Radiological and Stereological Evaluation of the Effect of Rifampin on Bone Healing in Critical-Size Defects

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Objectives: This study aimed to investigate the effect of rifampin with autogenous bone on bone regeneration in critical-size defects in the calvaria of rats.

Materials and Methods: In total, 40 rats were divided into 4 groups and a 5-mm diameter of calvarial defect was made in each rat's calvarium. Control group (C), bone defects were irrigated with sterile saline; rifampin group (R), bone defects were irrigated with rifampin. In the autogenous graft group (Ag), the autogenous graft was contaminated with saliva, and the defects were filled with an autogenous graft. In the autogenous graft + rifampin group (Ag+R), the autogenous graft was contaminated with saliva and was decontaminated with rifampin, and the defects were filled with the autogenous graft. The animals were killed at 4weeks. Bone formation was assessed by micro-computed tomography scanning and stereological analyses.

Results: The mean new bone volume was the greatest in the Ag/rifampin group (1.73 ± 0.17), followed by the Ag group (1.50 ± 0.05) (statistically significant difference at $P < 0.05$). The new bone volume was the lowest in the control group (1.05 ± 0.09); however, no difference was observed compared with the rifampin group (1.08 ± 0.07) ($P > 0.05$).

Conclusion: This study, despite its limitations, showed that rifampin with autogenous bone increased bone regeneration in rats with critical-size defects.

Key Words: Bone regeneration, critical-size defects, rifampin

(*J Craniofac Surg* 2016;27: 1481–1485)

Bone substitutes are widely used for reconstruction of bone defects in dentistry and medicine. Of the many graft materials available, autologous bone is ideal to achieve bone healing in bone defects.[1–5] Autologous bone grafts possess optimal osteoinductive, osteoconductive, and osteogenic properties for an ideal graft. On the other hand, they have some limitations; therefore, biomaterials are preferred in maxillofacial surgery.[6] However, good health status of the host bone is essential for optimal healing between the bone and the biomaterial; otherwise, the results can be unsatisfactory. The graft resorption rate depends on many factors, including graft type, vascularization, stabilization, and presence of infection.[7] Various additional materials (antibiotics, plasma materials, etc) are combined with graft materials to prevent infection of the surgical site and to increase the effectiveness of the bone graft. Also, chemical and antibiotic solutions are commonly used for disinfection in oral and maxillofacial surgery.[8]

Rifamycin is a semisynthetic antibiotic that is obtained from natural rifamycin B. It is bactericidal against gram-positive and gram-negative microorganisms, and is used to disinfect wounds.[7–9] Rifamycin has been used to locally wash fistulas, maxillary sinuses, areas of osteomyelitis, and abscesses in oral and maxillofacial surgery. Rifamycin may be applied topically, is well tolerated on bone tissue, and has been shown to decrease the chances of infection.[10] Although many authors have used various antibiotic agents mixed with bone grafts,[7,8,11–13] there is insufficient information about the effects of local antibiotic application on the healing of autogenous bone block grafts. The purpose of this study was to evaluate the effects of rifamycin solution on autogenous bone grafts used to repair critical-size defects.

METHODS

Surgical Procedures

Eight-month-old female rats ($n = 40$) with an average weight of 250 g were randomly assigned to 4 groups. Rats were anesthetized with an intraperitoneal injection of 10 mg/kg ketamine HCl (Ketalar; Pfizer, Istanbul, Turkey), and a semilunar incision was made in the calvarium, allowing reflection of a full-thickness flap in the anterior direction. A 5-mm-diameter calvarial defect was made using a trephine on low-speed bur under continuous saline irrigation. The autogenous bone was contaminated with the rat's saliva for 20 seconds, after which the graft was decontaminated with rifamycin solution (Rif 250 mg; Abdi Ibrahim, Istanbul, Turkey) for 10 minutes. The operation flaps were then closed with 3/0 silk suture material. This study was approved by the Bülent Ecevit University Animal Care and Use Committee.

Experimental Groups

The following experimental groups were used:

Control group (C): bone defects were irrigated with sterile saline. The flap was closed without any further action.

Rifampin group (R): bone defects were irrigated with rifampin. The flap was closed without any further action.

Autogenous graft group (Ag): the defects were filled with a contaminated autogenous graft. The flap was sutured using silk sutures.

Autogenous graft + rifampin group (Ag+R): the defects were filled with decontaminated autogenous graft with rifampin.

