Perioperative glucocorticosteroid treatment delays early healing of a mandible wound by inhibiting osteogenic differentiation

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A B S T R A C T

Aim: The purpose of this study is to investigate the effects of dexamethasone on repair of a critical size defect of the mandible in male Sprague-Dawley rats.

Materials and methods: Fifty rats were divided into 2 groups: saline control and dexamethasone-treated groups. A 1 mm × 3 mm full-thickness bone defect was created at the inferior border of the mandible. Saline or dexamethasone was administered once a day for 5 days after postoperative palinesthesia. On days 1, 3, 6, 10 and 17, after cessation of drug administration, 5 samples from each group were analysed. The bone defect healing process was examined and analysed by stereology, radiology, histology and histochemical staining for total collagen, tartrate-resistant acid phosphatase staining for osteoclasts and immunohistochemical staining for the COX-2, RUNX2 and osteocalcin antigens.

Results: The dexamethasone-treated rats exhibited significantly lower radiopacity properties compared to the control rats. Histological staining revealed that the osteogenic differentiation and maturation of a callus in the defect region was significantly delayed from day 1 to day 10 in the dexamethasone group after cessation of drug administration compared to the control group. Consistent with the histological data, the level of total collagen protein was significantly lower in the dexamethasone group than in the control group. However, there was no significant difference between the 2 groups at day 17. Immunohistochemical analysis of COX-2, RUNX2 and osteocalcin expression showed that, at day 1, COX-2 and RUNX2 expression in the dexamethasone group was significantly lower than in the control group. There was no significant difference in osteocalcin expression between the two groups at each time point. There was no significant difference in the number of osteoclasts between the two groups.

Conclusion: In a model of bone healing of a mandible defect, dexamethasone-treated rats exhibited impaired osteogenic differentiation and maturation due to the inhibition of COX-2, osteogenic gene, RUNX2 and collagen protein expression, which resulted in delayed bone repair. Although perioperative short-term therapy did not exhibit long-term effects on wound healing of the maxillofacial bone, the application of glucocorticoids should be cautiously considered in the clinic.

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Introduction

The perioperative application of glucocorticosteroids (GCs) is commonly used in oral and maxillofacial surgery to minimize oedema and decrease pain. In surgical extraction, several studies have reported that GCs significantly reduce postoperative pain, swelling and trismus after third molar surgery because of their ability to suppress the inflammation response of wounded tissue.1–5 Patients undergoing orthognathic surgery5,6 and treatment of maxillofacial bone fractures7,8 also seem to benefit from perioperative GC treatment. Although the administration of GCs is thought to decrease postoperative oedema and pain, the problem of GCs delaying the healing process of bone wounds always causes concern for surgeons who wish to promote healing.

The process of bone wound healing is comprised of several overlapping phases. The process is initiated by an immediate inflammatory response that leads to the recruitment of mesenchymal stem cells and subsequent differentiation into chondrocytes that produce cartilage and osteoblasts, which form bone.9 As the result of an inflammatory response, a myriad of factors, including growth factors, cytokines, and prostaglandins (PGs), are released. These factors are likely to play an essential role in the initiation of the healing response that leads to the formation of new bone.10 The administration of GCs is thought to inhibit mast
cell production and secretion of PGs, interleukins-1 and -6 (IL-1 and IL-6), tumour necrosis factor-alpha (TNF-α), transforming growth factor-beta (TGF-β) and platelet-derived growth factor (PDGF). It is important to understand the adverse effects of steroidal anti-inflammatory drugs on bone wound healing. Since the 1950s, a number of studies have been conducted on the effect of GCs on bone healing. Not all animal studies have shown consistent results, but the inhibitory effect of GCs on fracture healing has been previously reported. The results of fracture healing in animal models treated with non-steroidal anti-inflammatory drugs (NSAIDs) or in mice lacking the cyclooxygenase-2 (COX-2) gene show that the inhibition or deficiency of COX-2 impairs the bone healing process. This mechanism may be particularly important during injury or inflammation when large amounts of prostaglandin E2 (PGE2) are produced by COX-2. In addition, the elevated COX-2 expression increases the osteoblastic potential of mesenchymal stem cells. COX-2 might exert its effect by regulating the transcription factor RUNX2, which is a crucial regulatory factor of the osteogenic differentiation of mesenchymal stem cells. It is well known that GCs can inhibit COX-2 expression and PG production; however, it remains to be investigated whether GCs delay bone wound healing by inhibiting COX-2 and RUNX2 gene expression. In published studies, the systemic administration of steroidal anti-inflammatory drugs (SAIDs) and NSAIDs has been demonstrated to delay healing in a long bone fracture model. However, it should not be assumed that these results will apply to cranial and maxillofacial bone wounds because cranial and maxillofacial bone differs significantly from the long bones in terms of development. Unlike the trunk and appendicular skeleton, which develops from the mesenchyme, cranial and maxillofacial skeleton is derived from the cranial neural crest ectomesenchyme. The embryonic origin of the bone influences the regeneration process of adult bone. The mandible, similar to the flat bones of the skull, develops primarily through the process of intramembranous ossification. In contrast, long bones develop by endochondral ossification. Therefore, investigating the effects of GCs on mandible wound healing is important for understanding cranio-maxillofacial bone wound healing.

