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ORIGINAL PAPER

Alternatives to autograft evaluated in a rabbit segmental bone defect

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Abstract
Purpose This study was designed to identify strategies for treating bone defects that can be completed on the day of surgery.
Methods Forty New Zealand white rabbits with unilateral rabbit radius segmental defects (15 mm) were treated with commercially available scaffolds containing either demineralised bone matrix (DBM) or a collagen/beta-tricalcium phosphate composite (Col:β-TCP); each scaffold was combined with either bone marrow aspirate (BMA) or concentrated BMA (cBMA). Bone regeneration was assessed through radiographic and histological analyses.
Results The concentration of nucleated cells, colony-forming unit-fibroblasts and platelets were increased and haematocrit concentration decreased in cBMA as compared to BMA ($p<0.05$). Radiographic analyses of bone formation and defect bridging demonstrated significantly greater bone regeneration in the defects treated with DBM grafts as compared to Col:β-TCP grafts. The healing of bones treated with Col:β-TCP was improved when augmented with cBMA.
Conclusions Scaffolds containing either DBM or Col:β-TCP with BMA or cBMA are effective same-day strategies available to clinicians for the treatment of bone defects; the latter scaffold may be more effective if combined with cBMA.

Keywords Rabbit · Bone marrow aspirate · Segmental defect · Tricalcium phosphate · Demineralised bone matrix

Introduction

The complications associated with impaired bone healing have been well documented [1]. The current gold standard to overcome deficiencies in bone healing is the use of autograft [2]. Significant advances have been made in the field of bone tissue engineering with the goal of replacing the use of autogenous bone grafts to avoid the negative effects associated with them. For example, biomaterials, cells and growth factors are manipulated to create tissue-engineered constructs that have both significantly improved our understanding of regenerating bone and demonstrated the potential of regenerative medicine (for review see [3–5]). Although these advances may result in ideal bone healing in the future, it is important for clinicians to be aware of bone healing alternatives to autograft that are currently at their disposal.

Among the tissue-engineering strategies being developed, those using either demineralised bone matrix (DBM) or beta-tricalcium phosphate (β-TCP) have been extensively explored [6–12]. Given the strong foundation supporting their effectiveness and regulatory status it is not surprising that scaffolds incorporating these materials are commercially available. Experimental investigations to improve their use are ongoing [8–11]; however, the overall objective of the vast majority of studies is to develop or optimise materials that have not yet received regulatory approval. Stem cell-based strategies, especially those using bone marrow aspirate (BMA), have been investigated to minimise the use of autograft [13, 14]. The

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means to concentrate a relatively less pure, heterogeneous mixture of cells from BMA is also being used in preclinical studies and is commercially available [8, 15]. Similar to the development of biomaterials, much of the research effort is directed towards the advancement of novel stem cell-based strategies rather than improving upon the use of approved methodologies. Collectively, the accessibility of both commercially available scaffolds and devices capable of enriching BMA supports the use of same-day strategies to improve the healing of bone defects.

Due to the paucity of experimental studies using clinically relevant, same-day strategies, the tools that are currently available to clinicians are not always clear. To this end, the objective of our studies was to evaluate bone regeneration using commercially available scaffolds containing DBM or β-TCP when combined with either BMA or concentrated BMA (cBMA).

Materials and methods

Experimental animals

This study was conducted in compliance with the Animal Welfare Act, implementing animal welfare regulations, and in accordance with the principles of the Guide for the Care and Use of Laboratory Animals. All animal procedures performed in this study were approved by the Institutional Animal Care and Use Committee at the US Army Institute of Surgical Research. Adult, female New Zealand white rabbits (~4 kg) were housed in a vivarium accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. A total of 40 surgical animals were used for this study, ten animals per group (n=10/group).