All animals survived the surgery and the postsurgical period. There was no evidence of wound dehiscence, infection, or abscess formation. All animals were killed 4 weeks after the operation.

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Received February 9, 2016.

Accepted for publication April 10, 2016.

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The authors report no conflicts of interest.

ISSN: 1049-2275

DOI: 10.1097/SCS.0000000000002762

Calvarias were removed and kept in formaldehyde prior to stereological examination and micro-computed tomography (CT) analysis.

Micro-CT Analyses

Samples were scanned using micro-CT (SkyScan 1174; Micro Photonics, Inc, Allentown, PA) with a spatial resolution of 15 μm, using 50 kV and 800 μA at a 0.7-degree rotation step for a total of 180 degrees. Three-dimensional images were taken using NRecon software, and the data were evaluated using CTAn software (Skyscan, Kontich, Belgium). Only the volume of mineralized new bone without graft materials was calculated (Fig. 1).

Stereological Analyses

Prior to stereological analyses, samples were decalcified in formic acid (5%) for 21 days. Samples were then fixed in 10% formaldehyde, dehydrated in a graded alcohol series, and clarified in xylol for light microscopic examination. After dehydration, samples were embedded in fresh paraffin and cut using a microtome (Leica RM 2135; Leica Instruments, Nussloch, Germany). Paraffin blocks were cut serially to a thickness of 7 μm, and every 20th section was selected for analysis. The first section was chosen at random, and all sections were sampled in a random manner. Sections were stained with hematoxylin-eosin and photographed using the stereology analysis system (Stereo Investigator 9.0; Microbrightfield, Williston, VT) with a light microscope (Leica M 4000 B; Leica Instruments) containing a digital color camera (Microbrightfield).

To estimate the volume of new bone area (Vn, Fig. 2), the unbiased Cavalieri method was applied using point-counting test grids. The point density of the point-counting test grids was designed to obtain an appropriate coefficient of error. Coefficients of error and coefficients of variation were estimated according to a formula developed by Gundersen and Jensen. The volume of each area was estimated using the following formula:

$$\text{Volume} = t \times a/p \times \sum p,$$

where t is section thickness, a/p is the area of each point on the point counting grid, and $\sum p$ is the total number of points touching the area of interest.

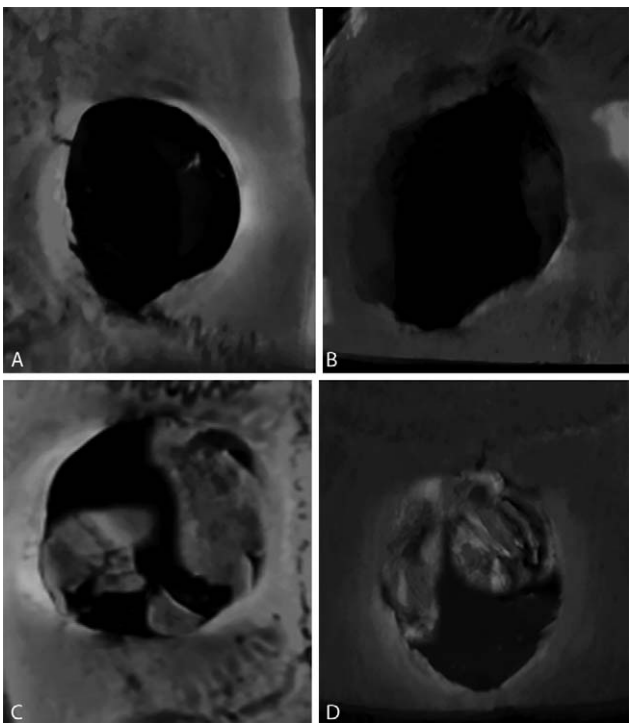

FIGURE 1. Three-dimensional images of defect region. (A) Control group on 4th week. (B) Rifampin group on 4th week. (C) Autogenous graft group on 4th week. (D) Autogenous graft + Rifampin group on 4th week.

