Bone Induction Capacity of the Periosteum and Neonatal Dura in the Setting of the Rat Zygomatic Arch Fracture Model

Derya Özçelik, MD; Tuğrul Turan, MD; Fevziye Kabukçuoğlu, MD; Kemal Uğurlu, MD; Özcan Öztürk, MD; Muzaffer Başak, MD; Mhjdat Bankaoğlu, MD

Objectives: Osteogenic properties of the dura and periosteum are thought to contribute to the regenerative capacity of membranous bone tissue. The purpose of this investigation was to elucidate (1) whether dura without underlying neural tissues can induce osteogenesis, (2) to what extent the periosteum participates in membranous bone healing, and (3) the difference between dura-induced and periosteum-induced osteogenesis.

Methods: A standardized 2-mm defect was created within the middle portion of each zygomatic arch in 30 Wistar albino rats. The rats were divided into 3 groups, 10 animals in each group. In group 1, the periosteum was removed and neonatal dura grafts were transplanted onto the zygomatic arch bone defect circumferentially. In group 2, the overlying periosteum was preserved. In group 3, the periosteum was removed. At 3 and 10 weeks, animals from each group were killed, and specimens were obtained. Data were collected from the 3-dimensional computed tomographic scans and histologic studies to compare the extent of bony repair.

Results: Fracture sites demonstrated osteogenesis associated with chondrogenesis in groups 1 and 2 and only limited osteogenesis with no chondrogenesis in group 3. In some animals in group 3, cortical bone ends underwent resorption. In groups 1 and 2, bone defects were obliterated by the formation of the mature compact bone at 10 weeks postoperatively. The difference between bone regeneration in these groups was not significant (P = .16). In group 3, the defects failed to heal by bony union, and in most of the samples the fibrous union was observed instead. The difference between groups 1 and 3 was significant (P = .03). The difference between groups 2 and 3 was not significant (P = .09).

Conclusions: The trend toward significance is in agreement with the current clinical practice of preserving periosteum in the manipulations of the membranous bone defects. Newborn dura can exert a potentiating effect on osteogenesis.

Arch Facial Plast Surg. 2003;5:301-308

The mechanism of membranous facial bone healing is still incompletely understood. Thaller and Kawanoto1 in 1990 showed osseous union in facial fractures in 10 patients who underwent a secondary reconstructive procedure. Prior to that, facial bone fractures were considered to heal by fibrous union. There have been no controlled studies investigating the effect of the periosteum on membranous bone healing. Similarly, the role of the dura mater in calvarial osteogenesis is presumed to be important, but precisely what that role is remains unclear.

Bony structures of membranous origin phylogenetically include the skull above the occipital line.2 The zygoma, which is a pure membranous bone, has proved to be an excellent model for the study of membranous bone repair.3,4 Zygomatic arch fractures do not require any fixation to keep the fragments in proper position. The fragments are stable, thereby eliminating the effects of mobility and method of fixation on healing.

Histologic evaluation of midfacial fracture repair in human studies has been found to be consistent with the results in an animal model,1 although it is possible that the rat may exhibit greater osteogenic potential than primates. The size of the bony defect leading to spontaneous healing varies by anatomic location, species, and age. Three- and 4-mm calvarial defects in adult rats have demonstrated osseous bridging and evidence of normal osseous repair throughout the defect.5

The mechanism of cellular differentiation attributed to the contact effect of different tissues is known as induction. An inducing cell in a bone model causes a responding cell to differentiate into an osteoblast.6 In 1997, Yu et al7 showed that dura without other central nervous sys-
tem components could induce osteogenesis within cutaneous tissue. Uddström and Ritsila investigated skull defects and evaluated the dura osteogenic capacity. They considered neonatal dura to be more potent than periosteum. Most commonly, the calvarium has been used to study the role of dura mater in the repair of bone defects. However, to elucidate the role of dura mater, complete isolation of the dura from its microenvironment was deemed appropriate. We therefore chose a zygomatic bone model. The experiment was designed to answer the following questions: (1) Can dura without underlying brain and its natural microenvironment induce osteogenesis? (2) To what extent does the periosteum participate in membranous bone healing? (3) What are the differences between dura-induced and periosteum-induced osteogenesis?

