Mechanical tension-stress induces bone morphogenetic proteins expression during distraction osteognenesis in rabbit mandible

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Title **Mechanical tension-stress induces bone morphogenetic proteins expression during distraction osteogenesis in rabbit mandible**

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ABSTRACT

Distraction osteogenesis is a unique and effective way to treat mandibular length inequality resulting from congenital and posttraumatic skeletal defects. The clinical and radiographic examinations, as well as, expression profiles of bone-related cytokines were investigated in the distraction region to reveal bone remodeling characters during mandibular distraction osteogenesis. There were marks different of mRNA expression of bone-related cytokines during the phase of active distraction and the phase of consolidation. The selective expression of each bone-related cytokines may provide useful insights into accelerated the bone maturation and treatment of poorly healing fractures in clinical cases.

Acknowledgement

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Thongchai Nuntanaranont

Content

Introduction

Distraction osteogenesis is a form of in vivo tissue engineering during which an osteotomy and controlled distraction are used to lengthen bone[1]. The gradual separation of cut bone edges results in the generation of new bone[2]. Its principle is based on the studies of Ilizarov, who showed that osteogenesis can be induced if bone is expanded (distracted) along its long axis at the rate of 1 mm per day. This process induces new bone formation along the vector of pull without requiring the use of bone graft[3]. Ilizarov used the canine tibia to study distraction osteogenesis by varying the stability of fixation, the energy of the osteotomy, and the rate and rhythm of distraction. He has postulated that all four factors are critical to osteogenesis[4]. Distraction osteogenesis was developed in the field of orthopaedic surgery and principally utilized for limb lengthening[5]. The use of distraction osteogenesis in the craniofacial skeleton was first reported by Snyder et al[6]. Who used monofocal distraction to lengthen canine mandible. They described the experimental mandibular lengthening, to repair a 1.5 cm bony defect at the mandibular body. Successful clinical bone lengthening in the craniofacial surgery was first described by McCarthy et al. in 1992[7]. The trial began in 1989 on bone lengthening involving the human mandible using an external device. Extraoral appliances were used to lengthen congenital hypoplasia mandible in children. Since then several clinical reports followed such as Havlik and Bartlett[8] ; Klein and Howaldt[9]. A wide variety of techniques are available to lengthen segments or entire maxillary and mandibular arches[10-13]. Because this method used local host tissue to regenerate new bone, it offers many potential advantages over bone grafting. Source of autografts are limited and may leave local morbidity at the donor site. Allograft may transmit unknown antigens, bacteria, or even viruses. Distraction osteogenesis can produce unlimited quantities of living bone directly from the osteotomy sites by controlled mechanical distraction. The new bone spontaneously bridges the gap and rapidly remodels to a normal macrostructure similar to the local bone[4, 14-16]. Age seems not to be a limitation so long as the patients have the potential to heal a fracture. According to this basis distraction osteogenesis has become a mainstay in bone tissue engineering and has significantly improved our armamentarium for reconstructive carnio-maxillofacial procedures[17].

However, although the biomechanical, histological, and ultra structural changes associated with distraction osteogenesis have been widely described, the mechanisms that regulate bone formation during distraction osteogenesis are not completely understood. The molecular mechanism governing the formation of new bone in the inter-fragmental gap of gradually distracted bone segments remain largely unclear. Mechanical stimulation by distraction osteogenesis may induce biological response of

new bone regeneration that is accomplished by a cascade of biologic processes that may include differentiation of pluripotential tissue, angiogenesis, mineralization, and remodeling. The most essential part is that it is unknown at present how the mechanical forces created by distraction are translated into biological signals to induce new bone regeneration in such a highly manner. Because bone morphogenetic proteins (BMPs) are potent inducers of osteogenesis in many experimental systems, they are obvious candidates for playing an important role in this process[18]. Among the members of the large BMP family, BMP-2, -4 and -7 have been showed to be especially important for osteogenesis[19, 20]. Therefore the present study was aimed to examine the temporal and spatial gene expression including BMP-2, -4, -7 and related bone formation markers of the regenerating tissue at different time point during distraction process of the rabbit mandible. The clinical, histological and radiographic appearance also were evaluated and analyzed with the concomitant BMPs expression pattern in each interval. The understanding of related biomolecular mechanism that mediates membranous distraction osteogenesis is the substantial knowledge to improve distraction osteogenesis and accelerate osseous regeneration.

Materials and Methods

Animals

This study was performed in accordance with the regulations and approval of the animal experiment ethic committee of Prince of Songkla University. Twenty skeletally mature, male New Zealand white rabbits aged 5-7 months, weighing 3-4.5 kg were used in the study. The animal were kept in single cages and fed with standard dried diet and water and libitum.

The experimental scheme was shown in Figure 1

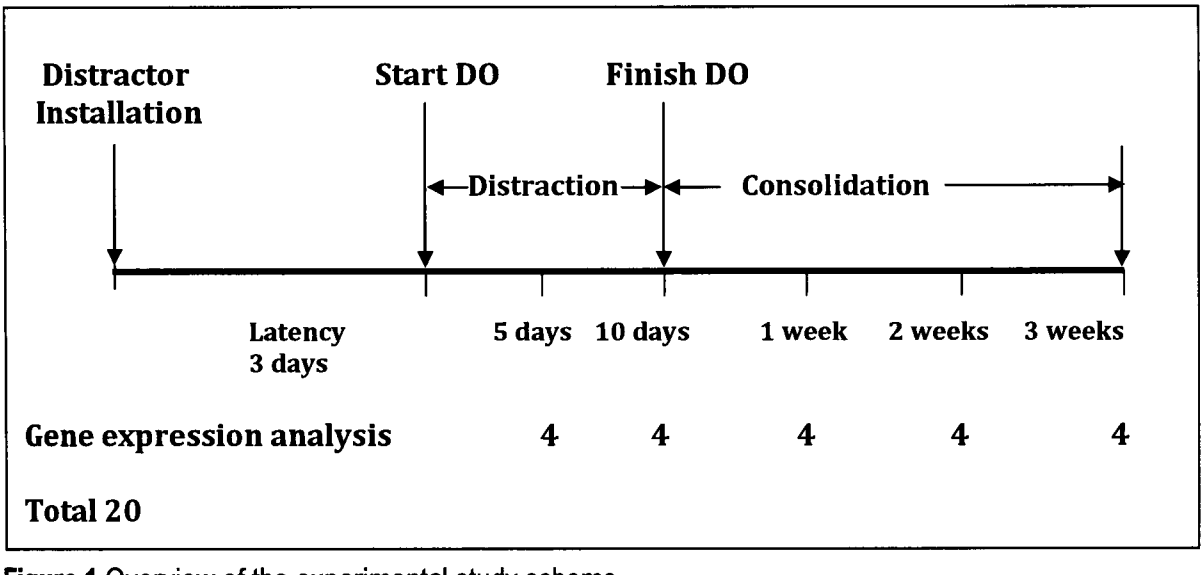


Figure 1 Overview of the experimental study scheme.

Surgical Procedure

The surgery for mandibular distraction osteogenesis was performed under aseptic condition. All rabbits were anesthesized with an intramuscular injection of Ketamine Hydrochloride (25 mg/kg) and Diazepam (5 mg/kg). The Penicillin G Sodium (0.5 million units) was administered preoperatively 20 minutes intramuscularly. The right submandibular area was shaved and disinfected with iodine solution. 1.0 ml of 2% Lidocaine with 1:100,000 epinephrine solutions were injected subcutaneously at surgical area. Submandibular incision was made with 3 cm long. The mandibular body was exposed by carefully dissection the plastysma muscle and reflection of periosteum. The facial artery and mental nerves were preserved. The osteotomy line was made straight downward to the lower border of the mandible between the premolar and the mental foramen. (Figure 2)

The osteotomy of the mandible was achieved by two steps: the first step was to perform a corticotomy by fissure bur at buccal and lower border aspects of the mandible, thus a bony slot was made in order to locate the distractor, moreover if the osteotomy had been completed before placement of the distractor, it would have been more difficult to fix the distractor between two unstable bone segments rather than keeping continuity of the mandible by an incomplete osteotomy at beginning. Then the distractor was located according to the bony slot (osteotomy line) and fixed by four self-tapping titanium microscrews. In addition, the vector of distraction was parallel to the long axis of the mandible and perpendicular to the osteotomy line. Hence the second step of the osteotomy was finished by a complete osteotomy using fissure bur and chisel after the placement of the distractor. Intraoperatively the distractor was activated up to about 3mm of lengthening for testing the distractor and completion of the osteotomy. (Figure 3) Then the distractor was drawn back without any lengthening. Every step in the operation was accompanied by thorough irrigation. Finally each layer of periosteum, plastysma muscle, subcutaneous tissue and skin was sutured.

