Cells Seeded on MBG Scaffold Survive Impaction Grafting Technique: Potential Application of Cell-Seeded Biomaterials for Revision Arthroplasty

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ABSTRACT: The objective of this study was to investigate the potential application of cell-seeded biomaterials for revision arthroplasty and the reconstruction of major joints using the impaction grafting technique. Using morselized cancellous bone graft as a porous scaffold, MG63 cells were seeded on the scaffold and impacted into an acetabulum cup model using a mechanical device constructed from data obtained during impaction grafting by an orthopedic surgeon. Immediately after impaction, cells were trypsinized from the scaffold and processed for cell survival rates using the double-stranded DNA PicoGreen® assay. Significant reductions in viable cells were observed between the fifth impact and both the first and second impacts ($p < 0.01$ and $p < 0.05$, respectively). Cell survival rate was 21.5% after five impacts. The biological performance of cell-seeded biomaterials may be enhanced by these surviving cells. Compared to allograft bone that is not osteogenic, a cell-seeded biomaterial might also be a suitable substitute for allograft bone for major joint reconstruction at revision arthroplasty. © 2006 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 24:501–507, 2006

Keywords: revision arthroplasty, impaction grafting; cell survival; allograft bone substitute; tissue engineering

INTRODUCTION

Despite the long-term pain relief and improved mobility provided by "the greatest single advance in orthopedic surgery,"[1] as many as one in five of the 500,000 hip arthroplasty operations performed worldwide fail within 10 years and require revision.[2,3] Revision hip arthroplasty is expensive, and the predicted outcome is typically worse than for the original procedure because of diminished femoral bone stock.[1–4] Furthermore, bone can be lost during the removal of the original prosthesis and bone cement, leaving femoral or acetabulum cavitary defects.[4] In the UK, approximately £40 million is spent on revision hip surgery annually,[5] and costs often include the procurement and processing of bone grafts. Typically three to six femoral heads (at £250 each) obtained from patients undergoing elective primary total hip or from cadavers are used per revision operation[6] using impaction grafting. Impaction grafting involves the compression of autologous bone or donor allograft bone into the defects to reconstruct the medullary canal and the acetabulum.[4,7–9]

Unfortunately, morbidity to the donor site, generally the iliac crest from where bone is often harvested, and limited bone supply prohibit the extensive use of autograft bone.[10,11] Risks of disease transmission and concerns about immunogenecity as well as legislation and regulations of bone grafts are additional problems hindering continued application of allograft bone.[6,9,12] Synthetic biomaterials may offer a seamless alternative, providing large supplies of pathogen-free

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material that are also versatile in structural configuration (powders, granules, solids, or porous blocks).[10,13–16] Furthermore, biomaterials such as tricalcium phosphate and hydroxyapatite exhibit excellent osteoconductive and biocompatibility properties,[17–19] which may be further enhanced by addition of osteogenic cells derived from the patient's autologous stem cells or growth factors such as bone morphogenetic proteins.[20] The successful application of cell-seeded bioceramics for bone filling and bone replacement would undoubtedly revolutionize orthopedic surgery with the potential for personalized medicine.

With this impetus, we have undertaken an in vitro study to investigate the survival rate of cells seeded on porous matrices after the application of impact forces generally employed in the operating room. We hypothesized that by virtue of the macrostructural architecture of a morselized cancellous bone graft scaffold, some cells would survive the effects of the impact forces.

MATERIALS AND METHODS

Fresh bovine cancellous bone from the proximal femora was milled using the Norfolk^{TM} Mark 2 bone mill with a stainless steel cutting blade with 6-mm holes (New Splints, UK) in the same manner morselized human allograft bone graft is produced in the operating room. The mean length of the morselized bone graft was 4.4 mm (range 2 to 10 mm). The graft material was defatted with ethanol and cleaned with copious water, leaving a porous structure of approximately 1 g/cc apparent density (Fig. 1). After cleaning, the graft material was placed in air-tight plastic bags and stored at -80°C until use.

