Guided Bone Regeneration
Simvastatin Coated AMCA Membranes

Simvastatin

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Dear...
Introduction

Large segmental bone defects in weight bearing bones pose a challenging task for orthopedic surgeons.

The methods used today for treating large fracture, with great bone defects include:

1. Bone auto graft: This method relies on the osteogenic properties of bone tissue. It consists of transporting bone tissue (bone segment or whole bone) from its original place to an area of bone defect, where the renewed blood supply will help the transported segment to survive and connect to the sides of the defect. This method is limited by the size of the defect as well as re-establishing proper blood supply.

2. Transport distraction osteogenesis, either monofocal (Ilizarov), or bifocal. This method relies on a mechanical device which transports a bone segment across an area of bone defect. The slow advancement allows for new bone formation eventually allowing closure of the defect sight (19). This process is time consuming, needs a complicated device designed especially to fit the area of bone defect, and is prone to infection.

3. Using adjacent bones (such as in the case of fibula pro tibia (1)).

4. Synthetic bone substitutes: This method has not been found to offer the long term load bearing function required in post traumatic segmental bone defect (PTSBD), (1).

Guided bone regeneration (GBR) is a new method being developed today in order to improve fracture healing. Using synthetic membranes as carriers, these membranes encourage osteoblast proliferation and migration, to form new bone matrix in the defect site (1). GBR has shown profound results.
Endogenous agents called osteoinductive growth factors have been found to promote bone regeneration. These factors include the bone morphogenic protein BMP. Synthetic agents such as simvastatin were found to induce some of these agents, thus promoting fracture healing.

Fracture healing

Fracture healing is a dynamic process that begins right after damage has occurred and goes on until full repair of defect is completed. This process involves the bone and adjacent tissue, including bone marrow, cortical bone, periost and soft tissue surrounding the bone.

The body uses one of two major techniques in fracture healing according to the position of bone fragments during the healing process (14):

1. Direct or primary cortical fracture healing, occurs only when there is anatomic reduction of the fracture fragments by rigid internal fixation and decreased interfragmentary strain. The process involves a direct attempt by the cortex to re-establish new havarsian systems by the formation of discrete remodeling units known as "cutting cones", in order to restore mechanical continuity. Vascular endothelial cells and perivascular mesenchymal cells provide the osteoprogenitor cells to become osteoblasts. During this process almost no periosteal response is noted (no callus formation).

2. Indirect or secondary fracture healing, this process takes place when anatomic reduction is not complete and involves two types of ossification, intramembranous and endochondral in the following manner:

   **Intramembranous ossification**: involves direct bone formation from committed osteoprogenitor and undifferentiated mesenchymal cells that reside in the
periostium, farther from the fracture site. It results in "hard callus" formation (no cartilage is formed). In this case endothelial cells from the bone marrow transform into polymorphic cells, which subsequently express an osteoblastic phenotype.

Endochondral ossification: involves recruitment of undifferentiated mesenchymal cells, which differentiate into cartilage, forming a "soft callus". Later on the soft callus becomes calcified, and is replaced by bone. Six identifiable stages are observed in this case:

a. Hematoma formation and inflammation.
b. Angiogenesis and formation of cartilage.
c. Cartilage calcification.
d. Cartilage removal.
e. Bone formation.
f. Bone remodeling.

This type of fracture healing is contributed by the periostium adjacent to the fracture, as well as external soft tissue.

Endochondral bone formation occurs in regions that are mechanically unstable.

Critical size defect is defined as the smallest intraosseous defect that a normal skeleton cannot bridge (4). In humans critical size defect is usually 20-25% the length of a longitudinal bone (13). In the case of rabbit mid-shaft critical size defects, different values were reported. Meining et al. reported 1cm to be the value in rabbits. It was shown that 1cm untreated defects fail to establish union, while treated ones do (4).

The value of 1cm was used as critical size defect in this study.
Membranes

Guided bone regeneration is a procedure in which a resorbable or non resorbable polymeric membrane (the carrier) is surgically introduced into the area of bone defect serving two purposes:

1. A barriers preventing ingrowth of muscle and connective tissue into the defect site (2).
2. A medium for osteogenesis and augmentation of the bone defect.

The stages seen in bone healing when a synthetic membrane is used, includes the following (2):

1. Bone defect fills with hemorrhage after membrane application.
2. At two weeks hematoma has been resorbed, new bone begins to grow from severed bone ends.
3. At 4 weeks, woven bone had filled the entire gap.
4. At 12-16 weeks, woven bone has remodeled into lamellar bone.

