Effect of MBCP block as carrier of rhBMP-2 in combination with ePTFE membrane on bone formation in rat calvarial defects

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ABSTRACT

Purpose: The carrier used as delivery agent for bone morphogenetic proteins(BMPs) should also act as a scaffold for new bone formation. Moreover, bone formation should be predictable in terms of the volume and shape. This study examined the osteogenic effect of macroporous biphasic calcium phosphate (MBCP) block combined with ePTFE membrane as a carrier for recombinant human bone morphogenetic proteins (rhBMP-2). In addition, the additive effect of ePTFE membrane on bone formation was evaluated.

Materials and Methods: Eight-millimeter critical sized calvarial defects were created surgically in 28 male Sprague-Dawley rats. The animals were divided into 2 groups containing 14 animals each. The defects were treated with either rhBMP-2/MBCP block (rhBMP-2/MBCP group) or rhBMP-2/MBCP block/ePTFE membrane (rhBMP-2/MBCP/ePTFE group). A disc-shaped MBCP block (3 mm height and 8 mm diameter) was used as the carrier for the rhBMP-2 and ePTFE membrane was used to cover the rhBMP-2/MBCP block. The histologic and histometric parameters were used to evaluate the defects after 2- or 8-week healing period (7 animals/group/healing interval).

Results: The level of bone formation in the defects of both groups was significantly higher at 8 weeks than that at 2 weeks ($P < 0.05$). The ePTFE membrane has no additional effect compared with the rhBMP-2/MBCP block only. However, at 8 weeks, rhBMP-2/MBCP/ePTFE group showed more even bone formation on the top of the MBCP block than the rhBMP-2/MBCP group.

Conclusion: These results suggest that the ePTFE membrane has no additive effect on bone formation when a MBCP block is used as a carrier for rhBMP-2. ***(J Korean Acad Periodontol 2008;38:325-334)***

KEY WORDS: rhBMP-2; MBCP block; ePTFE membrane; carrier.

Introduction

Dental patients commonly show a significantly resorbed ridge and insufficient bone volume for dental implants or conventional prosthodontic treatments due to the congenital and acquired bone defects caused by periodontitis, osteomyelitis, oral cancer, and trauma, etc. Therefore, reconstructive surgery to augment the resorbed alveolar bone is often performed on these bone defects so that it improves retention and stability of removable denture and enables dental implant placement. Appropriate augmentation of the alveolar bone ensures a predictable result and good esthetics. Currently autogenous bone is the most preferred material for this purpose. However, although highly effective, autogenous grafting has some limitations and problems in its application, which include inadequate supply and surgical morbidity, as well as donor site

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pain and infection. Moreover, significant volumetric resorption of the graft poses clinical problems in the case of block grafts from endochondral donor sites[1]. Therefore, there is a need for alternative biomaterials to autogenous bone.

The bone morphogenetic protein(BMP) is expected to be a good substitute for autogenous bone. BMP is the most promising osteoinductive protein for bone regeneration[2]. Since the discovery of BMPs[3], more than 20 BMPs have been identified. Several, including BMP-2, -4, -6 and -7, have been reported to have significant osteoinductive potential[4,5]. Among these, rhBMP-2 was found to have strong in vivo bone-inducing ability[5-7]. However, the application of BMPs alone is not enough to induce bone formation because the protein rapidly diffuses from the site of application. Therefore, the use of a carrier system is essential for delivering and slowly releasingrhBMP-2 during the period of time required for bone formation[8,9].

For clinical success using rhBMPs, the carrier should be easy to manipulate and be made into a specific shape. It also needs to provide sufficient firmness against soft tissue pressure during the healing period. Our previous studies searched for excellent rhBMP carriers, such as an absorbable collagen sponge(ACS)[9,10], ß-tricalcium phosphate(ß-TCP)[6,9,11], a fibrin-fibronectin sealing system(FFSS)[12,13] and a macroporous biphasic calcium phosphate (MBCP) block[14]. Each carrier material had its advantages and disadvantages. Although the ACS appeared to be an effective carrier in space-providing skeletal defects, it becomes victim to compressive forces when used for non-space-providing onlay indications. Osteoconductive and porous ß-TCP provided sufficient firmness and good biocompatibility. However, it is not moldable and has limitations in providing space.

