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Effect of hyperbaric oxygen therapy on bone prefabrication in rats

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Objectives: This experimental study aimed to create a prefabricated vascularized bone graft using the interconnected porous coralline hydroxyapatite ceramic by combining vascular bundle implantation, mesenchymal stem cells, and hyperbaric oxygen therapy (HBOT) administration in a rat model.

Methods: Forty-five male Sprague-Dawley rats were divided into three groups, each containing 15 rats. The hydroxyapatite ceramics were vascularized by the superficial inferior epigastric artery and vein in all groups. These vessels passed through the hole of the hydroxyapatite blocks. In Group 2, mesenchymal stem cells were administered into the hydroxyapatite. In Group 3, both mesenchymal stem cells and HBOT were administered. The presence and density of any new bone formation and neovascularization were evaluated by radiography, microangiography, scintigraphy, biochemical analysis, and histomorphometry.

Results: Neovascularization and bone formation were significantly greater in Group 3, in which both mesenchymal stem cells and HBOT were applied, than the other groups.

Conclusion: HBOT enhances neovascularization and osteogenesis, thus HBOT can provide optimal and faster prefabrication of a vascularized bone graft.

Key words: Bone grafting; hydroxyapatite ceramic; hyperbaric oxygen therapy; rat.

Bone defects often occur as a result of severe trauma, infection, tumors, or congenital causes. Vascularized bone flaps and autogenous bone grafts have recently been used in the treatment of bone defects.[1-3]

The use of autografts is an effective solution for reconstruction of bone defects. However, the process of obtaining an autograft causes morbidity and complications.[4] Allografts have the risk of carrying infectious diseases, and the sterilization procedures applied to prevent infectious diseases adversely affect the biological properties of allografts.[5,6] Therefore, demineralized bone matrices such as bioactive glass and ceramic biomaterials have been produced as alternatives to autografts and allografts.[1,2]

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To establish a 3-dimensional bone tissue structure, there must be a skeleton system, i.e., a cell carrier, for the osteoblasts to hold and proliferate. The most popular for bone prefabrication among these systems is hydroxyapatite (HA) ceramics. HA ceramics should be biocompatible, so that they enable root cells to stick to each other and proliferate. HA ceramics should also include a porous structure that facilitates vascular structures to proceed inward. The porosity in HA ceramics is more than 90%, and these pores are in touch with each other. For this reason, HA ceramics are the most commonly preferred biomaterial for bone prefabrication.[7-10] The width of the pores in the HA block vary between 100-500 µm.[11,12]

In the literature, there are many experimental and clinical studies related to bone prefabrication and prelamination. In these studies, bone growth factors and cytokines have been used for bone prefabrication.[13,14] However, the effects of hyperbaric oxygen therapy (HBOT) have not been studied experimentally. The purpose of this study was to investigate the impact of HBOT on bone prefabrication.

Materials and methods

In the first phase of the study, biocompatible HA ceramic blocks (Pro Osteon® 500R Porous Bone Graft Substitute, Interpore Cross International, Irvine, CA, USA) were shaped into cylinders (Fig. 1). We used 45 cylinder-shaped HA ceramic blocks, 0.8 cm long and 0.6 cm wide, each containing a tunnel 2 mm in diameter (Fig. 2).

In the second phase of the study, mesenchymal stem cells obtained from the femurs of rats were aspirated. Subsequently, these stem cells were cultured in media including 100 µg/mL penicillin and 25 µg/mL gentamicin at 37°C. After 14 days, the cultured mesenchymal stem cells were separated with trypsinogen and resuspended to $3x10^6$ cells/mL.[15]