These stages are similar to the ones described earlier in endochondral ossification (14).

A study of radial diaphyseal bone defects in New Zealand male rabbits (2) compared the effect of two resorbable poly-lactide membranes in the closure of bone defects: poly-L/D-lactide, and poly-L/DL-lactide. The difference between the two types has to do with polymer composition (LD, D, L units), and the distribution of these units in membrane. No evident radiographic or histological difference was found between the two compounds.

Comparing non resorbable ethyl cellulose (EC) membranes with resorbable chitosan (CH) membranes in mature New Zealand rabbits weighing 2.8-4.2kg (3) demonstrated
greater and faster bone growth in the EC group. EC membranes function better as osteoinductors, whereas CH membranes function better as osteoconductors.

A Swiss mountain sheep study (10), used perforated and non perforated poly (L/DL lactide) membranes, on segmental bone defects. The conclusion was that combining autogenous bone grafts along with the membrane promoted bridging of the defect in all models including perforated and non perforated membranes as well as single tube or double tube models. Control defects where no membrane was used, did not heal by 16 weeks of osteotomy.

Another Swiss mountain sheep study (11) used perforated (800-900µm pores) and non perforated poly-L/DL-lactide membranes, with or without autogenous bone grafts on segmental defects of the tibia. The conclusion was that pores allow soft tissue ingrowth and neovascularization into defect site, allowing for new bone formation and bridging of bone defect.

A 15 mini-pig model with radial osteotomy (13) revealed that bridging occurred after 6 weeks when defect was covered with resorbable poly lactide membranes. In the control group no membrane was used, and only one managed to form a complete regeneration while others showed some regeneration with persistent clefts in the middle of the defect even after twelve weeks of observation.

A study of 24 New Zealand rabbits was conducted along a period of 64 weeks (12), comparing GBR using poly-L-lactide membranes to primary healing without membrane. Controls (no membrane) showed no bridging of the bone gap, and developed synostosis of both ends of osteotomy to the adjacent ulna. Bone defects covered with membranes
showed complete bridging by 8 weeks. By 64 weeks cancellous and cortical bone could be seen bridging the original defect. This study suggests that membranes serve only as a physical barrier preventing soft tissue entry and disturbance of bone formation. In the cases of small dislodgement of membranes, fibrous tissue herniations into the gap could be seen, followed by blockade of bone formation.

AMCA membranes: ammonio methacrylate copolymer A (AMCA) is a positively charged compound used for slow release of drugs in the pharmaceutical industry (18). A former study of our group (18) was aimed to develop a polymeric membrane that enables adhesion, proliferation, and differentiation of mesenchymal stem cells (MSCs) on its surface. This study compared between ethylcellulose membranes (EC), and AMCA membranes in vitro. AMCA proved to be more efficient, allowing MSC adhesion, and formation of spindle cell monolayer with podia. In addition, using polyethylene glycol 400 (PEG 400) as a plasticizer, allowed for MSC proliferation and differentiation. Thus AMCA membranes with PEG 400 plasticizer were used in this study. These membranes are non resorbable membranes, product of the school of pharmacy, The Hebrew University Jerusalem. AMCA membranes are prepared using a solvent casting technique, which combines a polymer (AMCA 85%), a plasticizer (PEG 400), and ethanol 20ml. Membranes are 130µm thick. In addition special AMCA membranes coated with simvastatin were prepared by combining simvastatin (0.36gr) in solvent casting.
Osteoinductive agents

Three groups of signaling molecules act in fracture healing (14):

1. Pro-inflammatory cytokines, such as IL-1, IL-6, TNF-α. They initiate the repair cascade by having a chemotactic effect on inflammatory cells, enhancement of extracellular matrix synthesis, stimulating angiogenesis, and recruiting endogenous fibrogenic cells to the injury site.

2. Transforming growth factor- beta (TGF-β) superfamily, including bone morphogenic proteins (BMPs). BMPs are secreted from osteoprogenitors and mesenchymal cells, osteoblasts, bone extracellular matrix and chondrocytes. They induce the differentiation of mesenchymal cells into chondrocytes and osteoprogenitors into osteoblasts.

3. Angiogenic factors such as vascular endothelial growth factor (VEGF), angiopoetin.

Statins

Statins are HMG-CoA reductase inhibitors, used as very effective cholesterol lowering agents.

In a retrospective study of a large population of elderly, predominantly male veterans (16), use of statins was associated with 36% reduction in fracture risk when compared with no lipid lowering therapy, and 32% reduction when compared with non-statin lipid lowering agents. Dose response was also shown.

