BMSCs Seeding in Different Scaffold Incorporation with Hyperbaric Oxygen Treat Seawater Immersed Bony Defect

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Research article

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Abstract

Introduction: The experiment was undertaken to estimate the effect of BMSCs seeding in different scaffold incorporation with HBO on the repair of seawater immersed bone defect. And future compared n-HA/PLGA with β-TCP/PLGA as scaffold in treatment effect of seawater immersed bone defect.

Methods: 60 New Zealand White rabbits with standard seawater defect in radius were randomly divided to group A (implant with nothing), group B (implanted with autogenous bone), group C (implanted with n-HA/PLGA/BMSCs, and Group D (implanted with β-TCP/PLGA/BMSCs). After implant, each rabbit receive HBO treatment at 2.4 ATA 100% oxygen for 120 minutes per day for 2 weesks. Radiograph, histological and biomechanical examination were used to analyze osteogenesis.

Result: X-ray analysis show that n-HA/PLGA/BMSCs and β-TCP/PLGA/BMSCs could accelerate the new bone formation, and the new bone formation in group C was lager than in group D or group A, and close to group B (P<0.05). After 12 weeks, in group A, defect without scaffold show a loose connect tissue filled in the areas. The medullary canal in group B was recanalizated. Defect in group C and D show a larger number of wove bone formation. The new wove bone formation in defect areas in group C was lager than D. The mechanical examination revealed ultimate strength at 12 weeks were group D>group C>group B>group A(P<0.05).

Conclusion: Scaffold of n-HA/PLGA and β-TCP/PLGA incorporation with HBO and BMSCs were effective to treat seawater immersed bone defect, and n-HA/PLGA was more excellent than β-TCP/PLGA.

Introduction

Healing bone defects was a difficult problem in orthopaedics. when bone defect was immersed by seawater, the repairing become more difficult. Autologous graft and allograft were possible methods. Autologous bone graft is regarded as gold standard in treating bone defect. However, the donor site bone source is limited, obtaining the autologenous bone was an invasive operation, which can lead to high donor site morbidity 1-2. Allograft have a potential of transmission of diseases and leading to immune response. Bone tissue engineering was a promising method that can overcome these problems mentioned above.

PLGA was widely used in bone tissue engineering due to its nontoxic and biodegrade as a scaffold. PLGA has been approved by Food and Drug Administration for human clinical appliations3. But it has poor mechanical strength and cell affinity which limits its using in bone tissue engineering4-6. In addition the acidic degradation can lead to inflammatory reation7-8. McBane JE reported that PLGA films were implanted subcutaneously in a rodent model which caused acute inflammatory response9. PLGA scaffold has been successfully repaired bone defect in SD10. However, PLGA isn’t an excellent scaffold in bone tissue engineering owing to its hydrophobicity and lack of bioactive properties and its degradation.
products leading to inflammation. Its properties can be improved by combinating with other materials. In our study there are two materials to be try:(1)n-HA,(2)β-TCP.

HBO therapy is that the patients expose to 100% oxygen under elevated pressure. Ahmed Jan, et al\textsuperscript{11} evaluated autogenous bone graft and autogenous bone graft with HBO by Micro-CT analysis and histomorphometric analysis and found that new bone formation was more in autogenous bone graft with HBO, suggesting that HBO can enable the bony healing of critical-sized bone defect. HBO therapy is thought to increase healing by increasing the amount of oxygen dissolved in blood. J.P. Grassmann et al.\textsuperscript{12} HBO therapy enhance bone repairing, which may attribute to an increase in angiogenesis.

Material And Methods

Experimental main material and reagents

BMSCs, MSC growth medium, trypsin were purchased from Cyagen Biosciences Inc (Guang dong, China). The n-HA/PLGA and β-TCP/PLGA scaffold (4 mm × 15 mm, cylinder) was provided by The Shandong Province key Laboratory of Medical Polymer Materials (Ji nan, China).

Cell culture

BMSC were obtained from Cyagen at passage 2. The BMSCs were were shawed and cultured in growth medium from Cyagen in humidified atmosphere containing 5% CO$_2$ at 37\textdegree C. The growth medium contained 440 ml MSC basal medium, 50 ml 10% FBS, penicillin/streptomycin mixture. The growth medium changed every 2-3 days. The cells were passaged 3 times at approximately 80\% confluence. Passage 5 BMSCs were digest and collected for determination and culture with scaffold.

Characterization of BMSCs

Flow cytometry was used to detect the MSC surface proteins such as CD29, CD44. Besides surface protein of msc, the maker of hematopoietic stem cells such as CD45, CD34 were test of the samples.

