%–50%, 2+; and 51%–100%, 3+ in the assessment score. While counting the fields, five fields under 100 magnifications were counted and averaged. Hematoxylin and eosin staining was used to assess trabecular bone development and osteoblastic density in the analyzed samples. Following the staining, the samples were evaluated under a light microscope, and the staining was rated semiquantitatively. In terms of osteoblastic density, a score of 0 indicated no staining; 1, 1–10 cells; 2, 11–20 cells; and 3, >20 cells. Moreover, 0, 1, 2, and 3 indicated no bone formation, faint staining, moderate staining, and robust staining, respectively (Table I). This assessment was based on five similar areas at 20 magnifications.

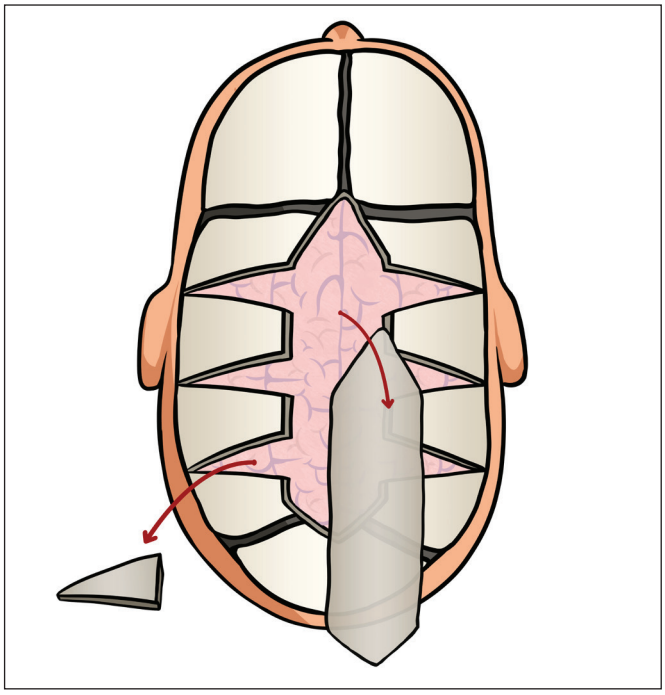

Figure 3: 2D drawing of removing the synostotic bone and Barrel–Stave relaxation osteotomies.

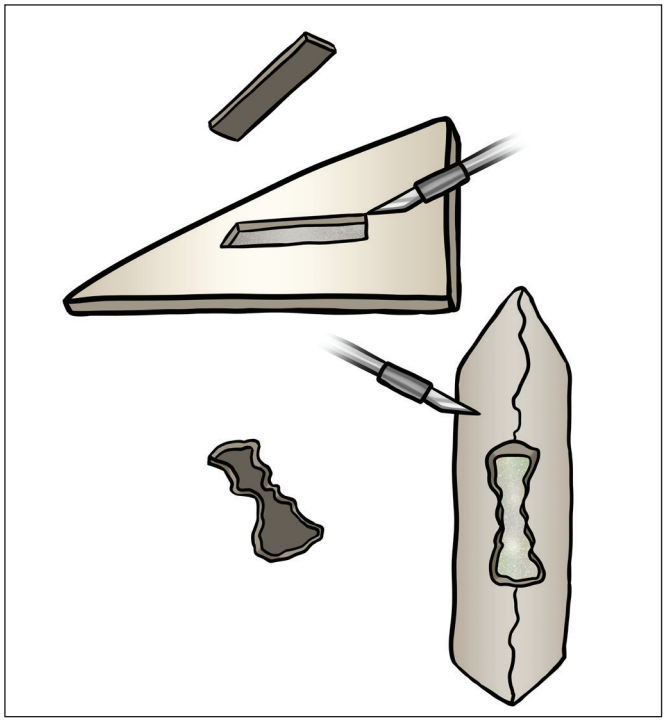

Figure 4: 2D drawing of tissue samples that are resected from the sagittal suture and the adjacent bone.