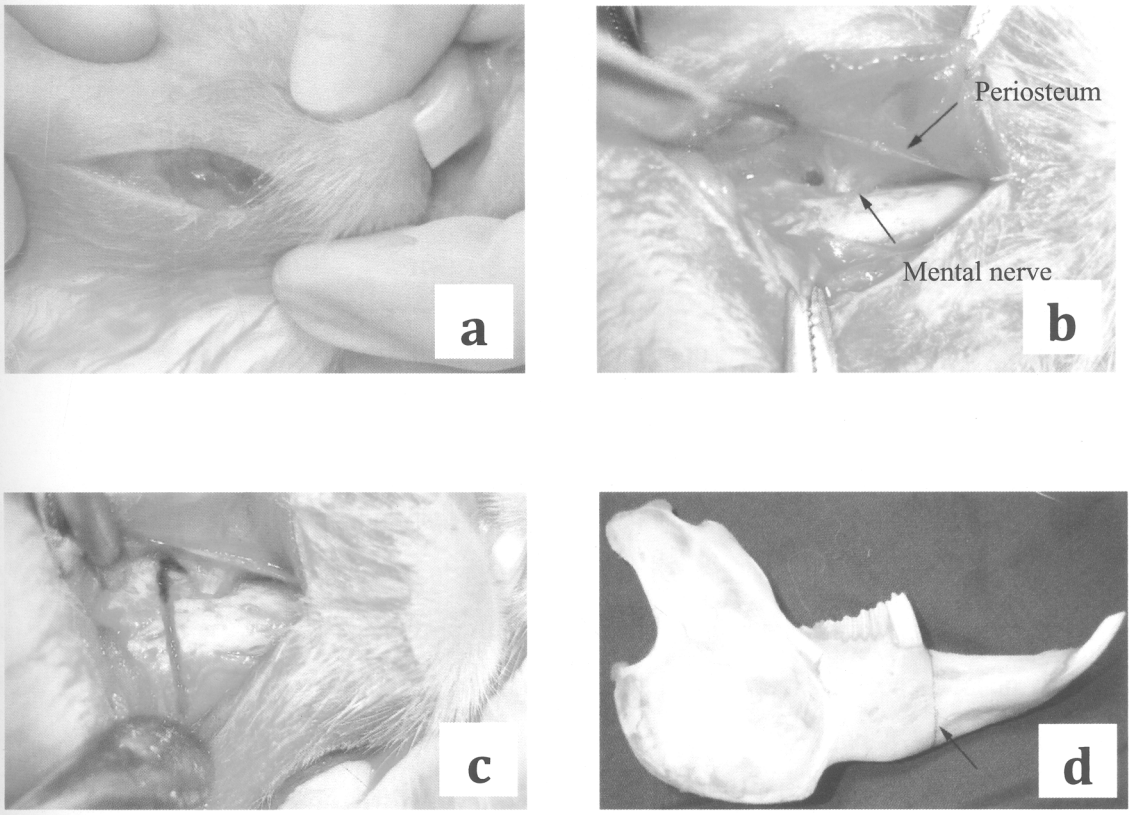


Figure 2

(a) A submandibular incision

(b) Exposure of buccal aspect of the mandible.

(c) A partial osteotomy (Corticotomy) was made just between the premolar and the mental foramen.

(d) A dry mandible showing the osteotomy line as the arrow indicated

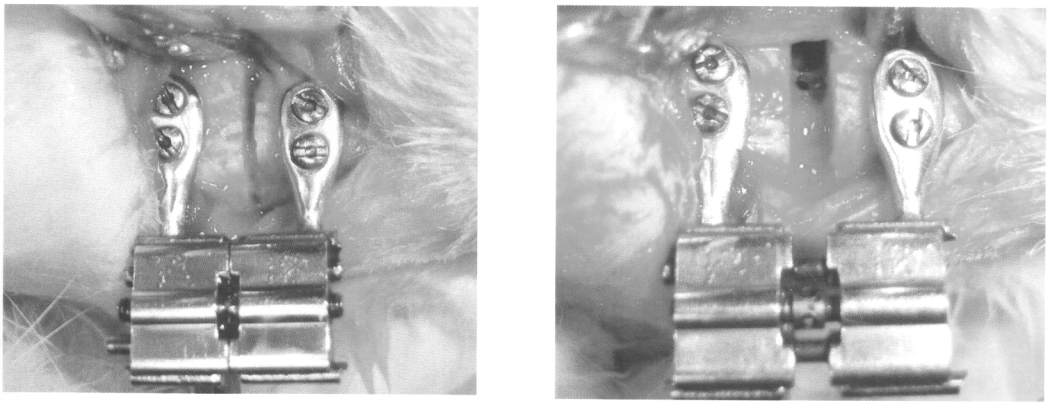


Figure 3. Customized distraction osteogenesis device was applied to the experimental site of the rabbit and was activate for test after complete osteotomy before drawn back to initial.

The rabbits returned to the normal diet immediately after surgery, which were fed by milled pellet. Postoperative care included intramuscular applications of Penicillin G Sodium (0.5 million units) and Acetaminophen 75mg per day for 3 days. After 3 days of latency period, distraction was started at the rate of 0.5 mm every 12 hours for 10 days and followed by 3 weeks of consolidation period. Four animals were sacrificed after 5, 10 days of distraction and at 1, 2, and 3 week of consolidation (n=20).The scarification was performed painlessly by an intravenous injection of Sodium pentobarbitone (1ml/kg) via the ear vein according to group.

Radiographic examination

After the sacrifice, the whole mandible was carefully dissected from the surrounding soft tissue, and then the mandible was separated into two hemimandibles at the symphysis by scalpel. Both two hemimandibles were taken the plain radiograph. The hemimandible was placed on an occlusal film with the lingual side contacting to the film. The lateral film of hemimandible was taken (10 mA, 50 KVP, 0.26 sec, 12 inch FFD) with an aluminum step-wedge. All of the films were taken by the same machine (Gendex, Illinois, USA) (Fig 4) and processed by an automatic film processor (Dent X 9000, DentX/Logetronics GmbH, Kornberg, Germany). Then the films were transformed into digital images by digital camera (JVC TK-C1380, Tokyo). The films were examined and recorded as descriptive study. Further quantitative study was performed under the software (Image Pro Plus 5.0, Media Cybernetics Inc. Slier Spring, USA) to measure the mean gray level which was represented the projectional bone mineral density (BMD) in the distraction gap (Fig 5).