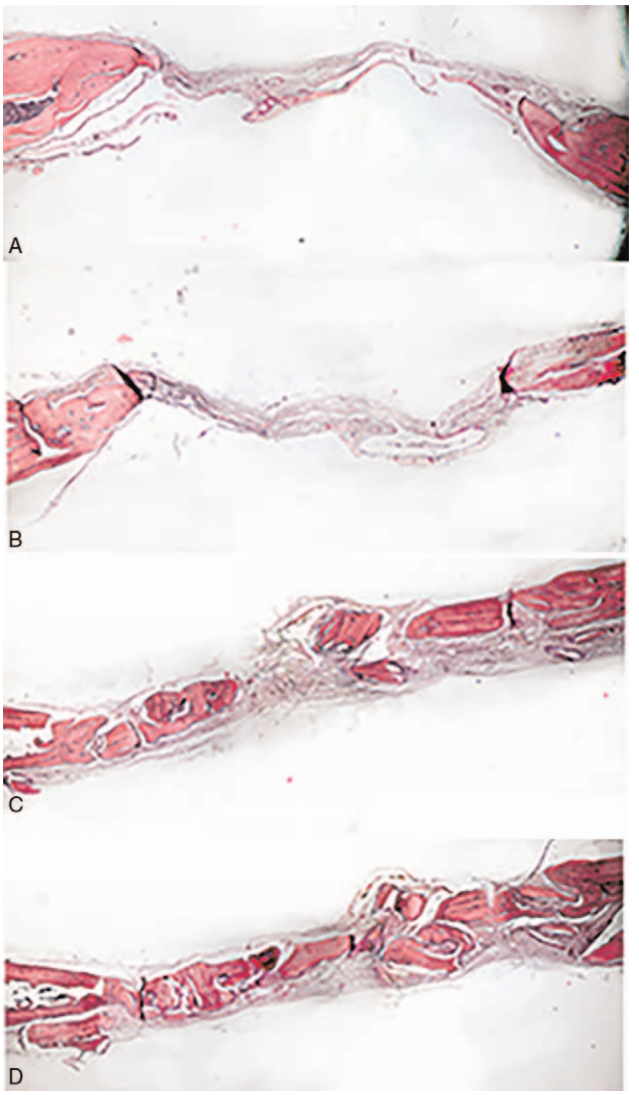

FIGURE 2. Histologic panoramic view of defect region. (A) Control group on 4th week. (B) Rifampin group on 4th week. (C) Autogenous graft group on 4th week. (D) Autogenous graft + Rifampin group on 4th week.

Statistical Analysis

The Shapiro–Wilk test was used to determine whether the data were normally distributed. Comparisons of the volume of new bone and connective tissue were analyzed using the Kruskal–Wallis nonparametric test, followed by post-hoc group comparisons with the Bonferroni-adjusted Mann-Whitney test when a non-normal distribution was identified. For the Bonferroni correction, $\alpha = 0.05/6 = 0.008$ was considered statistically significant. All tests were performed using a statistical software version 19.0 (SPSS, Inc, Chicago, IL).

RESULTS

Animals

All animals tolerated surgery well and survived the postsurgical period. No wound dehiscence, infection, or abscess formation was observed at any surgical site.

Micro-CT and Histomorphometric Findings

New bone volume (mm^3) is shown in Figure 3. The mean new bone volume was the greatest in the Ag/rifampin group (1.73 ± 0.17), followed by the Ag group (1.50 ± 0.05) (statistically significant difference at $P < 0.05$). The new bone volume was the lowest in the empty defect group (1.05 ± 0.09); however, no difference was observed between this and the empty/rifampin group (1.08 ± 0.07) ($P > 0.05$).

Micro-CT (%) findings are shown in Figure 4. The greatest new bone volume was observed in the Ag/rifampin group (10.22 ± 1.19); however, this value was not significantly different from that for the Ag group (9.29 ± 1.01) ($P > 0.05$). The lowest new bone volume was found in the empty defect group (1.15 ± 0.12); however, this was not significantly different from the value for the empty/rifampin group (1.20 ± 0.17) ($P > 0.05$). There was a significant difference between the Ag groups and non-Ag groups ($p < 0.05$).

The volume of connective tissue (mm^3) is shown in Figure 5. The mean connective tissue volume was THE lowest in the empty defect group (0.94 ± 0.09) and THE highest in the Ag/rifampin group (1.66 ± 0.23) ($P < 0.05$). There was no statistically significant difference between the empty defect group and the empty/rifampin group (1.03 ± 0.10), or between the Ag/infliximab group and the Ag group (1.73 ± 0.03) in terms of the volume of connective tissue ($P > 0.05$) (Fig. 5).