Bone marrow aspiration and concentration

BMA was collected in syringes containing anticoagulant (ACD- A, Anticoagulant Citrate Dextrose, Arteriocyte Medical Systems, Hopkinton, MA, USA) after access to the bone marrow compartment of the tibia and iliac crests of donor animals while under anaesthesia, similar to that described previously [16]. The ratio of ACD-A to aspirate was 1:6.5; a total volume of 29.98 ml±1.64 of ACD-A:aspirate was collected per animal. After the pooling and filtration of aspirates from two animals an aliquot of BMA (3 ml) was set aside for cell analysis and scaffold loading, and the remainder was used to generate cBMA using the Magellan® MAR01™ (Arteriocyte Medical Systems, Hopkinton, MA, USA) system as per the manufacturer's instructions. cBMA was also subjected to cell analyses and used to load scaffolds. Each batch of BMA or cBMA was used to load both a DBM and collagen-β-TCP (Col:β-TCP) scaffold to control for donor variability.

BMA and cBMA analyses

An aliquot of BMA or cBMA (~1 ml) was subjected to red blood cell lysis using 2 % acetic acid in water and the nucleated cells counted manually using a haemocytometer. The fibroblast colony-forming unit (CFU-F) assay was used as an indicator of progenitor cell content in BMA and cBMA, similar to that described previously [17]. A total of 4×10^5 nucleated cells were plated per well of a six-well plate in triplicate in media consisting of α-Minimum Essential Media, 10 % fetal bovine serum and 1 % penicillin/streptomycin in a humidified incubator (5 % CO_2, 37 ° C). Forty-eight hours after seeding media was changed, and media was changed every other day thereafter for 10 days. Cells were then washed with phosphate-buffered saline (PBS) and fixed with a 1:1 mixture of acetone:methanol for ten minutes at room temperature. The plates were allowed to air dry and stained with Giemsa to allow for counting of cell colonies. The CFU-F quantification was performed by an independent, blinded reviewer. An aliquot of each sample was also used for complete blood count analyses for haematocrit and platelet quantification.

Graft preparation

DBM grafts: Rabbit DBM powder (MAROMatch™, Arteriocyte Medical Systems, Hopkinton, MA, USA) was thoroughly rehydrated with either BMA or cBMA and a poloxamer reverse phase medium formulated into a gel-like form (MAROFuse™, Arteriocyte Medical Systems, Hopkinton, MA, USA) in a 1:1:1 ratio (1 cc MAROMatch™+1 cc MAROFuse™+1 cc BMA or cBMA). The preparation time for DBM grafts with BMA or cBMA was approximately five minutes. DBM grafts were then incubated for ten minutes prior to implantation at room temperature.

Col:β-TCP grafts: Col:-β-TCP composite scaffolds (Integra LifeSciences, Plainsboro, NJ, USA) were pre-cut to approximately 15 mm, placed in a 30-cc syringe joined by a stopcock to another syringe containing either 1 cc BMA or cBMA. The scaffolds were loaded with either BMA or cBMA by passing the aspirate through the syringes under negative pressure. Each graft material was incubated in either BMA or cBMA for ten minutes prior to implantation at room temperature.

Surgical procedures

After a ~40-mm incision over the mid-diaphysis of the radius, a 15-mm segment was removed using an oscillating bone saw with a copious amount of saline irrigation resulting in a critical-sized defect similar to previously described [16]. The segmental defect was then replaced with either (1) DBM+

BMA (DBM-BMA), (2) DBM+cBMA (DBM-cBMA), (3) Col:-β-TCP+BMA (Col:β-TCP-BMA) or (4) Col:-β-TCP+cBMA (Col:β-TCP-cBMA) and the soft tissue layers and skin were both closed with 4–0 Vicryl (Ethicon, Somerville, NJ, USA). Due to the radioulnar syndesmosis that forms the fibrous joint between the radius and the ulna, additional fixation of the defect was not required.

Radiographic analysis

Radiographs were taken immediately and four and eight weeks after surgery (MX-20 X-ray machine, Faxitron Corporation, Tucson, AZ, USA). Each radiograph was taken with an exposure time of 15 seconds and a tube voltage of 35 kV_p. Radiographs were scored for bone formation and bridging by five blinded observers (including an orthopaedic surgeon) as described by Bodde et al. [18] using the scoring system presented in Supplemental Table 1.

Histological examination

A subset of samples (n=5/group) was prepared for histological analyses. Each specimen was dehydrated in a graded series of alcohols and embedded in polymethyl methacrylate without decalcification. Specimens (5 mm) were sectioned along the vertical axis in the middle of the defect site, stained with modified Gomori's trichrome and scanned using a NanoZoomer Digital Pathology System. The histology slides were used to measure new bone formation using the ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

SigmaPlot© 12.5 (Systat Software, San Jose, CA, USA) was used to run Student's t tests, Mann–Whitney or analysis of variance tests with Tukey's comparison analysis when appropriate to determine differences. Statistical significance was determined when $p<0.05$.