MBCP consists of an intimate mixture of hydroxyapatite(HA) and ß-TCP at varying HA/ß-TCP ratios[15], and has been reported to have favorable osteoconductive properties[15-17]. MBCP has the required porous form, and can entrap rhBMP within its microporous structure so that the intrinsically diffusible rhBMP is retained prolonging its action. Moreover, the porous structure of MBCP allows the infiltration of cells. In addition, HA in MBCP provides sufficient mechanical strength to resist the compressive forces and maintains the volume of augmented bone. It was reported that a MBCP block might be a suitable carrier for rhBMP-2 to allow predictable bone formation in terms of the volumetric stability[14].

The main requirement for successful bone regeneration is the stability of the graft material in the defect site as well as the prevention of soft connective tissue ingrowth into the defect area. The use of a barrier membrane to cover and retain the graft material might satisfy both prerequisites. The placement of a biocompatible ePTFE membrane or some other type of biodegradable barrier over bone defects might help guide bone regeneration[18]. However, there has been some controversy regarding the additional benefits of the membrane on bone regeneration by rhBMP[19-23].

Therefore, this study examined the osteogenic effect of MBCP block combined with an ePTFE membrane as a carrier for recombinant human bone morphogenetic proteins (rhBMP-2) in a rat calvarial defect model. The additive effect of ePTFE membrane on bone formation was also evaluated.

Materials and Methods

1. Animals

Twenty-eight male Sprague-Dawley rats (body weight, 200~300g) were used. They were maintained in plastic cages in a room with 12h-day/night cycles, an ambient temperature of 21°C, and *ad libitum* access to water and a standard laboratory pellet diet. Animal selection and management, surgical protocol, and preparation followed the routines approved by the Institutional Animal Care and Use Committee, Yonsei Medical Center, Seoul, Korea.

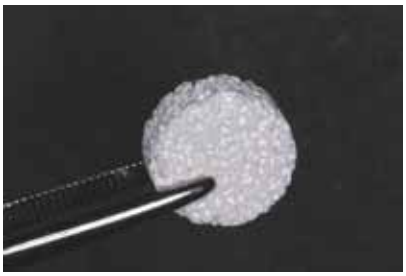


Figure 1. Disc-shaped MBCP block implant used (3mm in height and 8mm in diameter) in this study.

2. rhBMP-2 implant construction

The rhBMP-2(R&D Systems Inc., Minneapolis, MN, USA) was reconstituted and diluted in a buffer(sterile 4 mM HCl solution containing 0.1% bovine serum albumin) to produce a concentration of 0.025mg/ml. For the rhBMP-2/MBCP block implant, a disc-shaped MBCP block (3mm in height and 8mm in diameter; Biomatlante Inc., Vigneux de Bretagne, France) (Fig. 1) was loaded with 0.25ml of the rhBMP-2 solution. The rhBMP-2 /MBCP block implants were placed in the calvarial defects following a 5-min binding period.

3. Surgical procedures

The animals were generally anaesthetized by an intramuscular injection of ketamine hydrochloride (Ketalar®, Yuhan Co., Seoul, Korea) at 5mg/kg body weight. During surgery, routine infiltration anesthesia (2% lidocaine, 1:100,000 epinephrine, Kwangmyung Pharmaceuticals, Seoul, Korea) was used at the surgical site. The surgical site was shaved and scrubbed with iodine. An incision was made in the sagittal direction across the cranium and a full thickness flap was reflected to expose the calvarial bone. Then, a standardized, circular, transosseous defect, 8mm in diameter[24], was created on the cranium using a saline- cooled trephine drill (3i, Palm Beach Gardens, FL, USA). The animals were divided into two groups containing 14 animals each and allowed to heal for 2 (7 rats) or 8 weeks (7 rats). Each animal received one of two treatments: the rhBMP-2/MBCP block (rhBMP-2/MBCP group) or the rhBMP-2/ MBCP block/ ePTFE membrane (Gore-Tex®, W.L. Gore & Associates Inc.) (rhBMP-2/ MBCP/ePTFE group). The ePTFE membrane(about 10mm in diameter) was placed to cover the rhBMP-2/MBCP block and no additional device was placed for fixation of the ePTFE membrane. The periosteum and skin was closed and sutured for primary closure with 4-0 coated sutures (Polyglactin 910, braided absorbable suture, Ethicon, Johnson & Johnson Int., Edinburgh, UK).

