Effects of FGF-2 with hyaluronic acid, BCP, hyperbaric oxygen therapy on bone healing in irradiated calvarial defects of rats

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(Directed by Prof. Moon Kyu Chung, D.D.S., M.S.D., Ph.D.)

A Dissertation Thesis
Submitted to the Department of Dental Science
And the Graduate School of Yonsei University
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy in Dental Science

Heesuk An

December 2013

This certifies that the dissertation thesis

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December 2013

감사의 글

이 논문이 완성되기까지 끊임없는 지도와 격려로 지켜봐주신 정문규 교수님께 진심으로 감사를 드립니다. 또한 논문 심사에 세심한 지도와 따뜻한 조언으로 큰 가르침을 주신 이근우 교수님, , , 그리고 김성태 교수님께 깊은 감사의 말씀을 올립니다.

바쁜 가운데에서도 실험과정 동안 많은 시간을 할애하여 시편제작과 계측에 큰 도움을 주신 오성희 연구원과 방사선 종양학과의 선생님들에게도 감사의 뜻을 전합니다. 또한 지면으로 일일이 다 언급하지는 못하지만 논문이 나오기까지 많은 도움을 주신 모든 분들께 다시 한 번 감사드립니다.

여기까지 오기까지 커다란 사랑으로 보살펴 주시고 배려해주신 양가 부모님들께 진심으로 감사드리고, 마지막으로 항상 곁에서 커다란 힘이 되어주는 사랑하는 아내 주혜와 아빠에게 늘 미소로 응원해주는 첫째 정원이, 둘째 재용이에게도 고마움을 전하며 이 기쁨을 함께하고 싶습니다.

2013 12 월

안희석 드림

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Abstract

Effects of FGF-2 with hyaluronic acid, BCP, hyperbaric oxygen therapy on bone healing in irradiated calvarial defects of rats

Heesuk An D.D.S., M.S.D.

(Directed by Prof. Moon Kyu Chung, D.D.S., M.S.D., Ph.D.)

Purpose: For irradiated patients, the healing capacity of irradiated bone decreases markedly. Therefore, rehabilitation for patients who experienced radiotherapy requires more attention. The purpose of this study was to evaluate the effect of the grafted materials and hyperbaric oxygen therapy (HBO) on the bone healing in experimentally created calvarial defects in irradiated rats.

Materials & Methods: Sprague-Dawley rats (body weight about 300g, 4 weeks old, male) enrolled in the study were divided into the four groups. Rats were divided into the group with HBO and the group without HBO (NHBO). Each group had two subgroups which were FGF-2 with hyaluronic acid (HA) group and BCP block group according to the graft. Localized radiation with a single dose of 12 Gy radiation was applied to the calvarium. Four weeks after radiation, two symmetrical, circular defects of 6-mm-diameter were created in

the parietal bones of animal. The left defect was left empty as a control and the right defect was filled with the materials mentioned above. During 4 weeks of healing, 1-hour HBO was performed for the HBO groups for 5 times a week. After these 4 weeks of healing, the rats were sacrificed. The calvarial specimens were harvested for histomorphometric and micro-CT analysis.

Results: 1. FGF-2 with HA groups showed more new bone area compared with other groups. BCP block showed adverse effect on new bone formation. The effect of HBO on new bone area seemed minimal.
2. FGF-2 with HA groups showed more new bone length than other groups. BCP block seemed to have adverse effect on new bone formation. The effect of HBO on new bone length seemed minimal.
3. Generally, HBO groups showed more angiogenesis than NBO groups. However, when FGF-2 with HA was applied with HBO therapy, angiogenesis was not improved compared with HBO alone or FGF-2 with HA alone.

Conclusion: Within the limitations of this study, HBO had beneficial effects on angiogenesis. However, the effect of HBO on bone regeneration seemed minimal in the irradiated rat model of the present study. It showed that FGF-2 with HA enhanced bone generation and angiogenesis, but its efficacy was decreased with HBO. BCP block did not show any beneficial effect on bone regeneration.

Key words : radiotherapy, irradiated calvarial defect, bone regeneration, BCP block, fibroblast growth factor-2, hyaluronic acid, hyperbaric oxygen therapy

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I. Introduction

In Korea, the cancer has been one of the main causes of death and the incidence of that has been increasing gradually.[1] On the other hand, the 5-year

relative survival rate of 60.1% in the oropharyngeal cancer during 2006-2010 is higher than that of 41.1% during 1993-1995.[2] The improving survival rates could be attributable to early detection and improved treatments including a combination of surgical resection, radiotherapy, and chemotherapy.

For the treatment of head and neck cancer, radiotherapy in combination with surgical excision is generally used for the cosmetic and functional outcome. However, radiation therapy result in acute reactions including mucositis, xerostomia, and chronic reactions leading to reduced tissue healing capacity, avascular necrosis.[3]

The mechanism of retarded bone healing after irradiation has been explained that damage of the osteoprogenitor cells or reduced neovascularization may be a important factor, and that the normally balanced dynamic process of bone resorption and reformation may be disturbed.[4] It was shown that average change in bone formation appeared as -70.9% within a 4-week period after irradiation in rabbits.[5] The result that bone mineral density in the defect of irradiated rats was reduced, and the woven bone and immature marrow formed, indicating impaired bone was reported.[6] Likewise, for irradiated patients in the oral & maxillofacial field, their healing capacity of the bones has been markedly decreased. Thus, the rehabilitation after surgical resection and radiotherapy requires more attention when it is treated with implants and guided bone regeneration procedure. In those cases, means to improve reduced healing potential are required. It may involve improving the capacity of the graft materials or the management on the subjects.

Biphasic calcium phosphate (BCP) which is one of synthetic bone substitutes is composed of hydroxyapatite and β-tricalcium phosphate. BCP has been showing favorable results from previous studies.[7,8] However, BCP doesn't have any osteoinductive capacity. This is the one of drawbacks of synthetic bone substitutes. Recently various attempts were made to enhance the osteogenic potential of these synthetic bone substitutes. It is one of the methods to coat it with peptides such as BMP, oligopeptide, Epigallocatechin-3-gallate (EGCG). These coating methods showed favorable results in the previous studies.[9,10,11] Another drawback of BCP is that this BCP bone substitute is supplied as a particulated form with which BCP could not be maintained properly in the defects without bony walls. The newly devised BCP block was fabricated with adding of collagen to make BCP particle not be dissipated. This BCP block has better capacity to maintain space for bone formation. However, adding collagen might delay the bone formation because the collagen should be resorbed first before new bone forms in the space.[12,13]

It has been demonstrated that Fibroblast growth factor-2 (FGF-2) is a effective angiogenic and osteogenic growth factor.[14] The authors concluded FGF-2 induced angiogenesis and new bone formation in calvarial defects of rats. FGF-2 was considered to contribute to wound healing by the mechanism of stimulating the proliferation of mesenchymal cells.[14] Anzai *et al.* asserted that FGF-2 could be effective not only in normal bone but also in irradiated bone.[43] They reported that the group with FGF-2 showed more new bone

formation than the control without FGF-2 in irradiated calvarium of rats.[43] In a clinical study, the local application of FGF-2 with periodontal surgery showed favorable outcomes with significant bone fill in comparison with control group.[16]

Hyaluronic acid (HA) found in the extracellular matrix throughout the body is a naturally occurring biodegradable polymer, responsible for structural properties of tissues as a component of extracellular matrix.[17] It is also known as hyaluronan or hyaluronate. It is usually applied in a pure form or in a biofunctionalized form.[18] Its biocompatibility and relative ease of use make it a popular injectable dermal filler,[19] and it has been also applied in the management of osteoarthritis, replacing dysfunctional synovial fluid.[20] Also it has other roles as follows. 1) Effect on angiogenesis: A novel composite biomaterial based on HA-hydrogel has been developed to promote angiogenesis in order to improve wound healing.[18] 2) Osteoconductive potential: HA promotes the bone regeneration in the way of chemotaxis, proliferation and differentiation of mesenchymal cells.[21] With the characteristics of hygroscopicity and viscoelasticity, it plays an essential role in regenerative process by maintaining spaces and the structural integrity of tissues.[23] Furthermore as a resorbable material, it is eventually replaced by host bone.[18] 3) Carrier function : HA may be carried out as biomaterial scaffold for other molecules, such as FGF-2, employed in GBR techniques and tissue engineering.[22]

Radomsky et al. introduced a viscous gel composed of FGF-2 and hyaluronate as a novel treatment of injection into fractured bones.[15] In the

fibulae of baboons with experimentally created a bilateral 1-mm-gap, a single administration of this formulated gel to the defect site accelerated fracture healing as proof of increased callus formation and physical solidity than at the untreated sites.[15]

Hyperbaric oxygen therapy (HBO) could be regarded as an method for the management on the irradiated subjects. It has been known that irradiated bone had hypocelluar, hypovascular, and hypoxic properties.[45] In addition, it is asserted that osteoblastic activity might be regulated by oxygen tension.[46] The therapy proceed with inhalation of increased oxygen to permit systemic diffusion and delivery of that.[24] It is usually carried out in a chamber with 100% oxygen at the absolute atmospheric pressure between 2.0 and 2.5 ATA for 90-minute session.[24] The volume of oxygen combined to hemoglobin in blood is generally around 20 % and the one dissolved in plasma is elevated with pressure.[24] The P_{O2} in the irradiated tissues is normally 5 to 15 mmHg. The P_{O2} in the tissues after hyperbaric oxygen therapy was elevated to 20 to 35 mmHg.[25] It is known that inhalation of oxygen greater than 1 ATA will promote to produce reactive oxygen species, playing a role as signaling molecules for various growth factors, cytokines, and hormones.[26] It is also considered that hyperbaric oxygen-mediated oxidative stress stimulates the differentiation of circulating stem/progenitor cells and the growth of new blood vessel by local endothelial cells.[26] These results have provided a basis for efficacy of HBO. However, there is a controversy about the efficacy of HBO. In animal studies, HBO showed that it promoted bone formation in normal

bone[27] and angiogenesis in irradiated bone.[44] To the contrary, others argue that there has been limited evidence to support its benefit.[47,48] In a clinical study, it was ineffective to treat the mandibular radionecrosis in a randomized, place-controlled trial.[28] Generally HBO has no serious problems, but several side effects, such as reversible myopia, barotrauma can occur.[29] The average costs of HBO are between $300 and $400 per each 90-minute session.[29]

The purpose of this study was to evaluate the effect of the grafted materials and HBO on the bone healing in experimentally created calvarial defects in irradiated rats.