High doses of orally administered simvastatins have been shown to improve fracture healing in a mouse femur fracture model (15). A mouse study comparing between subcutaneous injection of simvastatin and local simvastatin treatment of a fracture
showed that local treatment contributes to stronger fracture healing in a statistically significant manner as opposed to subcutaneous injection (15).

Former studies discovered that statins induce the expression of osteoinductive growth factors such as (bone morphogenic protein- BMP, transforming growth factor- TGF-β, and glucocorticoids), causing increased bone formation:

1. Simvastatin stimulates mineralization in rat BMSCs (bone marrow stromal cells). Northern blot analysis showed an increase in BMP expression during incubation with effective doses of simvastatin ($10^{-8}$-$10^{-7}$ M). (5)

2. Simvastatin tilts the reciprocal relation between osteogenesis and adipogenesis toward osteogenesis. Osteogenic and adipogenic cells both arise from the multipotential precursors (BMSCs). Statins promote the expression of BMP2 in a dose dependent manner. This induces osteoblast differentiation, proliferation, and maturation, as well as new bone formation in vitro and in vivo (6). BMP2 also inhibits adipocyte differentiation (6). Lipophilic statins (simvastatin, mevastatin in particular) cause the effect mentioned above while hydrophilic statins (pravastatin) fail to do so (8).

3. Simvastatin inhibits the formation of down chain reactants in the activity of osteoclasts, thus inhibiting osteoclast formation and activity (7).

4. Ju Hyeong Jeon et al. showed that simvastatin releasing implanted devices, induced bone growth in rat calvaria.

Former studies of our group succeeded in combining AMCA membranes with simvastatin. An efficient release curve was demonstrated (figure 1).
Hypothesis: in this study I hypothesized that combining simvastatin with AMCA (Ammonio Methacrylate Copolymer type A) membranes will improve bone growth in GBR. Like prior studies from our group a 12 mature New Zealand male rabbit model was used, with a critical size defect created in a single radial bone.

Figure 1. Simvastatin release curve from AMCA membranes demonstrated by a dissolution study. Note that thicker membranes are associated with slower release (a), higher concentrations of simvastatin are associated with faster release (b).
Methods and materials:

This study is an analytical prospective single blinded controlled study. 12 mature New Zealand male rabbits (weighing 3.5 kg) were randomly divided into experimental and control groups (6 rabbits in each group). For the control group AMCA membranes were used. For the experimental group simvastatin coated AMCA membranes were used.

Surgical procedure:

Prior to surgery animals were sedated using a mixture of ketamine (30mg/kg), xylasine (3mg/kg) and atropine (1mg/kg), injected IM. Following, animals were anesthetized using pentothal (30mg/kg), diluted in Hartman solution. Cefamizine 0.5gr IM was given as a prophylactic dose. The left forelimb was sterilized with chlorehexidine, and shaved. Subcutaneous injection of Lignocaine 1% was given in the operation site. The Henry technique was used in order to reveal the medial third of the radial bone. A 1cm segment was removed from the radius along with the surrounding periost, using an electric saw (thus creating a critical size defect). The ulna was preserved functioning as a natural splint. The operation field was washed with normal saline, and a synthetic cylinder shaped AMCA membrane was inserted to replace the area of missing bone. The membrane was fixated to both sides of osteotomy using hystoacryl (figure 2).

Figure 2. AMCA membrane is introduced into the area of bone defect (A). AMCA membrane is closed in a cylindrical shape around the area of bone defect (B).
After membrane insertion, fascia was reattached using vicryl 3/0, and skin using ethylon 4/0. The wound was seasoned with Opsite spray. During the operation a continuous drip of Hartman solution was given IV.

Post surgery:
After the operation rabbits were returned to their cages without limitation to normal everyday activity.

During the 5 day period post operation, rabbits were monitored daily for weight loss, wound swelling, or any sign of infection. In addition, injections of antibiotics (cefamezine, 0.5mg) and analgesia (rymadil) were given daily.

In the following weeks, a regular checkup was done twice a week, to insure animals were not suffering from infection or weight loss. Sutures were removed two weeks post operation.

Post operative follow up:

1. Rabbits underwent forelimb X-ray scans (AP, Lateral) 2, 4, 6, and 8 weeks post surgery. In each session a mixture of ketamine (50mg/kg), and xylazine (5mg/kg) were injected IM to sedate the animals.

