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ORIGINAL ARTICLE

Effects of caffeic acid phenethyl ester on wound healing in calvarial defects

HAKKI OGUZ KAZANCIOGLU[1], MEHMET CIHAN BEREKET[2], SEREF EZIRGANLI[1], MEHMET SERIF AYDIN[3] & SERTAC AKSAKALLI[4]

[1]*Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, Bezmialem Vakif University, Istanbul, Turkey,* [2]*Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, 19 Mayis University, Samsun, Turkey,* [3]*Department of Histology, Faculty of Medicine, and* [4]*Department of Orthodontics, Faculty of Dentistry, Bezmialem Vakif University, Istanbul, Turkey*

Abstract

Objective. The aim of this study is to analyze histologically the effect of CAPE on bone healing of Critical Size Defect (CSD) in rat calvaria. ***Study design.*** Thirty-two 3-month-old male rats were used. The animals were randomly divided into four groups. Group A received isotonic saline solution, Group B received CAPE (50 mmol/kg) locally, Group C received CAPE (100 mmol/kg) locally and Group D received CAPE (10 mmol/kg/day i.p. for 28 days) systematically. A 5-mm diameter calvarial defect was created in the right side of the parietal bone without damaging the underlying dura mater. Twenty-eight days after the surgery, all the animals were sacrificed. The original defect area was removed from the animal's calvarium bone en bloc. Beginning at the center of the surgical defect, serial sections of 6 μm thick were cut longitudinally. The sections were stained with hematoxylin and eosin for analysis under a light microscope. The sections were analyzed for the presence of inflammatory infiltrate, connective tissue formation and new bone formation. Computer-assisted histomorphometic measurements were carried out with an automated image analysis system. ***Results.*** The total new bone areas were significantly greater in group D than in all groups and group C was statistically insignificant from the other groups ($p < 0.05$). Group B had a greater, but not statistically significant ($p > 0.05$), amount of total regenerated bone area than the control group. ***Conclusion.*** The results indicate that 100 mmol/kg topical and 10 mmol/kg/day systemic application of CAPE increases bone healing, especially with systemic application.

Key Words: *propolis, wound healing, CAPE, critical size defect, histopathology*

Introduction

Bone loss may be caused by trauma, congenital defects, bone atrophy or tumor excision. Regeneration of bone defects is a significant component of all clinical dental practice. In addition, the treatment of some fractures and the restoration of extensive bone defects may require the use of bone regeneration procedures to enhance the possibility of a successful treatment [1]. The repairing of bone defects has attracted the interest of researchers in several fields of health.

Critical size defect (CSD) can be defined as the smallest size bone defect that will not completely heal during the natural lifetime of an animal [1–3]. Fibrous connective tissue fills the defect and aggregates on the margins of the defect, resulting in limited bone regeneration.

Experiments in bone regeneration use the rat calvarium defect model [3,4]. Currently, bone healing stimulation has been achieved with the application of chemical stimuli such as biomaterials and bone morphogenetic proteins. Also used are physical stimuli such as ultrasound, electromagnetic fields and, more recently, low-level laser therapy (LLLT), ozone therapy and propolis application [4–11].

Propolis is a natural plant product that honeybees collect from buds of several species of trees. One of its active components is caffeic acid phenethyl ester (CAPE) that, at a concentration of 10 μM, completely

Correspondence: Assistant Professor Dr Hakki Oguz Kazancioglu, Bezmialem Vakif University, Faculty of Dentistry, Department of Oral and Maxillofacial Surgery, 34093, Fatih, Istanbul, Turkey. Tel: +90 212 453 17 00. Fax: +90 212 533 23 26. E-mail: dt_oguz@yahoo.com

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blocks both production of reactive oxygen species (ROS) in human neutrophils and the xanthine/xanthine oxidase system [6]. Previous studies have showed that CAPE also exhibits antioxidant [6], anti-inflammatory [7], antiviral [8], immunomodulatory [9] and anti-cancer effects [10], as well as wound-healing acceleration [11] properties.