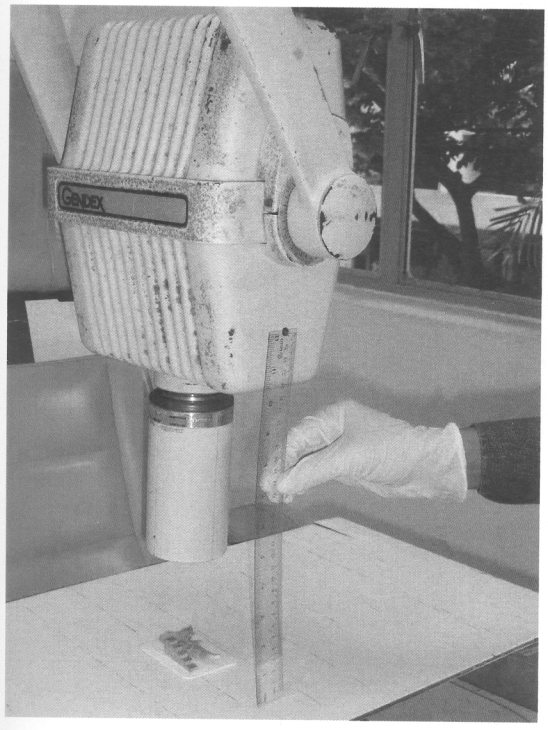


Figure 4. The lateral film of hemimandible, a ruler was used to keep the same distance between the X-ray tube and the film. An aluminum step-wedge used The X-ray machine (Gendex, Illinois, USA).

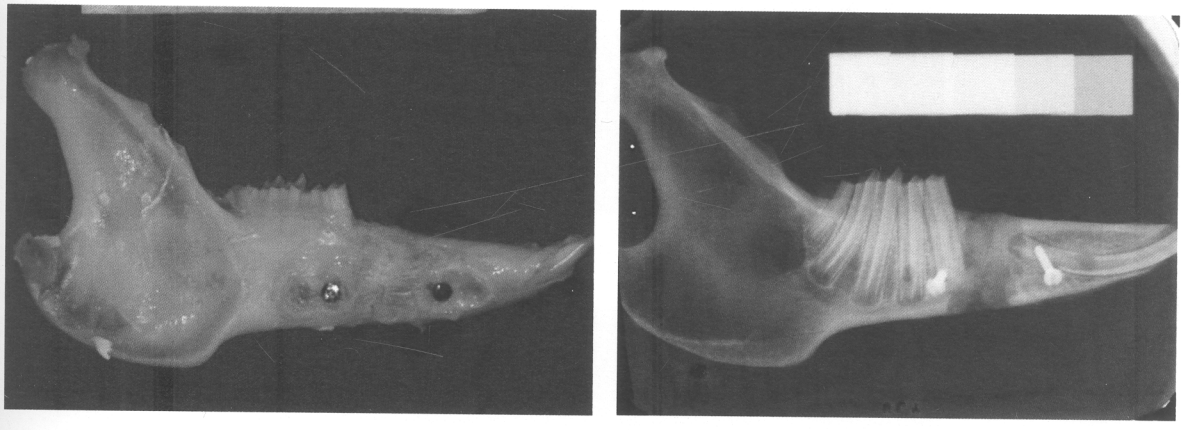


Figure 5. Radiograph of the distracted mandible.

Bone samples collection

After taken the plain radiograph, the lower half of the regenerative tissue in the distraction gap was collected by using the 0.8-cm diameter trephine bur on the rotary headpiece (figure 6), including the opposite non-distracted hemimandiblein the same area for the gene expression analysis by real-time reverse transcription polymerase chain reaction. All the specimens were saline-soaked and frozen at -80° for preservation before additional testing.

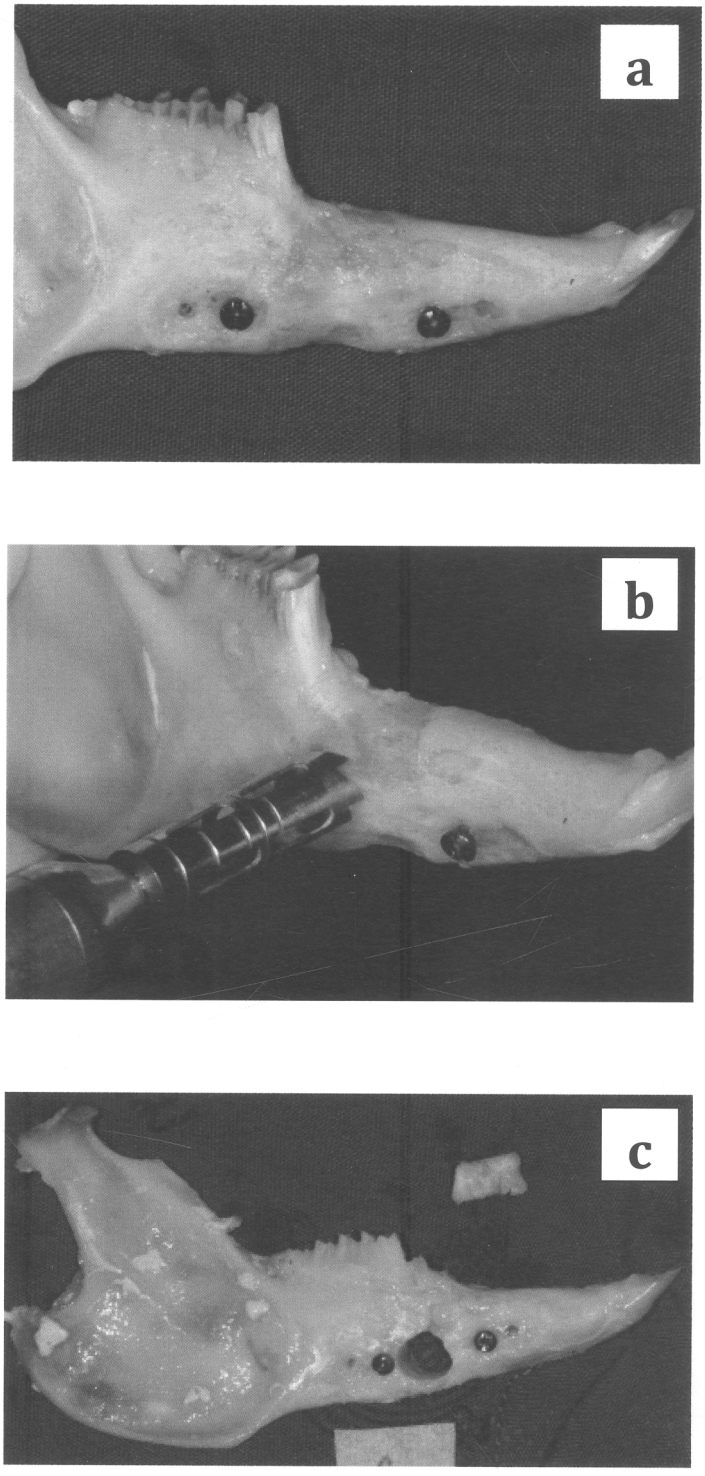


Figure 6.

(a) The distracted hemimandible with 2 screws, the regenerative tissue in the distraction gap was between the screws

(b) 0.8-cm diameter trephine rotary bur was used to harvest the specimen in the lower half of the gap

(c) Specimen was punched out for gene expression analysis

RNA extraction and real-time RT-PCR

The frozen bone samples were milled to a fine powder under liquid nitrogen in a freezer mill (Bone Mill, Metuchen, N. J, USA). Total RNA were extracted with RNeasy Micro Kit(Qiagen, Valencia, CA, USA) according to manufacture's instruction. Total RNA of each sample was measured by using a spectrophotometer (Biochrom Ltd, Cambridge CB4, England) at 260 nm and 280 nm for estimating RNA concentration and purity. The extracted RNA will be stored in Rnase-free water at -80° C until be used.

