REPAIR OF CRANIAL BONE DEFECTS USING ENDOCHONDRAL BONE MATRIX GELATIN IN RAT

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Abstract - *Bone matrix gelatin (BMG) has been used for bone induction intramuscularly and subcutaneously by many investigators since 1965. More recently, some of the researchers have used BMG particles for bone repair and reported various results.*

In present study for evaluation of bone induction and new bone formation in parietal defects, BMG particles were used in five groups of rats. The BMG was prepared as previously described using Urist method. The defects were produced with 5-mm diameter in parietal bones and filled by BMG particles. No BMG was used in control group. For evaluation of new bone formation and repair, the specimens were harvested on days 7, 14, 21 and 28 after operation. The samples were processed histologically, stained by H&E, Alizarin red S staining, and Alcian blue, and studied by a light microscope.

The results are as follows:

In control group: Twenty-eight days after operation a narrow rim of new bone was detectable attached to the edge of defect.

In BMG groups: At day 7 after operation young chondroblast cells appeared in whole area of defect. At 14th day after operation hypertrophic chondrocytes showed by Alcian blue staining and calcified cartilage were detectable by Alizarin red S staining. The numerous trabeculae spicules, early adult osteocytes and highly proliferated red bone marrow well developed on day 21. Finally typic bone trabeculae with regulated osteoblast cells and some osteoclast cells were detectable at day 28 after operation. In conclusion, BMG could stimulate bone induction and new bone formation in bony defects. So, it seems that BMG could be a good biomaterial substance for new bone induction in bone defects.

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Key Words: Endochondral bone matrix gelatin (Ec BMG), bone induction, new bone formation, parietal, rat.

INTRODUCTION

Senn was the first researcher who substituted exogenic (bovine) muriatic acid demineralized bone matrix for a blood coagulum and investigated the healing of skull defects in 14 normal dogs (1). One year later, Miller used decalcified bone chips as grafts for bone repair (2).

Recently, some researchers used demineralized matrix for repair of bone defects (2,3,4,5). In 1957 Ray and Holloway (3) and in 1963 Hejna and Ray (4) reported an enhanced repair of rat skull defects filled with demineralized matrix and showed bone growth from the bony rim. Ray and Holloway reported the concept of new bone formation by induction (3). Urist (1965) was the first to conduct a series of important experiments in which he was able to demonstrate that HCL-treated demineralized bone matrix (DBM) induced the formation of bone (5). Reddi (1972, 1976) used a simple method for preparation of DBM and reported early stages after implantation of DBM in subcutaneous tissue and demonstrated that the proliferation cells induced by the implants first differentiated to cartilage (6, 7). Scott et al (1990) emphasized that the matrix of endochondral bone differs from the matrix of intramembranous bone (8). They said that the best grafting for craniofacial defects were intramembranous demineralized bone matrix (Im BMG) allografts (8). In another study, Scott et al suggested that intramembranous bone matrix contained osteoinductive factors which could only induce direct, not endochondral, bone ossificatoin (9). In the above mentioned kind of ossification, bone replaced with connective tissue proper, no cartilage intermediate was formed. In most of the previous researches, DBM and its derived material used for bone induction were implanted in muscle or subcutaneously (6,7,10,11). A literature review revealed only a few studies about the process and type of ossification by BMG in skull bone defects (12, 13, 14). The aim of this study was to determine the process and type of bone ossification by using endochondral bone matrix gelatin (Ec BMG) as described by Urist (5) at parietal bone.

MATERIALS AND METHODS

Preparation of Bone Matrix Gelatin(BMG)

Allogenic BMG was prepared according to the method of Urist (5,14,15) as briefly described follow:

Twenty male 5-6 weeks-old Sprague Dawley rats (120-150 gr body weight) were killed by lethal dose of chloroform. Diaphyseal shafts of long bones (femur and tibia) were collected and dissected free of muscle, bone marrow exactly removed and bones were cut into chips.

Liquid nitrogen was used to freeze the bone shafts in this procedure to avoid possible denaturation of proteins. The bone's lipid was removed by chloroform/methanol (1/1), demineralized in 0.6 N HCl and extracted successively with solutions of 2.0 M $CaCl_2$, 0.5 M ethylenediamine tetra acetic acid (EDTA), 8.0 M LiCl and water (55 degrees centigrade) to remove soluble proteins. The bone chips using liquid nitrogen were pulverized in a sample chamber heicho and sieved. All the process was performed exactly in sterile condition.