Ⅱ Materials and Methods

2.1. Experimental animals & materials

The study was carried out on Sprague-Dawley rats (body weight about 300g, 4weeks old, male) and approved by the Institutional Animal Care and Use committee of the Yonsei Medical Center, Seoul, Korea. All experimental procedures proceeded according to the guidelines for animal experiments of Yonsei University, College of Dentistry.

One of the graft materials was a gel of 0.07% FGF-2 (Genoss Co., Suwon, Korea) with 2% HA (Genoss Co., Suwon, Korea) and the other was a BCP block. The molecular weight of HA is 3,000 kDa. A BCP block [Osteon^{TM} II collagen (Genoss Co., Suwon, Korea)] was cut to the dimension of the defect with 6-mm diameter & 1.5-mm height. The BCP block is reported to be composed of synthetic bone graft and bovine Type I collagen. HA collagen membrane (Genoss Co., Suwon, Korea) was used bilaterally.

Rats were divided into the group with HBO and the group without HBO (NHBO). Each group had two subgroups which were FGF-2 with HA group and BCP block group according to the graft. Localized radiation with a single dose of 12 Gy radiation was applied to the calvarium. Four weeks after radiation, two symmetrical, circular defects of 6-mm-diameter were created in the parietal bones of animal. The left defect was left empty as a control and the right defect was filled with the materials mentioned above.

Group 1 : (FGF-2 + HA) without HBO

Group 2 : BCP block without HBO

Group 3 : (FGF-2 + HA) with HBO

Group 4 : BCP block with HBO

Table 1. Experimental design

Group	Number	Control (Left defect)	Experimental (Right defect)	Hyperbaric oxygen therapy
1	n=5	no graft	0.07% FGF-2 + 2% HA	No (NHBO)
2	n=5	no graft	BCP	No (NHBO)
3	n=5	no graft	0.07% FGF-2 + 2% HA	Yes (HBO)
4	n=5	no graft	BCP	Yes (HBO)

2.2. Protocol for animal irradiation

After the induction of general anesthesia with intraperitoneal injection of an anesthetic cocktail composed of Rompun® (xylazine, Bayer, Leverkusen, Germany) and Zoletil® (tiletamine and zolazepam, Virbac Lab., Carros, France). Rats were immobilized with a customized fixation device (Figure 1) and the radiation fields were verified using an external beam simulator (Nucletron, Veenendaal, the Netherlands). They received localized radiation with a single dose of 12 Gy radiation to the calvarium. The irradiated protocol of calvarium of rats was as follows. The sedated rat was placed in the fixation device to keep the rat in the prone and head-first position and the head of the rat was immobilized. Using a 2D simulator (Simulix Evolution, Nucletron, Veenendaal, Netherlands), the position of the calvarium in the irradiation field was verified, and the treatment center was marked. The rat and the fixation device were transferred to a 6-MV medical linear accelerator (Elekta Synergy, Elekta Oncology systems Ltd, Stockholm, Sweden) commonly used for treatment in humans. A 0.5cm tissue equivalent bolus was placed on top of the rat's head to ensure electronic equilibrium. Using a 2cm x 2cm field size, a radiation dose of 12Gy in a single fraction was delivered to the calvarium of the rat in two parallel-opposed, equally weighted lateral fields.

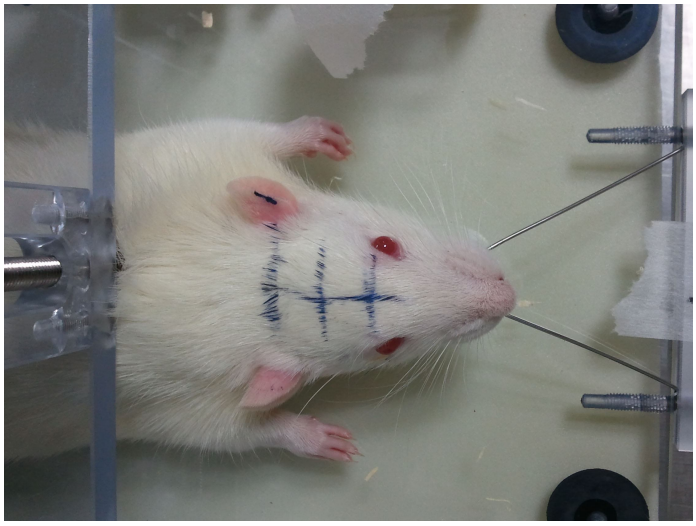


Figure 1. Customized fixation device for local irradiation

2.3. Surgical procedure

Four weeks after radiation, the surgical procedures were conducted under general anesthesia through the intraperitoneal injection of an anesthetic mixture composed of Rompun® and Zoletil®. The surgical area was shaved, and the skin was washed with 70% ethanol. An intraperitoneal injection of 0.9mL of lidocaine (1:100,000 epinephrine, Yuhan Co., Seoul, Korea) was administered to control bleeding and provide additional anesthesia. A 20-mm-long midline incision was made in the scalp along the sagittal suture and the flaps were reflected laterally. Then two symmetrical, circular(6-mm diameter), transosseous bone defects were created in both parietal bones with a surgical trephine bur under saline irrigation (Figure 2). The midsagittal suture was not included in the bone defect to avoid the damage to the dura mater. The left experimental

defects were left empty as a control and the right defects were filled with FGF-2 with HA or BCP block. The skin flaps were repositioned and sutured with non-resorbable suture material, the sutures were removed after 10 days.

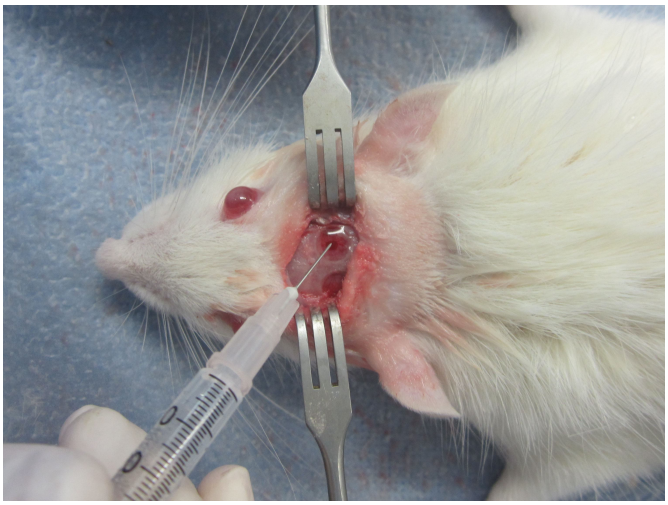


Figure 2. Intraoperative view of the defects

All rats under the study were given free access to food pellets and tap water, housed and taken care of at the animal experimental laboratory of Yonsei University, College of Dentistry, Seoul, South Korea.

The animals were sacrificed after these 4 weeks of healing. For euthanization, the rats were perfused transcardially with 4% paraformaldehyde under general anesthesia. The skin was removed from the calvarium and the calvarial specimens were harvested for histomorphometric and micro-CT analysis. Tissue samples were fixed in 10% formalin for 24 hours. And the

specimens were decalcified by soaking in formic acid-hydrochloride acid for 24 hours and embedded in paraffin.

2.4. Hyperbaric oxygen therapy procedure

During 4 weeks of healing from the graft surgery, the rats belonged to HBO groups were placed in a experimental hyperbaric chamber and exposed to 100% oxygen at 2.4 atmosphere absolute (ATA) for one hour per day for 5 times a week. The other NHBO groups were left at room as usual. The HBO therapy was begun from the next day of operation in chamber designed for experiment (Figure 3).

Figure 3. Experimental hyperbaric chamber for HBO

2.5. Histological analysis

All specimens were decalcified with 10% EDTA at 4 ℃ for one month. The decalcified specimens were first embedded in paraffin wax, and then a series of 5-㎛ thick sections were prepared. The specimens were stained with Hematoxylin-Eosin (H&E) stain. In addition, antigen retrivals were performed with 0.1M citrate buffer and immuno-histochemistry with CD31 (Santa Cruz Co.) as a primary antibody was performed. The stained specimens were observed with Olympus BX51 microscope. New bone area & length were measured with Tomoro scope eye image analysis system after taking images with TDI digicam HQ camera in H&E stain (x40) (Figure 4, 5). Blood vessels were counted at both defect side of 1-mm width in IHC with CD31 (x100) (Figure 6).