METHODS

The study was performed on 30 Wistar albino rats (aged 15 months, weighing 300-350 g). The rats were divided into 3 groups, 10 animals in each group, and zygomatic bone defects were created in each group. In group 1, neonatal dura grafts were transplanted onto the zygomatic arch bone defects circumferentially (20 zygomas) after removing the overlying periosteum of the zygomatic arch. In group 2, the overlying periosteum was preserved (20 zygomas). In group 3, the periosteum was removed along the entire length of the arch (20 zygomas). In groups 2 and 3, no graft replacement was made.

Institutional guidelines regarding animal experimentation were followed. The rats were housed in an air-conditioned animal facility with 12-hour light-dark cycles and free access to food and water.

Twenty additional Wistar albino rat neonates were used for harvesting the dura grafts within 48 hours after birth. The animals were anesthetized with intramuscular ketamine injection (0.002 mg/kg). A skin flap based on its lateral border was elevated, and craniectomy was performed using microsurgical scissors over the entire length of the cranium, carefully preserving the underlying thin dura. Under a dissecting microscope, a piece of dura approximately $10 \times 10$ mm was dissected off the calvarium (Figure 1). Then it was immediately transferred to the zygomatic bone defect of the adult rat.

In 30 adult rats then, a standardized 2-mm defect was created within the midportion of each zygomatic arch (Figure 2). General anesthesia of the adult rats was achieved with intramuscular ketamine injection (10 mg/kg). Through lateral incisions, both zygomatic arches were exposed. Using an osteotome, a 2-mm defect was created. In group 1, the arch was wrapped in dura graft (Figure 3A, right zygoma). The bony surface of the dura was placed in contact with the bone whenever possible, considering the fragility and small quantity of the neonatal dura mater. This procedure was not a complete wrapping procedure; therefore, guided-tissue regeneration was not a factor in bone regeneration in this model. Two interrupted 10-0 nylon tacking sutures were used to keep the dura graft in place. After placement of the dura graft on one side, the skin incision was closed with interrupted 4-0 silk suture. The periosteum was preserved in group 2 (Figure 3A, left zygoma) and completely resected along the arch in group 3 (Figure 3B, left zygoma).

At 3-week intervals, 3 rats from each group were killed, and at 10 weeks the remaining animals were killed with an intracardiac administration of ketamine solution. Assessments included gross observation of the surgical sites regarding the fracture gap stability, a radiologic assessment by 3-dimen-
sional computed tomographic (CT) scans, and a histologic assessment.

Three-dimensional CT scans were obtained at 3 and 10 weeks postoperatively. The scans were used for determining small differences in bone regeneration between the groups, as previously described.\(^9\) One-millimeter section thickness was used to minimize the artifacts.\(^10,\)\(^11\)

Histologic assessment was also performed. At 3 weeks in 9 rats and 10 weeks in 21 rats postoperatively, the operative areas were reexposed through lateral incisions and the zygomatic arches removed en bloc. The specimens were placed in 10% formalin fixative for 1 day, decalcified in nitric acid, embedded in paraffin, and sectioned horizontally to incorporate the entire defect within the slide. Four-micron-thick serial sections of each block were prepared. Three to 5 sections were examined for each zygoma specimen. Tissue sections were examined with hematoxylin-eosin staining and evaluated for the extent of bony repair in each group by a pathologist blinded to group assignment.

Statistical analysis was performed using the Welch approximation t test. A P value less than .05 was considered statistically significant.