2. Before each x-ray, blood samples were taken to measure the systemic levels of simvastatin.

3. 8 weeks post surgery rabbits were anesthetized using ketamine (50mg/kg) and xylazine (5mg/kg), and then sacrificed using an overdose of IV Phenobarbital 300mg. Limbs were extracted preserving all of the radius and ulna, including olecranon.

4. Extracted limbs were sent to μ-CT scans.

5. Finally, extracted limbs were sent to histological sectioning.
Assessment of variables:

X-ray scans:

Two x-ray scans were obtained from each rabbit at 2, 4, 6, and 8 weeks post surgery. These scans were evaluated for two variables, according to the methods described in an earlier study of our group (4):

1. Area of new bone formation (in cm²). Since rabbits may defer in bone size, or quality of scan, each calculated area was normalized to the width of the narrowest point of the olecranon.

2. Density of new bone formation. This variable was also normalized to the density of the central point of the olecranon.

The Osirix DICOM viewer (Osirix Imaging Software) was used to measure the variables mentioned above.

Each variable was analyzed using the anova with repeated measure technique in order to assess:

1. The time effect (change over time).

2. Treatment effect (the AMCA+simvastatin effect).

3. Interaction between time and treatment (is the change over time dependent on treatment?)

In the case of time effect, and time/treatment interaction effect the greenhouse-geisser test was used.

μCT:

One scan was made for each limb. This scan illustrates new bone structure and architecture. Osteotomy sight was divided into 4 transverse segments: proximal, proximal
medial, distal medial and distal quarters. Using the Osirix DICOM Viewer, bone density and bone surface area were measured in each segment, and in the entire sight.

**Histology:**

Following μ-CT scans, limbs were sent for histological sectioning and staining as follows:

1. Limbs were washed, while soft tissue remained intact around the bone.
2. Soaked in 4% formaldehyde solution for 24 hours.
3. Washed with regular running water for 4 hours.
4. Decalcification and dehydration.
5. Soaked in xylene solution for 6 hours.
7. After "block" is formed, using a microtome, longitudinal sections 5-7µm thick were taken.
8. Sections were dried in 50ºC for 24 hours.
9. H&E staining.

Histological sections were assessed according to a protocol developed in a former study of our group (4). Each section was divided into eight areas (fig. 3, table 1), and each area was given a grade from 0-6 (table 2).

![Figure 3. Illustration of osteotomy site.](image)
Area division of histological sections

<table>
<thead>
<tr>
<th>Area</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Internal fronts. These triangular areas are the proximal and distal osteotomy sites. Their triangular shape is due to new bone formation.</td>
</tr>
<tr>
<td>B</td>
<td>Internal bridge.</td>
</tr>
<tr>
<td>C</td>
<td>Internal gulfs, usually signify areas of membrane attachment in radioulnar space.</td>
</tr>
<tr>
<td>D</td>
<td>External gulfs, usually signify areas of membrane attachment under skin.</td>
</tr>
<tr>
<td>E</td>
<td>Ulnar reaction.</td>
</tr>
<tr>
<td>F</td>
<td>Radioulnar space.</td>
</tr>
<tr>
<td>G</td>
<td>Internal soft tissue.</td>
</tr>
<tr>
<td>H</td>
<td>External soft tissue.</td>
</tr>
</tbody>
</table>

Table 1

<table>
<thead>
<tr>
<th>Criteria for evaluation</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>No sign of bone formation</td>
<td>0</td>
</tr>
<tr>
<td>Initiation of bone formation</td>
<td>1</td>
</tr>
<tr>
<td>Small area of new bone</td>
<td>2</td>
</tr>
<tr>
<td>Advanced bone formation</td>
<td>3</td>
</tr>
<tr>
<td>New bone occupies most of the area</td>
<td>4</td>
</tr>
<tr>
<td>New bone occupies entire area</td>
<td>5</td>
</tr>
<tr>
<td>Modeling</td>
<td>6</td>
</tr>
</tbody>
</table>

Table 2. grading system for bone formation, as assessed by histological sections
Results:

Two rabbits did not survive the entire experiment period; one belonged to the control group while the other was in the experimental group. In addition two more rabbits suffered from fractures in the operated limb. These rabbits were excluded from the experiment, to prevent distortion of the results.

X-ray analysis:

Bone area:

New bone area in AMCA+simvastatin group was found to be superior to the AMCA group, but statistical analysis showed treatment and time/treatment interaction effect not to be statistically significant, with a p-value of 0.1 and 0.23 accordingly, (figure 4). Time effect alone was found to be statistically significant (p≤0.01). This only shows that AMCA and AMCA+simvastatin both efficiently induce new bone formation, disregarding comparison between the two.

