Carboxymethyl Chitosan Reduces Inflammation and Promotes Osteogenesis in a Rabbit Knee Replacement Model

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Abstract

Background: The major causes of failure after total knee arthroplasty (TKA) include prosthesis loosening and infection. This study aimed to investigatethe role of carboxymethyl chitosan (CMC) in knee arthroplasty.

Methods: A total of 20 New Zealand white rabbits that were divided into two groups (10 in the control group and 10 in the chitosan group) were included in the study. They underwent TKA surgery, and all were implanted with titanium rod prostheses; the prosthesis in the chitosan group was coated with CMC. After 12 weeks, all rabbits were euthanized, and the followinganalyses of some specific surface membrane tissues around the prosthesis were performed: X-ray analysis; micro-computed tomography scan; haematoxylin and eosin, Van Gieson, and Von Kossa staining; reverse transcription polymerase chain reaction; and Western Blotting.

Results: The result of CCK8 test showed CMC can promote cell proliferation and increase cell viability. Radiological result showed better amount of bone deposits and more bone formation in the chitosan group. HE staining result showed CMC reduces inflammation around the prosthesis. The VG and Von Kossa staining results showed CMC can promote bone deposition around prosthesis.And according to the results of PCR and WB, the OCN content was higher in the chitosan group, while the MMPs content was lower. The chitosan group has an increased OPG/RANKL ratio than the control group.

Conclusion: CMC can effectively inhibit the inflammatory response around the prosthesis and osteoclast activation and promote osteogenesis by interfering with the osteoprotegerin/receptor activator of nuclear factor kappa-B ligand/receptor activator of nuclear factor kappa-B signalling pathway.

Background

Artificial knee arthroplasty is an effective method for treating end-stage knee joint disease. Annually, millions of patients undergo this operation worldwide. Most patients have reconstructed joint function, which improves their quality of life[1]. The number of patients undergoing total knee arthroplasty (TKA) is increasing, and revision surgeries are also consistently developing[2]. However, complications including pain and dysfunction are inevitable. Revision surgery is relatively expensive, increasing the patients' medical burden. Moreover, revision surgery has less satisfactory results than the first TKA[3]. Therefore, decreasing the revision rate after the first TKA has become one of the most interesting topics in the field of joint surgery.

A current study shows that the most common causes of revision after the first TKA are infection and aseptic loosening[4]. Periprosthetic infections often occur in the early revision phase within 2 years[5]. The most common pathogen of infection is *Staphylococcus aureus*, which indicates that the main infection originates from the patient's skin or the surgical field and improper disinfection of the equipment[6]. Aseptic loosening of the prosthesis is significantly common years later in late revision phase 2[7]. The reasons for aseptic loosening in the early and late stages are poor bone growth and bone absorption, respectively. At present, apart from good techniques and continuous optimization of prosthesis materials, there is no optimal method for preventing failure after the first TKA. Infection can be primarily prevented by administering prophylactic antibiotics. Based onresearch involving a rabbit *Staphylococcus* animal model, Gosheger et al. proposed that a silver-coated artificial joint can reduce the incidence of postoperative infection[8].The prevention of aseptic loosening of the prosthesis is mainly to inhibit the inflammatory response[9]and interfer with the osteoprotegerin (OPG)/receptor activator of nuclear factor kappa-B ligand (RANKL)/receptor activator of nuclear factor kappa-B (RANK) pathway, which promotes bone growth, reduces osteolysis by inhibiting the function of osteoclasts[10]. Therefore, if a material can prevent infection, and enhance the contact between the bone tissue and prosthesis, which is very important for the success of joint replacement.

Chitosan is a high-molecular-weight polymer produced after deacetylation from keratin[11]. Keratin is widely found in the cell walls of fungi and shells of crustaceans and insects. Keratin molecules form chitosan through N-terminal deacetylation[12]. Chitosan's hydrophilic surface can also promote cell adhesion, proliferation, and differentiation. Carboxymethylchitosan (CMC) has recently been introduced. CMC not only retains the excellent properties of chitosan but also has stronger water solubility and biological activity[13]. The antioxidant effect of CMC is related to the hydroxyl and carboxyl groups in the polymer chain. They can easily absorb hydrogen atoms in free radicals to form stable polymer groups[14].