Cell seeding into scaffold

The PLGA/n-HA and gelatin/n-HA were treated by 75% achol, and then washed 3 times using phosphate buffered (PBS). BMSCs at 5 passage were suspended in growth medium at 2 × 10\textsuperscript{7} cells/ml. 200ul cells suspension was dropped on the top of the scaffold. The scaffold with cells was culture in cutie for 2 hours and then set in 24 well plate with growth medium. The medium was changed every 2-3 days.
60 New Zealand White rabbits (weighing 2.0–3.0kg), obtain from the animal experiments center of Anhui medical university (Hefei, China). Before the experiments, approval was obtain from the Ethical Committee for animal experiments of Anhui medical university. The rabbits were anesthetized by ear vein of 3% pentobarbital sodium (30mg/kg). Disinfection was done with iodine and 75% alcohol. A 3-5cm incision was created in the middle of radius. The tissue overlying the radius were dissected. A 15mm bony defect was made in the middle of radius. The forelims with 15mm bone defect were immersed in seawater for 3 hours. The rabbit with seawater immersed bone defect were divided into 4 groups (group A, group B, group C, and group D), with 15 rabbits in each group (n=15). The group C was implanted with n-HA/PLGA/BMSCs, the group D was implanted with β-TCP/PLGA/BMSCs, the groups B was implanted with autograft obtained from iliac crest, the group A was implanted with anything. After implantation, the rabbits were treated with 2.4 ATA 100% HBO for 90 minutes per day for 2 weeks. The rabbits were injected intramuscularly with penicillin every day for 3 days.

**Radiographic examination**

Radiographs of rabbit radius were examined at 4, 8, 12 weeks after surgery under anaesthesia. Radiological evaluation was done using the Lane and Sandhu scoring system13. The radiographs were evaluated by orthopedists. And the evaluation was under double blinded study.

**Histopathologic examination**

After 4, 8, 12 weeks, the rabbits were killed by euthanasia. Samples harvested from radius defect sites were fixed in 10% neutral buffered formalin for 48 hours. The samples were decalcified with 10% EDTA solution for 30 days, dehydrated in graded ethanol and embeded in paraffin. 5 um sectons were cut and stained with hematoxylin eosin.

**Immunohistochemistry stain of osteocalcin**

Immunohistochemical examination of OCN was performed by the slide was deparaffinized in xylene I-II respectively for 15 minutes and dehydrayed in gradea alcohol from 90%-70% for 3 munites. After that blocking was done with 0.5% H$_2$O$_2$ in methanol for 30minutes and washed by water for 5minutes. Pertreatment of slide was performed with citrate buffer in microwave cookI and cookII for each 5 minutes followed by blocking background target to block non-specific antigens and then incubated for 15minutes. Then it was given primary antiboby to OCN and incubated for 1 hour. The slide was given auniversal ink secondary antiboby to bine to the primary antiboby for 15minutes. Conuter staining was performed with haenmtaxylin for 1-2minutes.

**Bone mechanical strength test**

After surgery 12 week, the mechanical strength of radius was tested by the three-point bending test. The test was performed using a universal tensile testing machine (Instron, London, UK). The ultimate force in the bending test until the specimen was broken. The bones were placed horizontally on two rounded
supporting bars located at a distance of 30 mm, and the bending load was applied at the midpoint of the defect at the loading speed of 10 mm/ min until the specimens fracture. The biomechanical properties of the specimens were determined by ultimate loading (N) . The data were recorded as mean plus standard error of the mean.

**Statistical analysis**

All data are presented as mean±SD. Comparisons between groups were done by one-way analysis of variance (ANOVA). The SPSS 19.0 was using for statistical analysis. The differences were considered as statistical significance at the level of $P < 0.05$.

**Result**

**Radiological examination**

X-ray examinations were done to evaluate the development of bone regeneration in the defect, which were displayed in figure 1. At 4 weeks after surgery, litter callus formatted in group A. The shadow of autogenous bone in group B was visible and callus formatted in the ends of bone defect. The shadow the area of bone defect in groups C and D was filled with bony callus, which was observed as the cloudy shadow. The area of cloudy shadow in group C was larger than in group D. After 8 weeks the bony callus started to be absorbed, and cortical bone began to form in group B, C and D. While the cortical bone was hard to see in group A. In group B, the autogenous bone can not be seen due to be absorbed. At 12 weeks after surgery, group B exhibited significant bone formation and bony union. In the terms of bone formation and union, Group C was close to group B, and excellent to group D. The defect in group A was still evident, which indicated that the critical bone defect can’t repaired by itself. The radiographic scoring of the x-ray was measured with the result listed in table 1. The assessment of repair defect demonstrated a statistically significant improve bringing of defect with C compared to D. The group C and D had a higher scores than group A($P<0.05$), but lower than group B($P<0.05$).