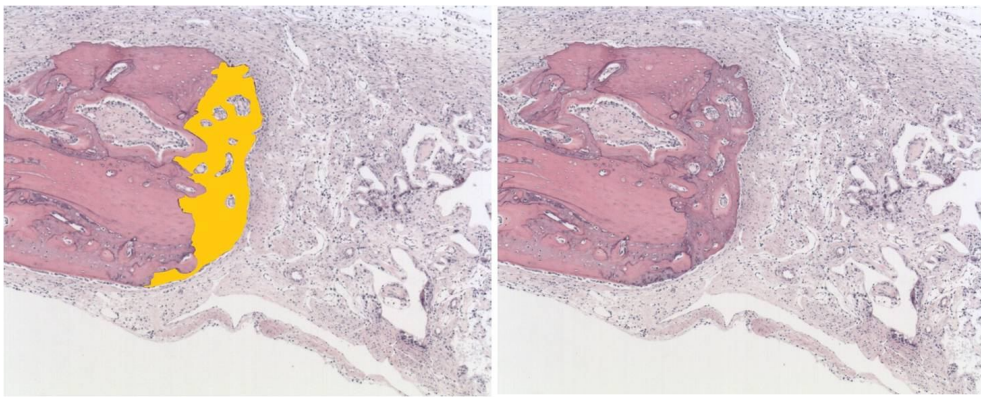


Figure 4. Method of measuring new bone area

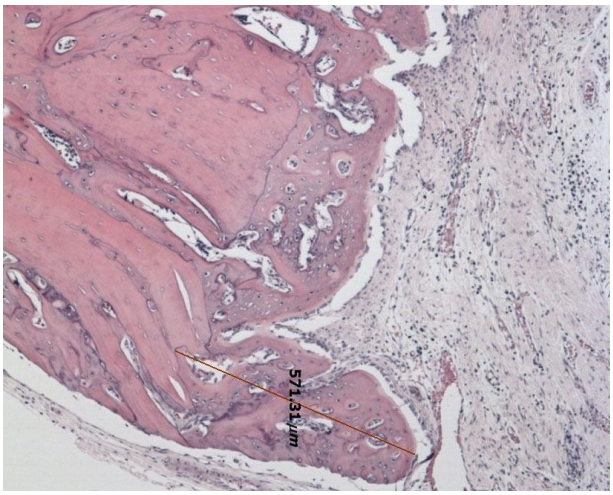
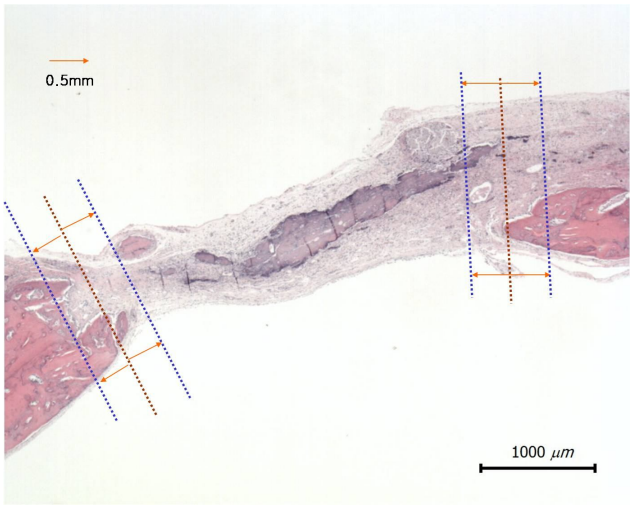
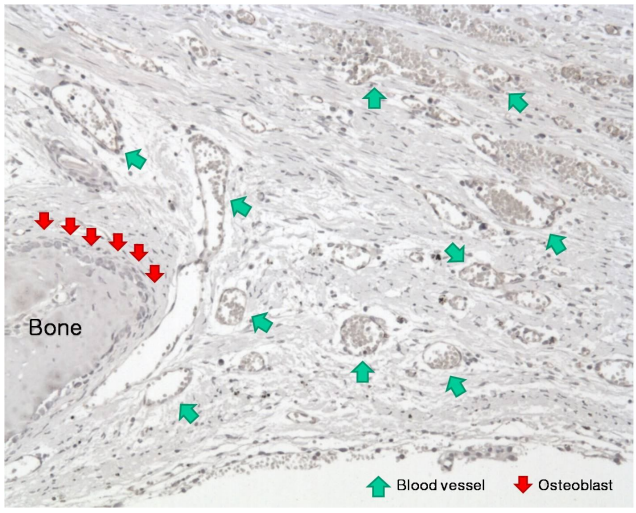


Figure 5. Method of measuring new bone length



(a) Both defect sides to measure



(b) IHC with CD31, x100

Figure 6. Method of counting blood vessel

2.6. Histometrical analysis

Before decalcification of the specimens, a 3-dimensional micro-CT image was taken for each rat using a micro-CT scanner (Skyscan 1173, Kartuizersweg 3B 2550 Kontich, Belgium). The exposure parameters were 130 kV and 45μA. This was reconstructed with a 3D-software to obtain volumetric information.

2.7. Statistical analysis

All data were recorded as mean ± standard deviation.

Ⅲ Results

3.1. Histometrical analysis

(1) New bone area

Table II. New bone area

Group			New bone area (mm^2)
NHBO	Group 1	HA + FGF-2	0.34 ± 0.09
	Group 2	BCP	0.17 ± 0.11
	Control	no	0.29 ± 0.12
HBO	Group 3	HA + FGF-2	0.36 ± 0.26
	Group 4	BCP	0.17 ± 0.04
	Control	no	0.30 ± 0.06

FGF-2 with HA groups showed more new bone area compared with other groups. BCP block showed adverse effect on new bone formation. The effect of HBO on new bone area seemed minimal.

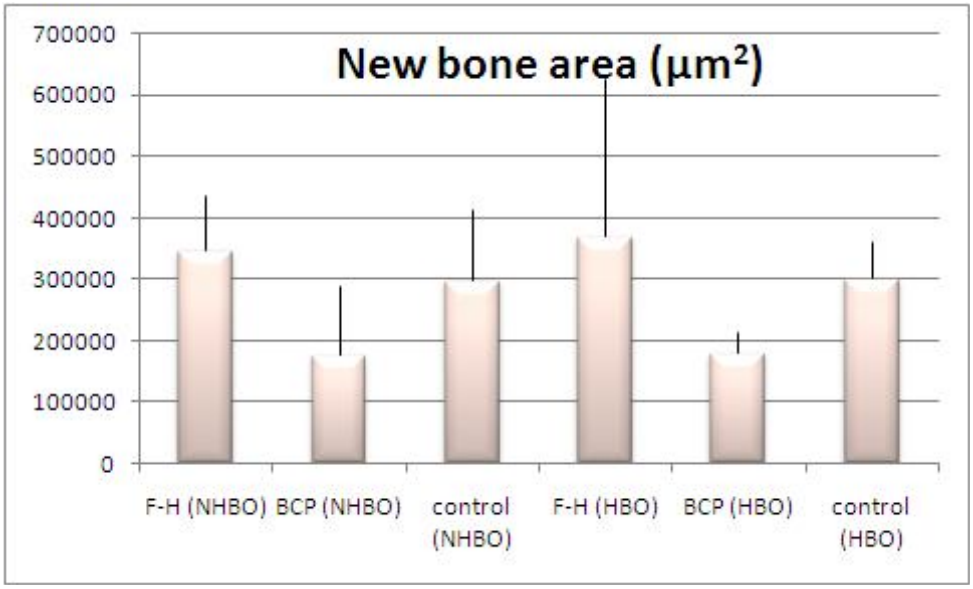


Figure 7. New bone area

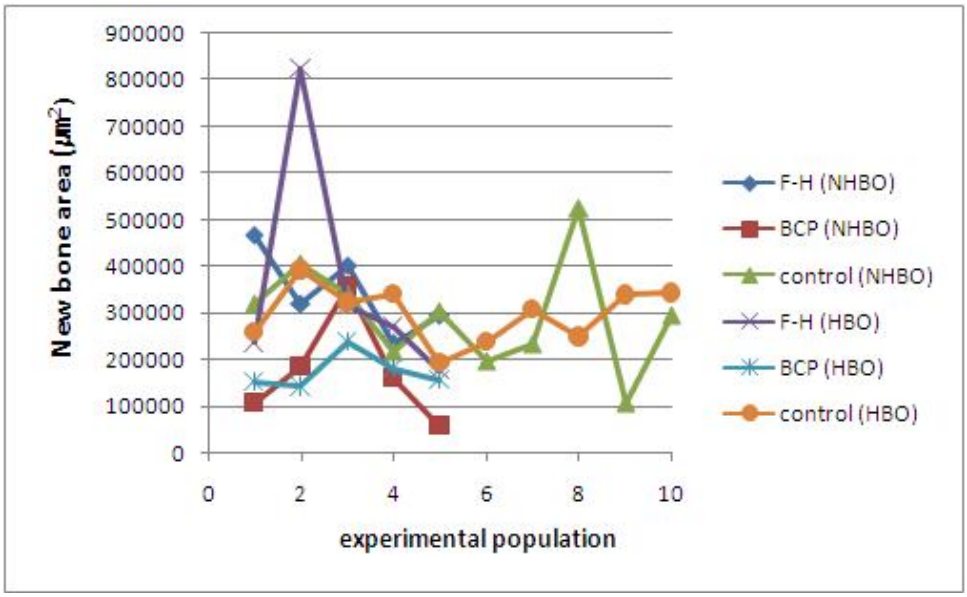


Figure 8. Scatter diagram of new bone area

(2) New bone length

Table III. New bone length

Group			New bone length (mm)
NHBO	Group 1	HA + FGF-2	1.00 ± 0.34
	Group 2	BCP	0.51 ± 0.18
	Control	no	0.81 ± 0.19
HBO	Group 3	HA + FGF-2	0.93 ± 0.26
	Group 4	BCP	0.58 ± 0.15
	Control	no	0.84 ± 0.30

FGF-2 with HA groups showed more new bone length than other groups. BCP block seemed to have adverse effect on new bone formation. The effect of HBO on new bone length seemed minimal.