SEM to Display Samples

After being fixed for at least 1 day in 2.5% glutaraldehyde produced with a 0.1 M phosphate buffer at 7.2 pH, the samples were subjected to two 15-min changes and then rinsed with phosphate buffer (pH 7.2). Thereafter, it was run through a series of alcohol concentrations of 70%, 80%, 96%, and 100% for 15 min each. The samples were dried at the critical point after being maintained in amyl acetate for 15 min twice, and they were then fixed to the staples using double-sided adhesive tapes by breaking them as a whole or in various portions. The samples were photographed and studied in the JEOL JSM6060 SEM at 5–10 KV after coating with gold in the Polaran SC 502 coating apparatus.

AAS to Determine Calcium

In the laboratory, bone samples were first fixed in glutaraldehyde for 24 h before being categorized. For SEM and AAS experiments, the identified samples were prepared independently. For AAS experiments, samples were washed with phosphate buffer and then left in an oven at 60°C. After 2 days of storing the dried samples in concentrated nitric acid at room temperature, the solution was diluted with distilled water. Finally, the concentrations of calcium in the solutions were determined and measured in mg/gr.

Statistical Analysis

IBM SPSS Statistics for Windows, version 20.0 (IBM Corp., Armonk, NY, USA) was used for data analysis. As descriptive statistics, mean ± standard deviation values were used for quantitative variables according to the data distribution. Cross-tables and chi-square tests were used to control the relationships after checking the normality assumptions of data. In comparisons between the two groups, t-tests were used to analyze normally distributed data, and Mann–Whitney U tests were used to analyze non-normally distributed data. In all tests, a p-value ≤ 0.05 was considered significant.

RESULTS

The mean age of the 28 included patients was 11 months (4 months to 5 years). Fifteen (54%) patients were male, whereas 13 (46%) were female. First, hematoxylin and eosin staining was used to assess trabecula formation and osteoblastic density, which are both indicators of bone formation, for histopathological investigation of synostotic and parietal bone samples collected during surgery. No significant difference was found in trabecular bone formation scores between the groups (p>0.05) (Table I). However, the osteoblastic density was significantly higher in group I than in group II (p<0.05) (Table II). The scores of the osteopontin antibody staining in cells with membranous and cytoplasmic staining significantly increased in group II (p<0.05) (Table III). Further, to assess calcium accumulation in bone samples, AAS studies revealed a significant difference in favor of group I compared with group II (p<0.05) in the mean calcium accumulation (Table IV).

DISCUSSION

Before birth, the calvarial bones reunite with suture lines, and with their gentle condition, they facilitate brain development (5). As a disruption in suture lines during this period presents an issue that affects the entire craniofacial skeleton, the development of craniofacial bones should be evaluated comprehensively. The calvarial skeleton is composed of bone structures generated by intramembranous ossification of mesenchymal cells (4). Calvarial osteogenesis starts with the condensation of mesenchymal cells, which then develop into osteoprogenitor cells. These cells proliferate and finally become osteoblasts, which form the bone matrix (29). The transformation of progenitor cells directly on the bone surfaces or in the surrounding mesenchymal tissue causes osteoblastic differentiation in calvarial bones (2,23). Osteoblasts transform from mesenchymal precursor cells to preosteoblasts during new bone formation and eventually become functional. Their main function is to synthesize the extracellular matrix, regulate gene expression for bone calcification, and increase or decrease osteoclast functions (7,10). Bone sialoprotein, alkaline phos-

Table I: Change of Trabecular Bone Formation Scores

			Trabecular Bone Formation Scores			p
			1+	2+	3+	
Group	Study	n	2	11	15	0.76
		%	3.6	19.6	26.0	
	Control	n	1	13	14	
		%	1.8	23.2	25.0	
Total		n	3	33	44	
		%	5.4	42.9	51.8	

p: *P-value.*

Table II: Change of Osteoblastic Density Scores

			Osteoblastic Density Scores			p
			1+	2+	3+	
Group	Study	n	4	6	18	0.02
		%	7.1	10.7	32.1	
	Control	n	9	11	18	
		%	16.1	19.6	14.3	
Total		n	13	17	26	
		%	23.2	30.4	46.4	

p: *P-value.*

Table III: Change of Osteopontin Staining Scores

			Osteopontin Staining Scores				p
			0+	1+	2+	3+	
Group	Study	n	8	7	11	2	0.00
		%	14.3	12.5	19.6	3.6	
	Control	n	2	2	3	21	
		%	3.6	3.6	5.4	37.5	
Total		n	10	9	14	23	
		%	17.9	16.5	25.0	41.1	