While a meta-analysis of the clinical literature shows that short duration and treatment-dose of GCs have no long-term effects on the wound healing of the maxillofacial bone, the effect of GCs on the histological healing process remains unknown. This study aimed to investigate whether the perioperative administration of clinically relevant doses of GCs could delay the healing process of mandible in a rat mandible osteotomy model. In addition, the osteogenic molecular expression changes in bone wound regeneration were detected.

Materials and methods

Experimental animals

A total of 50 adult male Sprague-Dawley rats, weighing 357.470 ± 8.9350 g at 10 weeks old, were used in this study. The rats were housed individually in a temperature-controlled room with a 12-h light–dark cycle and had free access to food and water. The study conformed to the guidelines for the care and use of laboratory animals at Nanjing medical university. The animals were divided into 2 groups: a saline control group (n = 25) and a dexamethasone-treated group (n = 25). Subcutaneous injection of either sterile normal saline or dexamethasone sodium phosphate (dissolved in sterile saline, 0.4 mg/kg/24 h) was administered daily after postoperative palinesthesia. The subcutaneous injections continued for 5 days. On days 1, 3, 6, 10 and 17 after the cessation of drug administration, 5 rats from each group were sacrificed and analysed.

Mandible defect repair model

After rats were anaesthetised by intraperitoneal injection of 10% chloral hydrate (3.5 mg/kg) and placed on a stereotaxic frame, a 1.5-cm incision was made along the inferior border of the mandible. The soft tissues were dissected to expose the body of the mandible. Using a slow-speed handpiece (5000 rpm), a 1 mm × 3 mm full-thickness bone defect was created at the inferior border of the mandible. Subcutaneous tissue and skin were sutured layer to layer with 4-0 silk.

Sample preparation

Rats were sacrificed at the assigned time points by cervical dislocation under anaesthesia. Mandibles were collected and fixed in 4% paraformaldehyde/PBS for 48 h at 4 °C. After X-ray imaging, samples were decalcified in 10% EDTA, pH 7.0 for 3 weeks at 4 °C. The solution was changed twice weekly. Mandibles were then processed for paraffin embedding. Serial 5-μm sections were obtained and processed for staining with haematoxylin and eosin (HE), for histochemical analysis of total collagen, or for immunohistochemical analysis of COX-2, RUNX2 and osteocalcin expression as described below.

Radiographic densitometry analysis

The defect-bearing mandible was radiographed, and the average radiopacity density at the osteotomy site was quantified using an image-analysis software package (JID-801, JIEDA Scientif-ic, Nanjing, China).

Histochemical staining for total collagen

Total collagen was detected in paraffin-embedded sections using a modified method of Lopez-De Leon and Rojkind. Dewaxed sections were exposed to 1% Sirius Red in saturated picric acid for 1 h. After washing with distilled water, the sections were counterstained with haematoxylin. A percentage of the collagen positive area in the callus area of the defect site was quantitated using EMPIX imaging software (EMPIX Imaging Inc., U.S.A.).