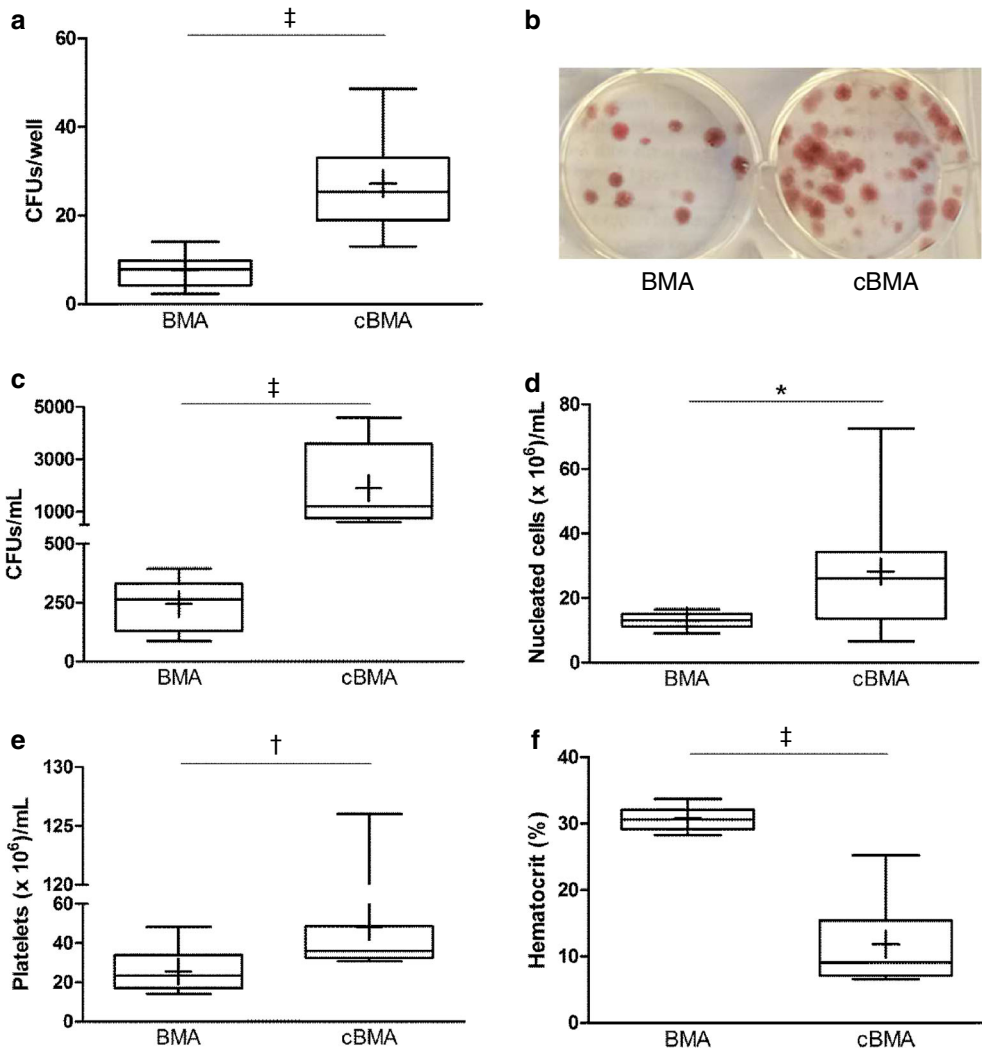

Fig. 1 BMA or cBMA were analysed for **a** CFUs/well, **b** representative CFU staining, **c** CFUs/ml, **d** the number of nucleated cells/ml, **e** platelets/ml or **f** per cent haematocrit. *Box plots* show the range and mean for each assay. *Cross* denotes the mean. *$p<0.05$, †$p<0.01$, ‡$p<0.001$