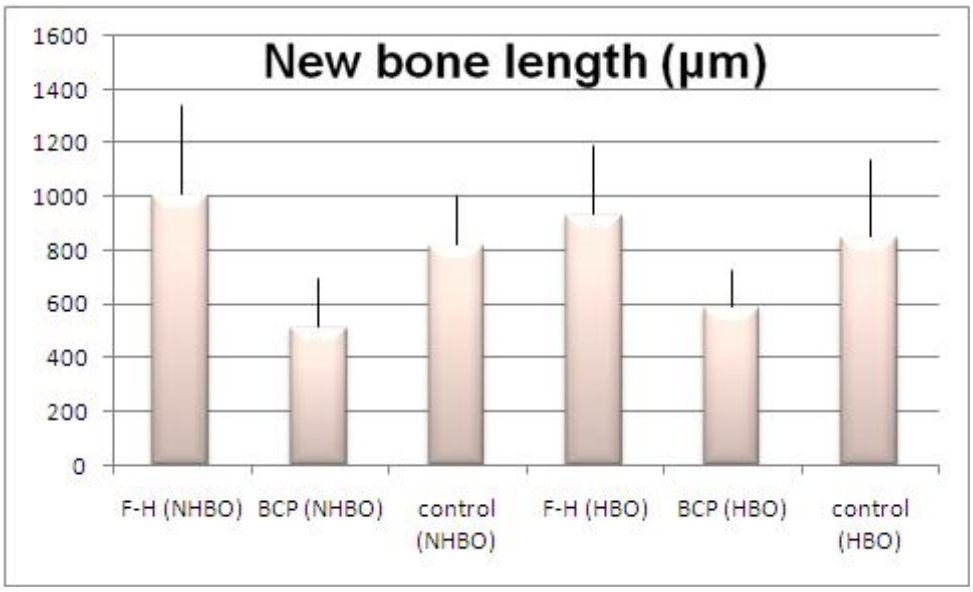


Figure 9. New bone length

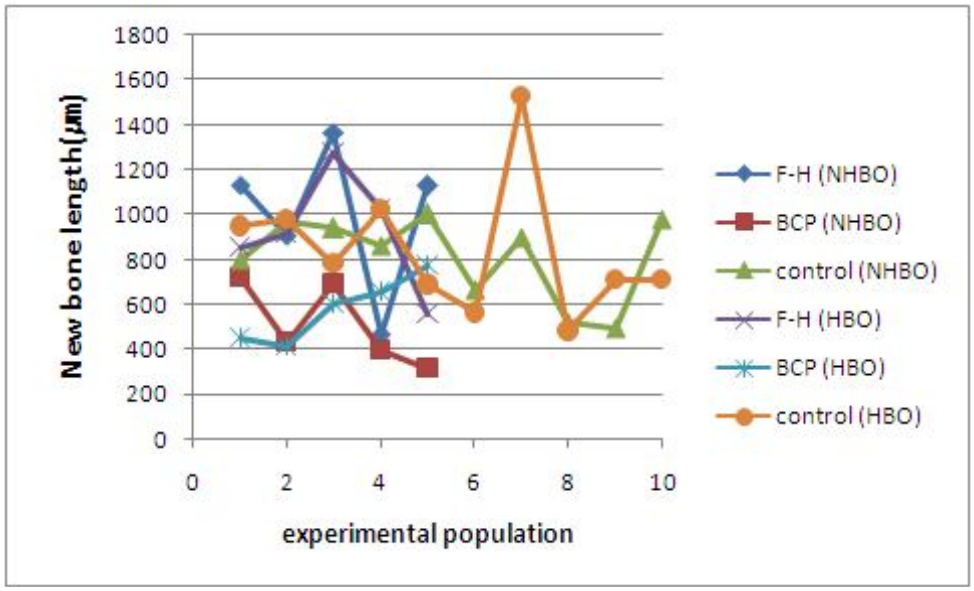


Figure 10. Scatter diagram of new bone length

(3) Numbers of blood vessel

Table IV. Numbers of blood vessel

Group			number (n)
NHBO	Group 1	HA + FGF-2	163.2 ± 43.0
	Group 2	BCP	161.8 ± 28.1
	Control	no	122.3 ± 27.9
HBO	Group 3	HA + FGF-2	153.2 ± 50.8
	Group 4	BCP	234.0 ± 41.1
	Control	no	177.3 ± 49.8

Generally, HBO groups showed more angiogenesis than NHBO groups. However, when FGF-2 with HA was applied with HBO therapy, angiogenesis was not improved compared with HBO alone or FGF-2 with HA alone.

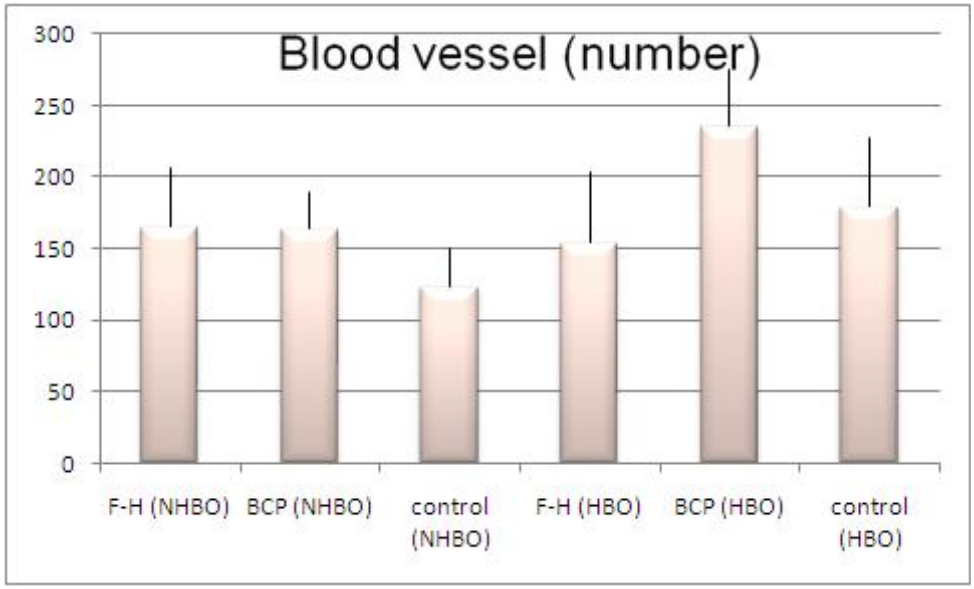


Figure 11. Numbers of blood vessel

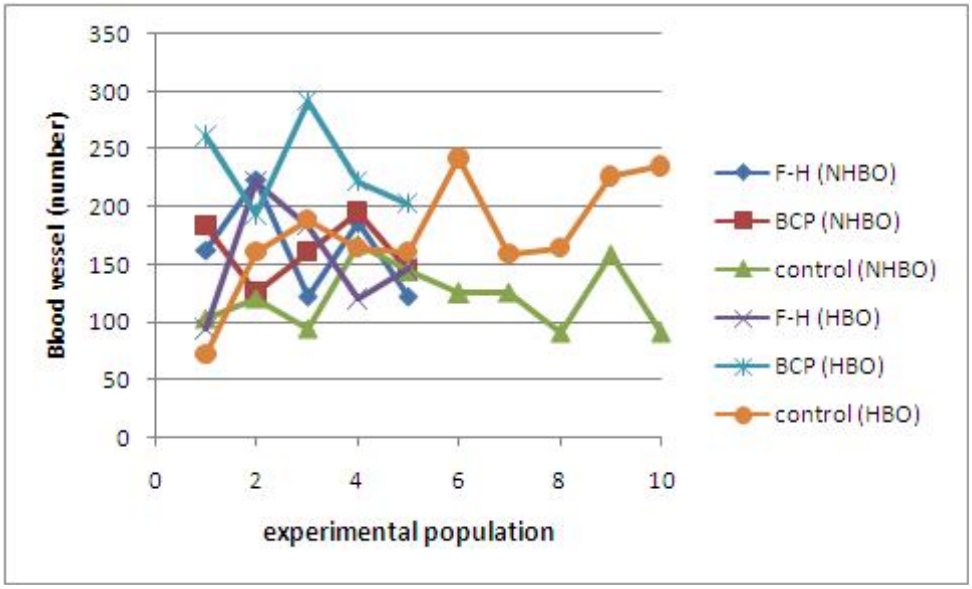
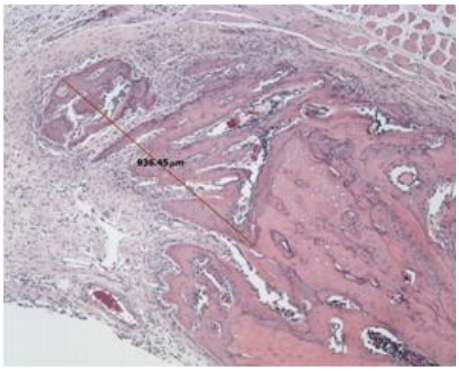
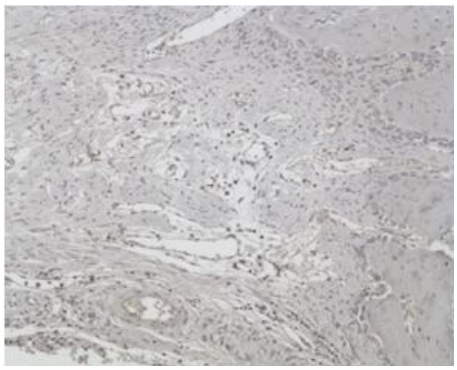