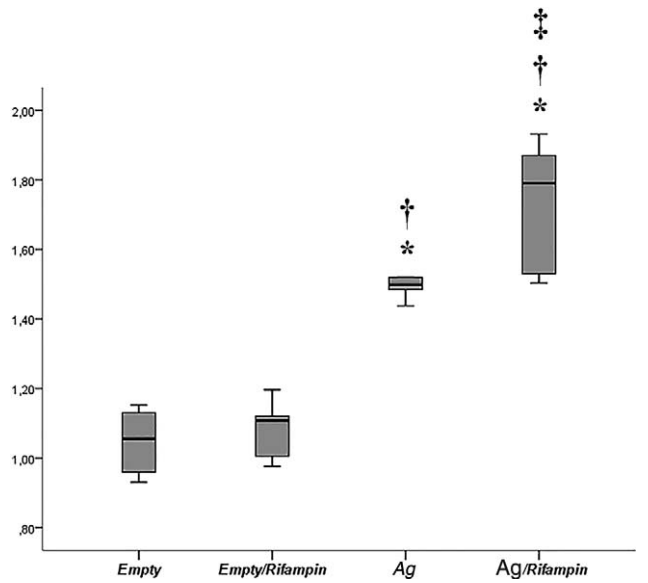

FIGURE 3. New bone volume (mm^3) in study groups. Data are presented as box and whisker plots. The median value is indicated by the line within the box plot. The box extends from the 25th to the 75th percentiles. Whiskers extend to show the highest and lowest values. *Statistically significant difference from Empty (Bonferroni-adjusted Mann–Whitney *U* test). †Statistically significant difference from Empty/Rifampin (Bonferroni-adjusted Mann–Whitney *U* test). ‡Statistically significant difference from Ag (Bonferroni-adjusted Mann–Whitney *U* test).

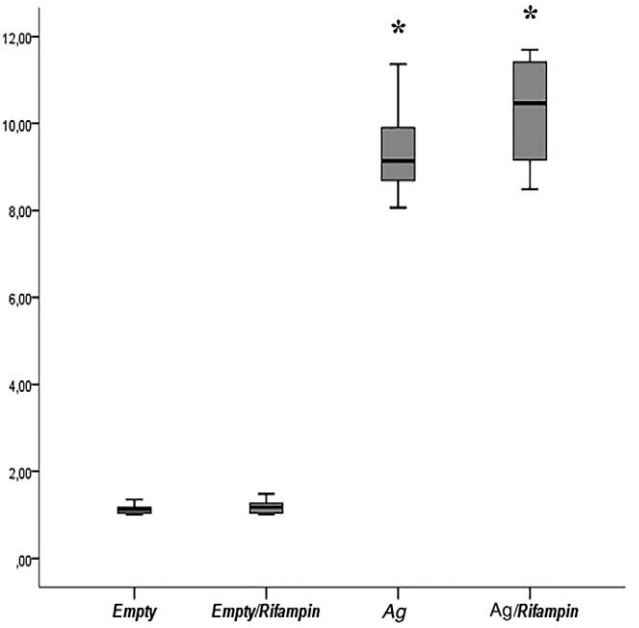

FIGURE 4. Percentage of new bone in micro-computed tomography (%) in study groups. Data are presented as box and whisker plots. The median value is indicated by the line within the box plot. The box extends from the 25th to the 75th percentiles. Whiskers extend to show the highest and the lowest values. *Statistically significant difference from Empty and Empty/Rifampin (Bonferroni-adjusted Mann–Whitney *U* test).

DISCUSSION

In the present study, we hypothesized that decontamination of an autologous graft with rifampin would augment and increase new bone formation in rats. To test this hypothesis, we filled 5-mm calvarial critical-size defects with autologous grafts decontaminated with rifampin. New bone volume was analyzed using micro-CT and stereological methods.

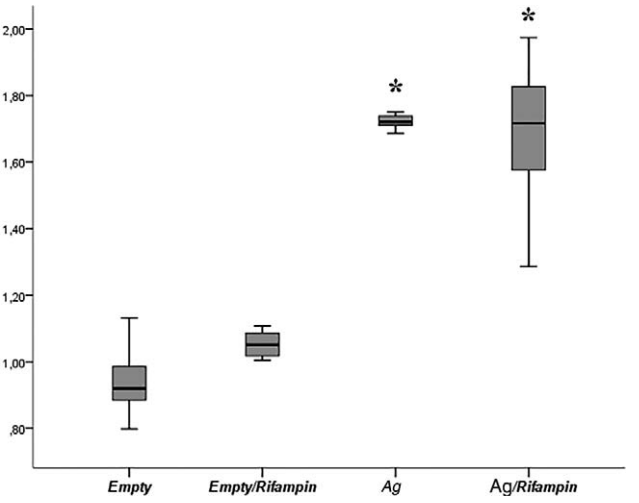

FIGURE 5. Connective tissue volume (mm^3) in study groups. Data are presented as box and whisker plots. The median value is indicated by the line within the box plot. The box extends from the 25th to the 75th percentiles. Whiskers extend to show the highest and lowest values. *Statistically significant difference from Empty and Empty/Rifampin (Bonferroni-adjusted Mann–Whitney *U* test).