Results

Cell analyses

The number of CFU-Fs/well was significantly higher in cBMA than BMA (27.22±3.30 vs 7.67±1.14, respectively; $p<0.001$) (Fig. 1a, b). The number of CFU-Fs/ml was also significantly higher in cBMA than BMA (1,900.18±3.30 vs 244.31±33.89, respectively; $p<0.001$) (Fig. 1c). One outlier in the cBMA platelet sample was 2.8 standard deviations from the mean and was therefore excluded from the statistical analyses. The number of nucleated cells per millilitre within cBMA was significantly higher than BMA ($28.20\pm6.75\times10^6$ vs $13.06\pm0.74\times10^6$, respectively; $p<0.05$) (Fig. 1d). The number of platelets per millilitre was increased from 25.53± 3.35×10^6 for BMA to $48.11\pm10.04\times10^6$ for cBMA ($p<0.01$) (Fig. 1e). The per cent haematocrit was decreased from 30.78 ±0.55 % for BMA to 11.84±2.14 % for cBMA ($p<0.001$) (Fig. 1f).

Animal surgeries

A 15-mm segment was removed from the mid-diaphysis of the radius and replaced with either DBM-BMA, DBM-cBMA, Col:β-TCP-BMA or Col:β-TCP-cBMA (Fig. 2a). No surgical complications, adverse inflammatory response, infection, fractures or graft rejections were observed.

Radiographic analyses

At the time of surgery Col:β-TCP could be observed; however, those treated with DBM appeared empty, as the demineralised tissue cannot be detected using radiographs (Fig. 2b). Despite the presence of residual Col:β-TCP, there appeared to be more bone in the DBM group at four and eight weeks, which were evaluated using a standard scoring system [18]. Defects treated with DBM-BMA received a score of 3.62±0.10 and 3.7± 0.10 at eight weeks for bone formation (Fig. 3a) and bridging (Fig. 3b), respectively. All defects (10/10) had a score≥3 for both analyses. DBM-cBMA-treated defects received a score of 3.48±0.22 and 3.26±0.29 at eight weeks bone formation (Fig. 3a) and bridging (Fig. 3b), respectively. At eight weeks nine of ten and eight of ten defects treated with DBM-cBMA received a score≥3 for bone formation and bridging by eight weeks, respectively. None of the defects that were treated with Col:β-TCP-BMA achieved a score≥3 for bone formation or defect bridging by eight weeks; average values for the group were 1.56±0.15 and 0.84±0.15, respectively. Defects treated with Col:β-TCP-cBMA achieved a score≥3 in four of ten and two of ten animals at eight weeks for bone bridging and formation; average values for the group were 2.54±0.29 and 2.16 ±0.37, respectively. Within Col:β-TCP, but not DBM, there was a main effect of aspirate type for both bone formation and bridging (cBMA>BMA; $p<0.05$). Within DBM, but not Col:β-TCP, there was an increase in both bone formation and bridging between four and eight weeks ($p<0.05$). A summary of the average and individual radiograph scores are found in Supplemental Table 2.

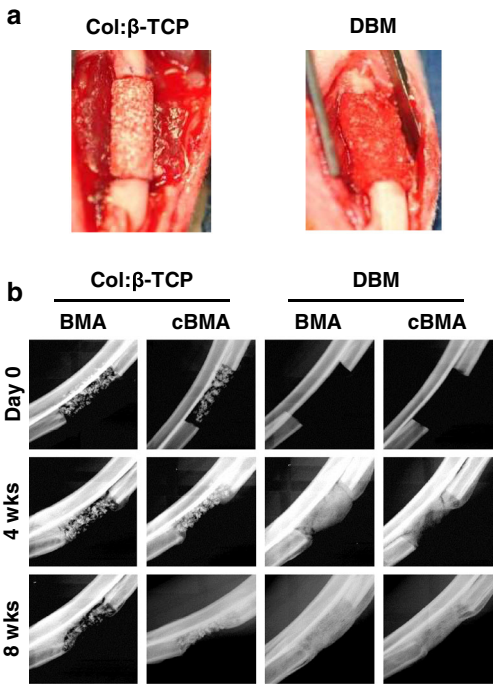

Fig. 2 **a** Photographs of Col:-β-TCP or DBM scaffolds that were loaded with either BMA or cBMA placed in a 15-mm rabbit radial defect. **b** Representative radiographs of radial segmental defects implanted with Col:β-TCP or DBM scaffolds containing BMA or cBMA at the time of surgery (day 0), 4 or 8 weeks after surgery

Histological examination

Histology sections (Fig. 4a–d) were used to quantify new bone formation within the area of the defect in a subset of samples ($n=5$/group). The results showed a significant increase in per cent bone in the DBM group as compared to Col:β-TCP (0.331 vs 0.229; $p<0.05$); however, no differences were seen between the BMA and the cBMA groups (0.289 vs 0.272; $p>0.05$).