In the third phase of the study, mesenchymal stem cells ($5x10^6$ cells/mL) were implanted into each of the cylinder-shaped HA ceramic blocks. A solution containing dexamethasone (0.1 µ), sodium beta glycerophosphate (10 mM), and vitamin C phosphate (80 mg/mL) was prepared to demonstrate osteogenic differentiation of mesenchymal stem cells.[16,17] HA blocks were kept for 2 days in this solution. Three random test groups were created, each with 15 Sprague-Dawley rats. The superficial inferior epigastric artery and vein were passed through the tunnels created in the HA ceramic blocks for all groups. The mesenchymal stem cells were implanted into the HA ceramic blocks in Groups 1 and 2. After mesenchymal stem cell implantation in Group 3, HBOT was performed with a hyperbaric oxygen tank designed for experimental studies (Fig. 3). Starting on the date of the operation, 100% oxygen at 2.5 ATA (1 ATA=1 atmosphere absolute=760 mmHg), two 10-min each air breaks and 90-min sessions were used for the rats in Group 3. This protocol was used 4 times daily on the day of operation and 2 subsequent days; thereafter, it was used twice daily for the next 7 days.

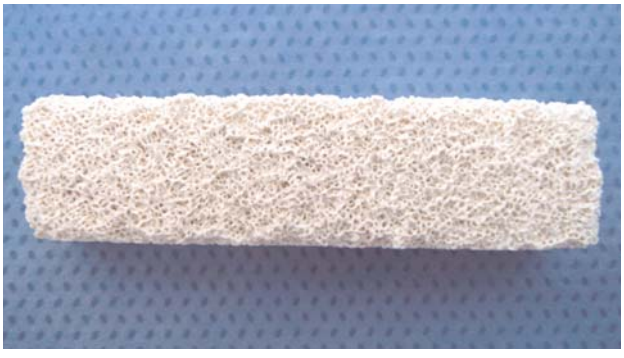

Fig. 1. Hydroxyapatite ceramic bloc.

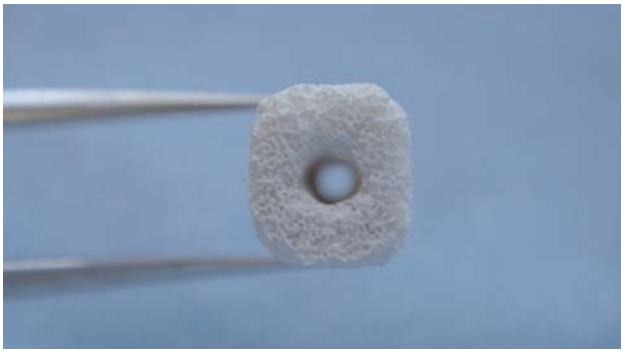

Fig. 2. Tunnel with a 2 mm diameter inside hydroxyapatite ceramic bloc.

Fig. 3. Hyperbaric oxygen tank.

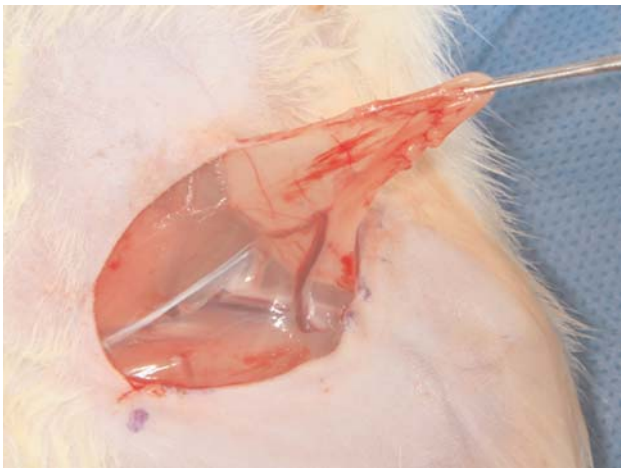

Fig. 4. The superficial epigastric artery and vein.

Surgical procedure

In the surgical phase of this study, the hairs on the rat's lower extremity were removed with a razor to reach the superficial inferior epigastric artery and vein. The transverse incision was made over the anterior side of left thigh. Subsequently, the superficial inferior epigastric artery and vein were reached by blunt and sharp dissection (Fig. 4). The superficial epigastric artery and vein were passed through the tunnel in the HA blocks (Fig. 5). HA blocks were then placed in the subcutaneous pouch.

The second phase of the surgical procedure was performed 2 weeks later in all groups. HA blocks were reached by reopening the incision line. The HA blocks in all groups were covered with silicone to prevent vascular invasion from the surrounding tissue.