p: *P-value.*

Table IV: Assessment of Calcium Accumulation

Group	n	Average	Mean Rank	Total Rank	U	Z	p
Control	28	113.57	17.04	477.00			
Study	28	151.32	39.96	1119.0	71.00	-5.26	0.00
Total	28						

p: *P-value,* ***u:*** *Mann-Whitney U statistics,* ***z:*** *Z-score*

phatase, osteocalcin, osteopontin, and type 1 collagen are all extracellular matrix proteins that can be indicators of mature osteoblasts (22). On the contrary, osteoclastic differentiation is a more complex procedure that involves sequential molecular steps. Unlike osteoblasts, they are derived from monocytes/macrophages and are located on the bone surface together with osteoblasts (7). They create a sealed interface between themselves and the bone structure with their folded cell surfaces. The pH of the environment must be kept low to enable the digestion of the mineral and protein components of the bone beneath. Therefore, osteoclasts are responsible for the secretion of acids and lysosomal enzymes and bone demineralization (7). Osteocytes are the most abundant cells in the established skeletal skeleton. Aged osteoblasts responsible for extracellular matrix formation develop into osteocytes and play important roles in the density of the organic components of the matrix (8). On the contrary, the extracellular matrix is formed by the combination of organic and inorganic structures that exist outside the cellular components and serves as a bed for this component. The organic part of the matrix is made up of collagen, non-collagenous proteins, proteoglycans, cytokines, and growth hormones, which accounts for 70% of the bone matrix (20). The inorganic part of the matrix is composed of calcium, calcium salts, fluoride, sulfate, and potassium, which is responsible for the compressive strength of the bone (3). Osteopontin is an aspartic acid-rich extracellular matrix protein that is highly phosphorylated and glycosylated, hydrophilic, negatively charged, secretory, organic, and non-collagenous (28). The regulation of bone cell adhesion, regulation of osteoclast function, and matrix mineralization are its key functions in biomineralization (1). Although osteopontin increases in the extracellular matrix of the bone during bone formation and remodeling, studies have demonstrated that bone formation and calcium deposition can proceed even in the absence of osteopontin (10,19). Osteopontin is assumed to be required for bone remodeling; however, the cells responsible for its secretion remain unknown (11,26). Its primary function in bone formation is to balance bone formation/resorption, particularly through osteoclast activation, in addition to conveying information about the functions of the cells that secrete it (25). Extracellular phosphates and kinases must phosphorylate osteopontin before it can bind with mineralized tissue (10). Osteopontin accelerates bone resorption by osteoclasts via integrin $\alpha v_{\beta 3}$, whereas the CD44 receptor promotes osteoclast adherence to the bone (21). The binding of osteopontin to integrin αv, a membrane receptor on osteoclast cells, stimulates osteoclasts and amplifies the osteolytic effect (24). According to Chellaiah et al., rats with inhibited osteopontin secretion showed significantly lower CD44 expression on the osteoclast surface, hypomobile osteoclasts, and decreased osteoclast activity, bone resorption, and trabecular gaps in the cancellous bone (6). Furthermore, the examination of the mechanical resistance of their bones showed that these rats had increased bone hardness and strength (6). Mature osteoblasts are believed to secrete osteopontin, and osteopontin is effective in modulating the cells that secrete it. In some experimental studies, osteopontin was suggested to play a significant role in bone mineralization, and it is ineffective in osteoblast development (12). However, studies have indicated that preosteoblasts and mesenchymal stem cells inhibit the response of cytokines and bone mineralization (13,16). In our study, the osteopontin score of group I was significantly lower than that of group II, but the trabecula formation, which indicates bone formation, was not different be-