Discussion

The primary objective of this study was to identify clinically relevant strategies capable of enhancing bone repair. Grafts were prepared using currently available scaffolds composed of materials that have been well studied, namely DBM and β-TCP, and supplemented with either BMA or cBMA. The major findings of the study are that (1) DBM grafts were capable of achieving union by four weeks post-surgery, regardless of whether BMA or cBMA were used, and (2) the

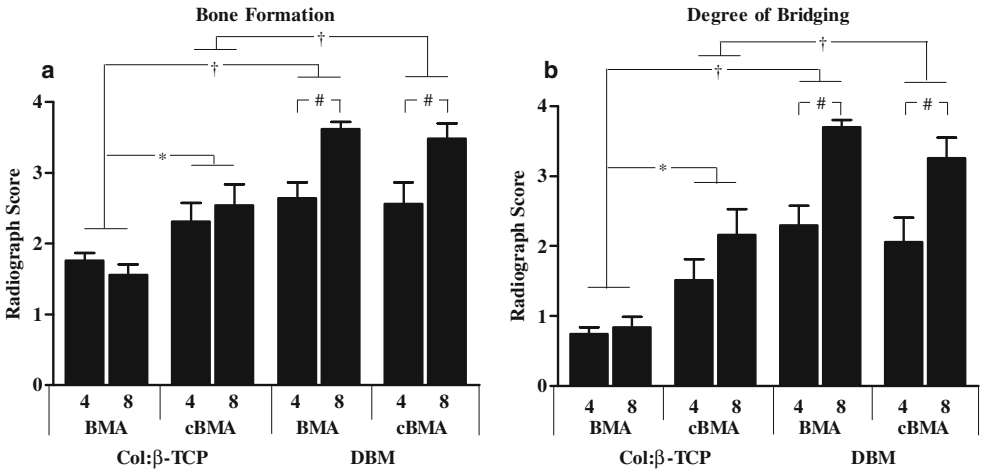

Fig. 3 Quantification of bone formation (**a**) and degree of bridging (**b**) as determined from the scoring of the radial radiographs at four and at eight weeks for the Col:β-TCP and DBM groups with BMA or cBMA. Results are mean±SEM; *$p<0.05$, † $p<0.01$, ‡ $p<0.001$

supplementation of Col:β-TCP composite grafts with cBMA improved bone healing as compared to those supplemented with BMA.

At first glance a logical speculation is that the differences in bone repair may be attributed to the main component (i.e. DBM or Col:β-TCP) contained within each scaffold, especially since both types of scaffolds were loaded from the same batches of BMA or cBMA. DBM has been shown to be osteoinductive because it retains measurable levels of bone morphogenetic protein (BMP) and drives stem cell differentiation and new bone formation [19–22]. β-TCP is an osteoconductive material [23] that supports new bone deposition but does not drive cellular differentiation. However, it is also important to take into account the differences in the delivery strategy for each material; Col:β-TCP composite grafts were delivered as strips that were cut to match the defect, while DBM was delivered in a putty form. Future studies that include a direct comparison between β-TCP and DBM using the same form of delivery will be useful to better understand critical parameters for bone repair.