DNase I-treated total RNA (0.5μg) or water blank (no RNA) were reverse transcribed using the QIAGEN OneStep RT-PCR (QiagenInc, USA) for cDNA synthesis. RT reaction (5 μl) was amplified in triplicate by real-time PCR (Applied Biosystems 7300) in a final volume of 25 μl using SYBR Green Master Mix reagent at a final concentration of 1X (Applied Biosystems, USA) . Beta-Actin , a constitutively expressed housekeeping gene, was also amplified under the same conditions and used to normalize reaction. The primers for the rabbit specific genes were acquired according to the published papers as shown in Table 1

Table 1. Sequence of primers and probes (1st seqence)

	PRIMER	PROBE
BMP2	TGCGGTCTCCTAAAGGTCGA	CGGGTCCCGGCCACCATG
	GGAAGCAGCAACGCTAGAAGA	
BMP4	CCGCAGCCTAGCAAGAGC	CCGTCATCCCGGATTACATGCGG
	CCTGACTGGAGCCGGTAAAG	
BMP7	GGAGCGCTTTGACAACGAG	CGTTCCGCATCCGCGTGTACC
	CCAAGTGCTCCTGCAGCA	
bFGF	ATCAAAGGTGTGTGTGCAAACC	CCAGCAGTCTTCCATCTTCCTTCATAGCAA
	AAGCACTCGTCTGTGTAACACATTTAG AA	
TGF beta-1	AGCCACTGCCCATCGTGT	CTACGTGGGCCGCAAGCCCA
	CACGATCATGTTGGACAGCTG	
VEGF	GGGCTGCTGCAATGATGAA	TCCTCGGTGGGCACACACTCCA
	GATCTGCATGGTGACGTTGAA	
Beta-Actin	CGAGATCGTGCGGGACAT	AGCGCCACGTAGCACAGCTTCTCCTT

Histological examination

The upper part of the specimen was fixed in 10% formalin for 2 weeks and decalcified with 50% formic and 20% sodium citrate then dehydrated in increasing concentrations of alcohol until 100% is reached, finally embedded in paraffin. 10 μm thickness of sections were sectioned (horizontal direction, so that the whole distraction gap was present in every section) and stained with hematoxylin and esion for light microscopy (Carl Zeiss, Axioskop 40, Germany).

Statistics analysis

The data will be analyzed by descriptive statistic for the qualitative data. The quantitative data will be assessed by one way ANOVA and post comparison test. Statistical significance was set at $p<0.05$.

Results

The animals tolerated well with the anesthesia and completed the experimental process uneventfully. There was no accidental death of rabbit encountered. The animals recovered well from anesthesia, a period of anorexia for a couple of days was frequently observed. After this period they were able to eat independently without any disturbance from the distraction device. Clinically, obvious crossbite and overgrowth of the lower incisors developed in all rabbits. After completion of activation, the animals were able to take regular food for the remaining part of the study. No significant weight loss was observed. Ten consecutive days of distraction were achieved in all animals without any failure of the distraction device.

Clinical and radiographic appearance

<u>At 5 days of distraction</u>

The distraction gap of the bone cut was open to half way of decided length and filled with very soft red regenerative tissue. (Figure 7a) The 5- mm radiolucent gap was established. No radiopaque area was showed in the distraction gap.(Figure 7b) The histologic section showed numerous of spindle shaped mesenchymal cells, which resembled to fibrosblast cell, was seen throughout the expanded gap. These spindle cells with dense collagen production arranged themselves parallel to the distraction vector as the distraction begun. In some area, the mesenchymal cells formed buds that led to thedevelopment of a primitive microvasculation. In this stage, the predominant histologic appearance was a rich fibrillar matrix of collagen. Neither osteoid nor osteoblast was observed in the expanded gap. In some areas, the collagen bundles became denser and less vascular almost resembling the tendon. Finally, the spindle-shaped fibroblasts and the collagen bundles, which oriented parallel to the distraction force, established bridging of both bone surfaces. (Figure 7c,d,e)

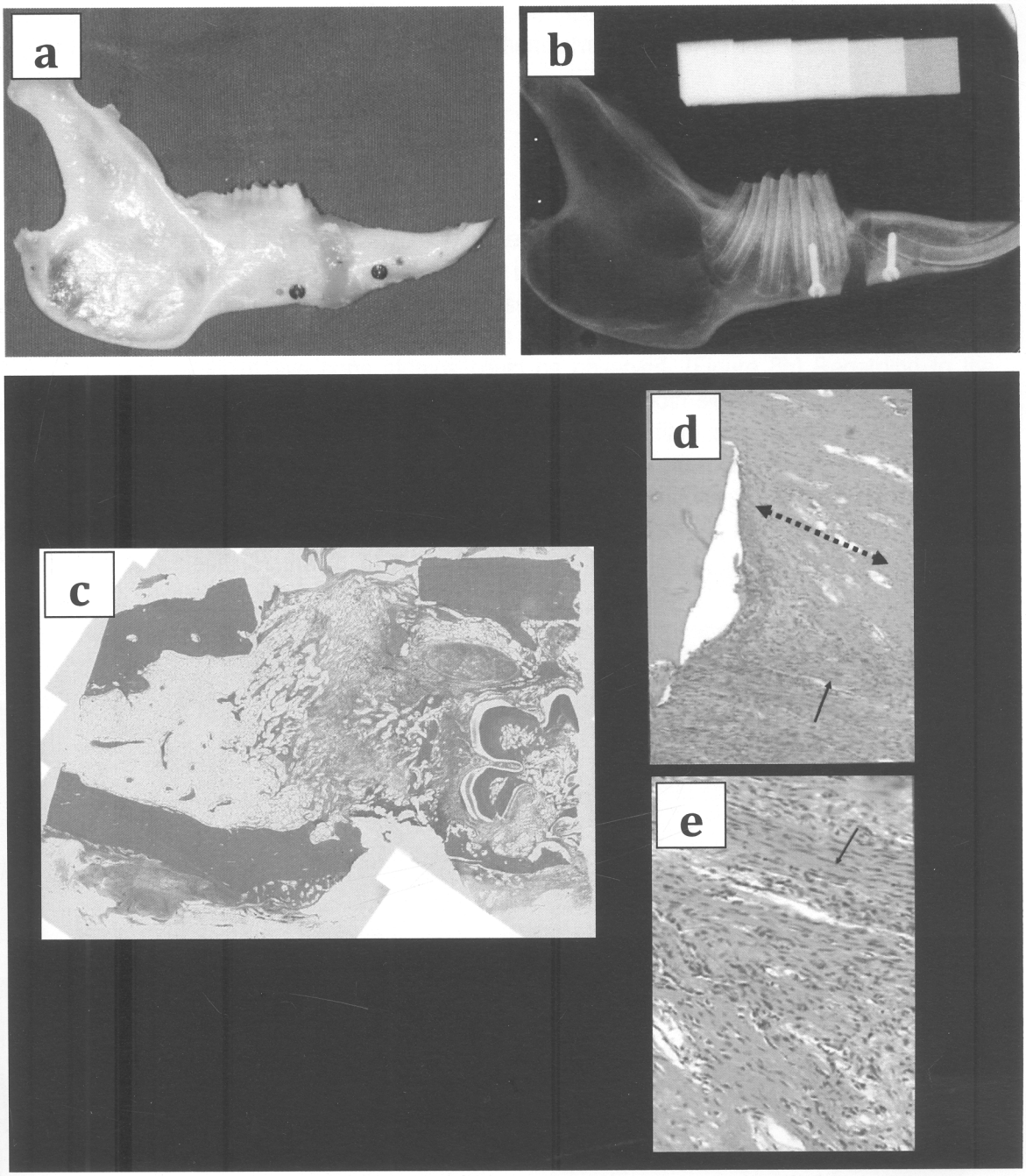


Figure7.