Figure 4. new bone area over time in the AMCA vs. AMCA+simvastatin group.
Bone density:

New bone density in AMCA+simvastatin group was found to be superior to the AMCA group, but statistical analysis showed treatment and time/treatment interaction effect not to be statistically significant, with a p-value of 0.41, 0.63 accordingly (figure 5). Time effect alone was found to be statistically significant (p\(\leq\)0.01). These results are concordant with the area analysis.

![Graph showing new bone density over time in the AMCA vs. AMCA+simvastatin group.](image)

Figure 5. new bone density over time in the AMCA vs. AMCA+simvastatin group.

In an attempt to reduce the influence of the small sample size on the results, an aparametric approach using the Mann Whitney test was chosen. This test compares the two groups separately in each time point for each variable. None of the time points submitted a significant p value (table 3).

<table>
<thead>
<tr>
<th>Week</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actual area</td>
<td>.114*</td>
<td>.343*</td>
<td>.200*</td>
<td>.200*</td>
</tr>
<tr>
<td>Actual density percent</td>
<td>1.000*</td>
<td>.686*</td>
<td>.486*</td>
<td>.486*</td>
</tr>
</tbody>
</table>

Table 3. p values of the comparison between the results for area (second row), and density (third row) using the Mann Whitney test.
Another aparametric approach using the Friedman test was used to assess the time effect separately for each group. This study attained significant p values (table 4). As mentioned earlier this only shows that AMCA and AMCA+simvastatin both efficiently induce bone growth, but does not compare the two groups.

<table>
<thead>
<tr>
<th></th>
<th>AMCA</th>
<th>AMCA+simv</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actual density percent</td>
<td>0.7</td>
<td>0.041</td>
</tr>
<tr>
<td>Actual area</td>
<td>0.007</td>
<td>0.044</td>
</tr>
</tbody>
</table>

Table 4. p values for the Friedman test assessing the time effect for each group individually.

A former study by our group used 5 rabbits in order to examine the effect of AMCA membranes on fracture healing. Both radii were osteotomized in each rabbit. Sequentially one was treated with an AMCA membrane while the other was not treated and served as control. Since the former study was done in the same conditions as the current study (same surgery method, anesthesia, follow up and euthanasia), its results may be comparable to the current study. Thus I combined the area results together in the following manner:

1. Adding the control group (limbs without membrane), and creating a three group comparison of actual area: control (former study), AMCA (current study), AMCA+simvastatin (current study). This combination proved bone area in AMCA+simvastatin group to be superior to the AMCA group, and the control group (figure 6). Time and time/treatment interaction effects gave p values of 0.27 and 0.32 accordingly. Treatment effect gave an almost significant p value of 0.061.
2. Adding the AMCA group from the previous study, to the current AMCA group, and remaining with a two group comparison of actual area. In this case time effect is not significant (p=0.19), but both time/treatment interaction effect and treatment effect are almost significant (p=0.079, 0.068 accordingly), (figure 7).

Figure 6. three group analysis of actual area: combining the results of a prior study, a three group comparison is shown, including control (no membrane), AMCA, and AMCA+simvastatin.

Figure 7. extended two group analysis of actual area: combining the results of a prior study, a larger AMCA group was created. This figure shows the comparison between the new AMCA group and the AMCA+simvastatin group.
The above results show that increasing sample size decreases the p value, i.e. the probability to conclude there is an effect when in reality there isn't. This combination is not sufficient to conclude that the hypothesis is correct, but it could indicate that if I were to conduct a larger scale experiment with more rabbits, I might prove that simvastatin truly and significantly increases the induction of new bone formation.

One problem with this combination though, is that results are not completely comparable to the current study, since the two forelimbs of each rabbit were involved, thus making mobilization and recovery a bit different.

\(\mu\)-CT:
The \(\mu\)-CT images shown below (fig. 8 and 9) show new bone formation in purple for AMCA and AMCA+simvastatin accordingly. These images were measured for new bone volume (BV) and bone surface area (BS). In order to reach a standardized value, both variables were divided by the total volume (TV) of the osteotomy sight (cylinder consuming all the volume of the osteotomy sight).

The student t-test was used in order to compare the AMCA and AMCA+simvastatin groups for both variables. Comparison of standardized bone volume (BV/TV) gave a p-value of 0.37, while the comparison of standardized bone surface area (BS/TV) gave a p-value of 0.18.