tween the two groups (Figure 5A-C). SEM analysis of the tissue samples revealed that trabecular formation occurred in both groups, but the bone structure in group I was less organized, the trabecular formation grew coarser, and the trabecular space shrank (Figures 6A, B; 7A, B; 8A, B). Furthermore, the calcium levels in the analyzed samples were significantly higher in group I, which was consistent with the findings of Chellaiah et al. (6); this implied that the high calcium levels could be attributed to the low osteopontin expression in group I. De Pollack et al. investigated the difference in the new bone formation in the prematurely closed suture line compared with that in normal bones, and they assessed bone formation/resorption balance and osteoblastic activity according to the age of the patients (9). They claimed that bone formation outpaced resorption between the ages of 3 and 6 years, but the new bone formation slowed with age (9). However, the premature closure of sutures may be explained by the fact that production outpaced resorption (9). In addition, the intact bone and osteoblasts from the prematurely closed suture line were generated in cell cultures. Consequently, no significant difference was found in the expressions of alkaline phosphatase and osteopontin in the osteoblast culture of the samples from the prematurely closed suture line and normal bones. Although studies have linked higher osteoblastic cell proliferation to metabolic bone disorders (17,18), De Pollock et al. suggested that greater osteoblastic cell proliferation is not asso-

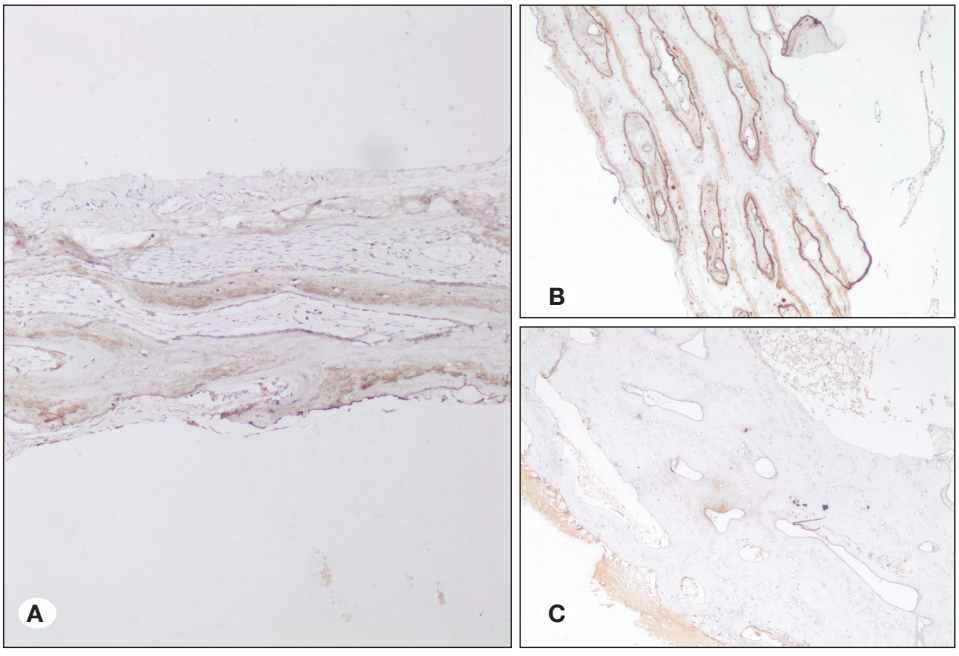

Figure 5: Immunohistochemical staining of tissue samples
A) Immunohistochemical staining of tissue samples in group II with osteopontin and +2 osteopontin score at ×40 magnification
B) Immunohistochemical staining of tissue samples in group II with osteopontin and +3 osteopontin score at ×100 magnification
C) Immunohistochemical staining of tissue samples in group I with osteopontin and +1 osteopontin score at ×100 magnification.