- **(a)** The distraction gap was open up to 5 mm and filled with reddish regenerative soft tissue.
- **(b)** The radiograph demonstrated a radiolucent gap between the osteotomized bone stumps. No any radiodensity was noted throughout the expanded gap in this stage.
- **(c)** The histologic study showed the distraction gap which predominantly filled with fibroblast like cells. Very few area of newly formed bone was observed. (H&E stain; original magnification x5)
- **(d)** The spindle-shaped fibroblast interspersed among bundle of collagen was demonstrated. The collagen bundles which tended to align themselves to the parallel manner with the direction of distraction (dot arrow) were obviously noted (arrow). The cut bone surface was also seen on the left. (H&E stain; original magnification x20)

(e) In the higher magnification revealed the spindle-shaped cell resembling fibroblastic cell with dense collagen production which organized themselves parallel to the vector of distraction (arrow). (H&E stain; original magnification x40)

At 10 days of distraction

The distraction gap of approximately 10 mm filled with soft tissue was noted. The regenerated tissue in gap had the brownish-red color, and this made it easily distinguished from the normal adjacent bone stump. The consistency of the newly formed tissue was similar to fibrous connective tissue. (Figure 8 a) The radiograph showed the distraction gap corresponded to the clinical finding. The initial radiopacity near both of the native bone stumps could be observed. (Figure 8b) Regarding to the histologic section, longitudinal new bone trabeculae were seen on both sides near the osteotomized bone surfaces, as well as the area adjacent to the periosteum. The fibrous tissue and collagen were seen mainly in the central region of the gap. The osteoblastic cells were found resting on the primary bone spicules, whichresulted from the incorporation of the collagen bundles and the osteoid. These longitudinal new bone trabeculae were seem try to bridge the expanded gap by extending from the cut bone surface and periosteum (Figure 8c, d)

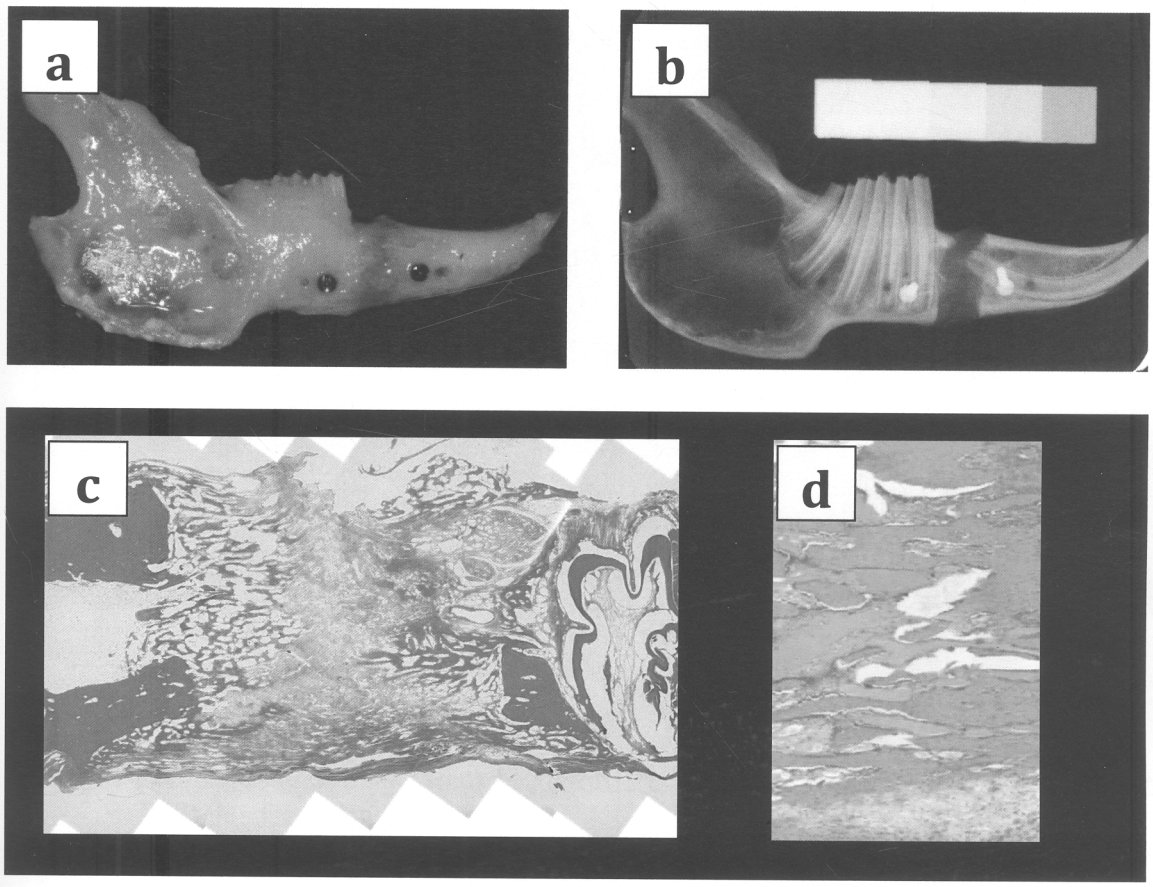


Figure 8

(a) Clinical feature of the hemimandible after 10-day of distraction. The newly formed fibrous-like tissue filled up in the gap was demonstrated.

(b) Radiographic showed the successful expanding gap of nearly 10 mm. The increasing of radiopacity particularly adjacent to both bone stumps could be noticed.

(c) Histologic section of the distraction gap showed the beginning of the new bone formation extending from both bone stumps in the parallel manner to the distraction vector toward the middle fibrous area.(H&E stain; original magnification x5)

(d) New bone trabeculae in the distraction gap appeared to align parallel to the distraction vector. (H&E stain; original magnification x20)

At 1-week consolidation

The regenerative tissue in the distraction gap in this stage had the appearance resemble to the callus-like tissue. The consistency of the tissue was increased similar to the fibrous tissue consistency. (Figure 9a) The radiographic examination showed markedly increasing of the radiopacity of the distraction gap especially from both bone stumps. (Figure 9b) The histologic finding also demonstrated rapidly increased of the newly formed bone in the distraction gap. (Figure 9c)

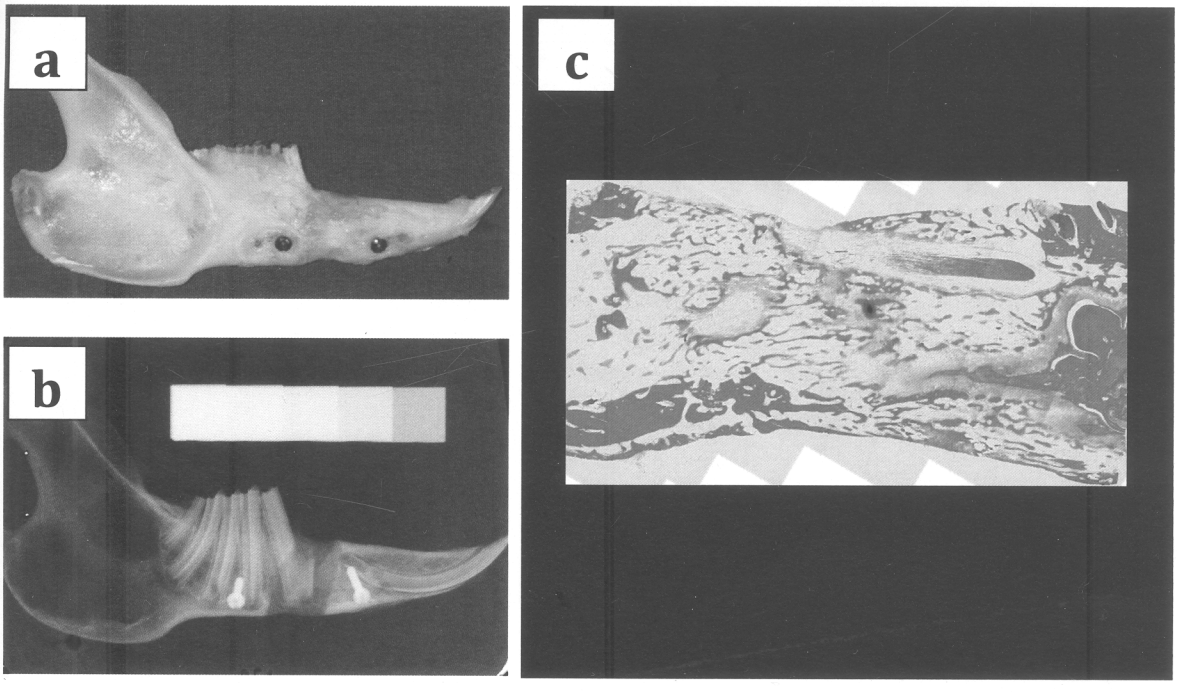


Figure 9

(a) Clinical appearance after 1 week of consolidation phase showed the callus-like texture of the tissue in the distraction gap.