To determine the impact forces employed by the surgeon we used a metal hammer with a modified linear variable differential transducer (LVDT) to make an accelerometer, a stainless steel hemispherical impactor (43 mm diam), a load transducer (15 kN capacity; Novatech, UK), and a data acquisition system (Spike2 Version 5.08). The surgeon replicated the impaction technique as best as he could using a wooden acetabulum cup model (48 mm in diameter) with several 1-mm holes to facilitate fluid perfusion during impaction (Fig. 2). The cup was filled with 12 g of hydrated bone graft and impacted while impact forces were measured using the load transducer and the accelerometer. This was repeated about five times, and the recorded data provided information on the number of impacts used on a single layer of morselized graft. Using conservation of energy equations for potential and kinetic energies, average impact forces were calculated and used to develop the impaction system (Fig. 2), which simulated the surgeon's hammer strikes by dropping a 1.4-kg steel cylinder from a height of 0.63 m.

An osteosarcoma cell line, MG63, was used to seed the morselized cancellous bone graft scaffold and to determine cell survival after impaction. Stored frozen cells were plated in T75 flasks in high glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, 100 U/mL and μg/mL penicillin/streptomycin, 2.5 μg/mL fungizone, and 2 mM L-glutamine. Medium was first changed after 7 days culture and twice a week thereafter. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2 with up to two to four passages. Then the cells were harvested using 0.25% trypsin/EDTA and counted using a haemocytometer before seeding on 100 mm^2 Petri dishes at 5×10^6 cells per dish density. All cell culture medium,

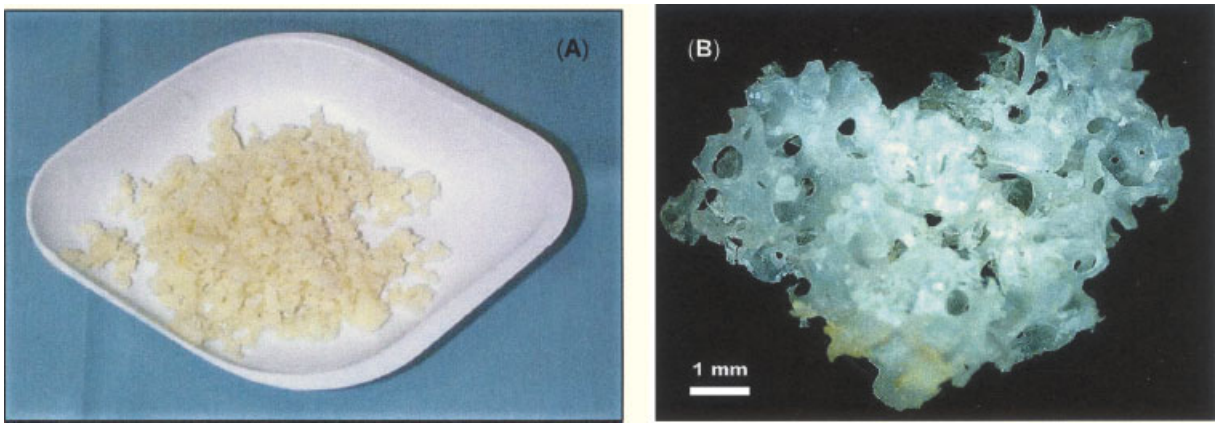

Figure 1. Photographs of morselized cancellous bone graft after cleaning and defatting (A), and the architectural structure of a representative scaffold, apparent density = 1 g/cc (B). [Color scheme can be viewed in the online issue, which is available at http://www.interscience.wiley.com]

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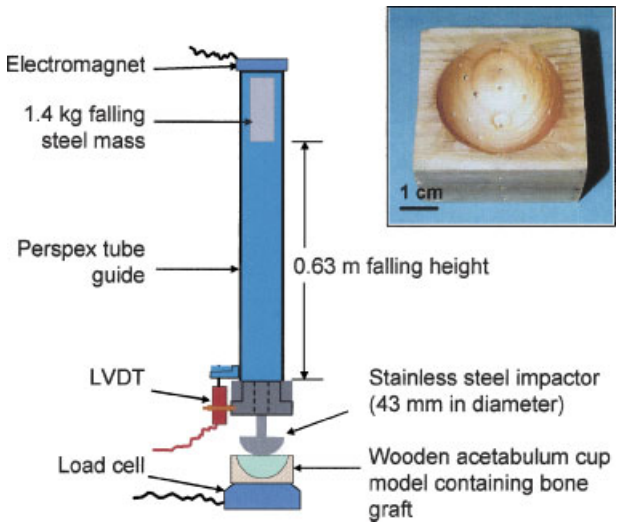