Figure 12. Numbers of blood vessel

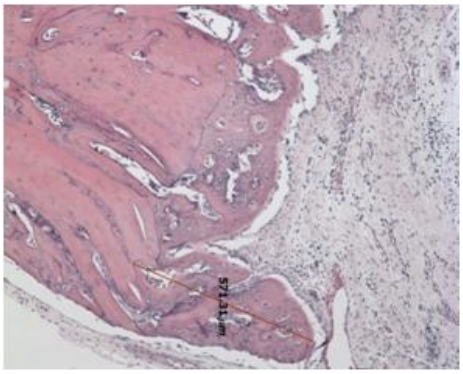
3.2. Histomorphological analysis



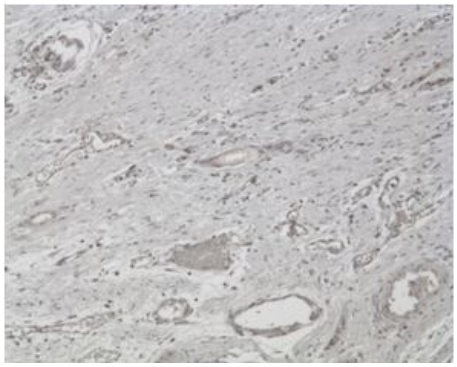
(a) experimental defect
(H&E, x40)



(b) experimental defect
(IHC with CD31, x100)



(c) control defect
(H&E, x40)



(d) control defect
(IHC with CD31, x100)

Figure 13. Histological images of FGF-2 with HA (NHBO) group at 4 weeks

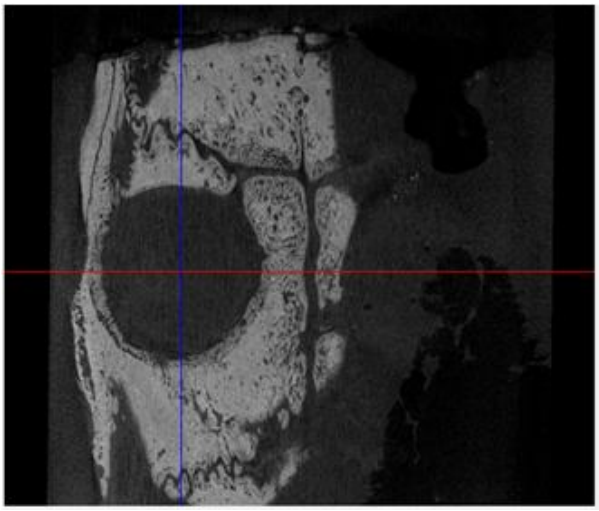
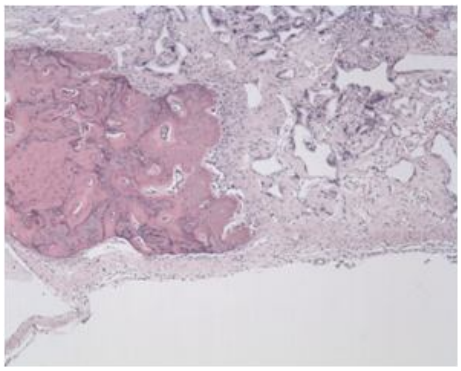
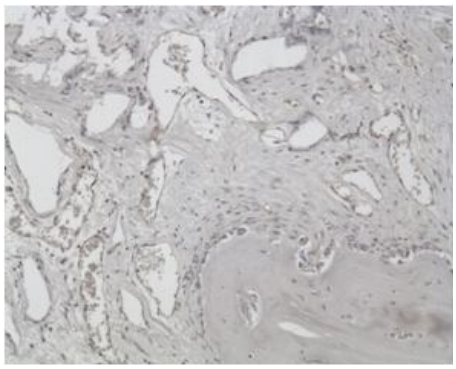


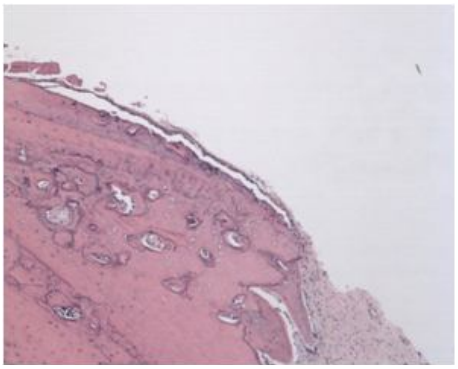
Figure 14. An example of micro-CT images of experimental region in FGF-2 with HA (NHBO) group at 4 weeks



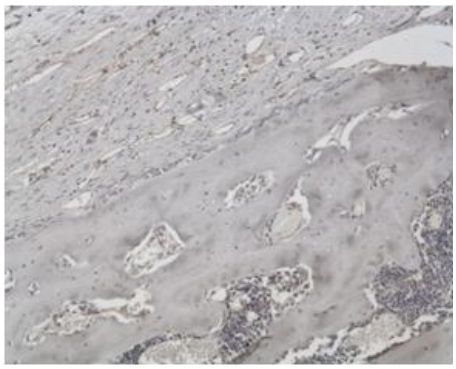
(a) experimental defect
(H&E, x40)



(b) experimental defect
(IHC with CD31, x100)



(c) control defect
(H&E, x40)



(d) control defect
(IHC with CD31, x100)

Figure 15. Histological images of BCP (NHBO) group at 4 weeks

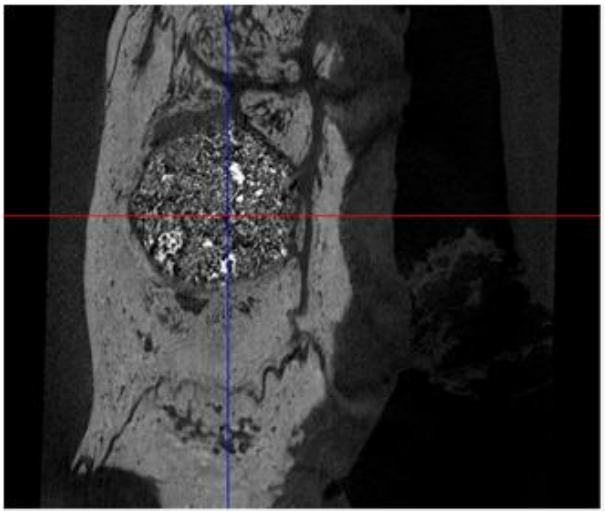
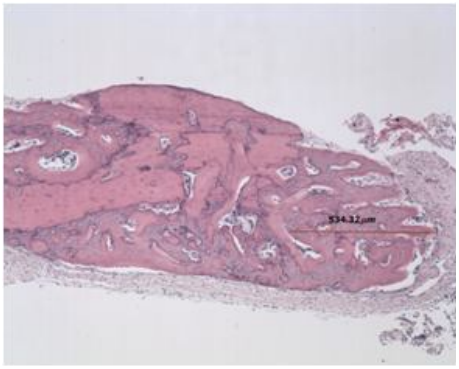
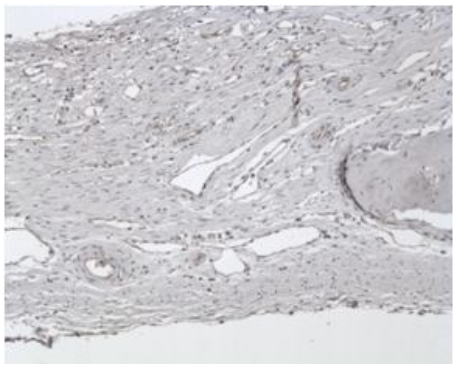


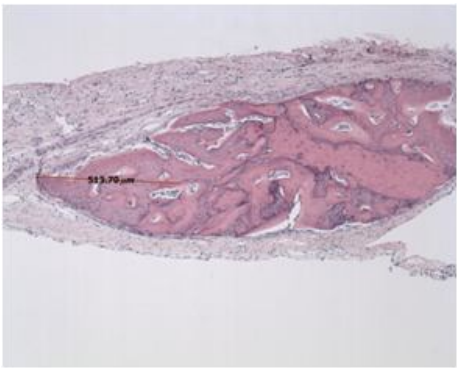
Figure 16. An example of micro-CT images of experimental region in BCP (NHBO) group at 4 weeks



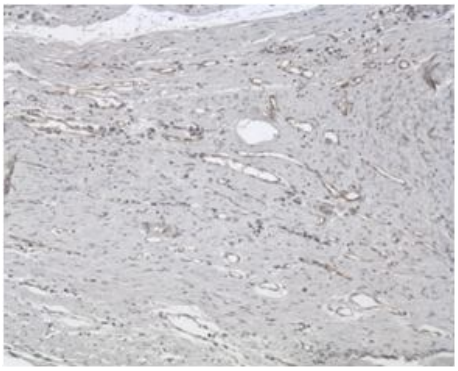
(a) experimental defect
(H&E, x40)



(b) experimental defect
(IHC with CD31, x100)



(c) control defect
(H&E, x40)



(d) control defect
(IHC with CD31, x100)