4. Histologic and histomorphometric procedures

The animals were sacrificed by CO_2 asphyxiation at 2 and 8 weeks post-surgery. Block sections including the surgical sites were removed and fixed in 10% neutral buffered formalin solution for 10 days. All samples were decalcified in EDTA-HCl for 7 days and embedded in paraffin. Three micrometer thick coronal sections through the center of the augmented area were obtained at 80um intervals, and stained with hematoxylin and eosin (H&E). The most central section from each block was selected for the histologic and histomorphometric evaluation. After conventional microscopic examination, computer-assisted histomorphometric measurements were done using an automated image analysis system (Image-Pro Plus®, Media Cybernetics, Silver Spring, MD, USA) coupled with a video camera on a light microscope (Olympus BX50, Olympus Optical Co., Tokyo, Japan). The sections were examined at ×10 magnification. A digitizer was used to trace the defect outline versus new bone formation, and the percentage of bone fill was determined. The value of each measurement was automatically calculated by the image

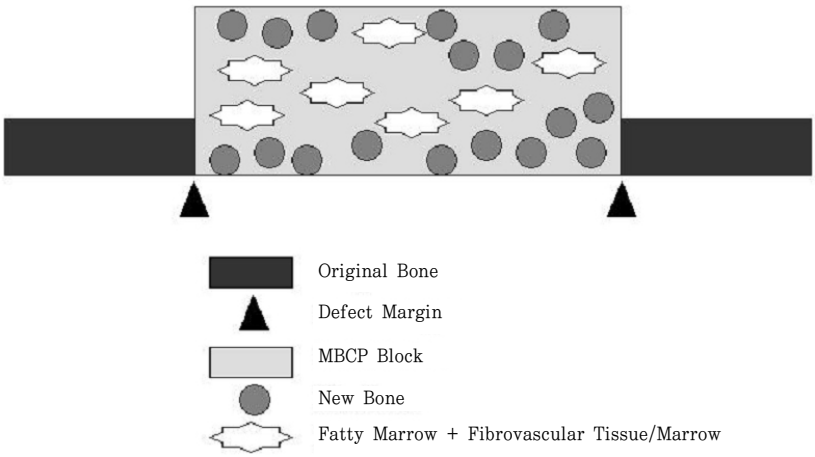


Figure 2. *Schematic drawing of calvarial osteotomy defect showing histomorphometirc analysis.*

analysis system. The following histomorphometric parameters were measured for each section (Fig. 2).

1) Augmented area (mm^2) was measured as all tissues within the boundaries of the MBCP block, including new bone, the residual biomaterials, fatty marrow and fibrovascular tissue/marrow.
2) New bone area (mm^2) was determined by the newly formed bone area within the total augmented area.
3) Bone density (%) was determined by the percentage of newly formed bone area within the total augmented area: Bone density (%) = (New bone area / Augmented area) ×100

5. Statistical Analysis

Histomorphometric recordings from the samples were used to calculate group median and range. The Wilcoxon two sample test was used for statistical analysis of the difference between two groups. P-value <0.05 was considered statistically significant.

Results

1. Clinical observation

Wound healing was generally uneventful and similar for all groups. There were no macroscopic signs of infection.

2. Histologic observation

At 2 weeks, new bone formation was observed in the bottom of the MBCP block (Fig. 3). Bone formation was significantly enhanced at 8 weeks. The new bone appeared more lamellar at 8 weeks than that at 2 weeks. A large number of osteocytes, osteblasts, and osteoclasts were observed in the area of new bone formation. The incremental lines, fatty marrow and concentric ring of the Haversian system were also observed in this area. The pattern of newly formed bone moved from outside to the inside of the defect (Fig. 4).

There was no significantly different appearance by the membrane compared with rhBMP-2/MBCP block only. At 8 weeks, new bone formation was significant and more enhanced than at 2 weeks (Fig. 5, 6). In addition, an even bone formation pattern under membrane was observed (Fig. 6-B). However, in the rhBMP-2/MBCP group, the bone formation pattern was irregular at the top of MBCP block (Fig. 4-B). The new bone formation pattern was similar in the central and base parts (bottom of defect) of the both groups (Fig. 4-C, D and Fig. 6-C, D).