Evaluation methods

Microangiography

Microangiography was applied to five rats from each group on day 30. The left carotid arteries of rats were exposed by left oblique cervical incision. The proximal segment of the carotid artery was cannulated with a 20 G epidural catheter after connecting the distal segment of carotid artery. Heparin 1 mL (5000 IU) (Nevparin 5000 IU/mL, Mustafa Nevzat Pharmaceuticals, İstanbul, Turkey) was injected. Subsequently, a solution was prepared using lactated Ringer (75 mL), barium sulfate (20 mL), and bovine gelatin (5%). This solution was heated to 36 °C and infused with low-pressure technique through the catheter after 15 min. Following the infusion, the rats were kept in the refrigerator at 4 °C. Radiographs were taken in mammography (at 23 KV, 12 mAs dose) after 12 hours.

Scintigraphy

Bone scintigraphy was applied for the evaluation of neovascularization and osteoblastic activity in the HA blocks in all groups. Technetium (Tc) marked with 99m methylene diphosphonate (^{99m}Tc MDP) was used as radiopharmaceutical agent. On day 30, 2 microcurie

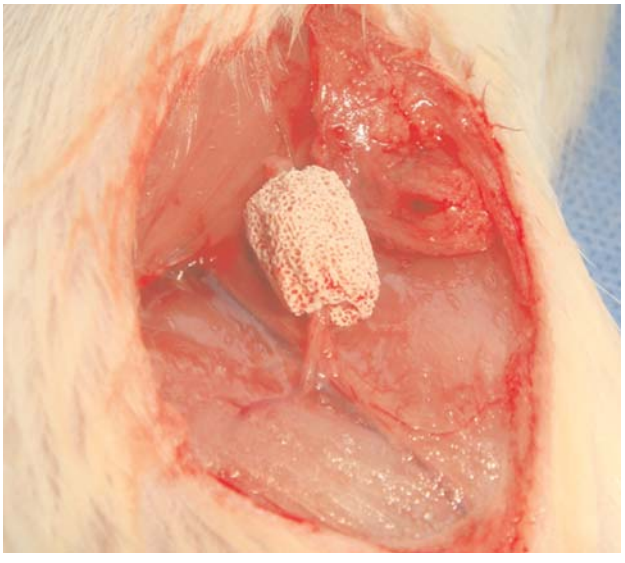

Fig. 5. The superficial epigastric artery and vein in the tunel of hydroxyapatite block.

^{99m}Tc MDP was infused into the left jugular vein of rats in all groups, and spot images were obtained after 2 hours. Static images were taken with a 256x256 matrix zoom for 5 min. Blastic activity of the HA block was measured and compared with the measured blastic activity of the symmetrical right thigh.

Biochemical analysis

The osteocalcin and alkaline phosphatase levels of HA blocks in all groups were measured with biochemical methods on day 45 after operation. The osteocalcin activity was measured using Sephadex G-25 column (NAP-25 column, Amersham Pharmacia Biotech AB, Uppsala, Sweden) and 10% formic acid. The alkaline phosphatase activity was measured using p-nitrophenyl phosphate.[16]

Histological examination

After the tissue samples were taken on day 45, they were fixed in 10% formalin at 4°C for 24 hours. Subsequently, tissue samples were washed with distilled water and decalcified by leaving them in nitric acid for 72 hours. Five-micrometer pathological sections were obtained from paraffin embedded blocks after decalcification of the bone samples. These samples were stained with hematoxylin-eosin (H-E) and examined with x10 magnification under a light microscope.

Statistical evaluation

Mann-Whitney U test was used for comparison of all test groups. All values were obtained using SPSS 10.0 software (SPSS Inc, Chicago, IL, USA). The mean standard deviation and skewness values in each group were calculated because of the limited number of rats in test groups. In this manner, the reliability of Mann-Whitney U test was checked.

Results

Microangiography

The degree of neovascularization in all groups was assessed by microangiography. There was no neovascularization in Group 1. In Group 2, neovascularization was seen only in the center of HA ceramic blocks. However, neovascularization starting from the center of HA ceramics and extending to the periphery was observed in Group 3. Therefore, maximum neovascularization was clearly detected in Group 3 (Fig. 6).