None of the results mentioned above show statistical significance, even though standardized values were higher in the AMCA+simvastatin group.
Histological analysis:

This variable is semi quantitative (ordinal), thus the empiric Mann-Whitney test was applied. Table 5 shows the percentage of specimens to achieve each score (see table 2). Note that each zone (table 1, figure 3) is analyzed individually. This analysis was done according to Mosheiff et. al (4). Zones B-D were not found to have a statistically significant difference. Zone A was found to have a statistically significant difference (p=0.057), favoring the AMCA group. This is not consistent with our x-ray, and μ-CT results.

Figure 8. μ-CT image of the osteotomy site (purple) in the AMCA group. The image shows new bone formation 8 weeks post osteotomy when an AMCA membrane was used without simvastatin.

Figure 9. μ-CT image of the osteotomy site (purple) in the AMCA+simvastatin group. The image shows new bone formation 8 weeks post osteotomy when an AMCA+simvastatin membrane was used.
Figure 10 demonstrates the average score for all zones per rabbit. The overlap between AMCA and AMCA+simva groups illustrates why no significant p value was obtained.

<table>
<thead>
<tr>
<th>Score</th>
<th>Zone A</th>
<th>Zone B</th>
<th>Zone C</th>
<th>Zone D</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0%</td>
<td>75%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>1</td>
<td>25%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>2</td>
<td>0%</td>
<td>0%</td>
<td>25%</td>
<td>0%</td>
</tr>
<tr>
<td>3</td>
<td>75%</td>
<td>0%</td>
<td>25%</td>
<td>0%</td>
</tr>
<tr>
<td>4</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>50%</td>
</tr>
<tr>
<td>5</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>50%</td>
</tr>
</tbody>
</table>

Table 5 - percentage of rabbits found to receive each score in each group. One rabbit from the AMCA+simva group could not be assessed for zones A and B. One rabbit from the AMCA group could not be assessed for zone D.

Systemic simvastatin levels:

Systemic simvastatin levels were taken before each X-ray. Our equipment could not detect any level of simvastatin in rabbits' blood at any time point.
Discussion:

This study focused on the effect of simvastatin coated AMCA membranes on GBR. To serve this purpose a prospective randomized controlled trial, of 12 New Zealand male rabbits was used.

To assess new bone growth X-ray and μ-CT scans as well as histologic sections were analyzed. A positive trend toward new bone formation was demonstrated, favorable for simvastatin coated AMCA membranes:

1. The statistical methods used to analyze X-ray scans (the anova with repeated measures, and the Mann-Whitney test) demonstrated better bone growth for the AMCA+simvastatin group, but the results failed to achieve statistical significance. Time effect achieved statistical significance, which proves membranes are sufficient for bone growth induction, but does not refer to simvastatin effect. In an attempt to tackle the small sample size, results from a former study, done in the same conditions, were combined with current results. This combination enlarged the control group and yet decreased the p value, indicating that AMCA+simvastatin membranes may truly improve bone growth significantly.

2. μ-CT scans demonstrated new bone formation in a qualitative and concrete manner. Sufficient bone growth and bridging was seen (figure 8, 9). Quantitative analysis did not achieve statistical significance favoring the AMCA+simvastatin group. This could partly be due to the small sample size. In this case though, I could not apply older results to increase sample size; since this is the first study to use μ-CT in our series.
3. Histological sections were evaluated by a technician who specializes in bone histology. No significant difference was found between the two groups, except for zone A favoring the AMCA group. This assessment is problematic, since it depends on the process of sectioning, fixation, and staining, as well as finding the best section. Adding to that the assessment is subjective. Perhaps a repeated assessment by a person from our group may land other results.

Systemic levels of simvastatin were undetectable. This indicates that simvastatin released from membranes (figure 1) acts focally without systemic distribution.

In an attempt to understand the contradictive results of radiology and histology, two problems were characterized:

1. X-ray analysis did not give a significant p-value favoring AMCA+simvastatin.
2. Histological analysis did not coincide with X-ray analysis.

The first problem can be explained by different theories:

1. Simvastatin does not improve bone growth in GBR when combined with AMCA membranes, and the hypothesis is incorrect.
2. Simvastatin was not sufficiently released from membranes, or was not used in the proper concentrations in order to induce significant bone growth. This theory is partly erroneous because former studies have shown proper release of simvastatin (figure 1). Perhaps a higher concentration is needed.
3. Sample size was too small. Combining former results with current results in order to increase sample size gave a better p value (almost significant). This of course
does not prove the hypothesis to be correct, but it implies that a larger scale study may be needed for further assessment.