Although CAPE has antioxidant effects, its effect on bone healing and remodeling has not been investigated to date. The purpose of the current study was to analyze histologically the effect of CAPE on bone healing of CSD in rat calvaria.

Methods

Sample

Thirty-two 3-month-old male rats (300–330 g) (*Rattus norvegicus albinus*; Wistar) were used. The experimental protocol was approved by the Institutional Review Board and Animal Use Committee of the Bezmialem Vakif University (protocol = 2012/356). The study was conducted in accordance with the accepted guidelines for the care and use of laboratory animals in research. Throughout the study all animals were maintained under a controlled light/dark cycle (12:12 h, light on at 8:00 AM) at an ambient temperature of 21 ± 2°C and relative humidity of 55–60%. They were fed standard laboratory pellet food and drinking water was available *ad libitum*.

Synthesis and dose of CAPE

The systemic dose of CAPE was chosen on the basis of the Grunberger et al. [12] study. The synthesis technique of CAPE was previously reported by Yilmaz et al. [13]. In the current study, ready-made CAPE (Sigma, St. Louis, MO) was dissolved in absolute ethanol. Further dilutions were made in saline and administered either intraperitoneally (i.p.) or locally.

Experimental design

The animals were randomly divided into four groups as follows:

Group A: The control group (n = 8), receiving isotonic saline solution (an equal volume of CAPE, i.p.);

Group B: The local CAPE group 1 (n = 8), receiving CAPE (50 mmol/kg);

Group C: The local CAPE group 2 (n = 8), receiving CAPE (100 mmol/kg); and

Group D: The systemic CAPE group (n = 8), receiving CAPE (10 mmol/kg/day i.p.) for 28 days.

Pre-surgically, flat shaped-pure titanium barriers with a diameter of 6 mm and a thickness of 0.3 mm were molded commercially. All the barriers were cleansed in a series of alcohol solutions in an ultrasonic bath to remove possible contaminants. Afterwards, they were sterilized by autoclave.

Surgical procedures

All operations were conducted under sterile conditions. Each rat was anesthetized by an intramuscular injection of 3 mg/kg zylazine hydrochloride (Rompuns, Bayer, Leverkusen, Germany) and 35 mg/kg ketamine hydrochloride (10% Ketasol®, Richter Pharma AG, Wels, Austria). The dorsal part of the cranium was shaved and, aseptically, an incision ~20 mm long was made along the sagittal suture of the scalp, through the skin, musculature and periosteum, to expose the calvarium. A 5-mm diameter CSD was created in the right side of the parietal bone without damaging the underlying dura mater. A trephine bur was used with a low speed handpiece during profuse irrigation with sterile saline. All the defects were grafted with a circular gelatin sponge (2 mm thick, 5 mm diameter). In the control group (Group A), this gelatin sponge was mixed with saline solution. Defects in the local CAPE groups (Groups B and C) were grafted with gelatin sponges mixed with CAPE solutions containing 50 and 100 mmol/kg, respectively. Afterwards, the defect areas were closed with titanium barriers (Figure 1). The borders of the barriers were glued to the bone with N-butyl-2-cyano-acrylate (Histoacryl®, B.Braun, Melsungen, Germany). The skin was carefully sutured with resorbable 3/0 polyglactin 910 sutures (Vicryl; Ethicon, Somerville, NJ). In the systemic CAPE group (Group D), CAPE (10 mmol/kg/day) was

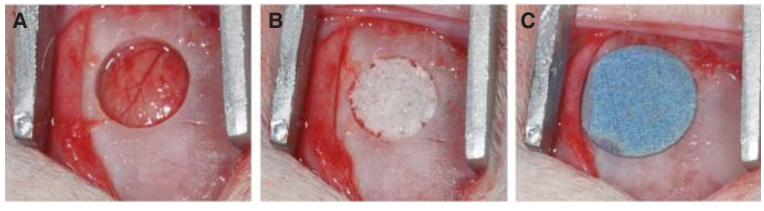

Figure 1. (A) A 5 mm diameter critical-size defect was created on the parietal bone; (B) The defect was grafted with a circular gelatin sponge; (C) The defect area was closed with titanium barriers.

administered i.p. for 28 days. All surgical procedures were performed by the same operator (H.O.K.).