Implantation of the BMG

Thirty male 5-6 weeks old Sprague Dawley rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (11)(Abbot Laboratories, North Chicago, Illinois). The skin covering of parietal bones was shaved and dissected. Full thickness cranial defects, 5-mm in diameter, were produced with a dental bur in parietal bone of rats, rinsed with Lactate Ringer's solution during and after operation for cooling and clearing of any remained debris. In experimental groups the defect were filled with 5 mg allogenic BMG and coated by Surgy Cell. In control group only sterile Surgy Cell was used. The day of implantation is designated as day 0. The animals were held by standard condition. Specimens for histologic study were harvested at days 7, 14, 21 and 28 using a dissecting microscope.

Histologic preparation

For harvesting of specimens the animals were perfused through the heart first with cold saline and then with freshly prepared 4% paraformaldehyde in sodiume phosphate buffer (pH= 7.4). The harvested specimens were repeatedly fixed in 4% paraformaldehyde, decalcified by EDTA (pH= 7.4), embeded in hard paraffin and sectioned at 5 micron thickness (16). Sections at intervals of 80 micron were stained with hematoxilin and eosin (H&E), Alcian blue, and Alizarin red S staining. The slides were viewed microscopically and photos obtained.

RESULTS

Clinical and physical examinations

All animals rapidly recoverd after surgery and remained in good health throughout the course of the experiment. There was no evidence of infection or other complication in any animal. 28 days postsurgically, control (unfilled) defects did not exhibit bone formation gross anatomically, as they were soft to palpation. In contrast, defects filled by BMG were hard to palpation after 14 days of operation.

Histologic findings

Control group: After 21 days defects location were filled by fibrous connective tissue (CT). A narrow rim of new bone (NB) appeared around the edge of defects occasionally. A few new bone claster (NB) could be seen near the host bone (Fig. 1).

BMG group: Healing of BMG group was characterized by endochondral ossification. At 7 days after BMG implantation, cartilage tissue (Car) with numerous chondroblasts were interposed between BMG particles (Fig. 2).

In addition, specific cartilage staining (Alcian blue) showed the positive results.

On day 14 after implantation typic chondrocytes (CHO) with their territorial cartilage matrix (Cal Car) appeared (Fig. 3). Also, undecalcified paraffin sections of Alizarin red S staining showed calcium deposition (CD) between BMG particles (Fig. 4). Numerous trabeculae spicules (TB) of woven ,primary, bone and highly proliferated red bone marrow (BM) well developed, but no hyaline cartilage was apparent on day 21. At day 28, typic bone trabeculae (TB), greatly active regulated cuboidal osteoblasts (ob), numerous osteocytes (o), some multinucleated osteoclasts (oc), marrow cavity and rich red marrow cells (mc) were observed in BMG group (Fig. 5). BMG particles were absorbed almost entirely (Fig. 6).

DISCUSSION

Repair of bony defects in human, specially in the craniofacial region, repersent a major challenge for the surgeons. Traditionally, autogenous cancellous bone from a source such as the iliac crest is considerd to be the best material for bone grafting (17, 18), but this kind of grafting material is restricted and unavailable in sufficient amount, and effectiveness of this graft is often compromized by non-union, stress fracture and rejection of grafts (16).

Recently some of the reasearchers prepared bone derived osteoinductive particles by various methods from bones (3, 6, 19, 20) which could induce new bone via endochondral ossification. Rabbi used DBM for skull bone repair in rabbits (19, 20). Dauglas used DBM in horses skull (20) and Sato used Bone Morphogenic Protein (BMP) in dogs skull (22) with different results.