(b) The radiopacity was significant increase in the distraction gap especial the area near the bone stumps

(c) The rapidly increase of the newly formed bone in the distraction gap extending from both bone stumps was observed when compared to the previous stage. (H&E stain; original magnification x5)

At 2-week consolidation

The regenerative tissue in the distraction gap had same appearance nearly similar to the normal bone callus texture. The harder in consistency also was observed. (Figure 10a) The radiopacity in the distraction gap kept on increasing, leaving only small radiolucent band in the middle of the gap. (Figure 10b) New bone formation which extending paralleled from the both bone stumps was nearly completed filled up the gap except the small area of the fibrous tissue in the middle region. (Figure 10c)

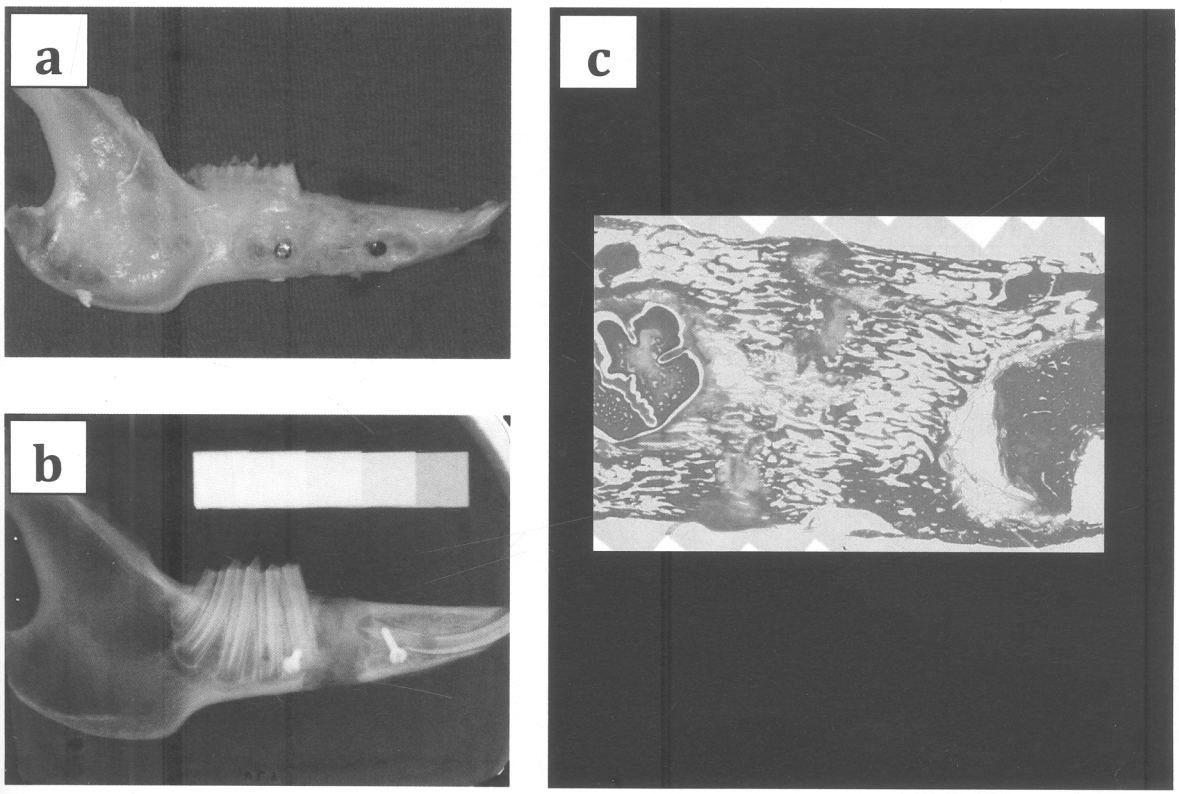


Figure 10

(a) Clinical appearance and consistency after 2 week of consolidation phase of the regenerative tissue in the distraction gap revealed more callus-like tissue in the distraction gap.

(b) The radiopacity in the distraction gap was still progressively increased from both bone stumps. The only small radiolucent band in the middle area was seen.

(c) The newly formed bone in the distraction gap was increase and nearly filled up the entire gap except the fibrous middle zone. (H&E stain; original magnification x5)

At 3-week consolidation

The regenerative tissue in the distraction gap showed nearly completed mature cortical bone appearance including the color, texture and consistency. (Figure 11a) The radiographic examination demonstrated the distraction gap that was filled up completely with the radiopacity. The new established upper and lower radiographic cortical bone structure of the expanded gap can be noticed. (Figure 11b) The histologic section showed the more mature regenerated bone throughout the distraction gap. The fatty bone marrow and cortical bone structure can be observed. (Figure 11c)