The second problem has to do with the complexity that goes into preparing and assessing histological sections:

1. During microtome sectioning, and preparation staining, deformities could occur in the histological structure.

2. Choosing the right preparation, which preserves the true histological structure, could be a tricky task.

3. The assessment of these sections, even though fully distinguished by Mosheiff et. al (4), remains subjective. Hence further assessment by a person more familiar with the technique might be necessary.
Summary:

Guided bone regeneration (GBR) is a technique in which a cylindrical synthetic membrane is introduced into an area of long bone defect, thus encouraging the bridging of the bone defect. Synthetic membranes play two roles in this process: a physical barrier from external tissue which interferes with bone regeneration (2), and a medium upon which new bone formation occurs (18).

Earlier studies showed that simvastatin decreases fracture risk, and encourages new bone formation through the induction of osteoinductive growth factors such as bone morphogenic protein (BMP).(5, 6, 15, 16).

The hypothesis of this study is that simvastatin coated AMCA membranes significantly improves GBR.

This study is an analytical prospective single blinded controlled study aimed to test the hypothesis. 12 mature New Zealand male rabbits were used.

Ammonio Methacrylate Copolymer type A membranes (AMCA), were chosen for GBR, as they proved to be a good surface for mesenchymal stem cell (MSC) attachment, proliferation and differentiation (18).

Rabbits were randomly assigned into two even groups: one received AMCA membranes coated with simvastatin, and the other received regular AMCA membranes.

During the operation a 1cm defect was created in the radius of the left forelimb, and the AMCA membrane was introduced and fixated to osteotomy sites, forming a cylindrical shaped envelope around the area of bone defect.
Post operation, X-ray scans of the operated limbs were performed every two weeks until the 8th week, then rabbits were sacrificed, and limbs were extracted to undergo μ-CT and histological analysis.

X-ray scans were analyzed for area and density of new bone formation. These scans showed significant bone growth in both groups, more in the AMCA+simvastatin group, but did not achieve statistical significance (p<0.05) favoring the AMCA+simva group.

The results of a former study by our group were combined with current results in order to increase the sample size. Combining the two studies expanded the AMCA group. This technique succeeded in decreasing the p value to almost 0.05, but on its own is not sufficient to conclude that the hypothesis is correct. Still it can imply that a larger scale experiment is needed in the future.

Systemic levels of simvastatin were measured in rabbits during the experiment, and were found to be undetectable. Former in vitro studies showed sufficient release of simvastatin from membranes (figure 1). This indicates that simvastatin induces local effect, without any systemic effect. The question was raised whether sufficient concentrations of simvastatin were used in the current study in order to induce a significant effect.

Histological analysis was not consistent with X-ray analysis and did not show superiority for the AMCA+simva group. Further analysis of histological sections by a person more familiar with our grading system (4) may be needed. Since this analysis is rather subjective, a more objective and accurate assessment was obtained by μ-CT analysis. μ-CT scans demonstrated new bone formation qualitatively. A positive trend toward GBR and new bone formation was noticed using simvastatin coated AMCA membranes. Statistical analysis did not achieve a significant p-value favoring AMCA+simvastatin.
In conclusion, to this point simvastatin does not significantly improve GBR. For future research the following steps need to be taken:

1. A larger scale experiment is needed, i.e. a large sample size.

2. Higher concentrations of simvastatin may induce a more significant effect.
סיוכם:

צמינון עטס מודרנטת יהינה שיטה בה נשענו שימוסו במעצבה סינתטית בצלינדר ברוח יילנדה. על מות הצילינדר בצורת סינתטית בممבראנה נעשה שימוש בה שיטה הנה מודרנת עצמה צמיחת ר, מנת על אורכית עצם חרר קיים בו האזור של מבודדת סביבה ליצור של ושיחוי מחדש צמיחה המאפשר דבר החסר האזור. האור צמיחת בתהליך שני תפקידים שניים משחקות סינתטיותemmבראנות: 1. רקמות צמיחת שמונע מכאני מחסום ועי החסר האזור לתוך סמותו כהעצם צמיחת ובו (2).