Figure 2. Schematic representation of the impaction system. Insert is a photograph of the wooden acetabulum cup model with 1mm holes for fluid drainage during impaction. [Color scheme can be viewed in the online issue, which is available at http://www.interscience.wiley.com]

regents, and plasticware were purchased from Invitrogen Corporation (Paisley, UK). Before seeding all morselized cancellous bone graft matrices were sterilized by immersion in 70% ethanol for 2 min, then rinsing with phosphate-buffered solution (PBS, pH 7.2) twice, followed by immersion in DMEM supplemented with 100 U/mL and μg/mL penicillin/streptomycin for 1 min, and finally two PBS washes before storage at −20 C. Before cell-seeding on matrices, all grafts were soaked in DMEM overnight. The seeding procedure was carried out in two steps to ensure attachment of cells into the graft matrices. First, 5×10^6 cells in 1 mL DMEM were dropped on top of a heap of matrices in the Petri dish and cultured for 45 min. Second, the heap was spread out into a monolayer, and 10 mL DMEM was added. The seeded cells were then allowed to grow on the morselized cancellous bone graft matrices for 48 h at standard culture conditions.

Forty-eight hours after incubation, five portions of 12 g of cell-seeded matrices were placed in five autoclaved wooden acetabulum cup models, and each filled cup was subjected to one, two, three, four, or five impacts. Axial deformation of the graft scaffold was measured using the LVDT, and compressive forces exerted both by the material and the cup were measured using the load cell (Fig. 2); 2×6 g of cell-seeded morselized cancellous bone graft were not impacted, and used as controls. To remove bias in the viability assay, codes were used to label cups, and the person doing the assay was not allowed to see the impaction procedure. Immediately after impaction, the compressed cell-seeded morselized cancellous bone graft matrices were removed from the cups and transferred into 50-mL centrifuge tubes.

For cell viability assay, compressed graft matrices were transferred into 50-mL centrifuge tubes containing 5 mL 0.25% trypsin/EDTA solution and incubated for 3 min to detach cells from the matrices. This was followed by the addition of 5 mL DMEM with 10% FCS to the tube to neutralize the enzyme, washing with vigorous shaking before the medium was collected in 15-mL centrifuge tubes and centrifuged at 250 *g* for 5 min. The pellet was resuspended in 1 mL DMEM for further analysis with PicoGreen® (Molecular Probes, Eugene, OR). In brief, 150-μL cell suspension from each sample was added in triplicates in 96-well plates and incubated overnight to allow cell attachment. The medium was then removed, and wells were washed three times with carbonate buffer (pH 10.2). After adding 100 μL 0.1% Triton X-100/carbonate buffer (Sigma, UK) into each well, cells were fully lysed by four cycles of freezing (at −80°C) and thawing (at 37°C). Fifty microliters of cell lysates from each sample were then transferred in triplicates into 96-well plate. DNA standards (Salmon testes, Sigma, UK) were diluted in 0.1% Triton X-100/carbonate buffer to serial concentrations from 8 μg/mL to 0.25 μg/mL, and 50 μL of each concentration was transferred in duplicates to 96-well plate. PicoGreen® reagent was diluted 1:50 in Tris-EDTA buffer, 50 μL of which was added to each well of samples and standards. The fluorescence intensity was measured using a fluorescent plate reader (Tecan GENios, Maennedorf, Switzerland) at an excitation wavelength of 485 nm and emission wavelength of 538 nm. Cell numbers were thus expressed as DNA content (μg/mL), and cell survival rates were expressed as a percentage of DNA content relative to the control DNA content.

To examine cell attachment and deformed matrices after impaction, representative control and impacted cell-seeded graft matrices were randomly collected and prepared for observational analysis using a JEOL 840A scanning electron microscope (SEM). Sample specimens were fixed in 3% glutaraldehyde in 0.1 M Cacodylate buffer at 4°C for 24 h, and then dehydrated by immersion in increasing concentrations of ethanol from 50 up to 100% before drying in a critical point drier (CPD, E3000, East Sussex, England). The dried specimens were fixed onto a stub and coated with gold in a Polaron SEM sputter coating system before analysis on the SEM.