In the craniomaxillofacial region, extensive defects cannot heal spontaneously and often lead to dysfunction and deformity.[14] Bone defect models are the preferred approach to investigate the efficiency of biomaterials and stimulatory factors on healing.[15] The critical-size rat calvarium defect model was used in this study because it has proven useful in several studies of osteopromotive substances. This model is an inexpensive and simple way to evaluate bone regeneration. In rats, 5-mm calvarial defects are regarded as critical-size defects.

We chose to use 2 methods to analyze our samples and used stereology and micro-CT analysis to improve the reliability of our results. Histologic examinations are limited to 2-dimensional slices, which may fail to identify small islands of bone formation. In 1984, Sterio[16] described several modifications to the approaches used to estimate the quantity of objects in three-dimensional space. Estimating microscopic parameters in a three-dimensional space and using micro-CT for three-dimensional imaging increases the reliability of morphological measurements.[17]

Stereologic and micro-CT analyses are examined in a three-dimensional space, so for these methods, error chance is decreased compared with conventional histology. The values obtained from micro-CT and stereological evaluations were similar. Micro-CT results showed that new bone formation was greater in both of the rifampin + autogenous bone groups than in the autogenous bone control group.

Bone defects generally occur in the maxillofacial region as a result of surgical removal of large cysts or tumors or surgical treatment of osteomyelitis. Various graft materials have been used to restore maxillofacial defects. Bone grafts are primarily employed to serve as a filler and scaffold to facilitate bone formation and wound healing.[1–3,8] The graft healing rate depends on graft type, the degree of vascularization, stabilization, and presence of infection. Contamination of the graft is related to infection and may impair osteogenesis.[7] Various studies have focused on preventing graft infection.[18–21]

Chlorhexidine, rifamycin, and tetracycline are used to decontaminate collected bone graft particles,[13] although chlorhexidine may have a negative impact on osteogenesis.[22] But an optimal decontamination solution would destroy microorganisms without damaging bone and osteoprogenitor cells.[23] Antibiotic solutions have been used successfully to decontaminate graft particles without negative effects on osteogenesis.

Both local and systemic antibiotics have been used to treat infection in bone grafting procedures,[21] but in the literature, local antibiotics are preferred over systemic antibiotics.[18–23] Local antibiotics more effectively reduce the risk of initial surgical infection and have fewer systemic adverse and toxic effects.[7] Some authors have reported harmful effects of local antibiotics,[19,24] although Yaman et al[19] demonstrated that rifampin, neomycin, and cefazolin sodium were safe for bone histologically.

The effect of local antibiotic applications on bone was researched by authors. Petri[11] suggested that antibiotic-supplemented bone allograft material may also simplify healing in oral and maxillofacial surgery. Gomes et al[18] reported that doxycycline and minocycline induced proliferation of osteoblasts in vitro. Witso et al[20] showed that autogenous particulate bone treated with rifampin can carry the drug to the grafting site.

Rifampin has been used as an irrigation solution since 1963.[8] It is now used in various oral and maxillofacial surgeries.[7,8,10] Yaman et al[19] reported that rifampin is the most suitable agent for decontamination introduced into bone grafts during oral surgery.[19] Sivolella et al[10] reported that autogenous bone with rifampin is a valid grafting material for the reconstruction of minor bone defects. Kaya et al[8] used allogeneic, alloplastic, and heterogeneous bone graft substitutes treated with rifampin in rat tibia bone defects, and reported that topical rifampin application may accelerate the bone repair process and that the combination of rifampin and allogeneic bone grafts can also reduce new bone formation in osseous defects.

We evaluated the effect of autogenous bone decontaminated with rifampin in critical-size defects. The mean new bone volume was the greatest in the Ag + R group in histomorphometric and micro-CT analysis according to our results. However, there was no significant difference between the nontreated and rifampin groups.