This study was designed to investigate the effect of BMG on the healing of cranial bone defect in rats. The defect was 5-mm in diameter and full thickness of the parietal bone and the periosteum was entirely disrupted

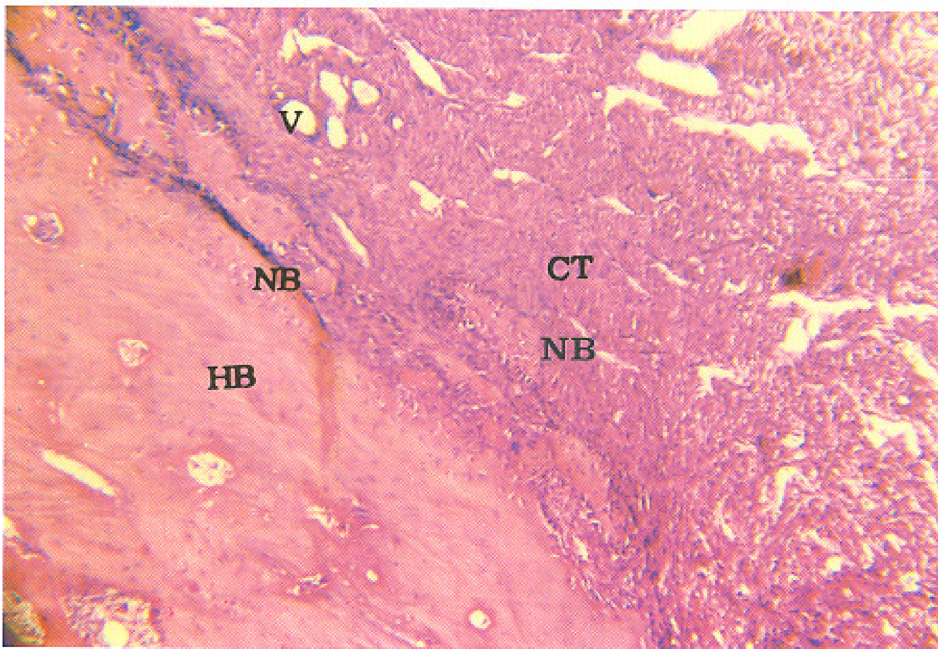

Fig 1. Photomicrograph of control (unfilled) calvaria defect at day 21 after operation. A thin margin of new bone (NB) attached to host bone (FIB) appeared. Also, a few new bone clusters near the defect margin and blood vessel which invaded to fibrous connective tissue (CT) are present. H & E staining original magnification × 100.

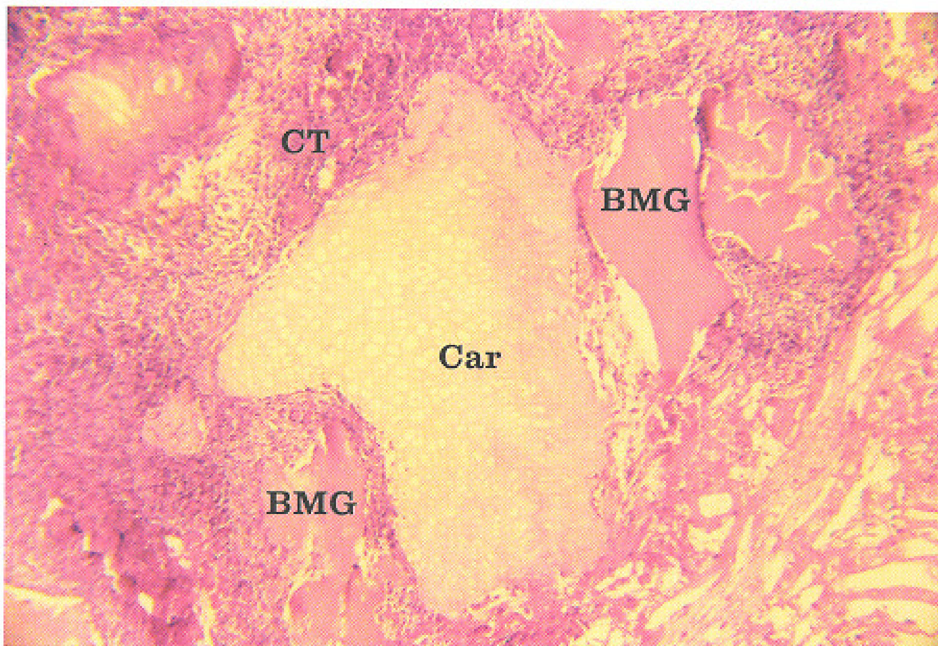

Fig 2. Photomicrograph of BMG induced hyaline cartilage in a rat calvaria defect at day 7 after operation. Hyaline cartilage between BMG particles and connective tissue (CT) is present. H&E staining. Original magnification ×100