RESULTS

CLINICAL EVALUATION

On clinical assessment by gross observation at 3 and 10 weeks postoperatively, the dura graft had become fully integrated into the surrounding soft tissues. At 3 weeks, the gap areas and fracture ends were covered with granulation tissue. Ten weeks postoperatively, the surgical sites showed complete hard tissue regeneration and stability in 12 (85%) of 14 sites in group 1 (Figure 4A); 10 (71%) of 14 sites in group 2 (Figure 4B); and 6 (43%) of 14 sites in group 3. In samples with partial or total lack of healing, fracture segments were connected by fibrous connective tissue (Figure 4C).

Three-dimensional CT scans were obtained at 3 and 10 weeks postoperatively. At 3 weeks, the pattern of healing was minimal; therefore, the results were not compared between groups. At 10 weeks, complete bone regeneration was observed in 12 (85%) of 14 sites in group 1; 7 (50%) of 14 sites in group 2; and 6 (43%) of 14 sites (43%) in group 3.

Results of the gross observation and the 3-dimensional CT analysis were comparable for groups 1 and 3. In group 2, bone healing in 3 animals was determined to be incomplete by CT but appeared complete on gross examination.

The 2 animals in group 1 with incomplete regeneration showed a diminution of 10% in the size of the original defect. Of the 4 incompletely regenerated animals in group 2, one showed a defect diminution of 55% to 75%, 2 showed a diminution of 35%, and 1 showed a widening of the original defect related to the degeneration of the fracture ends. Three of the incompletely regenerated animals of group 3 showed a diminution of 45% to 65% in the defect, 1 showed 95% diminution, and the remaining 4 animals showed widening of the original defect.

The amount of new bone formation for each sample is illustrated in Figure 5. Negative values represent widening of the gap due to necrosis and degeneration of the fracture ends. Mean values for bone regeneration for each group are listed in Table 1. The calculations were made according to the newly formed bone and not the final size of the defect; therefore, defect enlargement due to bone necrosis was not included in the calculations.

The Welch approximation analysis revealed a significant difference (P = .03) in the amount of bone formed in group 1 compared with group 3, which revealed fibrous union in most of the samples (Figure 6A-C). The difference between groups 1 and 2 was not statistically significant (P = .16) (Figure 6D-F). This implies that bone regeneration occurred equally well in the presence of either neonatal dura graft or the periosteum of the original tissue. Interestingly, the difference between the periosteum-removed and the periosteum-preserved groups...
was not significant (P = .09) (Figure 6G-I). The values are listed in Table 2.

HISTOLOGIC EVALUATION

Hematoxylin-eosin staining was performed at 3 and 10 weeks postoperatively. Histologic evaluation showed neonatal dura grafts to be viable. There was no evidence of adverse tissue reaction to the allograft. Specimens in group 1 at 3 weeks (n = 6) showed moderate inflammatory reaction. The defect was bridged with granulation tissue containing a significant quantity of osteoblastlike cells concentrated near the ends of the bone at the defect margin. These reactions were associated with osteoid formation. Some fibrochondroblastic hyperplasia was present in 2 of the 6 samples (Figure 7A). Immature bone formation with evidence of callus and vascular channel formation was detected (Figure 7B).

Specimens in group 2 at 3 weeks (n = 6) showed moderate inflammatory reaction and significant granulation tissue formation. Significant fibrochondroblastic hyperplasia was present in 4 of the 6 samples. Many osteoblastlike cells were associated with osteoid formation. Organization of bone with callus formation and intense vascularization appeared. Evidence of early bone formation in a subperiosteal location was observed (Figure 8).

Specimens in group 3 at 3 weeks (n = 6) showed some inflammatory reaction and minimal granulation tissue formation. There were small numbers of early bone nests with vascularization in 2 samples. In 4 of 6 specimens, necrotic bone ends were observed (Figure 9). Fibroblastic hyperplasia was present with no evidence of callus formation in these specimens.