To prevent post-operative infection, each animal was intramuscularly injected with 25 mg/kg Ceftriaxone for 3 days. In addition, 4 mg/kg of the intramuscular analgesic carprofen (Rimadyl, Pfizer, New York, NY) was administered every 24 h for 3 days, starting immediately after the operation. Healing progressed uneventfully in all animals and no post-operative complications were manifested.

Specimen preparation

Twenty-eight days after the surgery, all the animals were sacrificed by means of an overdose of 200 mg/kg IV pentobarbital (Pentothal, Abbott, Stockholm, Sweden). The skin was dissected and the area of the original surgical defect was removed from the animal's calvarium bone *en bloc* along with the surrounding tissues. This section was then rinsed in physiological saline, fixed in 10% formalin, dehydrated through a graded alcohol series, cleared in xylene and embedded in paraffin wax. Beginning at the center of the surgical defect, serial sections of 6 μm thick were cut longitudinally. The sections were stained with hematoxylin and eosin (H&E) for analysis under a light microscope (Nikon, Eclipse E 600, Tokyo, Japan).

Histological evaluations

Histological analysis of the samples was performed by a single examiner (M.S.A.) who was blinded to their group. The sections were evaluated for the presence of inflammatory infiltrate, connective tissue formation and new bone formation. Using an automated image analysis system, computer-assisted histomorphometic measurements were carried out. The sections were examined using a photo-microscope (Nikon Eclipse i5, Tokyo, Japan) coupled with a video camera attached to a light microscope (Nikon, DS-Fi1c, Tokyo, Japan) with an original magnification of X40. NIS Elements version 4.0 image analysis systems (Nikon Instruments Inc., Tokyo, Japan) were used for calculating the regenerated bone area (mm^2).

Statistical analysis

Data were analyzed using the commercially available software program IBM SPSS 20.0 (SPSS Inc., Chicago, IL). Mean values and standard deviations were used to calculate the amounts of regenerated bone. For detection of any statistically significant difference between groups, Kruskal-Wallis test with Dunn's post-hoc multiple comparison test was used. Statistical significance was set at 0.05. For each group, results are reported as medians and means ± standard deviation.

Results

No rat expired during the experimental period. No post-operative complications were noticed, such as inflammatory tissue responses or allergic reaction.

Histological analysis

The histologic specimens of all groups are shown in Figure 2. After the creation of the calvarial defects, no histopathological damage of dura mater was observed in any of the specimens. None of the defects had completely filled with regenerated bone and no cartilage tissue was found. Almost all of the CSDs were filled with connective tissue with collagen fibers parallel to the wound surface and a moderate number of fibroblasts. Newly regenerated bone tissue surrounded by a small number of osteoblasts was limited to areas close to the borders of the surgical defects. All groups exhibited an increase in the amount of regenerated bone and osteoblastic activity (Figure 3).

In the control group

Healing in all specimens was characterized by the loose connective tissues in the defect areas, as well as mineralized bone. This connective tissue comprised of a large number of collagen fibers parallel to the wound surface in addition to a moderate number of fibroblasts and blood vessels. The mineralized bones were minimal, originating from the defect margins.