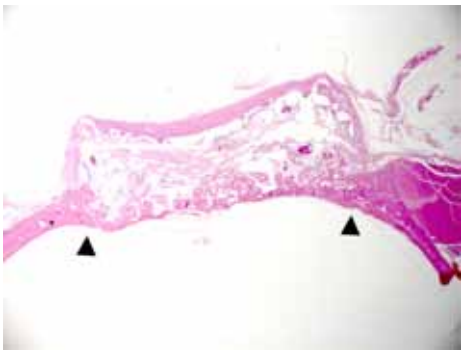
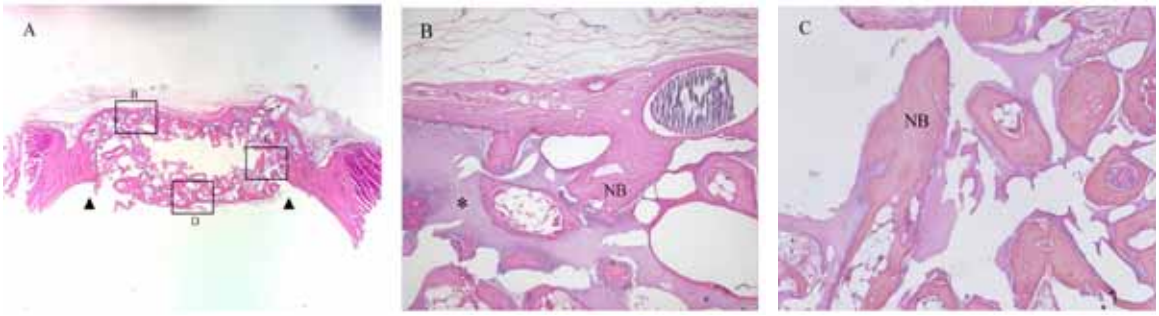


Figure 3. *Representative photomicrograph of rhBMP-2/MBCP group at 2 weeks. New bone formation was observed in the bottom of MBCP block and adjacent to the margins of the defect (▲=defect margin; H&E stain; original magnification ×10).*



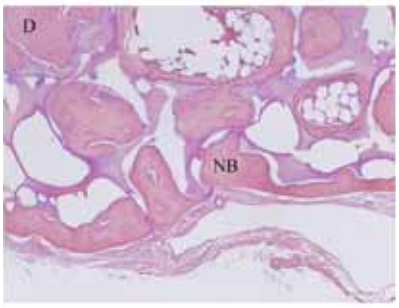


Figure 4. *Representative photomicrograph of rhBMP-2/MBCP group at 8 weeks (B, C and D; boxed area in A). At 8 weeks, more bone formation was observed in the base area comparing with 2 weeks. A lot of new bone was observed in top of MBCP block. However, bone formation pattern was irregular (▲=defect margin; *=MBCP remnants; NB=new bone; H&E stain; original magnification: A ×10; B, C and D ×100).*

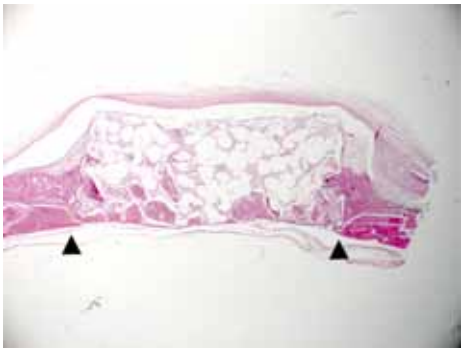


Figure 5. *Representative photomicrograph of rhBMP-2/MBCP /ePTFE group at 2 weeks. New bone formation was observed in the bottom of MBCP block and adjacent to the margins of the defect (▲=defect margin; H&E stain; original magnification ×10).*