Immunohistochemistry staining

For COX-2, RUNX2 and osteocalcin antibody staining, sections were de-paraffinised, and hydrated through graded alcohol into water. Antigen retrieval was conducted by heating for 10 min in a pressure cooker with citrate buffer. Endogenous peroxidase was blocked by incubation with 0.3% H2O2 in methanol for 30 min. Sections were incubated overnight at 4 °C with polyclonal rabbit anti-rat COX-2 (Bios, Beijing, 1:200 dilution), anti-RUNX2 and anti-osteocalcin (1:400 dilution) antibodies. Sections were washed in PBS and incubated for 1 h at room temperature with biotinylated anti-rabbit IgG. Staining was developed using 3,3-diaminobenzidine (DAB). The samples were counterstained with haematoxylin and mounted. The intensity of staining was semi-quantitated using Image-Pro Plus 5.0 imaging software.

Tartrate-resistant acid phosphatase (TRAP) staining

To detect bone-resorbing osteoclasts, TRAP staining was performed. Tissue sections were de-paraffinised and hydrated using xylenes and a graded alcohol series. The sections were preincubated with 50 mM sodium acetate and 40 mM potassium sodium tartrate buffer for 20 min and then incubated with TRAP substrate solution for 15 min at room temperature. Sections were
subsequently rinsed with running water for 5 min, counterstained with methyl green and mounted in Kaiser’s glycerol jelly.

**Statistical analysis**

All data in this study are expressed as the mean ± S.D. The paired t-test was used to evaluate the data. P-values less than 0.05 were considered significant.

**Results**

**General observation of mice and mandible defect following surgery**

All animals tolerated the surgical procedures well and were healthy during the entire experimental period. No wound infection or secondary fracture was found.

At day 1 (time point of drug administration cessation), soft calluses, which were easy to stick by dental explorer, were present in the defects of the PBS and dexamethasone groups. The calluses were easier to detach in the dexamethasone group than in the PBS group (Fig. S1A1). At day 3 and day 6, hard calluses were present in the PBS group. The number of hard calluses increased at day 6, but no hard calluses were found in the dexamethasone group (Fig. S1A2 and A3). At day 10, hard calluses began to appear in the dexamethasone group, and the defect region was completely bridged in the PBS group (Fig. S1A4). At day 17, remodelling of newly formed bone at the cortical bone gap was completed. There was no obvious difference between the two groups (Fig. S1A5).

**Radiographic evaluation**

The radiopacity density in the mandible defect sites was significantly different between the PBS group and the dexamethasone group. The values of radiopacity density at different time points were always lower in the dexamethasone group than in the PBS group (Figs. 1 and S1B).

**Histological observation of mandible defect healing**

At day 1 after the cessation of drug administration, compared to the PBS group, which had a large number of pre-osteoblast/osteoblast cells present in the calluses (Fig. 2A1), the calluses of the dexamethasone group exhibited a predominance of fibro-like mesenchymal cells and little pre-osteoblast/osteoblast cells (Fig. 2B1). At day 3, a small amount of trabecular bone was observed in the PBS group (Fig. 2A2). The trabecular bone was gradually thickened from day 3 to day 17 (Fig. 2A2–A5). There was a predominance of pre-osteoblast/osteoblast cells in the calluses of the dexamethasone group at day 3, which was similar to the histological results of the PBS group at day 1 (Fig. 2B2). At day 6 and day 10, the osteoid appeared in the dexamethasone group. Interestingly, between days 10 and 17, the trabecular bone formed rapidly in the dexamethasone group. At day 17, there was no obvious difference between the newly formed bone of the two groups (Fig. 2A5 and B5). No cartilaginous tissue was found during the healing process in either the PBS or the dexamethasone groups.

![Fig. 1. Radiographic findings. Histogram shows the radiopacity density in the mandible defect sites of the PBS and dexamethasone groups (*P < 0.05).](image)

![Fig. 2. Haematoxylin and eosin staining of representative sections at multiple time points. Photomicrographs (×20) show the mandible defect healing delayed from day 1 to day 10 in the dexamethasone group compared to the PBS group. No difference was observed at day 17 between the two groups. The arrow in B3 and B4 show osteoid. The abbreviations used are CB, old cortical bone, and NT, new growth trabecula.](image)
**Total collagen protein production during bone healing**

At day 1, negative collagen protein staining was observed in both the PBS and dexamethasone groups. At days 3, 6, 10 and 17, the area of positive collagen protein staining steadily increased in both groups. Collagen protein staining was significantly lower in the dexamethasone group than in the PBS group at days 3, 6 and 10 ($P < 0.01$). There was no significant difference between the two groups at day 17 (Fig. 3).