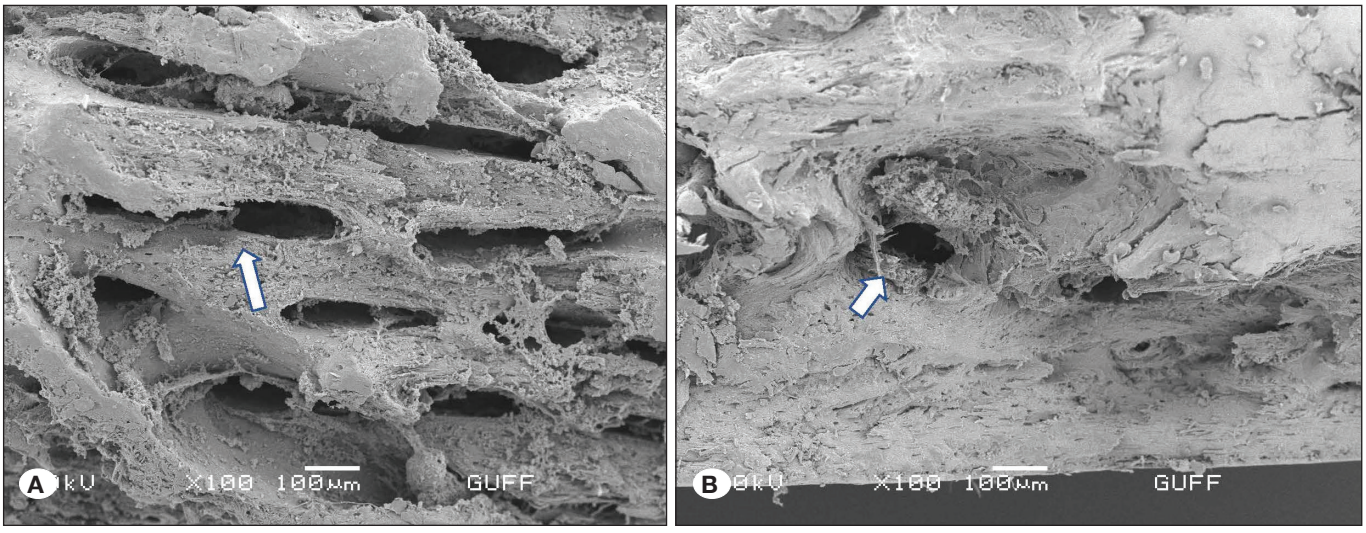

Figure 6: SEM of tissue samples demonstrating trabecular formations. **A)** SEM of tissue samples demonstrating trabecular formations. The trabecular formation is coarse, and the trabecular space is small in group I. **B)** SEM of tissue samples demonstrating trabecular formations. The trabecular formation is regular, and the trabecular space is wide in group II.

ciated with premature closure of sutures (9). They claimed that cell differentiation, rather than enhanced osteoblastic density, was more successful in preventing premature closure of sutures. In this study, alkaline phosphatase activity and osteocalcin production were used as markers of osteoblast maturation, and osteoblast maturation was higher in craniosynostosis bone samples. Although the osteoblastic density in synostotic bone samples was significantly higher in our study, the increased calcium density and decreased osteopontin expression in prematurely closed suture line suggested that the osteoblasts in the synostotic bone could not function normally and they might have impaired maturation (Figure 9A, B). This was because lower osteoblastic activation, osteopontin levels, and osteoclastic activity were observed in synostotic samples than in the parietal bones despite higher osteoblastic density. Bone resorption was reduced in the suture line that prematurely closed as osteoclastic activity decreased, and calcium accumulation in the tissue was increased. The low osteopontin staining achieved in our study was attributed to osteoblastic proliferation and diminished mature osteoblast differentiation, despite the high osteoblastic density in the synostotic bone. According to Kusuyama et al., reduced osteopontin levels also boost the responsiveness of preosteoblasts and mesenchymal stem cells to cytokines (16). This finding suggested that a compensatory mechanism was established in the attempt to activate osteoblast progenitors and osteoblast production in the prematurely closed suture line.

One of the study's limitations is that cell culture could not be used to accurately assess osteoblastic activity because the changes in cell phenotypes are more pronounced in long-term cultures and multiple passages. Furthermore, the study compared adjacent parietal bone tissues with mature bone formation to the prematurely closed suture line, which we believe simulates bone repair. In this study, we did not compare the sagittal suture and other mature sutures because