Scintigraphy

For quantitative evaluation of bone scintigraphy, the uptake of radioactivity of HA blocks in each group was compared with the symmetrical soft tissue of the HA blocks, and the average values of the groups were taken. There was no radioactivity in Group 1. However, radioactivity was detected in Groups 2 and 3. The radioactivity uptake in Group 3 was significantly higher than Group 1 and 2 ($p<0.05$) (Table 1).

Biochemical analysis

The mean levels of osteocalcin and alkaline phosphatase in HA blocks were significantly higher in Group 3 than Groups 1 and 2 ($p<0.05$) (Table 2).

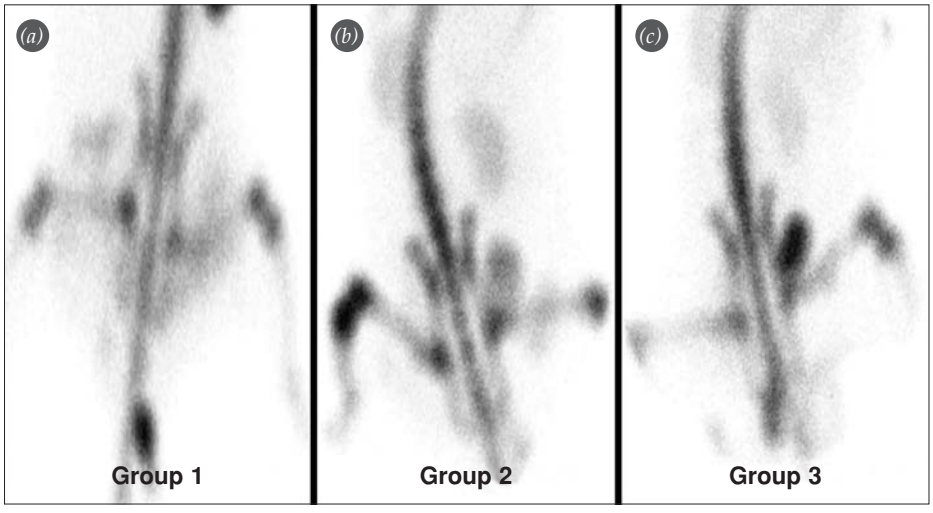

Fig. 6. (a-c) The degree of neovascularization in microangiography.

Histological evaluation

The maximum bone formation and neovascularization was observed in Group 3 by histological evaluation (Fig. 7).

Discussion

Tissue engineering is a new field of biotechnology that focuses on the development of biological equivalents in order to repair or replace damaged tissue. Studies in tissue engineering promise great hope for the future. In situations where the transplantation of an organ or tissue is the only option for the treatment of a disease, the main challenge is to secure a proper donor or an area of donor. Therefore, tissue prefabrication has attracted vast attention in recent years.

The basic structure in tissue engineering is stem cell. Stem cells renew themselves by dividing. They form tissues that serve specialized purposes, and they have differentiation abilities. Bone marrow includes hematopoetic stem cells that serve to develop all blood cells and mesenchymal stem cells that have the capability to form connective tissues. The main source of mesenchymal stem cells is bone marrow; however, mesenchymal stem cells may also be isolated from various other tissues such as muscle, bone, cartilage, fat, liver, cord blood, peripheral blood, and fetal bone. The physiological function of adult stem cells is to facilitate tissue homeostasis and tissue regeneration after injury. Under in vitro conditions, these cells may transform into many different cell types, such as adipogenic, chondrogenic, myogenic, and osteogenic cells. However, the mechanisms for change of different stem cells and progenitor cells are still unknown.[17-20]

Table 1

Radioactivity update rate in scintigraphy (mean)

	Group 1	Group 2	Group 3
Radioactivity update rate (cpp)	100	4300	22400

p<0.05 for Group 1 vs. Group 2, Group 1 vs. Group 3, and Group 2 vs. Group 3; cpp: count per pixel.

Table 2

Alkaline phosphatase and osteocalcine levels (mean)

	Group 1	Group 2	Group 3
Alkaline phosphatase (U/L)	0	600	950
Osteocalcine (Ng/mL)	0	34	42

p<0.05 for Group 1 vs. Group 2, Group 1 vs. Group 3, and Group 2 vs. Group 3.