Specimens in group 1 at 10 weeks (n = 14) showed that the vascular, inflammatory, and fibroblastic responses were almost completely resolved. Markedly decreased osteoblastic activity was demonstrated. In 5 of the 14 samples, diffuse osteochondroid activity or islands of chondroid tissue formation were detected (Figure 10A). There was evidence of cortical bone formation with haversian canal system in 12 of the specimens. Intense vascularity noted in the 3-week samples has subsided in these specimens (Figure 10B).

Specimens in group 2 at 10 weeks (n = 14) showed that the vascular, inflammatory, and fibroblastic responses and osteoblastic activity were almost completely resolved. In 10 of 14 samples, chondroid tissue formation was detected (Figure 11). Chondroid formation was spotty in some specimens and diffuse in others. There was evidence of cortical bone formation with haversian canal system in 7 of the specimens. No obvious callus was detected at this stage.

Specimens in group 3 at 10 weeks (n = 14) showed that the vascular, inflammatory, and fibroblastic responses and osteoblastic activity were almost completely resolved and the process had terminated. There was evidence of cortical bone formation with haversian canal system in 7 of the specimens. In 5 of the 14 samples, degenerated bone ends were observed (Figure 12). No chondroid tissue formation was detected in any of the specimens. Minimal granulation tissue was present. Dense fibrous connective tissue between the fracture ends was observed in 7 samples, which exhibited some lamellar bone formation as well.

Facial fracture repair was believed to occur via fibrous union until 1990, when Thaller and Kawamoto3 showed a stable osseous union. Fibrous healing occurs in facial bones if the gap between bone ends exceeds several millimeters, especially if the bone is thin or if soft tissue is interposed.1 Mostly, however, healing occurs via new bone formation.

In contrast to endochondral bone formation and healing by replacement of the preexisting cartilaginous matrix with woven bone, membranous bone development and healing does not involve a cartilage precursor. Mesenchymal cells differentiate directly into osteoblasts.3 However, in 1991, Rever et al3 demonstrated in a rabbit model that facial bone healing involves a cartilage precursor step in both the simple fracture and the fracture ostectomy models. In the present study, fracture sites exhibited diffuse distribution of osteogenesis with isolated islands of chondrogenesis in the presence of either dura graft or the periosteum. The osteochondroid matrix demonstrated in these groups was similar to the endochondral bone healing as well as to the bone morphogenetic protein–induced25 calvarial bone regeneration.

Without the periosteum, only limited osteogenesis with no chondrogenesis occurred, and cortical bone ends underwent resorption in most of the samples.

Table 1. Results of the Welch Approximation Statistical Analysis Testing If the Amount of Bone Regeneration Was Significantly Different Between Groups

<table>
<thead>
<tr>
<th>Compared Groups</th>
<th>t Value</th>
<th>P Value</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 vs 2</td>
<td>1.031</td>
<td>.16</td>
<td>Not significant</td>
</tr>
<tr>
<td>1 vs 3</td>
<td>1.958</td>
<td>.03</td>
<td>Significant</td>
</tr>
<tr>
<td>2 vs 3</td>
<td>1.362</td>
<td>.09</td>
<td>Not quite significant</td>
</tr>
</tbody>
</table>

Figure 5. Graph illustrating the bone regeneration for each sample (42 zygomas in 21 animals) in groups 1, 2, and 3 at 10 weeks after surgery. The amount of healing was individually calculated for each zygoma from 3-dimensional computed tomographic scan results. Negative values represent widening of the defect gap due to necrosis and degeneration of the fracture ends.