Figure 17. Histological images of FGF-2 with HA (HBO) group at 4 weeks

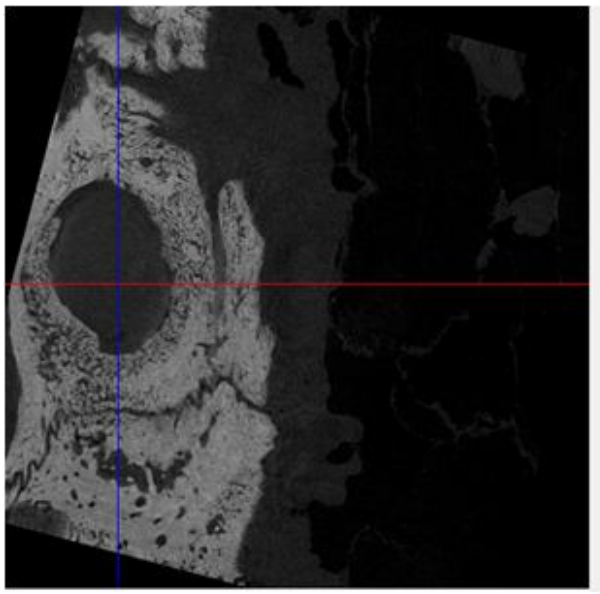
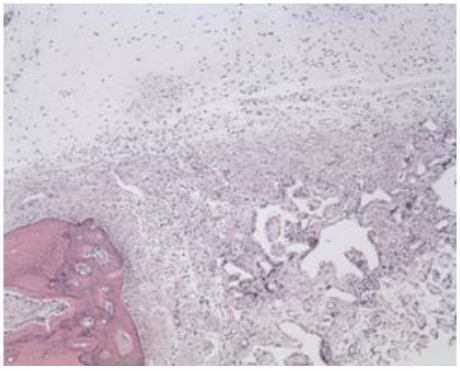
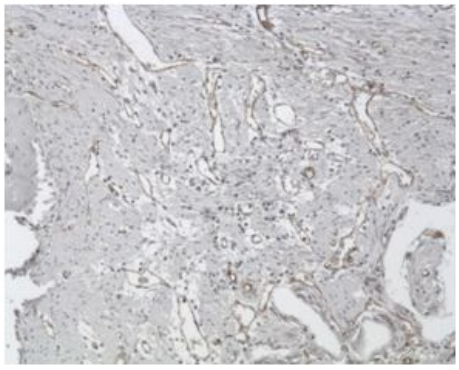


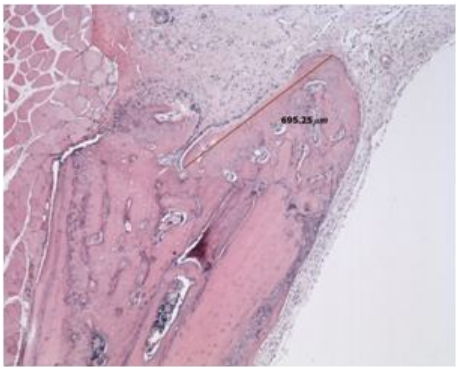
Figure 18. An example of micro-CT images of experimental region in FGF-2 with HA (HBO) group at 4 weeks



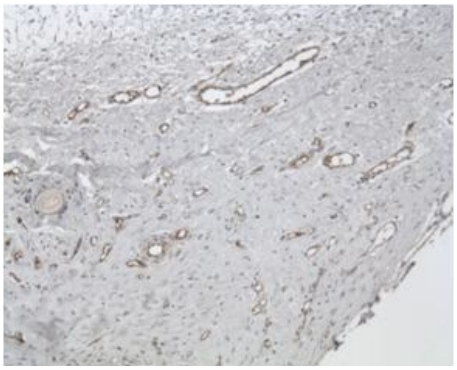
(a) experimental defect
(H&E, x40)



(b) experimental defect
(IHC with CD31, x100)



(c) control defect
(H&E, x40)



(d) control defect
(IHC with CD31, x100)

Figure 19. Histological images of BCP (HBO) group at 4 weeks

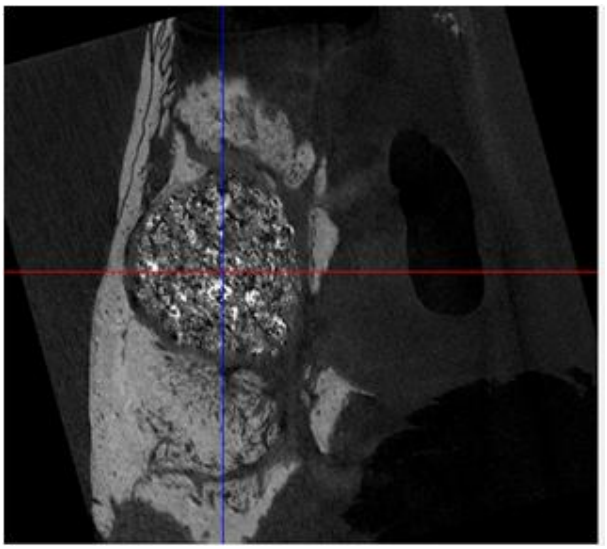


Figure 20. An example of micro-CT images of experimental region in BCP (HBO) group at 4 weeks

IV. Discussion

To rehabilitate irradiated patients in the oral & maxillofacial field with implants is not contraindicated anymore. It showed 92.6% of 5 year survival rates of implant in a clinical study.[50] But healing capacity of the bones has been markedly decreased on irradiated patients. More attention is needed to rehabilitate irradiated patients. Therefore, appropriate selection of bone substitute, treatment modality, treatment sequence are mandatory.

In the present study, FGF-2 with HA, BCP block was applied to the calvarial defect of irradiated rat. HBO therapy was also tried.

FGF-2 with HA was applied in the present study, because FGF-2 is considered to have effect on angiogenesis and osteogenesis. In other study, it was concluded that FGF-2 considered to contribute to wound healing by the mechanism of stimulating the proliferation of mesenchymal cells induced angiogenesis and new bone formation in calvarial defects of rats.[14] The result of the present study appeared similarly to that of other study, reported that the group with FGF-2 showed more new bone formation compared with the groups without FGF-2 in irradiated calvarium of rats.[43] In the present study, it was found that the groups with FGF-2 with HA showed increased blood vessels in the defect. This means that there is a possibility of inducing bone formation later. It is thought that to compare results at variety of healing period might be meaningful in a further study. And there was a study shown that bone formation decreased at the level of -70.9% within a 4-week period

after irradiation in rabbits.[5] Like the present study, as FGF-2 with HA did lead to angiogenesis in irradiated tissue, it could be regard to be effective in normal condition.

Hyaluronic acid (HA) is classified with molecular weight, which is above 2 x 10^6 Da as a high molecular weight, whereas below 3 x 10^5 Da is as a low molecular weight.[34] Whereas high molecular weight suppresses angiogenic effect, low molecular weight HA encourage an angiogenic response.[35] HA used in the present study is high molecular weight of 3 x 10^6 Da. The mechanism of HA to affect the angiogenesis was explained that it could induce early-response genes expression.[36] Pilloni *et al.* referred that HA of appropriate molecular weight with optimal concentration could promote osteoblast differentiation and bone formation.[37] In a similar experiment, it was demonstrated that it might be weight and dose-specific to enhance the osteogenic properties of bone graft materials owing to its stimulatory effects on osteoblasts.[38] So it deserves consideration to determine the dose and weight of HA before application depending on the purpose. In a animal study, HA was applied on extraction sockets of rats, and the application of it made the amount of bone trabeculaes and the number of blood vessel increased.[21] HA has a distinctive characteristics. It shows osteogenic properties itself, on the other hand it act as a scaffold. While de Brito Bezerra *et al.* used collagen sponge as a scaffold to evaluate the healing capacity of HA,[39] Hunt *et al.* used HA as biomaterial scaffold for other molecules.[22] Our study administered

the HA for two purposes, one thing was to observe the osteogenic effects of itself and the other was to use as a scaffold of FGF-2.

It was showed that FGF-2 with HA improved angiogenesis gene rally and specifically around defect margin where most of new bone formation occurs. New bone formation (area, length) was also improved by FGF-2 with HA. Even in the challenging bony defect of present study, FGF-2 with HA showed beneficial effects on bone healing. From the results of present study, FGF-2 with HA can be considered as an additive material when we rehabilitate irradiated bone.

This BCP block was applied in the present study because BCP block is not dissipated from defect as particulated BCP is. Most of the reconstruction after resective surgery and radiotherapy are performed on bony defect without bony walls. In this clinical situation, bone substitute cannot be maintained in the defect property. BCP block can be considered as a material of choice when we consider dissipation of bone substitute. However, BCP block is composed of BCP particles and collagen. This collagen is resorbed first. New bone forms in the space which is left after collagen resorption. BCP particles are resorbed after collagen resorption. Therefore, it requires more time for new bone formation when BCP block is applied than particulated BCP. In the present study, healing capacity of irradiated bone decreased into less than 50% of original capacity when we estimate based on the previous studies.[5] Four weeks of healing time for this challenging defect and bone substitute which requires more time for new bone formation were not enough to show any effects of

applied BCP block on bone healing. In the future study, more healing time for BCP block on this challenging defect. Increase of vascularity in BCP block groups can be considered as a process for collagen resorption, because more blood vessels were found around bone substitute than defect margin. It is not certain whether this healing process will be continued to new bone formation around bone substitute. More healing time is required to make sure this new bone formation in the future study. Less new bone area and length in BCP block groups than FGF-2 with HA groups can also be attributed to delayed new bone formation of BCP block. Different results are expected when 8 weeks of healing time are applied.