**Immunohistochemical analysis of COX-2, RUNX2 and osteocalcin expression during bone healing**

In the PBS group, the immunoreactive signal for COX-2 in callus tissue from day 1 samples was stronger than that from samples on other days. The expression intensity of COX-2 decreased slowly. In the dexamethasone group, the expression intensity of COX-2 from day 1 samples exhibited the weakest signal among samples from different time points. At day 3, COX-2 expression increased rapidly, and then its expression decreased slowly. At day 1, compared to the PBS group, COX-2 expression in the dexamethasone group was significantly inhibited. At other time points, there was no obvious difference between the two groups (Figs. 4A and S2). Similarly, at day 1, expression of the key transcription factor of early-stage osteoblast differentiation, RUNX2, in the dexamethasone group was significantly lower than in the PBS group. At day 3, RUNX2 expression increased rapidly, and then its expression level remained stable between days 3 and 10, and subsequently decreased significantly at day 17 in the dexamethasone group. After day 3, the RUNX2 expression level remained low in the PBS group (Figs. 4B and S3). Osteocalcin expression in calluses of the dexamethasone group at days 1 and 3 was lower than that in the PBS group, but no significant difference was evident between the two groups at each time point (Figs. 4C and S3).

**Dexamethasone could not change the osteoclast activity during mandible defect healing**

To investigate whether dexamethasone affected the remodelling of bone during bone wound healing, TRAP staining for osteoclasts was examined at multiple time points. The results showed that TRAP-positive osteoclasts were present during the early phase of bone healing. At day 1, poly-nuclear osteoclasts adhere to the cortical bone surface, resorbing the lamellar bone. The density of osteoclasts seemed to decrease in all regions during the bone healing process; however, the decrease was not statistically significant. At day 17, osteoclasts were present in new growth bone callus, remoulding the woven bone (Fig. 5). There was no significant difference in the number of osteoclasts between the two groups at each time point.

**Fig. 3.** Expression of total collagen protein. Representative photographs of total collagen staining in the PBS group (A) and the dexamethasone group (B) illustrate that collagen protein staining is significantly lower in the dexamethasone group than in the PBS group at days 3, 6, and 10 and has no difference at day 17. (C) The histogram shows the positive staining area of total collagen for the two groups at different time points. **$P < 0.01$; *P > 0.05$. Photomicrographs were taken at ×20 magnification.

**Fig. 4.** The expression intensity of COX-2 (A), RUNX2 (B), and osteocalcin (C). The intensity of staining was semi-quantitated using imaging software and shown in graph. *$P < 0.05$.**
Despite that glucocorticosteroids seem to be widely used in oral and craniomaxillofacial surgery, little has been published about their effect on bone repair after craniofacial bone wounding. Previous investigations into the effects of glucocorticosteroids on bone healing have focused on long bone defect models. In this study, we found that short-term systemic administration of dexamethasone impaired the early bone healing process in a critical sized mandible defect model. Our results are in agreement with those of two other groups that demonstrated impaired ulna fracture healing in rabbits and tibia fracture healing in rats treated with glucocorticosteroids. However, there are no significant influences on the healing status at late stages of healing. These findings are also in agreement with clinical observations, which show that short-term and treatment-dose glucocorticosteroids have no long-term effects on maxillofacial bone wound healing.

Histological data showed the same structure of the healing callus at day 17 between the two groups, but radiographic data indicated a lower density at the osteotomy site in the mandible of dexamethasone-treated rats when compared with controls. During bone wound healing, radiological healing often fall behind histological healing because the mineralization of new bone always build on the bone matrix. Although there was no obvious difference between the decalcified structures of two groups, the mineralization degree may be different at day 17. In fact, dexamethasone inhibits bone wound healing through its effects on calcium metabolism, in addition to its general inhibitory action on wound healing.