Significant differences within and between cell-survival groups were identified by analysis of variance (ANOVA- single factor) using SPSS™ (11.5, SPSS Inc, Chicago, IL). Tukey's test was performed post hoc and differences were considered significant at $p < 0.05$.

RESULTS

On average, the surgeon applied five impacts on a single layer of morselized cancellous bone graft in the acetabular cup, and the calculated average impact force varied between 600 and 900 N with

Table 1. Deformation of Matrices and Average Impact Forces with Increasing Numbers of Impacts Applied

Impacts Applied	n	Graft Deformation (mm)	Average Impact Force (kN)
1	5	12.241 ± 0.926[a]	0.690 ± 0.053
2	4	1.449 ± 0.177	5.865 ± 0.725
3	3	0.590 ± 0.322	19.809 ± 15.396
4	2	0.417 ± 0.324	28.935 ± 22.516
5	1	$0.238 \pm$ #DIV/01	$35.267 \pm$ #DIV/01

[a]Mean $\pm$ SD obtained from all cups at the same numbers of impacts applied.

deformation of material on the first impact in the order of 1 cm (Table 1). Less deformation or impactor penetration implied greater impact force; thus, the fifth impact exerted forces over 35 kN on the cell-seeded graft scaffold.

Two hours after seeding, osteosarcoma cells were seen attached to the graft surfaces (Fig. 3A and B). Although distribution was inhomogeneous, cells were observed within the macropores of the porous scaffold (Fig. 3B). After impaction, cells were still attached to the bone scaffold surfaces, which showed evidence of cracking caused by the two and five impacts applied during the impaction procedure (Fig. 3C and D, respectively). With five impacts, it appears some macropore remained open with cracks (arrows).

Cell counts and survival rates (Table 2) were significantly different between the control group (with no applied impacts) and all the other groups ($p < 0.001$) and between the groups with one and two impacts and the group with five impacts ($p < 0.01$ and $p < 0.05$, respectively). Relative to the control group, 39.7% of all the seeded cells were