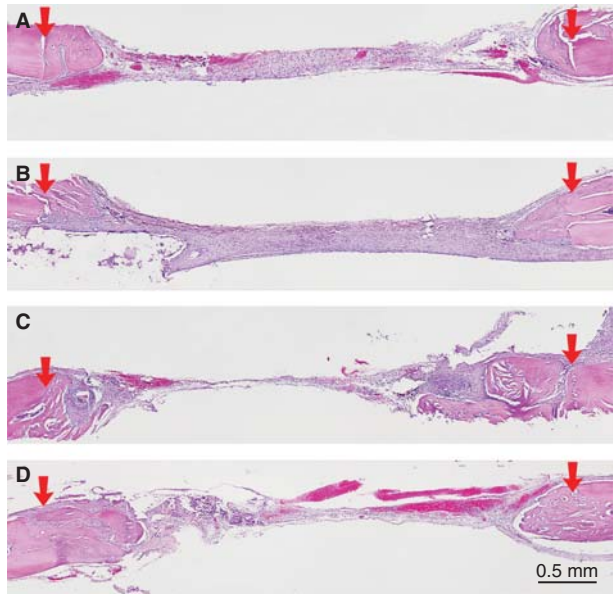

Figure 2. Panoramic views of the defect areas showing regenerated bone (red arrow) in all groups. (A) Control group. (B) Cape-50 local group. (C) Cape-100 local group. (D) Cape-systemic group (hematoxylin-eosin staining, original magnification ×40) with 0.5 mm bar.

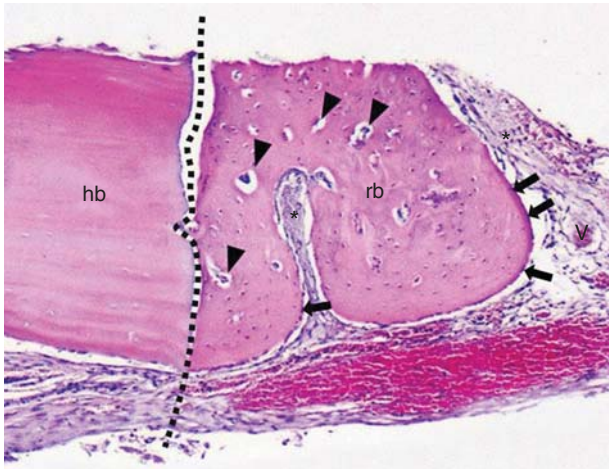

Figure 3. Regenerated new bone area and host bone area boundaries are observed (black line). There are many osteoblasts (arrow) and osteocytes (arrowheads) on new bone tissue.
*indicates fibrocellular connective tissue (star); hb, host bone; rb, regenerated bone; v, vessel (hematoxylin-eosin staining, original magnification ×100).

In the experimental groups

Histologic results similar to those of the control group were found in all the experimental groups. Osteoblasts were observed in the regenerated bone formation. In the central part of the defect was fibrous connective tissue with collagen fibers parallel to the wound surface, plus a moderate number of fibroblasts and blood vessels. Most specimens presented new bone formation throughout the length of the surgical defect. When compared to the control group, the regenerated bone areas seemed to be more extensive in each of the experimental groups, but also restricted in areas close to the original borders of the defect (Figure 4).

Histomorphometric analysis

The total new bone areas were significantly greater in group D than in all groups and group C was statistically insignificant from the other groups ($p < 0.05$) (Figure 3). Group B had a greater, but not statistically significant ($p > 0.05$), amount of total regenerated bone area than the control group (Table I).

Discussion

The present study aimed to investigate the effects of CAPE on bone formation during the process of bone healing and to evaluate the efficacy of CAPE on bone formation. Rats were chosen because surgical procedures on the calvarial bone of a rat are relatively simple to perform since the lateral borders of the rat calvaria have no major nerves or blood vessels that could cause extensive bleeding. In addition, the calvarial defect model has a number of similarities to the human maxillofacial region. Anatomically, the