HBO didn't show significant improvement in new bone formation. It is controversial to use HBO therapy. In some studies, HBO therapy induced more new bone formation.[49,51,52] In other studies, there has been limited evidence to support its benefit.[47,48] In the present study, HBO didn't demonstrate any beneficial effect on new bone formation. If the healing time had been more than 4 weeks, the result could have been different. In a previous study, healing time was 6 and 12 weeks and it showed favorable effects of HBO on new bone formation.[51] If the frequency and duration of HBO had been different, the result could have been different. In a previous study, HBO therapy was applied for 90 minutes per day 5 times a week for 4 weeks.[52] The result in that study showed more favorable effects on new bone formation. In the future study, other protocol of HBO should be tried. With considering different data from various HBO therapies, the effect of HBO on

new bone formation could be discussed better. Increased angiogenesis was found in HBO groups. It is not certain whether this increased vascularity would affect new bone formation favorably or not. However, from previous studies, increased vascularity might be associated with faster healing and more new bone formation. With longer healing time than 4 weeks, new bone formation in the rat which had more vascularity in 4 week could be ascertained in the future study.

When FGF-2 with HA was applied with HBO therapy, new bone formation was less than FGF-2 with HA alone. Angiogenesis in the group of FGF-2 with HA and HBO therapy was also less than FGF-2 with HA or HBO therapy alone. This result is in accordance with a previous study which showed unfavorable effects on healing when HBO and FGF-2 with HA were applied.[53] It was reported that bFGF was more effective compared with HBO to improve bone growth after radiation. In addition, it was reported that HBO didn't provide additive beneficial effect to bFGF therapy and explained that both modalities affect through a similar pathway. From the data of present study, applying simultaneously material or modality which have a similar effect could not be recommended.

In the future study, longer healing time, different protocol of HBO therapy, different type of BCP, should be considered. More valuable data would be obtained when compared with those of present study.

In the present study, there are several limitations. First, this study was performed with a small number of samples in each group. Second, it was

aimed to assess the factors influencing on bone healing in the irradiated calvarial defect. As the considerations were getting more, it was hard to control the variables. Considering the limitations, further long-term studies needs to be carried out.

V. Conclusion

Within the limitations of this study, the following conclusions can be drawn:

1. HBO had beneficial effects on angiogenesis.

2. The effect of HBO on bone regeneration seemed minimal in the irradiated rat model of the present study.

3. FGF-2 with HA enhanced bone generation and angiogenesis, but its efficacy was decreased with HBO.

4. BCP block did not show any beneficial effect on bone regeneration.

References

1. Statistics Korea [Internet]. Daejeon: Statistics Korea; 2012 [cited 2013 Oct 14]. Available at:http://kostat.go.kr
2. Jung KW, Won YJ, Kong HJ, Oh CM, Seo HG, Lee JS. Cancer Statistics in Korea: Incidence, Mortality, Survival and Prevalence in 2010. *Cancer Res Treat.* 2013 Mar;45(1):1-14.
3. Anderson L, Meraw S, Al-Hezaimi K, Wang HL. The influence of radiation therapy on dental implantology. *Implant Dent.* 2013 Feb;22(1):31-8.
4. Granström G, Jacobsson M, Tjellström A. Titanium implants in irradiated tissue: benefits from hyperbaric oxygen. *Int J Oral Maxillofac Implants.* 1992 Spring;7(1):15-25.
5. Jacobsson MG, Jönsson AK, Albrektsson TO, Turesson IE. Short- and long-term effects of irradiation on bone regeneration. *Plast Reconstr Surg.* 1985 Dec;76(6):841-50.
6. Hu WW, Ward BB, Wang Z, Krebsbach PH. Bone regeneration in defects compromised by radiotherapy. *J Dent Res.* 2010 Jan;89(1):77-81.
7. Nevins M, Nevins ML, Schupbach P, Kim SW, Lin Z, Kim DM. A prospective, randomized controlled preclinical trial to evaluate different formulations of biphasic calcium phosphate in combination with a hydroxyapatite collagen membrane to reconstruct deficient alveolar ridges. *J Oral Implantol.* 2013 Apr;39(2):133-9.
8. Mangano C, Perrotti V, Shibli JA, Mangano F, Ricci L, Piattelli A, Iezzi G. Maxillary sinus grafting with biphasic calcium phosphate ceramics: clinical and histologic evaluation in man. *Int J Oral Maxillofac Implants.* 2013 Jan-Feb;28(1):51-6.
9. Choi H, Park NJ, Jamiyandorj O, Choi KH, Hong MH, Oh S, Park YB, Kim S. Improvement of osteogenic potential of biphasic calcium phosphate bone substitute coated with two concentrations of expressed recombinant human bone

morphogenetic protein 2. *J Periodontal Implant Sci.* 2012 Aug;42(4):119-26.
10. Choi H, Park NJ, Jamiyandorj O, Hong MH, Oh S, Park YB, Kim S. Improvement of osteogenic potential of biphasic calcium phosphate bone substitute coated with synthetic cell binding peptide sequences. *J Periodontal Implant Sci.* 2012 Oct;42(5):166-72.
11. Shin YS, Seo JY, Oh SH, Kim JH, Kim ST, Park YB, Moon HS. The effects of ErhBMP-2-/EGCG-coated BCP bone substitute on dehiscence around dental implants in dogs. *Oral Dis.* 2013 Apr 8. [Epub ahead of print]
12. Clergeau LP, Danan M, Clergeau-Guérithault S, Brion M. Healing response to anorganic bone implantation in periodontal intrabony defects in dogs. Part I. Bone regeneration. A microradiographic study. *J Periodontol.* 1996 Feb;67(2):140-9.
13. Hartman GA, Arnold RM, Mills MP, Cochran DL, Mellonig JT. Clinical and histologic evaluation of anorganic bovine bone collagen with or without a collagen barrier. *Int J Periodontics Restorative Dent.* 2004 Apr;24(2):127-35.
14. Kigami R, Sato S, Tsuchiya N, Yoshimakai T, Arai Y, Ito K. FGF-2 angiogenesis in bone regeneration within critical-sized bone defects in rat calvaria. *Implant Dent.* 2013 Aug;22(4):422-7.
15. Radomsky ML, Aufdemorte TB, Swain LD, Fox WC, Spiro RC, Poser JW. Novel formulation of fibroblast growth factor-2 in a hyaluronan gel accelerates fracture healing in nonhuman primates. *J Orthop Res.* 1999 Jul;17(4):607-14.
16. Kitamura M, Akamatsu M, Machigashira M, Hara Y, Sakagami R, Hirofuji T, Hamachi T, Maeda K, Yokota M, Kido J, Nagata T, Kurihara H, Takashiba S, Sibutani T, Fukuda M, Noguchi T, Yamazaki K, Yoshie H, Ioroi K, Arai T, Nakagawa T, Ito K, Oda S, Izumi Y, Ogata Y,

Yamada S, Shimauchi H, Kunimatsu K, Kawanami M, Fujii T, Furuichi Y, Furuuchi T, Sasano T, Imai E, Omae M, Yamada S, Watanuki M, Murakami S. FGF-2 stimulates periodontal regeneration: results of a multi-center randomized clinical trial. *J Dent Res.* 2011 Jan;90(1):35-40.

17. Fakhari A, Berkland C. Applications and emerging trends of hyaluronic acid in tissue engineering, as a dermal filler and in osteoarthritis treatment. *Acta Biomater.* 2013 Jul;9(7):7081-92.
18. Reitinger S, Lepperdinger G. Hyaluronan, a ready choice to fuel regeneration: a mini-review. *Gerontology.* 2013;59(1):71-6.
19. Kim JE, Sykes JM. Hyaluronic acid fillers: history and overview. *Facial Plast Surg.* 2011 Dec;27(6):523-8.2013 Feb 15;92(2):1262-79.
20. Chang KV, Hsiao MY, Chen WS, Wang TG, Chien KL. Effectiveness of intra-articular hyaluronic acid for ankle osteoarthritis treatment: a systematic review and meta-analysis. *Arch Phys Med Rehabil.* 2013 May;94(5):951-60.
21. Mendes RM, Silva GA, Lima MF, Calliari MV, Almeida AP, Alves JB, Ferreira AJ. Sodium hyaluronate accelerates the healing process in tooth sockets of rats. *Arch Oral Biol.* 2008 Dec;53(12):1155-62.
22. Hunt DR, Jovanovic SA, Wikesjö UM, Wozney JM, Bernard GW. Hyaluronan supports recombinant human bone morphogenetic protein-2 induced bone reconstruction of advanced alveolar ridge defects in dogs. A pilot study. *J Periodontol.* 2001 May;72(5):651-8.
23. Bansal J, Kedige SD, Anand S. Hyaluronic acid: a promising mediator for periodontal regeneration. *Indian J Dent Res.* 2010 Oct-Dec;21(4):575-8.
24. Löndahl M. Hyperbaric oxygen therapy as adjunctive treatment of diabetic foot ulcers. *Med Clin North Am.* 2013 Sep;97(5):957-80.