Figure 6. Hematoxylin-eosin (H&E) staining. (A) Specimen in group 1 at 3 weeks (n=6) showing fibrous tissue interposed at the fracture gap. Mostly, however, healing occurs via new bone formation. (B) Specimen in group 2 at 3 weeks (n=6) showing moderate inflammatory reaction with minimal granulation tissue formation. Intense vascularity noted in the 3-week samples has subsided in these specimens (Figure 10B). (C) Specimen in group 3 at 3 weeks (n=6) showing some inflammatory reaction and minimal granulation tissue formation. There were small numbers of early bone nests with vascularization in 2 samples. (D) Specimen in group 1 at 10 weeks (n=14) showing moderate inflammatory reaction and minimal granulation tissue formation. Intense vascularity noted in the 3-week samples has subsided in these specimens (Figure 10B). (E) Specimen in group 2 at 10 weeks (n=14) showing moderate inflammatory reaction and minimal granulation tissue formation. Intense vascularity noted in the 3-week samples has subsided in these specimens (Figure 10B). (F) Specimen in group 3 at 10 weeks (n=14) showing moderate inflammatory reaction and minimal granulation tissue formation. Intense vascularity noted in the 3-week samples has subsided in these specimens (Figure 10B).
Periosteum can differentiate into osteoprogenitor cells and chondroblasts in response to altered microenvironment. Therefore, absence of the periosteum may account for impaired chondrogenesis in the periosteum-removed group. Isolated islands of cartilaginous tissue seen occasionally were thought to play no active role in repair process.

Burstein et al. described 3 layers of the periosteum: the inner cambial layer, the middle layer of "osteogenic reserve cells," and the outer vascular network.
of arterioles and venules that communicated with the internal network of trabecular vessels. The periosteum with its profuse vascular network was considered to play a major role in the prevention of necrosis of fractured bone ends. Necrosis detected in some samples seemed to correlate with the resected periosteum.

Three-dimensional CT measurements correlated with the results of the gross observation regarding the degree of healing in 39 of 42 samples. Three-dimensional CT correctly identified 93% of healed defects. Therefore, we think that 3-dimensional CT can reliably identify bone healing and can be used to help the surgeon plan surgical treatment as well as for the follow-up. Additionally, with the hematoxylin-eosin specimens, it would be possible to obtain some sort of relative volumetric estimation of the bone regeneration. Comparing 3-dimen-

<table>
<thead>
<tr>
<th>Group</th>
<th>Type of Surgical Procedure</th>
<th>Sample Size</th>
<th>Mean ± SEM Bone Regeneration, mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Dura grafted</td>
<td>14</td>
<td>1.742 ± 0.174</td>
</tr>
<tr>
<td>2</td>
<td>Periosteum preserved</td>
<td>14</td>
<td>1.485 ± 0.177</td>
</tr>
<tr>
<td>3</td>
<td>Periosteum resected</td>
<td>14</td>
<td>0.892 ± 0.397</td>
</tr>
</tbody>
</table>

Table 2. Mean Regeneration of the 2-mm Bone Defects Within Groups

sional CT measurements and the hematoxylin-eosin specimens’ volumetric estimation could give more information about the reliability of the 3-dimensional CT technique on bone regeneration. This subject might be the focus of further study.

Histologically, no difference between the dura-induced osteogenesis and periosteum-induced osteogenesis was detected. Osteoconduction of bone growth of the zygoma was distinguished from osteoinduction of mesenchymal cells of the dura and periosteum by evaluating the periosteum-removed group. In this group, the repair process was attributed to the small quantity of bony ingrowth from the fracture rims. However, decreased bone regeneration and fragment end resorption in this group emphasized the important role of the periosteum in healing. Our study therefore confirmed the importance of the periosteum, in accordance with current clinical guidelines that recommend its preservation in the manipulation of the membranous bone defects.

Immunologic response to components of the transplanted tissue was not observed. Hobart et al9 investi-
gated the role of dura in cranial bone regeneration in the immature animal and transplanted dura as an isograft. Through histologic evaluation, all grafts (ie, dura and periosteum) were found viable, well vascularized, and without signs of rejection. Similarly, Opperman et al18 transplanted coronal suture with underlying dura mater between isogeneic animals without signs of rejection reaction. In our specimens, the stromal and cellular composition at the recipient beds appeared to be normal as well.