<table>
<thead>
<tr>
<th>Group</th>
<th>4 weeks</th>
<th>8 weeks</th>
<th>12 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A</td>
<td>1.63 ± 0.31</td>
<td>2.62 ± 0.85</td>
<td>3.38 ± 0.76</td>
</tr>
<tr>
<td>Group B</td>
<td>4.95 ± 0.27</td>
<td>8.16 ± 0.35</td>
<td>10.76 ± 0.53</td>
</tr>
<tr>
<td>Group C</td>
<td>4.36 ± 0.48$^a$</td>
<td>7.53 ± 0.67$^a$</td>
<td>9.16 ± 0.36$^a$</td>
</tr>
<tr>
<td>Group D</td>
<td>4.12 ± 0.52$^{ab}$</td>
<td>7.36 ± 0.43$^{ab}$</td>
<td>8.56 ± 0.55$^{ab}$</td>
</tr>
</tbody>
</table>

Group A as control group, group B as autogenous bone group, group C as n-HA/PLGA, group D as β-TCP/PLGA. In the time point of 4, 8, 12 weeks after surgery, compare to group A and group B, $^aP<0.05$. compare to group C, $^bP<0.05$.

**Histological analysis**
The bone formation in radius defect was evaluated by HE, which were displayed in figure 2. The histological support the X-ray result. After 4 weeks after operation, there was no evident bone formation in group A; an amount of bone-like tissue formed in defect with group B, C and D; the scaffold in group C and D degraded partially. After 8 weeks, the woven bone filled the defect areas in group B, C and D; the scaffold in group B, C and D degraded completely. After 12 weeks, in group A, defect without scaffold show a loose connect tissue filled in the areas. The medullary canal in group B was recanalized. Defect in group C and D show a larger number of woven bone formation. The new woven bone formation in defect areas in group C was larger than D.

**Immunohistochemistry analysis**

Immunohistochemical analysis was used to detect the expression of osteocalcin (OCN) within the positively stained area (brown color), during the early phase of bone repairing at 4 weeks. OCN protein expression was markedly upregulated in the defect area at 4 weeks. The expression of OCN was seen in all groups as showed in figure 3. The result indicate that OCN involved in repairment process. In addition, the brown stain intensity of OCN in group B, C and D was denser than group A.

**Biomechanical results**

The result of biomechanical testing are display in table 2. The n-HA/PLGA and β-TCP/PLGA scaffolds enhanced the mechanical properties of the restored new bones as evidenced by a higher ultimate loading compared to the group A. However, compared to the group B, the biomechanics of repaired radius by nHA/PLGA and β-TCP/PLGA were not sufficiently strong.

<table>
<thead>
<tr>
<th>3-point bending test</th>
<th>Group A</th>
<th>Group B</th>
<th>Group C</th>
<th>Group D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ultimate strength (N)</td>
<td>51 ± 1.6</td>
<td>121 ± 4.2</td>
<td>112 ± 5.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>102 ± 2.3&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Group A as control group, group B as autogenous bone group, group C as n-HA/PLGA group, group D as β-TCP/PLGA group. Compare to group A and B, <sup>a</sup><i>P</i>&lt;0.05, compare to C, <sup>b</sup><i>P</i>&lt;0.05

**Discussion**

The repairment of bone defects using bone tissue engineering in animal model have been proved to be effective<sup>14</sup>. In practice, the bone defect maybe suffer from seawater immersing in naval operation. It is vital to address tissue engineering and HBO in the field of seawater immersed bone defects. Our study illuminate that the effectiveness of repairing bone defect with n-HA/PLGA /BMSCs or β-TCP/PLGA/BMSCs combined with HBO.

Stem cells play a vital part in tissue engineering. In our study, allogenic bone marrow MSCs were used due to following reasons: (1) autogenic MSCs was not recommended, because surgery on the illic bone would lead to injury and pain. (2) using allogenic MSCs didn’t lead to immunological rejection owe to lack of
expression of HLA class II antigens on MSCs.\(^{15}(3)\) in effectiveness of treating bone defect, there were similar between autogenic MSCs and allogenic MSCs. Soo-Hwan Kang\(^{16}\) compare autogenic MSC with allogenoc MSC in terms of bone regeneration in radius defect of the rabbits. On radiological, micro-CT and histological analysis demonstrated no evidence of immune reaction in allogenic MSCs group. Meanwhile, allogenic MSCs possess similar capacity for repairing defects compared to autologous MSCs. CD29 and CD44 were vital markers of BMSCs, BMSCs don’t express CD45 and CD34. The flow cytometry analysis demonstrated the cells at passage 5 was still MSCs.