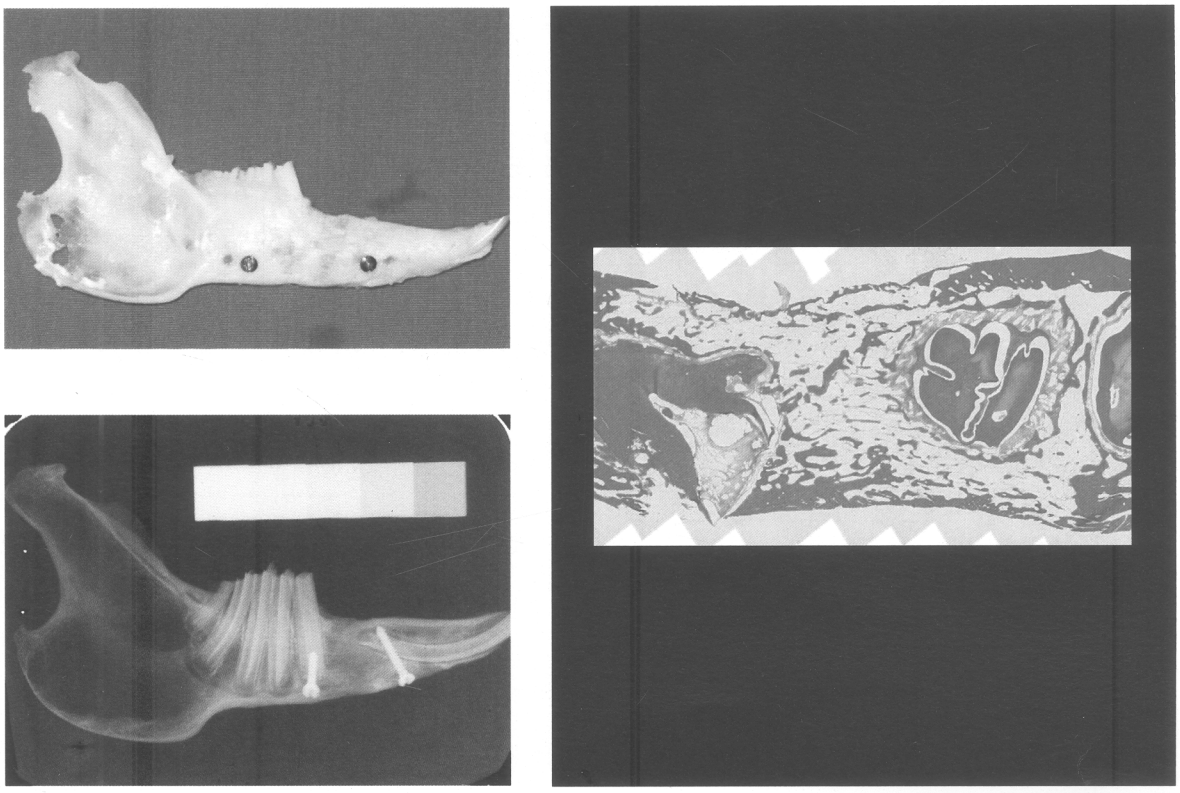


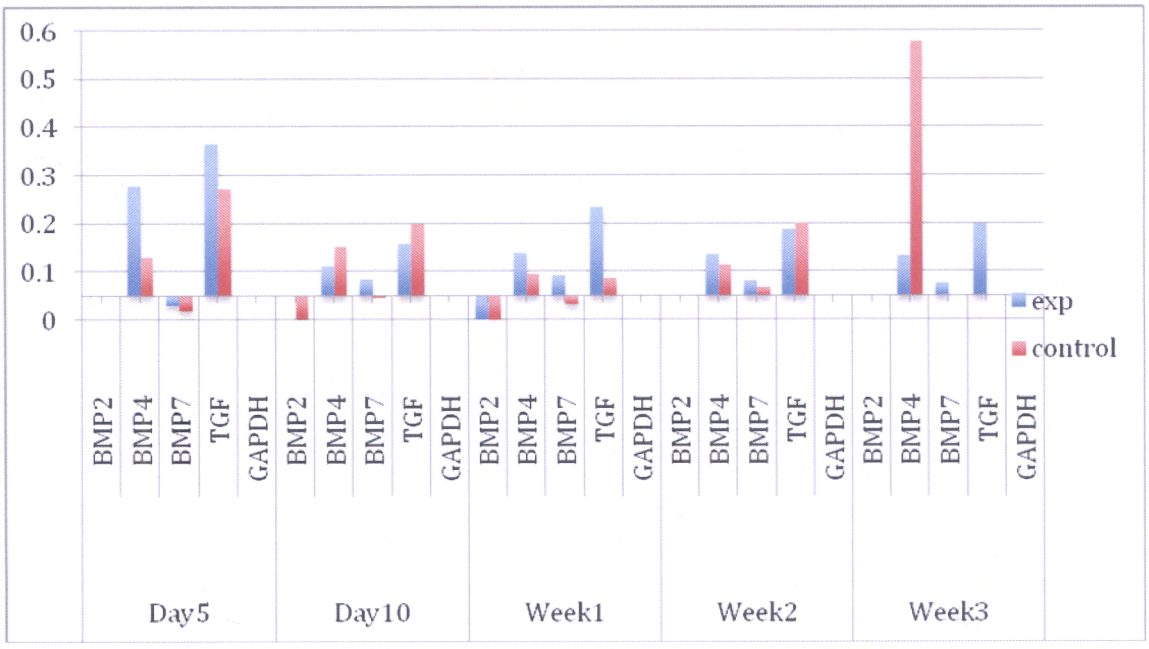
Figure 11

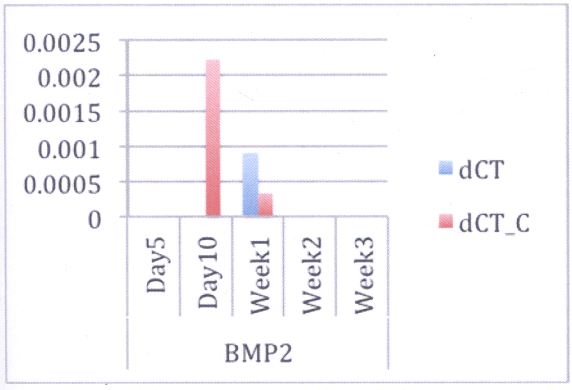
(a) Clinical feature of the distraction gap after 3 week of consolidation phase demonstrated normal outer cortical structure similar to the adjacent normal bone.

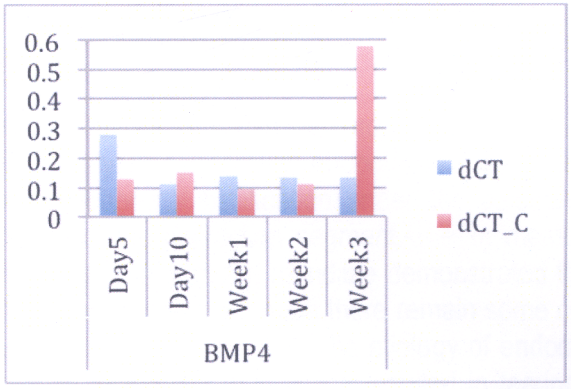
(b) The radiodensity was filled up completely throughout the distraction gap. The newly formed upper and lower cortical bone structure can be observed

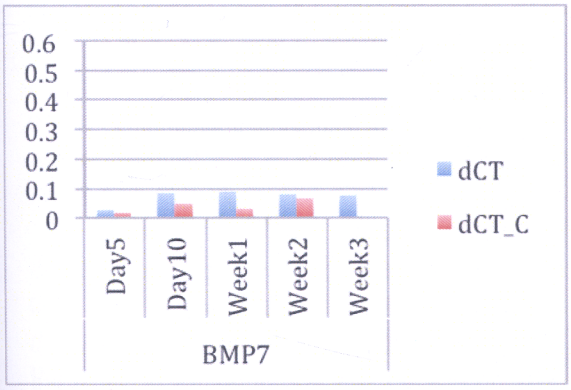
(c) The histologic section of the distraction gap demonstrated mature regenerated bone with the remodeled fatty marrow and cortical bone structure. (H&E stain; original magnification x5)

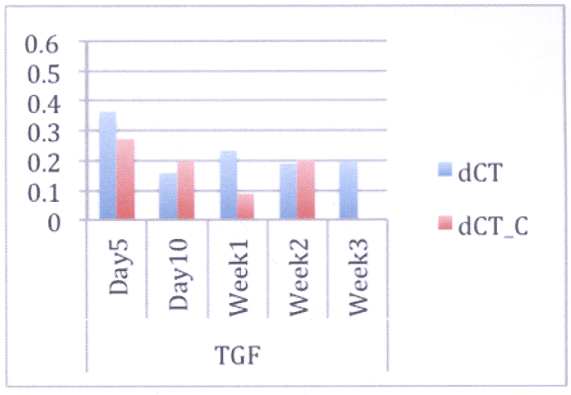
Figure 12. mRNA expression of BMPs and TGF-B1 by real-time RT-PCR











In control and experimental group, BMP family and TGF were detected in throughout the process of distraction osteogenesis which were distraction phase and consolidation phase.

Considering to the level of expression, the TGF was shown the highest value followed by BMP4 and BMP7.

During distraction phase (day 5 and day10), TGF revealed the highest level of expression (0.364 and 0.272), followed by BMP4 (0.279 and 0.112) and BMP7 (0.029, 0.084).

In early consolidation phase (week1), BMP4, 7 and TGF levels were increased markedly from control; however, the expression levels were not different from the end of the distraction phase.

During 2nd and 3rd week of consolidation phase, the expression levels of TGF were still highest among other groups (0.187, 0.199), while the expression levels were 0.135 and 0.133 in BMP4 and 0.080 and 0.076 in BMP7 respectively. Nevertheless the level of all experiment groups were gradually declined to the level of the control group in at end of consolidation phase.

Discussion

The technique of distraction osteogenesis involves the creation of new bone by gradual separation of two or more bony fragments following their surgical division. The development of distraction osteogenesis represents a significant alterative technique in the field of oral and maxillofacial surgery. According to the less invasive procedure when compared with traditional methods of maxillofacial reconstruction, the severe morbidity can be avoided. However while the basic factors that effect the distraction osteogenesis have been reported, the molecular expression during distraction process remains largely unclear. The present study investigated the effect of distraction osteogenesis to the expression of bone morphogenetic proteins in the regenerated tissue quantitatively in the distracted rabbit mandible.