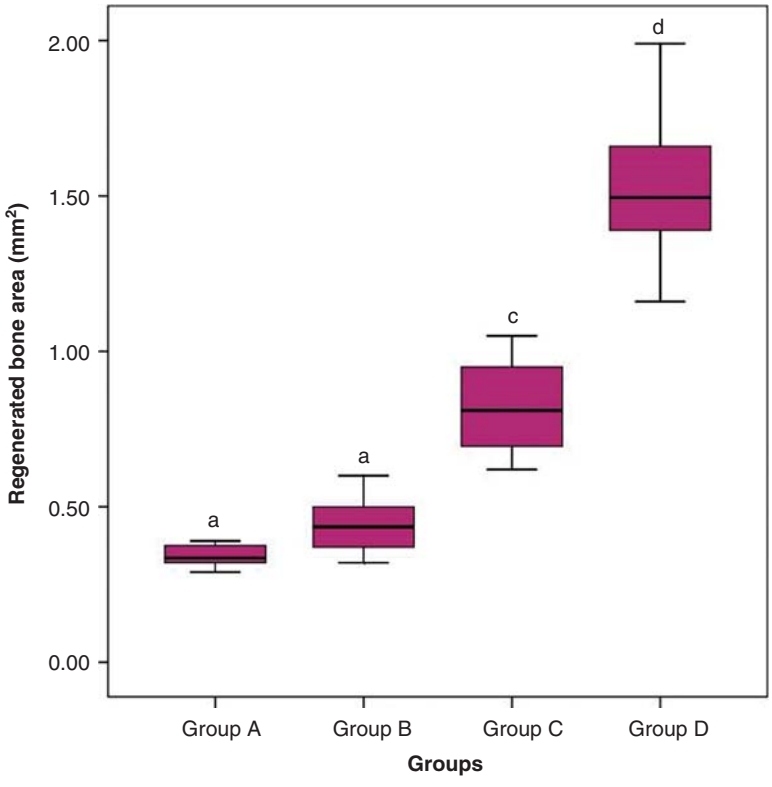

Figure 4. Graphs showing the regenerated bone analysis for each group; Dunn test $p < 0.05$: area (mm^2, Kruskal Wallis test- $\chi^2 = 27.5$, $p < 0.001$, group D vs all groups, group C vs all groups).

Table I. Results of the histomorphometric measurements.

Measurements	Control group Group A	Experimental groups			Results (Kruskal-Wallis)
		Group B (Cape-50 local)	Group C (Cape-100 local)	Group D (Cape-systemic)	
AREA (mm^2) X ± SD [Median]	$0.34 \pm 0.034^{\star}$ [0.33]	$0.44 \pm 0.092^{\star}$ [0.43]	$0.82 \pm 0.158^{\ddagger}$ [0.81]	$1.53 \pm 0.252^{\S}$ [1.49]	KW-$\chi^2 = 27.5$ $F = 94.32$ $p < 0.001$

X, Mean average; SD, Standard deviation; Dunn's test $p < 0.05$; Ss, statistically significant.
$^{\star,\ddagger,\S}p = 0.005$ ($p < 0.05$, Ss).

calvaria consist of two cortical plates with a region of intervening cancellous bone. Physiologically, the cortical bone in the calvaria resembles an atrophic mandible [14–16].

In experimental studies, the calvarial defect model has been regarded as the most selective experimental model of bone regeneration because poor blood supply and the membranous structure preclude any spontaneous healing. In the current study, the CSD model was used because it allows standardized production of defects that enable convenient analysis of newly-formed bone. Other advantages of the CSD model are that observations can focus on the bone healing process, parameters can be simply and accurately measured in each specimen, preparation of tissue specimens is easy and spontaneous healing would not occur. The literature on CSD reports some differences. Takagi and Urist [17] stated that an 8-mm diameter defect was not completely healed after 3 months. On the other hand, some other reports claim that 5-mm defect have to be considered as CSDs [18,19]. Therefore, in the current study we chose 5-mm as the CSDs, also termed trephine drill defects, as in other previous studies [18,19]. This was confirmed by the lack of bone regeneration at the control defects in the current experiment.