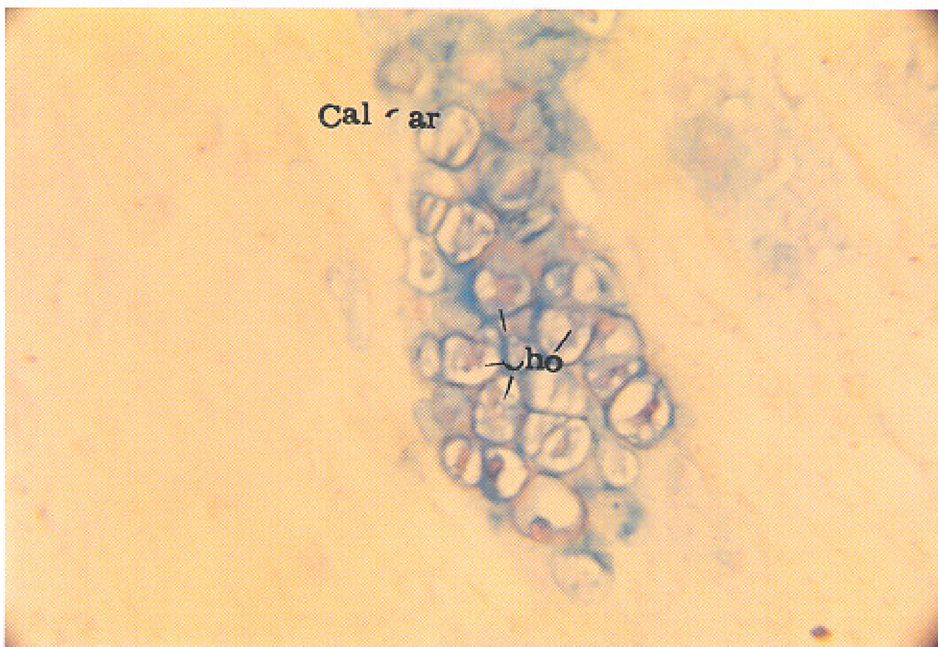

Fig 3. Photomicrograph of BMG induced endochondral bone ossification in a rat calvaria defect at day 14 after operation. Intrapted Calcified cartilage (Cal Car) with late chondrocytes (Cho) well showed. Alcian Blue staining. Original magnification ×400

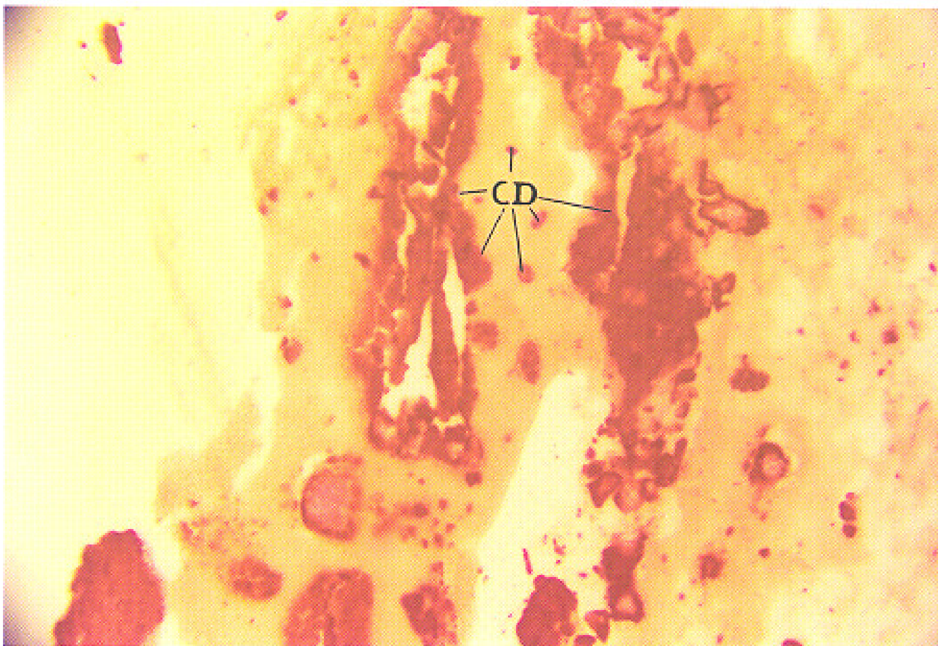

Fig 4. Photomicrograph of rat calvaria defect, used BMG at day 14 after operation, revealed calcium deposition (CD). Alizarin red staining. Original magnification ×100