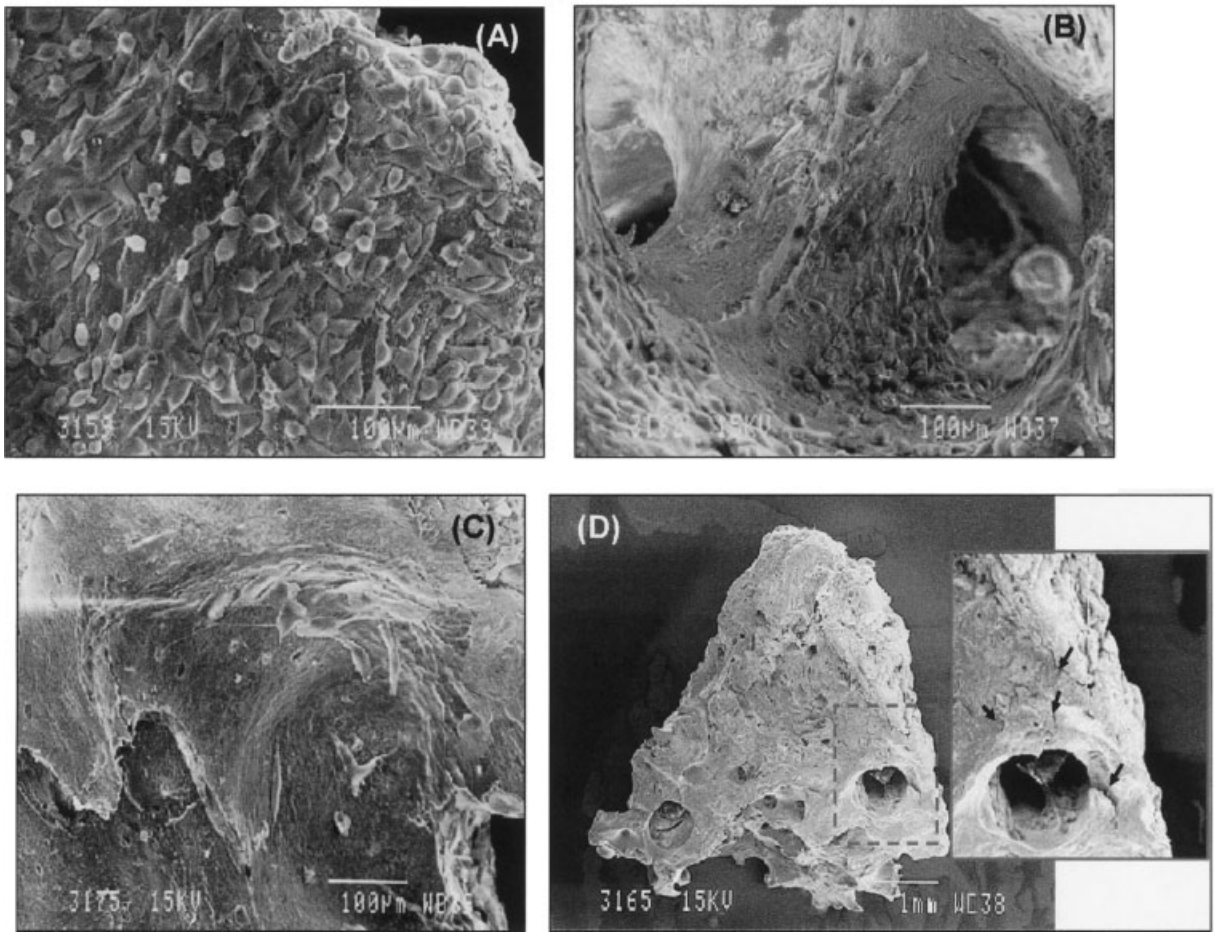

Figure 3. Photomicrographs showing cell adhesion on morselized cancellous bone graft scaffold surface (A) and within the pores (B) 2 h after seeding. After 2 (C) and 5 (D) impacts, cells were still attached to the matrices, which showed evidence of cracking and compression of the morselized cancellous bone graft scaffold, but with some pores evidently unobstructed (D insert).

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Table 2. Cell Survival Measured by PicoGreen® Assay Assessing the Amount of DNA from Intact/Viable Cells after Impaction

Impacts Applied	DNA Content (μg/mL)	DNA *Relative to Control* (%)
0	0.723 ± 0.165	100.0
1	0.436 ± 0.061^{a}	61.3
2	0.386 ± 0.162^{b}	54.3
3	0.343 ± 0.064	48.2
4	0.270 ± 0.026	37.9
5	$0.153 \pm 0.040^{a,b}$	21.5

Control matrix was significantly different from all other groups.

[a] 1 versus 5 $p < 0.01$.

[b] 2 versus 5 $p < 0.05$. Data presented as mean ± SD ($n = 12$ for the control and $n = 6$ for other groups).

destroyed after the first impact; by the fifth impact, however, over 21% of the cells were still viable.

DISCUSSION

The impaction grafting technique has improved the outcome of revision arthroplasty surgery,[1,6,21,22] but with increased need for these operations, the predicted shortages of allograft bone could become a realization.[12] Concerns over transmission of viruses such as hepatitis and HIV,[23,24] and other potential contamination, will inevitably increase the demand for more nonbiological or tissue-engineered biomaterials instead of allograft bone grafts. Furthermore, while allograft bone is osteoinductive and osteoconductive, osteointegration into the host bone is slow and incomplete, and biomechanical properties vary considerably, leading to inconsistent performance.[5] Therefore, several studies have been conducted to improve allograft supplies by combining harvested bone graft with synthetic biomaterials to form so-called synthetic bone graft extenders.[25,26] Others have opted to forsake allograft bone and to use synthetic biomaterials as the sole bone substitutes for impaction grafting.[5,21] As well as replacing bone, these efforts are also aimed at reducing subsidence, thereby improving the implant stability.[5] Subsidence is an important subject but is beyond the scope of this communication, and thus will not be discussed any further. However, in our view, should a significant number of seeded cells survive impaction, osteogenesis might occur quicker, and therefore implant subsidence may be reduced, consequently improving the stability of the prosthesis.