Our results supported those of previous publications. The results of this study may have been influenced by 2 factors. First, there may have been contamination of autogenous bone in the Ag group. Contamination and infection could have decreased osteogenesis in our study, resulting in significantly lower new bone volume in the Ag group compared with the Ag + R group. Another possible explanation may be the effect of rifampin on BMP-2, a bone morphogenic protein (BMP) that is involved in bone repair. Local rifampin application has been shown in the literature to increase BMP-2 and osteoblastic cells.[7] Therefore, rifampin may have increased osteogenesis in the Ag + R group in our study. However, the exact mechanism of this positive effect of rifamycin on BMP-2 remains unclear.

CONCLUSIONS

According to our study results, local rifampin has no detrimental effects on the healing of experimental defects in rat calvaria, and decontamination of autogenous bone with rifampin led to increased formation of new bone compared with that observed in the decontaminated autogenous bone group. Additional investigations are needed to further evaluate the effects of local antibiotic application on bone healing.

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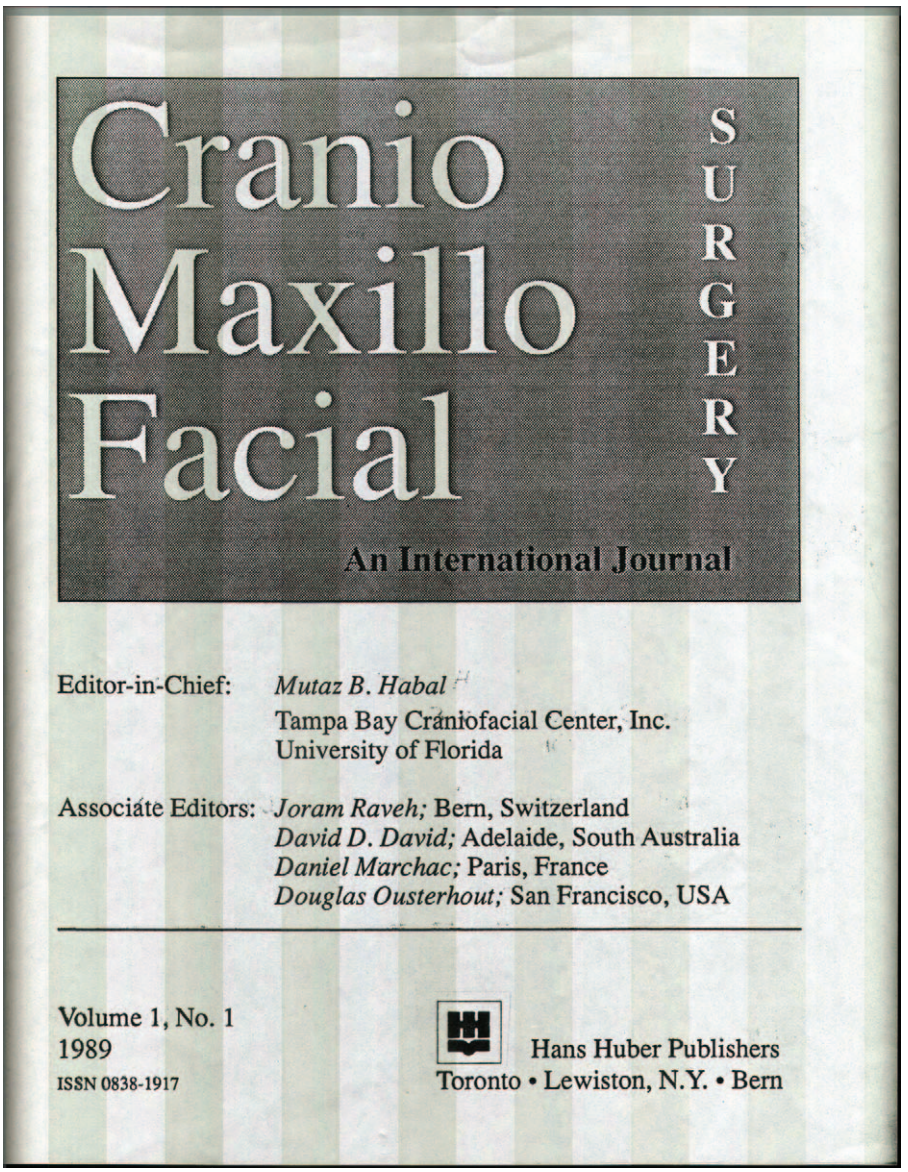

The original cover of the first issue never saw the light of day.