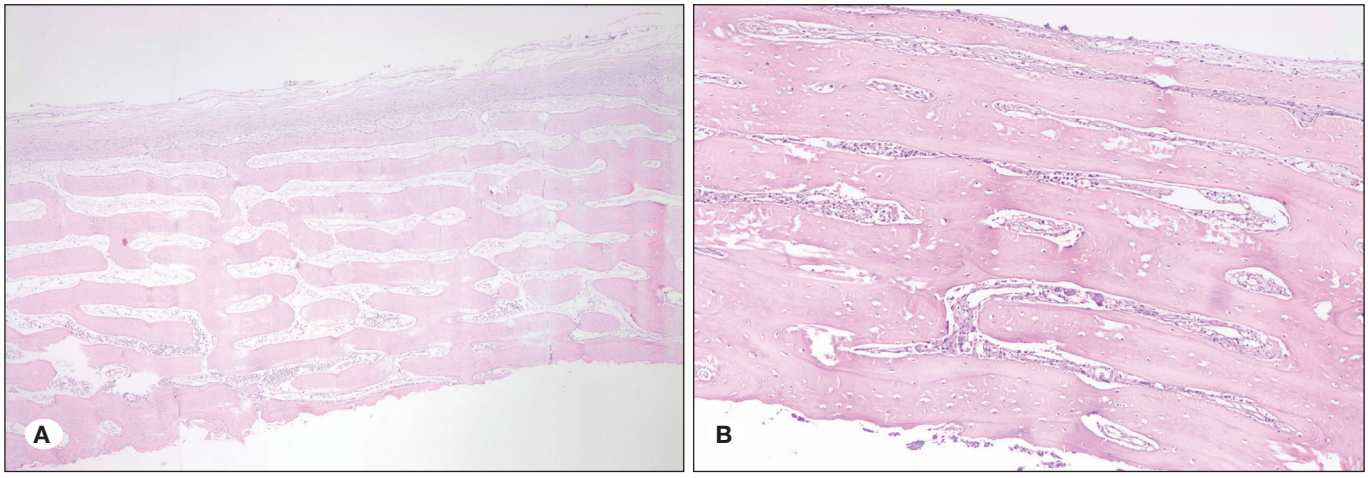

Figure 7: Hematoxylin and eosin (H&E) representation of trabecular formation in tissue samples. **A)** Hematoxylin and eosin (H&E) representation of trabecular formation in tissue samples in group II with ×100 magnification. **B)** H&E representation of trabecular formation in tissue samples in group II with ×40 magnification.

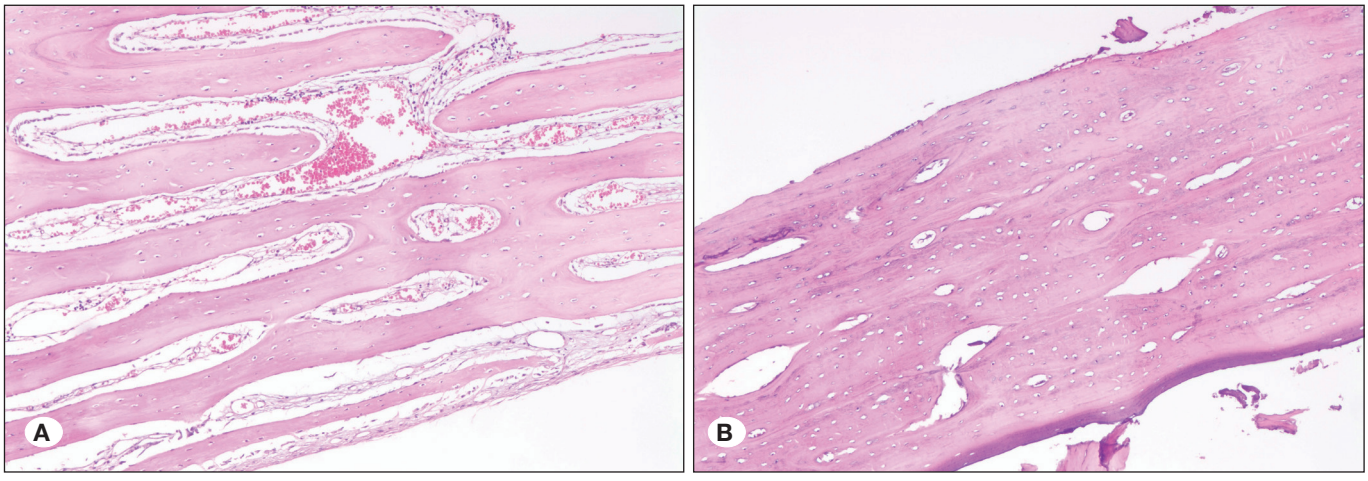

Figure 8: Hematoxylin and eosin (H&E) representation of trabecular formation in tissue samples. **A)** Hematoxylin and eosin (H&E) representation of trabecular formation in tissue samples in group I with ×100 magnification. **B)** H&E representation of trabecular formation in tissue samples in group I with ×40 magnification.