We used a cancellous bone scaffold for two reasons: to evaluate the surgeon's impaction technique using material similar to human morselized bone graft, and to provide a substrate that was friendly to osteoblastic-like cells.[27] Compared to osteoblasts, the robust MG63 cells were chosen because they adhere well to bone scaffold, and in our experience have been good surrogates for bone cells in cell-attachment studies. No effort was made to ensure a homogeneous distribution, and thus further work is planned to address this important aspect of cell seeding. We hypothesized that cell distribution particularly within the pores would alleviate cell death, thus ensuring a high rate of cell survival. A remnant of cells did indeed survive the high impact forces (exceeding 30 kN), presumably because they were protected within the macropores of the matrices. However, this protection has its limitations because in vivo other factors, including oxygen concentration, play a significant role on cell survival. However, although mature cells (such as osteoblasts) have higher metabolic activity, hence requiring high oxygen supply, the use of stem cells or less committed cells (such as osteoprogenitor cells) may ensure cell survival in a low-oxygen environment.[28] Therefore, instead of using osteoblasts to seed biomaterial matrices for impaction grafting purposes stem cells could be considered. Nevertheless, structure and biomechanical strength of the porous biomaterial will always be very important to cell survival upon impaction. Compared to the morselized cancellous bone graft used in this study, the brittle nature of hydroxyapatite or tricalcium phosphate bioceramics might result in more cell death upon impaction; therefore, toughening the bioceramic might preserve the architecture of the porous scaffold matrix and the seeded cells.

The calculated impact forces (700 N to 35 kN) are in agreement with other investigators,[29] so we are confident our impact forces are realistic. The seeded cells experienced the same forces applied in the operating room; hence, these findings could be translated with caution to the clinical setting. MG63 osteosarcoma cells were used, so we wonder if the nature of these cancer cells contributed to the observed survival rates. In addition, while our impact forces were realistic and the developed impaction system applied reproducible forces (Fig. 4), the impactor size was not changed during the procedure. Clinically, progressively larger impactors (in increments of 2 mm) are used to ensure adequate compression of the morselized

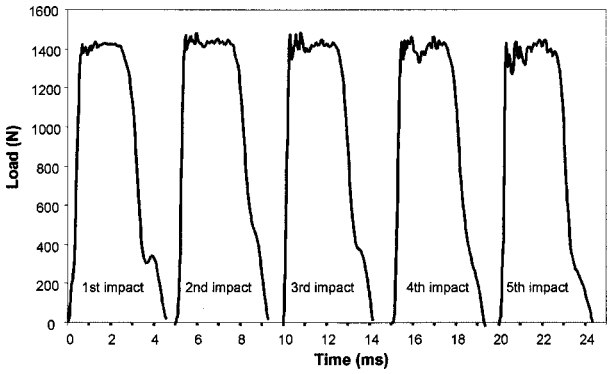

Figure 4. Reproducible impact forces employed by the impaction system on morselized cancellous bone graft. As the scaffold compresses with increasing impacts, impulse is reduced.

cancellous bone graft at the bottom as well as the rim of the acetabular cup.[6]

The objective of this study was to investigate the rate of cell survival with increasing impacts, and thus a single layer of graft was employed in a simple and yet informative model. Further studies will employ a model that will incorporate a layer of exothermic bone cement,[30] to mimic closely the clinical situation. In addition, local chemical effects of PMMA at the bone–cement interface will be considered. Because of its low monomer solubility, methacrylate monomer is prevented from being systemically absorbed in vivo; thus, it remains at the interface and diffuses into the local tissues where the unpolymerized monomer kills the local cells.[22,23] So the question remains, how many cells will survive in vivo?

We hope our study provokes more ingenious methods to preserve cell viability and development of cell-seeded biomaterials to substitute allograft bone. Our findings have raised questions, dealing with the development of impaction grafting models, the application of uncommitted cells, such as bone marrow stromal cells or osteogenic precursor cells, to seed porous biomaterials to substitute allograft bone grafts for revision arthroplasty, and evaluation of cell proliferation and differentiation after impaction loading.

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