The importance of delivering a sufficient number of progenitor cells to improve bone healing [17] provided the rationale for concentrating the BMA. In line with previous studies, the concentration of nucleated cells, CFU-Fs and platelets increased~2-, 3.5- and 2-fold with bone marrow concentration, respectively, so that a greater number of progenitor cells and platelets were delivered. The absolute values we observed for these parameters within BMA or cBMA are in general agreement with previously published work where rabbits [24], pigs [15] or humans [8] were used. Since the DBM grafts performed well regardless of whether BMA or cBMA were included, it was not possible to glean any additional information regarding the progenitor cell delivery within DBM. However, the finding that Col:β-TCP was improved with the use of cBMA allows for further speculation. A Pearson correlation test comparing the number of CFUs/well or CFUs/ml to bone formation within the Col:β-TCP demonstrated a significant correlation ($p<0.01$) with R^2 values of 0.50 and 0.36, respectively (data not shown). Due to the small sample size it is difficult to effectively determine the minimum number of progenitor cells required to heal this type of defect. Nonetheless, it suggests a relationship between the number of progenitor cells delivered and defect healing may exist under these circumstances, but may require a more challenging osteogenic model to fully elucidate.

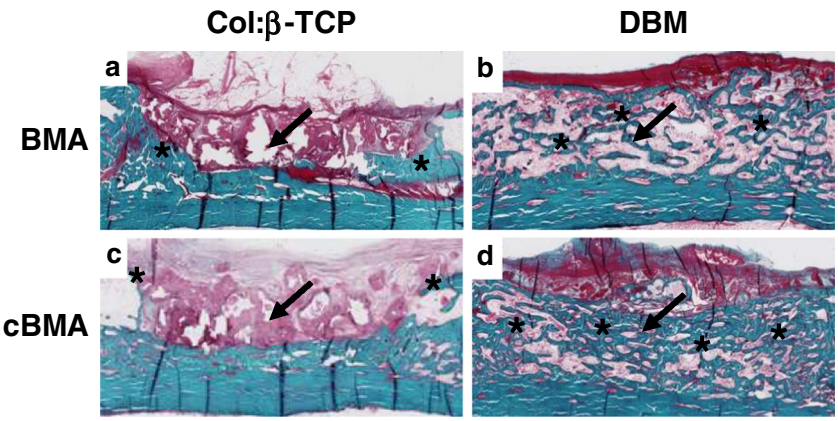

Fig. 4 Histology photographs of representative samples. Col:β-TCP samples with **a** BMA or **c** cBMA. DBM samples with **b** BMA or **d** cBMA. Photographs taken at ×1.25 magnification match the radiographs shown in Fig. 1. *Arrow* denotes the site of defect and * denotes new bone formation, also seen in *green*

Based on these findings, further evaluation of both scaffolds under conditions that may be more challenging and relevant to traumatic injuries is of great interest. This is especially relevant for better evaluating the importance of progenitor cells with DBM. One would expect the augmentation with cells to play a greater role in bone healing if the availability of stem cells and osteogenic growth factors in a defect is low. As just one example, poor fracture healing occurs when there is little soft tissue coverage and a subsequently lesser number of factors available for bone repair [25, 26]. Nonetheless, a conservative estimation based on our findings is that DBM grafts are capable of accelerating bone healing in this model where there is ample soft tissue coverage. Further, our findings are largely based on radiographic analyses using a standardised scoring method. It will be useful to follow up these semi-quantitative analyses with micro-CT, however, this comparison is complicated by the fact that residual β-TCP is difficult to distinguish from regenerated bone.

An obvious limitation to the study is that a group without BMA was not included therefore a definitive conclusion regarding the importance of BMA cannot be made. The goal of the study was to identify strategies currently available to expedite clinical translation. Since previous studies have already documented reduced healing in this model using similar scaffolds without augmentation [27–29], a control group containing either no scaffold or scaffolds without cells was not included to minimise animal use. Overall, based on the relative ease at which bone marrow aspiration can be obtained the cost/risk seems to favour its inclusion.

Many studies are directed towards maximising the regenerative potential of various combinations of biomaterials and stem cells while few experimental studies include both clinically relevant scaffolds and rapid cell isolation procedures. The ability of DBM grafts to achieve union by four weeks when supplemented with BMA suggests that this is one strategy available to clinicians for the treatment of difficult defects. Also, the current findings suggest that either material can be combined with cBMA to effectively heal bone.

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Conflicts of interest MAROMatch, MAROFuse, collagen-β-TCP scaffolds, and the Magellan® MAR01™ system and disposables were supplied by Arteriocyte Medical Systems. This study was funded in part by Arteriocyte Medical Systems.

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