2. hitting המ UIManager ליצאות עטס על פנים (18). מחקר שיש בו่วน simvastatin MORIR את הסוכן של הבכירות או כלפיות המבוגר. כל כ- ממציא מחקר ישנו花纹 simvastatin כ- שעום צמיחה של עצם עייה השראת בטוי מתקולים אוסטיאואדווקטילובית כ- מוקד (16, 15, 6, 5). bone morphogenic protein (BMP)

המの方 ההפתקר היא שמייבאנו אנא מoltipחת simvastatin מ“Andת להת😋 צמיחת העטס

המDDR עם analytical prospective single blinded controlled study את

והי בעדיה מסתעפ ההפתקר. ילש כ- השטוחה-ב-12 ארנה זו קני, נגורים, מסון, New Zealand

המ方 ממייבאנו 만나 ונבירה של שטוח ממקור, ammonio methacrylate copolymer type A (AMCA)

משוש שופחה בעבדות קודם (18) כי אם מוהלים מצע אפקטים ליחודיים, חולקה והתמיינת של תאי וגוס מונتعليق ממבראנה.

הארנה חולק באופי שרירותיUSHי קבודת: האחת קבלת ממבראנות אנא AMCA מצפתי ב-2 AMCA והשניה קבלת ממבראנות AMCA רגילות.

םשה ב- simvastatin והשיגה קבלת ממבראנות AMCA גם גילולה.

החייתו חזרה, ומבר סנייה של צורת ספורטני באור צמיחה של ציידי השטוח כ- simvastatin hystoacryl. ב-1cm מאמציו עטס הרדיוס באור שמלת. הממבראנות הסינון את אזור הצמיחה של פסיי הצמיחה על ידי שימשו ב-2, hystoacryl

שניצר מעטפת בזרת צילינדר מסיבי לארוז חסר העטס.

לאחר מחקר האורכה בצבע מבר סנייה את שלושות השריון ואת השימוש enfer קורא. לאחר מחקר

החייתו חזרו, והורשות המשועות מתקדמת בדיסקציה, ונשלח ל-µ-CT וهى טסונולוגית.
שחית הפגב針 수행ה החсход נמדד מגילומי המרה, והואר בבירור תוחר קלבוזה-^h.

.אולס ב-freuכא סטטיסטיות לא נמצי יוחזר מובחך (p<0.05) )קלבוזה-^h.

.סימבסטיאטין+AMCA

.סימבסטיאטין

ה쯔וקות של מחקר קודס ליבנקנשטיין עלי ידי קובצתיות שולב עי תוצאות המחקר النقدית, לע מות

לتحدיה את מדיל המחנה. השילוב ה-גילי הרחבר אט קבוצת-^h והבייאו לירידה של h-p.

לכמותו 0.05.

אמומת לא נותר חיסכון ומשייג תיניל עם הופות המחקר או כל, באב עפע הירידה-ב-^h.

יכולה לוביצר עלי כאשר שדנוגת יחות גודל במעל ייתח תוצאת מובחק סטטיסטיות.

בגמל קולי针 נלקח דד פרפריה מהארדנת לשמדיד בנומך. האל בר לשמדיד בנומך, אבל לא נמצע רומת

היתקנת גלייה במקושר הכיס לדרשון. יסמי קודס מהתראור אפקטיבי של

תואמהヴァידי ב-tiro ו-AMCA לשמדיד בנומך (הממחשה-1). ממצוא זה מצבי על כ-6-לע-^h שמדיד בנומך simvastatin ממחשה אפקט מוקמי

לא ספימה סטטיסטיות. העלוות השאלת האם היה שמונת ברמות ואחתות של simvastatin במחקר

הנוכחם, עלสมาคม לוחשות אחרק משמיעתי מוכובה סטטיסטיות.

האנליזה הגיסטורונית נתנה תוצאת לא האמות לימה ערכה בנומך ניקולמוי המרה,ך שלא נראת כל

יתר לקבוצת-^h. ראיי ליען כי ערכה ויהנה סובייקטיבית, על כ-יתן הערכה

נוסף על-אэм יוחר מנהס בİŞיט הידייה (4) יוחר תצאות יוחר מתאיומתו. אנליזה-^h ב-µ-CT

מאמציי הערכה יוחר ואבייקטיבית מודיכת.

סוקור-^h ב-µ-CT דגימה ביברה אינומית או יוצאת העמות החודש. הצפה המגמה דהיבית בחוליק

עודמי העמות המודיכת, כאש היה שימש בשכבה אורתכ ת-µ-CT. אנליזה

.סימבסטיאטין AMCA מוצמק ב-

.הסטטיסטיות לא נותר p-verte ל-AMCA+simvastatin

לסכים分かる, סימבסטיאטין תורח לעב חכי כימאני פימות עמות מודיכת, יושג צויר לקט במדים הבחנים על

מונת לשתלך ואת החסמה:

.1 ניסיון דומא עי מדומ יוחר רהב.

.2 שימשו ביריכוזים יוחר גודליים של simvastatin יוחר יוחר אפקט יוחר מובחק.
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