Calvariectomy in children younger than 2 years and in immature experimental animals has been shown to be associated with complete reossification when the dura mater was left intact.19 Hobar et al,5 in 1993, showed that the neonatal dura mater possesses the ability to promote parietal bone regeneration that cannot be replicated with the transplanted adult dura. Although these findings implicate the osteogenic potential of the neonatal dura mater, the molecular mechanisms governing these events remain unknown. Mehrara et al,20 in 1999, indicated that the dura mater underlying the developing calvarial bone expressed fibroblast growth factor 2 and transforming growth factor β1. The spatial and temporal expression of these growth factors implicated them in the regulation of calvarial bone formation.

In our study, whether the bone defect was repaired by osteochondroblastic differentiation of the dura cells or by the action of the dura-derived growth factors on mesenchymal cells is not known. The main interest of our study was to demonstrate the bone induction capac-

---

Figure 10. Photomicrographs of a group 1 histologic specimen (dura group) 10 weeks postoperatively (hematoxylin-eosin, original magnification ×125). A, Bridging of the fracture ends by new bone (arrow) is visible, with preexisting bone on the left and right of the figure. In this specimen osteochondroid tissue formation can be detected as diffuse in nature rather than an isolated island. B, New bone formation is observed at the edges of the preexisting bone. Although it is not easy to identify in a decalcified and longitudinal section of the zygoma, evidence of cortical bone formation with the haversian canal system can be seen. The vascular inflammatory and fibroblastic responses subsided.

Figure 11. Photomicrographs of a group 2 histologic specimen (periosteum-preserved group) 10 weeks postoperatively (hematoxylin-eosin). A, Osteochondroid activity is visible. New-woven bone is forming from the ends of the existing bone and extending into the defect. However, it does not bridge the gap at this time (original magnification ×125). B, Isolated areas of the chondroid reaction belonging to a different specimen can be noted here under higher magnification (original magnification ×310). Cells exhibit a distinct homogeneous basophilia.

Figure 12. Photomicrograph of a group 3 histologic specimen (periosteum-resected group) 10 weeks postoperatively. This specimen shows necrotic bone end (hematoxylin-eosin, original magnification ×125).
ity of dura mater in a noncalvarial fracture model. We found that neonatal dura mater has an osteogenic potential even in the microenvironment different than calvarium, and its osteogenic capacity seemed to be as potent as that of the periosteum. A better understanding of the differentiation of dura cells exposed to different conditions will provide clinical benefit in 2 important areas of craniofacial surgery: cranial bone regeneration and pathogenesis of craniosynostosis.

Müke and Meyer-Glauner, 21 in 1977, concluded that calvarial regeneration is entirely dependent on the extraskeletal dura mater. Mossaz and Kokich 19 did not concur with this statement but suggested that the lack of dural continuity causes an alteration in the integrity of the dura mater, which ultimately results in a decreased rate of bone deposition following a calvariectomy procedure. Our study suggests that newborn dura mater without underlying brain tissue can exert a potentiating effect on the repair of midfacial bone defects. It implies that the influence of dura mater on osteogenesis is not directly mediated by the dural-neural tissue interaction. Additionally, a change of environment does not appear to change this ability of the dura mater, although it has been suggested that transfer of the dura can alter biomechanical forces critical to bone formation. It is possible that dura mater transmits its own inductive signals for bone formation. Additional studies are needed to clarify the biochemical mechanisms that coordinate the dura mater–derived bone induction.

Accepted for publication February 5, 2003.

This study was presented at the 22nd National Congress of the Turkish Society of Plastic Surgeons in competition session, Izmir, Turkey, September 30, 2000.

Corresponding author and reprints: Derya Özçelik, MD, Sezai Selek sok, Hersek apt, 4/B, D:2, 80200, Nisantasi, İstanbul, Turkey (e-mail: deryaozcelik68@yahoo.com).

REFERENCES