25. Marx RE, Ames JR. The use of hyperbaric oxygen therapy in bony reconstruction of the irradiated and tissue-deficient patient. *J Oral Maxillofac Surg.* 1982 Jul;40(7):412-20.
26. Thom SR. Hyperbaric oxygen: its mechamisms and efficacy. *Plast Reconstr Surg.* 2011 Jan;127 Suppl 1:131S-141S.
27. Nilsson P, Albrektsson T, Granstrom G, Rockert HO. The effect of hyperbaric oxygen treatment on bone regeneration: an experimental study using the bone harvest chamber in the rabbit. *Int J Oral Maxillofac Implants.* 1988 Spring;3(1):43-8.
28. Annane D, Depondt J, Aubert P, Villart M, Géhanno P, Gajdos P, Chevret S. Hyperbaric oxygen therapy for radionecrosis of the jaw: a randomized, placebo-controlled, double-blind trial from the ORN96 study group. *J Clin Oncol.* 2004 Dec 15;22(24):4893-900.
29. Tibbles PM, Edelsberg JS. Hyperbaric-oxygen therapy. *N Engl J Med.* 1996 Jun 20;334(25):1642-8.
30. Hollinger JO, Kleinschmidt JC. The critical size defect as an experimental model to test bone repair materials. *J Craniofac Surg.* 1990 Jan;1(1):60-8.
31. Develioğlu H, Saraydin S, Bolayir G, Dupoirieux L. Assessment of the effect of a biphasic ceramic on bone response in a rat calvarial defect model. *J Biomed Mater Res.* 2006 Jun 1;77(3):627-631.
32. Nussenbaum B, Rutherford RB, Krebsbach PH. Bone regeneration in cranial defects previously treated with radiation. *Laryngoscope.* 2005 Jul;115(7):1170-7.
33. Chen X, Matsui Y, Ohno K, Michi K. Histomorphometric evaluation of the effect of hyperbaric oxygen treatment on healing around hydroxyapatite implants in irradiated rat bone. *Int J Oral Maxillofac Implants.* 1999 Jan-Feb;14(1):61-8.

34. Liao YH, Jones SA, Forbes B, Martin GP, Brown MB. Hyaluronan: pharmaceutical characterization and drug delivery. *Drug Deliv.* 2005 Nov-Dec;12(6):327-42.
35. Rooney P, Kumar S. Inverse relationship between hyaluronan and collagens in development and angiogenesis. *Differentiation.* 1993 Aug;54(1):1-9.
36. Deed R, Rooney P, Kumar P, Norton JD, Smith J, Freemont AJ, Kumar S. Early-response gene signalling is induced by angiogenic oligosaccharides of hyaluronan in endothelial cells. Inhibition by non-angiogenic, high-molecular-weight hyaluronan. *Int J Cancer.* 1997 Apr 10;71(2):251-6.
37. Pilloni A, Bernard GW. The effect of hyaluronan on mouse intramembranous osteogenesis in vitro. *Cell Tissue Res.* 1998 Nov;294(2):323-33.
38. Huang L, Cheng YY, Koo PL, Lee KM, Qin L, Cheng JC, Kumta SM. The effect of hyaluronan on osteoblast proliferation and differentiation in rat calvarial-derived cell cultures. *J Biomed Mater Resi.* 2003 Sep 15;66A(4):880-4.
39. de Brito Bezerra, Mendes Brazão MA, de Campos ML, Casati MZ, Sallum EA, Sallum AW. Association of hyaluronic acid with a collagen scaffold may improve bone healing in critical-size bone defects. *Clin Oral Implants Res.* 2012 Aug;23(8):938-42.
40. Vanden Bogaerde L. Treatment of infrabony periodontal defects with esterified hyaluronic acid: clinical report of 19 consecutive lesions. *Int J Periodontics Restorative Dent.* 2009 Jun;29(3):315-23.
41. Ballini A, Cantore S, Capodiferro S, Grassi FR. Esterified hyaluronic acid and autologous bone in the surgical correction of the infra-bone defects. *Int J Med Sci.* 2009;6(2):65-71.

42. Park JK, Yeom J, Oh EJ, Reddy M, Kim JY, Cho DW, Lim HP, Kim NS, Park SW, Shin HI, Yang DJ, Park KB, Hahn SK. Guided bone regeneration by poly(lactic-co-glycolic acid) grafted hyaluronic acid bi-layer films for periodontal barrier applications. *Acta Biomater.* 2009 Nov;5(9):3394-403.
43. Anzai J, Kitamura M, Nozaki T, Nagayasu T, Terashima A, Asano T, Murakami S. Effects of concomitant use of fibroblast growth factor (FGF)-2 with beta-tricalcium phosphate (β-TCP) on the beagle dog 1-wall periodontal defect model. *Biochem Biophys Res Commun.* 2010 Dec 17;403(3-4):345-50.
44. Marx RE, Ehler WJ, Tayapongsak P, Pierce LW. Relationship of oxygen dose to angiogenesis induction in irradiated tissue. *Am J Surg.* 1990 Nov;160(5):519-24.
45. Marx RE. Osteoradionecrosis: a new concept of its pathophysiology. *J Oral Maxillofac Surg.* 1983 May;41(5):283-8.
46. Tuncay OC, Ho D, Barker MK. Oxygen tension regulates osteoblast function. *Am J Orthod Dentofacial Orthop.* 1994 May;105(5):457-63.
47. Pitak-Arnnop P, Hemprich A, Dhanuthai K, Pausch NC. A systemic review in 2008 did not show value of hyperbaric oxygen therapy for osteoradionecrosis. *J Oral Maxillofac Surg.* 2010 Oct;68(10):2644-45.
48. Fritz GW, Gunsolley JC, Abubaker O, Laskin DM. Efficacy of pre- and postirradiation hyperbaric oxygen therapy in the prevention of postextraction osteoradionecrosis: a systematic review. *J Oral Maxillofac Surg.* 2010 Nov;68(11):2653-60.
49. Jan A, Sándor GK, Brkovic BB, Peel S, Evans AW, Clokie CM. Effect of hyperbaric oxygen on grafted and nongrafted calvarial critical-sized defects. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod.* 2009 Feb;107(2):157-63.

50. Mancha de la Plata M, Gías LN, Díez PM, Muñoz-Guerra M, González-García R, Lee GY, Castrejón-Castrejón S, Rodríguez-Campo FJ. Osseointegrated implant rehabilitation of irradiated oral cancer patients. *J Oral Maxillofac Surg.* 2012 May;70(5):1052-63.
51. Jan AM, Sándor GK, Iera D, Mhawi A, Peel S, Evans AW, Clokie CM. Hyperbaric oxygen results in an increase in rabbit calvarial critical sized defects. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod.* 2006 Feb;101(2):144-9.
52. Jan A, Sándor GK,, Brkovic BB, Peel S, Kim YD, Xiao WZ, Evans AW, Clokie CM. Effect of hyperbaric oxygen on demineralized bone matrix and biphasic calcium phosphate bone substitutes. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod.* 2010 Jan;109(1):59-66.
53. Wang X, Ding I, Xie H, Wu T, Wersto N, Huang K, Okunieff P. Hyperbaric oxygen and basic fibroblast growth factor promote growth of irradiated bone. *Int J Radiat Oncol Biol Phys.* 1998 Jan 1;40(1):189-96.

국문 요약

FGF-2 , , 고압산소요법이 방사선 조사를 받은 백서 두개골 결손부의 골 치유에 미치는 영향

(지도: 정 문 규 교수)

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목적: 임플란트를 이용한 치료는 일반적인 경우뿐만 아니라 두경부 종양으로 인해 수술 및 방사선 조사를 받은 환자의 재건 수술에서도 요구되는 경우가 많다. 심미적이고 기능적인 회복을 위하여 임플란트 식립과 골이식이 동반되어야 하는 경우도 증가하고 있다. 그러나 방사선 조사를 받은 악골의 치유능력은 정상조직에 비해 현저히 떨어지므로 특별한 주의가 요구된다. 본 연구의 목적은 방사선 조사로 인해 골 생성능이 저하된 실험용 쥐의 두개골에 원형 결손부를 형성하여, 골이식재에 따른 골 재생 결과를 비교해 보는 것이다. 또한 고압산소요법의 실시 유무가 골 재생에 어떠한 영향을 미치는지 알아보고자 한다.

방법: 4 4 . 고압산소요법을 적용한 군과 적용하지 않은 군으로 나누고, FGF-2(유아세포 성장인자) , (BCP block) . 12 Gy , 4

간 기다렸다가 쥐의 두개골에 2 . 2
개의 골결손부를 형성하였는데, 한쪽에는 아무것도 넣지 않고 나머지 한쪽에는
FGF-2 BCP block . 4 주
간의 치유기간을 가졌고, 그 기간 동안 고압산소요법 실험군에는 고압산소요법을
1 5 . 4 , 조
직학적 분석을 시행하였다.

결과: 1. FGF-2 와 하이알루론산을 적용한 군이 다른 군보다 더 많은 신생골 면적을 나타냈으며, BCP block . 신생골 면적에 있어서 고압산소요법으로 인한 효과는 미미하게 보였다.

2. FGF-2 와 하이알루론산을 적용한 군은 다른 군보다 신생골 길이가 길게 나타났으며, BCP block . 신생골 길이에 있어서 고압산소요법으로 인한 효과는 미미하게 나타났다.

3. 대체적으로 고압산소요법을 적용한 군은 적용하지 않은 군보다 더 많은 혈관생성을 보였다. FGF-2 와 하이알루론산을 적용한 군과 함께 적용하였을 때는, FGF-2 와 하이알루론산을 단독으로 적용하거나 고압산소요법만을 단독으로 적용한 것에 비해 혈관생성 능력이 향상되지 않았다.

결론: , 방사선 조사를 받은 쥐의 두개골 결손부 모델에서 고압산소요법은 혈관생성에는 유용한 효과를 보였으나, 골 재생에는 적은 효과를 보였다. FGF-2 와 하이알루론산을 적용하는 것은 골 재생 및 혈관생성에 효과적이었으나, . BCP block 은 골 재생에 있어 유용한 효과를 보여주지 못했다.

핵심어 : , , , (BCP), 섬유아세포 성장인자(FGF-2), , 고압산소요법