The reason for HBO improved bone healing in the study were enhancing vascularization, upregulating expression of osteogenic markers, and downregulating expression of pro-inflammatory cytokines\(^{17}\).

Ideal scaffolds of tissue engineering have the characteristic: good biocompatibility, appropriate degradation. PLGA approved by FDA in certain clinical applications\(^{18}\), has been widely used in treatment bone defect. While PLGA have some disadvantages. Limited abilities of osteoconduction and osteoinduction hinder using in bone tissue engineering. However, the biggest disadvantage of PLGA is its acidic degradation products, which have lower pH values and easily cause inflammatory reactions in the implantation area. A lower pH value surrounding damage the proliferation of the cells, and the inflammatory reaction can trigger the release of cytokines by the host, damaging bone formation\(^{19-20}\). The PLGA are acidic degradation products, n-HA and β-TCP are alkaline degradation products\(^{21}\). n-HA and β-TCP can mediate the pH which produced by the acidic degradation products of PLGA. Therefore, they can avoid aseptic inflammation, which can provided a suitable microenvironment for new bone formation. Shu He, et al\(^{20}\) provide that nHA can neutralize the pH value due to the PLGA degradation. n-HA/PLGA revealed more strengthening effects in adhesion and osteogenic differentiation of BMSCs compared to PLGA\(^3\).

A critical-sized bone defect is defined as the length of defect more than 1.5 times diameter of bone\(^{22}\). The length of the radius defect was 15 mm for the rabbit bone defect created in our experiment. The length was according to the bone defect model created by Yi Wu\(^{23}\). In the experiment of Yi Wu, the rabbits of the 15mm-radius defect in the control group which implanted without any material can not repair spontaneously. In our experiment, sixty New Zealand white rabbits were created seawater immersed bone defect model in their radius. After different therapeutic measures for correspondent groups, the injured limbs were gradually recoveryment. In our study, after 4 weeks of surgery, bone defect area in groups C and D have low density cloudy callus. After 12 weeks, there was a excellent connection and integration with broken ends, which displayed the composite of n-HA/PLGA and β-TCP/PLGA have an excellent repairment capacity for seawater immersed bone defect. There was no achievement of complete bone union in group A. The three-point bending test showed that the mechanical properties in n-HA/PLGA and β-TCP/PLGA group were closed to autograft group.

**Conclusion**
In the experiment, the hostological and radiological study show the scaffold were degraded gradually, new callus was formed gradually, the radiographic result show that the scaffold /MSCs incorporation with HBO was successfully repair the 15 mm bone defect by 12 weeks after surgery. Scaffold of n-HA/PLGA/BMSCs and β-TCP/PLGA incorporation with HBO were effective to treat seawater immersed bone defect, and than n-HA/PLGA was more excellent than β-TCP/PLGA.

Abbreviations

BMSCs(Bone marrow mesenchymal stem cells), HBO(Hyperbaric oxygen), β-TCP (Beta tricalcium phosphate), PLGA (poly lactic-co-glycolic acid)

Declarations

Ethical Approval and Consent to participate

The experimental animal ethics committee of Anhui medical university(approval number LLSC2013031)

Consent for publication

I declare that I have obtained explicit permission from all co-authors to submit this paper in its current form

Competing interests

The authors declared no conflicts of interest.

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Authors' contributions

Design of the work the acquisition, analysis, or interpretation of data for the work.

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References


Figures

Figure 1

Radiographs in A group after operation 4 weeks (A), 8 weeks (E), 12 weeks (J); B group after operation 4 weeks (B), 8 weeks (F), 12 weeks (K); C group after operation 4 weeks (C), 8 weeks (G), 12 weeks (L); D group after operation 4 weeks (D), 8 weeks (H), 12 weeks (M)
Figure 2

Hematoxylin and eosin staining for new bone tissue after operation 4 weeks (A), 8 weeks (E), 12 weeks (I); B group after operation 4 weeks (B), 8 weeks (F), 12 weeks (J); C group after operation 4 weeks (C), 8 weeks (G), 12 weeks (K); D group after operation 4 weeks (D), 8 weeks (H), 12 weeks (L)
Figure 3

Immunohistochemical analysis for bone tissue after operation 4 weeks. There are positive tissue expression of OCN on extracellular matrix (black arrow)