In recent years, studies have been conducted on stem cell therapy as a substitute for organ transplants. It was shown in these studies that stem cells may repair the damaged areas when they are implanted in areas with bone and nerve tissue damage. Moreover, it has been also proven that bone marrow cells obtained from experimental animals under the right circumstances have proliferation and differentiation

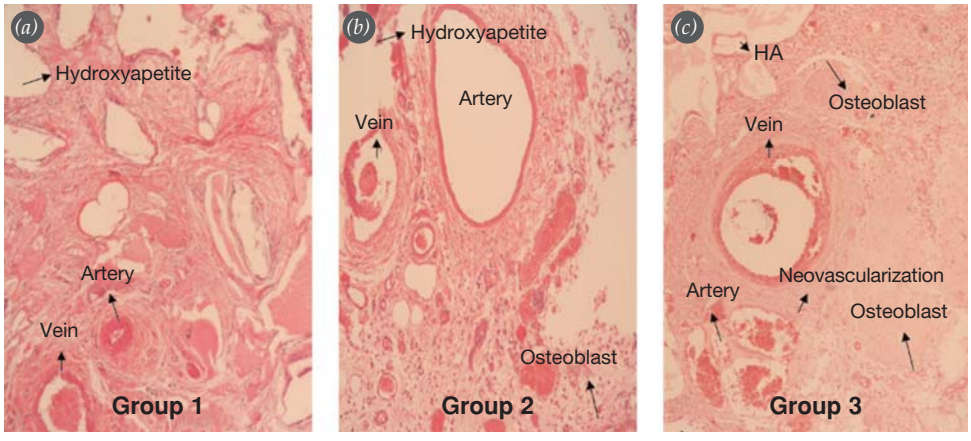

Fig. 7. (a-c) Histopathological appearance of bone formation and neovascularization (H-E x10).

ability.[21-25] Bone defects remain a challenge, even in the presence of new surgical techniques. Therefore, the use of cultured osteoblast cells in the treatment of bone defects has been increasing.

There are two main effects of hyperbaric oxygenation. The first is the mechanical effect on the gases in the body, and the other is increased partial oxygen pressure in the blood. However, another effect of HBOT is vasoconstriction within the first 72 hours.[26-28] In response to this effect, the rate and amount of neovascularization continue to increase in the subsequent hours. It is possible to have an amount of oxygen that is sufficient for the needs of the body to be dissolved in plasma with HBOT. The oxyhemoglobin passes from the arterial system to the venous system when the rate of dissolved oxygen in the plasma exceeds 6%. In this fashion, plasma has the capacity to carry an oxygen supply sufficient even for tissues with reduced blood flow.

Alkaline phosphatase activity and osteocalcin levels are the two most important biochemical components for the assessment of bone prefabrication.[29] Alkaline phosphatase is located at the cell membrane of osteoblast, and it has a correlation with direct osteoblast activity. Osteocalcin is synthesized by osteoblasts, and osteocalcin is the key factor pointing to the presence of bone tissue. In our study, the highest levels of alkaline phosphatase and osteocalcin were observed in Group 3.

We implanted stem cells into HA ceramics because HA ceramics are biocompatible and osteoconductive, and HA ceramics are polymerized within a clinically acceptable time. The vascularization of HA ceramics is necessary for stem cells to survive and proliferate. The superficial inferior epigastric artery and vein were used in our study as the vascular pedicle for the formation of new vascular structures and for these new vascular structures to reach inside HA ceramics.

The most important problem in bone prefabrication is late and inadequate vascularization. For this reason, we investigated the efficiency of HBOT on bone prefabrication in this study. In addition to having a mechanical impact and increasing the solubility of oxygen, HBOT also increases and accelerates capillary proliferation. This experimental study shows the positive impact of HBOT in bone prefabrication.

In conclusion, HBOT provided early and increased vascularity in bone prefabrication that was performed by applying mesenchymal stem cell implantation and vascular pedicle. Therefore, HBOT can be added to treatment protocols in bone prefabrication as a positive stimulating factor.

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