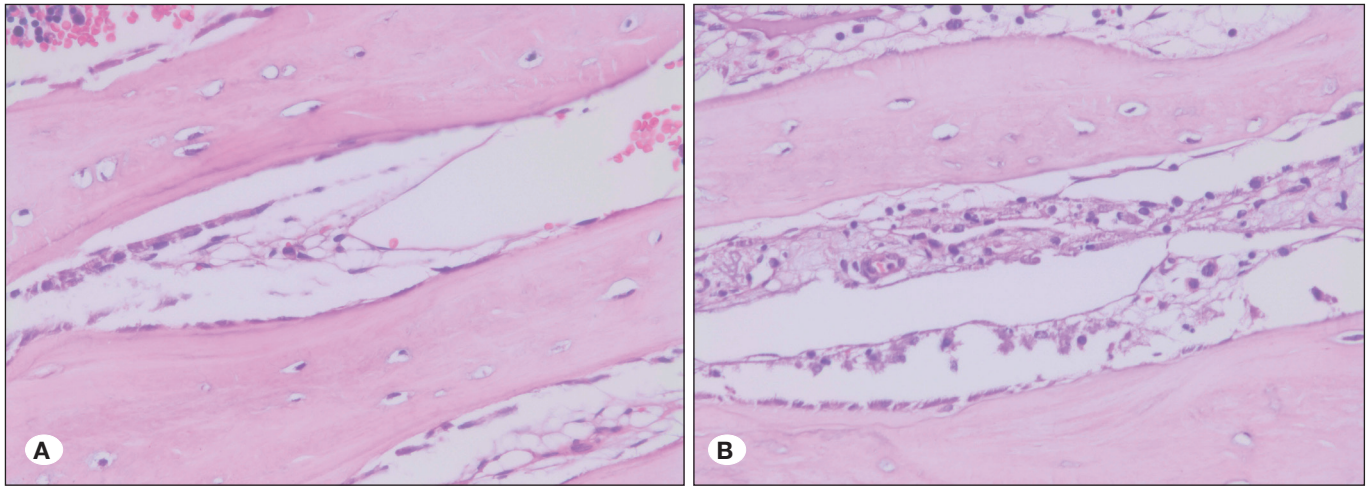

Figure 9: Hematoxylin and eosin (H&E) representation of osteoblastic density in tissue samples. **A)** Hematoxylin and eosin (H&E) representation of osteoblastic density in tissue samples. At ×400 magnification, group II received a +1 score. **B)** H&E representation of osteoblastic density in tissue samples. At ×400 magnification, group I received a +3 score.

of the type of surgical procedure. Further studies should include a large cohort and compare the parietal bone of a healthy patient with the synostotic suture of another patient. Studies conducted to date regarding the premature closure of sutures have always suggested that it may be attributed to the unilateral rise in the formation phase in the bone formation/resorption balance because there was no study on resorption.

Our findings demonstrated that despite the high number of osteoblasts present during premature closure of sutures, their differentiation was reduced, and decreased osteopontin expression inhibited osteoblasts, resulting in bone mineralization. This situation suggested that bone mineralization occurred at the suture line as the formation was slow and the resorption was less.

CONCLUSION

Mineralization or demineralization of the bone is caused by an imbalance in bone formation or resorption. In our study, the osteoblastic maturation rate decreased in sagittal synostosis, bone resorption was slower than new bone formation, and the remodeling rate was low in sagittal synostosis. However, further research elucidating the behavior of osteoblasts acquired from the early closure of sutures will help clarify molecular mechanisms.

ACKNOWLEDGMENTS

The determination of calcium by atomic absorption spectrometry of the bone samples in our study was performed by Research Assistant Ahmet Furkan Kayis at Ankara Gazi University, Faculty of Science, Department of Chemistry. In addition, data analyses were evaluated by Ece Turker, and medical illustration was made by Olcay Kurt to better understand the surgical procedure and how the study was performed.

AUTHORSHIP CONTRIBUTION

Study conception and design: CC, GF, TH
Data collection: CC
Analysis and interpretation of results: DF, ZS, DAM
Draft manuscript preparation: CC
Critical revision of the article: TH
Other (study supervision, fundings, materials, etc...): GF, TH, CC, ZS, DAM, DF
All authors (CC, GF, TH, DAM, DF, ZS) reviewed the results and approved the final version of the manuscript.

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