Histological evaluation showed that the creation of calvarial defects caused no damage to the underlying dura. Healing was characterized by thin fibrous connective tissue in which a large numbers of collagen fibers and fibroblasts were oriented parallel to the wound surface. The calvarial bone defects were also filled by some blood vessels.

The basic principle for guided bone regeneration involves firstly the placement of a barrier membrane to secure the blood clot and secondly create a secluded space around the bone defect, thus enabling the bone-forming cells to have access without competition from other tissues. The borders of barriers were glued to the bone with Histoacryl® to prevent soft tissue migration and stabilization [20]. Orda et al. [21] used Histoacryl® for adhered autoplastic peritoneal patches to hepatic and splenic wounds. They did not observe any signs of acute inflammatory problem in the epithelial and mesenchymal tissues and complete recapsulation of the liver parenchyma was noted until the 7th day of experiment.

To enhance wound healing, several treatment modalities such as bisphosphonates, propolis, LLLT and hyperbaric oxygen therapy have been researched [4,5,22–25]. Propolis, recommended as topical therapy for wounds since ancient Roman and Greek times [24], is widely used in folk remedies because of its anti-inflammatory properties that speed up the healing process without side-effects [22–25]. These properties are associated with its chemical components, which vary depending on seasonal conditions [26,27]. CAPE, first extracted from honeybee hives in 1988 by Grunberger et al. [12], is an active component of propolis. According to Song et al. [24], CAPE is one of the compounds responsible for the anti-inflammatory action of propolis and acceleration of the healing of surgical wounds in rats. It exhibits anti-inflammatory effects by modulating the arachidonic acid cascade and by inhibiting leukotriene and prostaglandin production via inhibition of the cyclooxygenase and lipoxygenase pathways [28–30].

Several studies have reported that CAPE contributes to the acceleration of the wound healing process. Ha et al. [31] studied the effect of CAPE on osteoclasts and bone remodeling and showed that CAPE significantly inhibited RANKL-induced osteoclast formation in mouse calvariae *in vivo* and proposed that CAPE might be useful as a therapeutic agent for treatment of bone destructive diseases. Elmali et al. [32] aimed to determine the *in vivo* effects of intra-articular injections of CAPE on cartilage in an experimental rabbit osteoarthritis model and reported that intra-articular injection of CAPE may protect cartilage against the development of experimentally-induced osteoarthritis. Cicek et al. [33] studied both the effects of radiation emitted by mobile phones on bone strength and of CAPE on the changes induced by radiation. They conclude that radiation and CAPE can significantly improve bone strength, possibly as a result of the antioxidant property of CAPE [34]. Similarly, Altan et al. [35] stated that propolis has hastened new bone formation in the expanded suture in rats.

Propolis is well tolerated topically, although rare cases of allergy do occur. The main reason for CAPE being selected in the current study was its known strong anti-inflammatory, antioxidant, immunomodulatory and antimitotic properties [6–11,30]. Topical application of 50 mmol/kg CAPE on rat calvarial bone defects appeared to have had some beneficial effect on bone healing, but this was not statistically significant. Topical application of 100 mmol/kg and systemic application of 10 mmol/kg/day i.p. CAPE caused a statistically significant increment in bone formation both on the edges as well as in the central region of the surgical wound compared to the bone growth exhibited by the control and 50 mmol/kg local CAPE groups. When topical application of 100 mmol/kg and systemic application groups compared each other, the systemic application group has superiority against topical application.

To our knowledge, the current study is the first published report describing changes in bone in CSD during the bone healing process, demonstrating that CAPE application increases bone healing and osteoblast formation.

In conclusion, this is the first systematic study in animals showing that CAPE improves the wound repair process. The results indicate that 100 mmol/kg topical and 10 mmol/kg/day systemic application of CAPE increases bone healing, especially systemic application and that the mechanism of the action of systemic CAPE application should be investigated further.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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