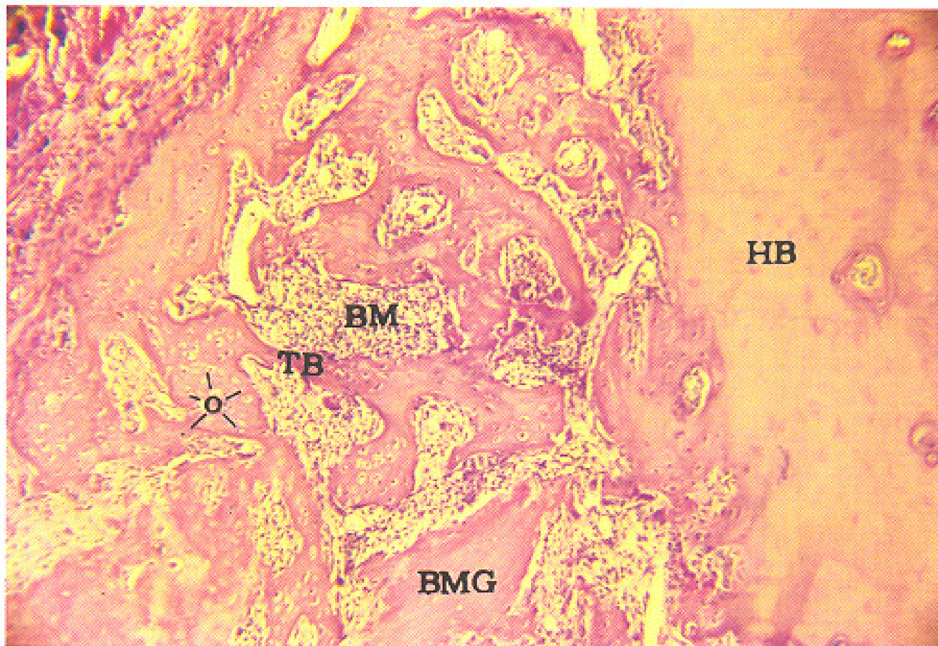

Fig 5. Photomicrograph of new bone formation in a rat calvaria defect at day 21 after operation. A) Note trabeculae spicule of woven bone (TB), most osteocyte (o) and well-developed bone marrow (BM) which exhibit adjacent to host bone (HB). H&E staining. Original magnification ×100