Short-term therapy with glucocorticosteroids may be routinely administered to reduce postoperative pain and swelling after oral and craniomaxillofacial surgery. It has been shown that glucocorticosteroids inhibit collagen production and osteoprogenitor and osteoblast cell activation. In the current study, relatively large differences in the level of total collagen protein between the dexamethasone group and the control group were found at the early and middle healing stages (Fig. 3). In addition, osteoprogenitor and osteoblast differentiation of mesenchymal cells were delayed in the dexamethasone group (Fig. 2). These results suggest that dexamethasone delays the early mandible healing process by reducing the synthesis of collagen and inhibiting the osteogenesis maturation of the callus.

Anti-inflammatory glucocorticoids are potent inhibitors of cyclooxygenase, a key regulator of PG synthesis. There are two COX isoenzymes encoded by separate genes, COX-1 and COX-2. COX-1 is usually constitutively expressed, while COX-2 is rapidly and transiently induced. Both COX-1 and COX-2 are expressed in osteoblasts, and COX-2 is the main enzyme regulating the production of PG. Treatment with steroidal anti-inflammatory drugs or NSAIDs shows that inhibition or deficiency of COX-2 impairs the long bone healing process. Mice lacking the COX-2 gene also show that COX-2 plays an important role in tibia and craniofacial fracture repair. In the current study, we examined the expression intensity of COX-2 using immunohistochemical staining. At day 1 after cessation of dexamethasone administration COX-2 expression in the dexamethasone group was significantly inhibited compared to the PBS group. Over time, the inhibitory effect of dexamethasone on COX-2 expression diminished. Therefore, inhibiting COX-2 expression may be one of the molecular mechanisms by which dexamethasone delays the early mandible healing process.

COX-2 participates in the initial step of osteogenesis and the latter step of osteoblast maturation. In addition, it regulates gene expression related to bone formation, such as RUNX2 and osteocalcin. COX-2 also participates in the osteogenic differentiation of human bone marrow-derived mesenchymal stem cells (MSCs). Additionally, we found that dexamethasone suppressed the osteogenic differentiation of callus mesenchymal cells. Similar to the COX-2 expression profile, the key transcription factor of osteoblast differentiation, RUNX2, was expressed at a significantly lower level in the dexamethasone group than in the PBS group at the early healing stage. In our study, differentiation could not be shown by immunohistochemical staining for osteocalcin expression, but HE and collagen staining results showed that dexamethasone delayed the latter step of osteoblast maturation. In future studies, it is necessary to extract RNA from calluses and to compare the mRNA levels of osteogenic genes using quantitative real-time PCR.

In addition to osteoblasts and osteocytes, osteoclasts are also involved in the process of bone wound healing. Two peaks of increased osteoclast population occur during bone wound healing. The first peak, during the inflammatory phase, is necessary to remove all the dead and necrotic bone from the wound gap. The second peak, during the remodelling phase, corresponds to the resorption of the redundant mineralized callus tissue. The osteoclasts are a potential modulator of the effect of glucocorticoids on osteoblasts. Bone remodelling is characterized by tethering the activities of the two cells. Additionally, glucocorticoid therapy is the most common cause of secondary iatrogenic osteoporosis. Thus, functional osteoclasts were examined in our study. The results suggest that short-term therapy with dexamethasone seems to have no effect on activating osteoclasts in mandible wound healing. Collectively, in the current study, the delay of the mandible wound healing process by dexamethasone depends on its inhibitory effects on osteogenic differentiation and maturation of MSCs.

In summary, we have demonstrated that glucocorticoids could delay the healing process of mandible in a rat mandible osteotomy model. Perioperative short-term therapy did not result in long-term effects on maxillofacial bone wound healing. In glucocorticoid-treated rats, the bone healing of a mandible defect exhibited impaired osteogenic differentiation by inhibiting the expression of COX-2 and the osteogenic gene, RUNX2, resulting in delayed bone repair. These results should be carefully considered before the use of glucocorticoids in clinical oral and craniomaxillofacial surgery.
Conflict of interest

We have no potential conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.injury.2012.04.014.

References


35. Reihan Q, Lane NE. Effect of glucocorticoids on bone density. Medical and Pediatric Oncology 2003;41:212–6.