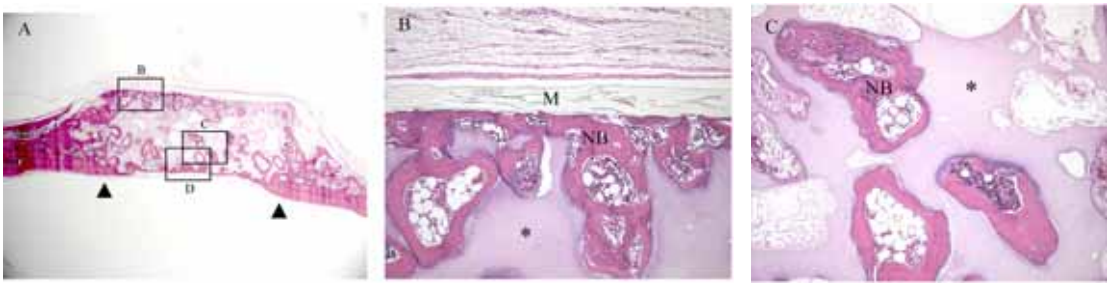


Figure 6. *Representative photomicrograph of rhBMP-2/MBCP/ePTFE group at 8 weeks (B, C and D; boxed area in A). In the top of MBCP block, a lot of new bone was observed and the appearance of the new bone was more lamellar than at 2 weeks and the pattern of bone formation beneath membrane was more even than in the rhBMP-2/MBCP group (▲=defect margin; ✻=MBCP remnants; NB=new bone; H&E stain; original magnification: A ×10; B, C and D ×100).*

3. Histomorphometric analysis

Table 1, 2 and 3 show the results of histomorphometric analysis. Total augmented area and new bone area were not significantly different between two groups at 2 and 8 weeks. In terms of new bone area and bone density, the ePTFE membrane generally showed a slight beneficial effect on bone formation at 2 weeks, although this was not statistically significant as shown in Table 2 and Table 3. Regarding the bone density, there were statistically significant differences between 2 weeks and 8 weeks results in both groups ($P<0.05$). However, there were no significant differences between the two groups at 2 and 8 weeks of examination.

Table 1. Total Augmented Area (Median & Range; mm^2, n=7)

Group	2 weeks		8 weeks	
rhBMP-2 / MBCP	13.45	7.21	19.69	10.7
rhBMP-2 / MBCP / ePTFE	18.57	7.31	17.93	12.64

Table 2. New Bone Area (Median & Range; mm^2, n=7)

Group	2 weeks		8 weeks	
rhBMP-2 / MBCP	1.43	0.53	4.52	3.86
rhBMP-2 / MBCP / ePTFE	3.03	3.84	4.88	7.85

Table 3. Bone Density (Median & Range; %, n=7)

Group	2 weeks		8 weeks	
rhBMP-2 / MBCP	10.69	6.05	25.06	15.14*
rhBMP-2 / MBCP / ePTFE	14.27	14.75	26.35	40.72*

* Statistically significant difference when compared to 2 weeks ($P<0.05$).

Discussion

This study investigated the additive effect of ePTFE membrane on bone regeneration after the implantation of a rhBMP-2/MBCP block in a critical-sized rat calvarial defect model. The defects were implanted with either a rhBMP-2/BMCP block or a rhBMP-2/MBCP block/ePTFE membrane. The level of healing was evaluated histologically and histomorphometrically after a 2- and 8-week healing period.

In our previous study[24], we demonstrated the bone regenerative effect of rhBMP-2 delivered with a MBCP block in a rat calvarial defect model. The rationale behind the study was to utilize the favorable bone healing capacity of the MBCP block, which has an osteoconductive effect, and the volumetric predictability over the healing time because of the low resorption rate and the resistance of the block type against soft tissue compression. The favorable osteoconductive effect of the MBCP in bone healing has been well documented[15-17]. It was suggested that the higher local calcium and phosphate concentrations in MBCP might have a stimulatory effect on bone formation. In addition to the osteoconductive effect of MBCP, there are many reports showing the osteoinductive effect of MBCP when implanted in ectopic sites in various species[25-27].