The mode of ossification of the regenerated tissue is varied depend on the site of bone in origin. In long bone fracture healing, new bone is mainly formed through endochondral ossification. On the other hand, new bone is formed through endochondral ossification under the peiosteum and through intramembranous ossification at the center of the distraction segment.[21, 22] In membranous bone distraction osteogenesis, Karp et a[23] in the lengthening of dog mandible demonstrated that new bone was formed predominantly by intramembraous ossification, although there remain some conflicts. Since endochondal ossification has been reported in some studies.[24, 25] The etiology of endochondral bone formation may be influent by the inappropriate distraction rate, which resulted in tearing of the microcirculation followed by impairment of the oxygen tension.[24, 26] In the present study, some small foci of the cartilage were found in some specimens in the active distraction period and early consolidation phase but the majority of ossification pattern still was the intramembranous ossification in origin. This finding probably resulted from the good stability of the well-designed distraction device and appropriated distraction protocol used in the present study.

The majority of studies concerning the expression pattern of bone-related cytokine in distraction osteogenesis process were described in qualitatively evaluation. In the present study, real time RT-PCR was used to detect the temporal gene expression of bone morphogenetic proteins during distraction osteogenesis in rabbit mandible. The real-time RT-PCR is a combination of three steps: 1) the reverse transcriptase – dependent conversion of RNA into cDNA, 2) the amplification of the cDNA using the PCR and 3) the detection and amplification of the products in real time[27]. The quantification of real-time PCR is achieved by measuring the amount of fluorescence increases as the PCR proceeds. The PRC primers bind to sites adjacent to the region to be amplified and a probe binds to the region

amplified. The probe is labeled with both a fluorescent reporter molecule and a fluorescence-quenching molecule. When the reporter and quencher molecules are both bound to the probe, fluorescence is quenched. It assays are characterized by a wide dynamic range of quantification of 7-8 logarithmic decades, a high technical sensitivity (<5 copies) and a high precision (<2% standard deviation)[28, 29]. The benefit of this procedure over conventional methods for measuring RNA include its high sensitivity, large dynamic range, no –post PCR steps, and the potential for high throughout as well as accurate quantification. Furthermore, many of the key proteins, such as cytokine and transcription factors, are found in such low abundance that real-time RT-PCR quantification of their mRNAs represents the only technique sensitive enough to measure reliably their expression in vivo[30, 31]. Bone Morphogenetic protein (BMP) is a factor that can induce ectopic bone regeneration. It was found by Urist[32] in 1965 and was purified by Wozney et al[33] in 1988. BMPs belong to the transforming growth factor-beta (TGF-beta) super-family, which conserves 7 cysteine residues in a mature active form.[33-36] BMPs have been reported to be expressed in development and fracture healing. They are potent inducers of bone growth and repair in many experimental systems, and also have been confirmed to be the important contributing local factors in regulating bone formation in distraction osteognesis both in long bone and craniofacial skeleton.[2, 37-40]

The TGF and BMP family of morphogens are the most important molecules driven the activities of bone regeneration. Different BMPs exhibit different temporal patterns of expression during distraction osteogenesis. BMPs promote the migration of monocytes and mesenchymnal cells, differentiate chondroblasts to osteoblasts, and promote the synthesis of particular proteins in osteoblasts and chondroblasts.[41, 42] BMP 2 plays a strong role in differentiating undifferentiated cells to osteoblasts and chondroblasts. BMP 3 is the most abundant BMP in demineralized bone, accounting for approximately 65% of the total. BMP 3 also play a role in stopping the differentiation of osteoblasts, because the excess expression of BMP3 was reported to have no effect on the alkaline phosphatase activity level and did not promote the expression of osteocalcin.[38] BMP 4 plays a role like BMP 2 and is concerned with tooth development.[43] BMP 5 is concerned with osteognesis at the body trunk. Most of BMP 6 is concerned with endochondral ossification.[44] BMP 7 is expressed between the fingers at the embryo stage.[45] The previous studies reported the elevated signals of BMP 2 and 4 detected in the chondrogenic precursor cells of the subperiosteal immature callus as early as 4 days after the osteotomy and decline to pro-operative levels at the end of the latency period.[38, 39] Along with the process of distraction, expression of BMP 2, 4, 5, 6, 8 are markedly induced and maintained at a high level for as long as distraction is continued and then gradually decrease during the consolidation phase.[2, 37-40] Their signals are detected widely among the chondrogenic and osteogenic cells and also their precursor cells at the fibrous interzone. BMP 3 is not observed conspicuously during distraction but is strongly expressed at week 1 and 2 of consolidation.[40] Regarding to the BMP 7, it probably play a similar role as BMP2 and BMP 4 in distraction osteogenesis process[38], while other experiments only detected weak or even no expression.[2, 39, 40]

BMP2, 4, and TGF-beta were reported to reach the expression peak during the active distraction phase to stimulate uninterrupted bone formation in response to strain cause by distraction and were gradually disappears in the consolidation phase[39, 46, 47]. This similar pattern of cytokines response to the distraction process was demonstrated quantitatively in the present study. The peak level of expression of BMP4 and TGF were detected rapidly as early as in day 5 of active distraction phase and gradually declined to the same level as the controlled bone in the end of consolidation period. When compared to the non-operated control bone, BMP4 posted the highest level of response with elevated level of approximately two-fold at 5 days of active distraction period and returned to control bone level at the end of the consolidation phase. In the present study, low level of BMP 7 was found through out the distraction process. But the expression peak was found late than the other

groups at 10-day of completion distraction. Then the expression signal was continued until the end of consolidation period.

In the case of long bone distraction osteogenesis, Sato et al[39] in a rat femur distraction model reported the following pattern of BMPs expression. BMP 2 and 4 always expressed during distraction but the expression of BMP 6 began to decrease on 10-day distraction. BMP 7 was not expressed. All expression of BMPs decreased to the level before distraction on 7 days after the completion of distraction. The present study using mandible as the membranous bone distraction showed that the selective BMPs have the expression pattern accorded to the long bone distraction. The expression of BMP 2 and 4 was observed continuously from the beginning of distraction process which was similar to the expression reported in long bone distraction. The expression of BMP 7 resemble to the expression reported in long bone on the point of low level of expression. The different of the expression when compared to long bone distraction is the expression of each BMPs was maintained for 2 weeks after completion of distraction.

According to the temporal pattern of expression, as soon as distraction was applied, the pattern of expression of all bone-related cytokines was rapidly rising up. This situation was occurred before any new bone formation was evident by either radiography or histology. Thus the temporal pattern of these cytokines expression is consistent with theirs role in the regulation of new bone formation. When distraction was discontinued, the cytokines expression appears to be quickly declined. When the callus tissue matured in the late consolidation phase, the expression was decrease to the normal level same as the nonoperated side. The temporal pattern of this expression strongly suggests that cellular BMP production is directly or indirectly enhanced by the mechanical stimulus provided by distraction process. This evidence suggests that the change in mechanical tension-stress created by distraction osteogenesis process leads to increase BMPs expression. Thus BMPs and other bone-related cytokines such as TGF-beta are the key factors that play a role in the signaling pathway linking the mechanical forces created by distraction to the cellular response and eventually the new bone formation.

The understanding of the mechanism of distraction osteogenesis at the protein and gene level particularly endogenously expression profiles of bone-related cytokines during distraction process are the crucial steps to improve the outcome of new bone generation. The clearly insight into the molecular mechanism during distraction will provide the foundation for future targeted therapeutic manipulation designed to improved distraction osteogenesis process and accelerate bone healing. In addition investigation of the expressed patterns of growth factors during distraction osteogenesis will elucidate the suitable candidate gene for therapeutic intervention of new bone formation. Base on this knowledge, the administration of appropriated recombinant bone morphogenetic proteins or growth factors in the right timing will accelerate bone induction process in distraction osteogenesis process and resulted in improving distraction rate and shortening the consolidation period. In addition this principle can be applied to obtain the best outcome in the other modality of osseous regeneration.

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