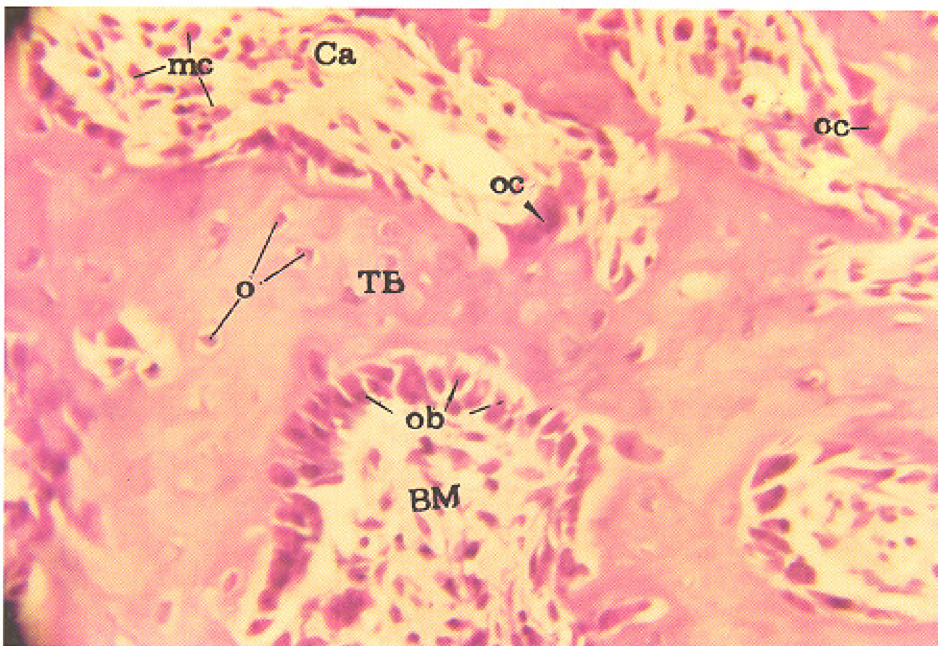

Fig 6. Photomicrograph of new bone formation in rat calvaria defect at day 28 after operation. Note trabeculae bone (TB) with lacunated osteocyte (o), active osteoblasts (ob), many osteoclasts (oc), highly proliferated bone marrow and numerous mononuclear cells (mc). H&E staining. Original magnification ×400

and not replaced. The BMG could induce new bone formation in multi-step cascade (23, 24). The formation of new bone began with chemotaxis of osteoprogenitor cells and their attachment to BMG (24). This process is followed by proliferation and differentiation of attached cell to chondroblast (25). Bone begins to form when the cartilaginous matrix undergoes calcification, replacement by osteoid and substitution by osteoblasts. Approximately in most studies, new bone formation in the implants was woven bone, although small amount of lamellar bone was present (25). There are many possible reasons for this event, including the characteristic of host mesenchymal cell biology, differences in mechanical stress on grafted tissue (26), and architecture variation of implanted materials (26). Reddi et al reported the large amount of cartilage, which formed in the center of implants by day 5 and 7 after implantation.They also emphasized that at this stage, the center of implant had not yet vascularized (5). Capillary ingrowth on day 9 is followed by start of bone formation on day 10 (5). Results of our study were agreeable with findings of Reddi et al (26) and Hosny (27) who reported bone formation intramuscular and in parietal bone defects by manner of endochondral after 7 days. Daoglas et al also reported intramembranous ossification using Ec BMG. They showed initiation of bone formation 5 weeks after BMG implantation only in one case (21). These findings probably resulted from insufficient capability of DBM particles because of lack of inducer component for any reason. We believed that BMPs component of BMG particles had a principal role in direction of ossification.

In the present study, 7 days after operation, endochondral ossification in parietal defect was observed. This cartilage tissue located between BMG particles which indicated that the differentiation of mesenchymal cells was dependent on BMP components of BMG. It can be a reason for guidance of BMG component in differentiation proceedings of mesenchymal cells. In this line, only Dauglas et al reported intramembranous ossification using Ec DBM (21). Dauglas also said that the initiation of bone formation began 5 weeks after Ec DBM implantation only in one animal. A lack or weakness of BMP component of DBM could cause this finding .Using Ec DBM individually or in combinations with Im DBM, Rabbi et al (12, 13) reported endochondral ossification. Also, Reddi (28) believed that BMPs enclosed chondrogenic proteins. Our confidence established on the point that Im BMG only could stimulate chemotaxis of mesenchymal cells and differentiation of mesenchymal cells to chondrocyte enrolled by endochondral BMG components.

Specific staining of cartilage (Alcian Blue) at 14th day after implantation revealed hypertrophic and calcified chondrocytes at implants region. The occurrence confirmed by Alizarin red S staining. The findings of many investigators support our results (12, 13). The results of Douglas (21) are in contrary with our findings. The different reasons may be related to method of BMG preparation and inactivity of BMG component or animal models.

Twenty-one days after operation, the numerous trabeculae spicules, early adult osteocytes and highly proliferated red bone marrow well developed. Finally typic bone trabeculae with regulated cuboidal osteoblast cells, some multinucleated osteoclast cells, marrow cavity and rich marrow cells appeared at day 28 after operation, which indicated good bone repair. Hosny et al (25) reported same results after 72 days by DBM and Rabbi et al (12, 13, 29) used DBM for parietal defects and studied the specimens after 14th day of operation. Also, Peng et al (16) reported endochondral ossification in thyroid cartilage defects 28 days after DBM implantation. Conclusion of this study estabilished on an endochondral ossification by BMP component of EC BMG.

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