It was expected that the use of a MBCP block as a rhBMP-2 carrier would result in predictable new bone formation in terms of the volume and shape. In a clinical point of view, a carrier for the delivery of BMPs should serve as a scaffold for bone forming cells while providing a space for bone formation to occur. In addition, the space should be maintained for a relatively long period in order to allow sufficient maturation of the newly formed bone. In terms of clinical bone tissue engineering, provision of the volume and shape of the bone tissue is a key factor for treatment success. In the previous study investigating the effect of rhBMP-2/MBCP block on bone formation in rat calvarial defects after 2 and 8 weeks healing period, the new bone area in the rhBMP-2/MBCP block (2 weeks;3.0±0.9mm^2, 8 weeks;5.5±2.2mm^2) was significantly larger than that in the MBCP block alone (2 weeks;1.0±0.6mm^2, 8 weeks;3.0±0.9mm^2). Therefore, it was suggested that the MBCP block is an effective carrier system of rhBMP-2 with the volumetric stability[24]. Therefore, in this experiment, a MBCP block only group without rhBMP-2 was not included because the aim was to confirm the regenerative effects of the rhBMP-2/MBCP block and to determine whether or not the placement of an ePTFE barrier membrane would have a synergistic effect.

In this study, there was significant better enhancement of bone formation in the defects of both the rhBMP-2/MBCP group and rhBMP-2/MBCP/ePTFE group at 8 weeks than at 2 weeks. However, although rhBMP-2/MBCP block induced new bone formation in the augmented defect, the placed ePTFE barrier membranes had no positive effect on bone regeneration.

There has been some controversy regarding the additional benefits of the membrane on bone regeneration by rhBMP[19-23]. An earlier study demonstrated that the initiation and rate of bone healing beneath the osteopromotive membranes can be enhanced significantly by the implantation of rhBMP-2. However, membrane placement per se significantly hampered the osteoinductive capacity of the BMP[21]. On the other hand, several studies[22,23] have reported the synergistic effect of a combination of rhBMP-2 and osteopromotive membranes, which led to rapid, complete graft integration and size maintenance without extensive regenerative bone resorption and graft size reduction. Moreover, rhBMP-2 appeared to accelerate the remodeling of the graft in the absence of a membrane. However, all these studies used easily degradable materials, such as ACS and poly($_{D,L}$-lactide-co-glycolide) (PLA/PGA) as rhBMP-2 carrier. In contrast, the MBCP block has a volumetric characteristic that maintains the shape during bone formation by rhBMP-2. Therefore, when used as a carrier for rhBMP-2, the MBCP block itself can have a similar

function to a membrane. This is confirmed in a previous study, which reported a decrease in the biological resorption of the MBCP block at 1 month after implantation in dogs as a result of the protective effect of the newly formed lamellar bone on the surface and in the core of the block[28]. In our experiment, this effect of the MBCP block was confirmed by the fact that the ePTFE membrane generally showed a slight beneficial effect on bone formation at 2 weeks after rhBMP-2/MBCP block implantation than at 8 weeks, although this was not statistically significant (Table 2 and Table 3). However, a synergistic effect of ePTFE might not be observed at 8 weeks because new bone was already induced by rhBMP-2 on the surface of the MBCP block in the initial healing stage, which might inhibit the in-growth of soft tissue into the defects (Fig. 3 and Fig. 4).

The slight beneficial effect of the ePTFE membrane observed in the initial healing stage (2 weeks) appeared to be partly because the membrane created an environment that prevents the invasion of competing cells from the overlying soft tissue and partly because the membrane can have the advantage of keeping the rhBMP-2 implant in place. However, although the cell occlusivity of the membrane has been mentioned as a critical determinant for guided tissue regeneration[29], the real importance of cell occlusion for optimal regeneration is unclear[30]. Recent studies have suggested that tissue occlusion does not appear to be a critical determinant for GTR but may be a requirement for optimal GTR[31,32]. Therefore, as shown in our results, the tissue occlusion of the ePTFE membrane might have only an accessory effect when the space for bone regeneration is obtained using other materials, such as a MBCP block. However, if an absorbable and non-spacemaking material such as ACS is used as a rhBMP carrier, it would be advantageous to apply a membrane to obtain volume maintenance because barrier-membrane placement may be useful for graft retention as well as for predetermining the final shape of the regenerative site.

In conclusion, these results showed that the use of a rhBMP-2/MBCP block, regardless of combined use of an ePTFE membrane, can achieve bone augmentation with significant bone formation. This suggests that the MBCP block is a suitable carrier of rhBMP-2 and might be effective in maintaining the space needed for guided bone regeneration. In addition, these findings showed that the ePTFE membrane has no synergistic effect on bone formation when a MBCP block is used as a carrier for rhBMP-2.

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