Despite the above mentioned characteristics of chitosan, studies on the use of chitosan in TKA have not yet been conducted. Rabbit knee replacement model was a mature model which can modulate most of mechanical and biological characters of real TKA[20]. So we established the rabbit TKA model to simulate the early reaction stage of joint prosthesis implantation with the use of chitosan to investigate its effect on the prosthesis surroundings.

Methods

Animals and materials

（1）A total of 20 adult male New Zealand rabbits, were randomly divided into the chitosan group and the control group, with 10 rabbits in each group. The rabbits were purchased from the Hubei Provincial Center for Disease Control and Prevention (Wuhan, Hubei, China). The average weight of the rabbits was 2.83 kg. The animals and procedures were approved by the Ethics Committee of Wuhan Fourth Hospital，and conducted following the relevant guidelines and regulations (Approval Number:20191947).

(2) Titanium rod prostheses purchased from Stryker Co. Ltd., USA, were cylindrical in shape (length, 2 cm; diameter, 0.5 cm) and sterilized using a high-pressure steam before use.

(3) Chitosan hydrogel was purchased from Shijiazhuang Yishengtang Medical Product Co. Ltd. in China. CMC was used as the main component and was configured with a physiological equilibrium solution with a concentration of 25 mg/ml. It was a colourless transparent viscous liquid that was stored in a sterile

area and sealed in a 4°C refrigerator for single use.We diluted it according to the results of the CCK8 test and selected the concentration with the best cell proliferation ability for the experiment.

(4) Wuhan Fourth Hospital provided animal cages and an experimental operation platform.

Detection of the value-added ability of carboxymethyl chitosan

A total of 0 (control group), 0.1, 0.2, 0.5, 1, 5, or 25 mg/mL carboxymethyl chitosan was added to cells in each well of a 96-well plate, and the plate was placed in an incubator for 24 h. A total of10μL of cck8 reagent was added to each well, and the plate was incubated at 37 °C with 5% $CO2$ for 4h. After incubation, the solution was mixed thoroughly, and the absorbance at 450 nm was measured with a microplate reader. Cell proliferation rate = absorbance value of experimental group-absorbance value of blank group / absorbance value of the control group-absorbance value of the blank group.The concentration with the least effect on cell activity was chosen for subsequent experiments.

Animal surgery

All rabbits were adaptively fed for 1 week before surgery. The animals were anaesthetized intramuscularly with xylazine hydrochloride 0.2 ml/kg. The rabbit was placed in a supine position on an operating table. The right knee was used as the surgical side. The sterile towel covered the surgical area, and the medial skin incision and paracondylar approach were used to expose the articular cartilage along the subcutaneous tissue and the joint capsule layer. A hole was drilled in the femoral intercondylar fossa posterior cruciate ligament to open the medullary cavity, and the medulla was expanded from 0.3 cm to 0.5 cm along the longitudinal axis of the femur with a depth greater than 2 cm. The titanium rod of the chitosan group was immersed in the CMC solution for half an hour and subsequently implanted in the femoral bone marrow cavity. Each layer of tissue was closed after the medullary cavity was flushed with saline(FIGURE 1).

Postoperative management

The principle of aseptic operation was followed, and 400,000 units of intramuscular injection of penicillin was administered twice a day for the first 3 days after operation. None of the animals had any lower extremity fixation.

Animal sacrifice and tissue harvest

All 20 animals were euthanized with excess doses anesthetic phenobarbital after 12 weeks. The anesthetic dosage of phenobarbital was 40mg/kg, with the concentration as 2%. Synovial tissue on the ipsilateral side of the knee joint was extracted and placed in 10% zinc formalin to prepare paraffin sections. The titanium rod segment with the right distal femur was collected, and the bone was cut off using a hacksaw 2 cm away from the knee joint surface. The distal part was stored frozen at -80°C, and hard tissue sections were prepared. The residual boundary membrane tissue around the two titanium rods was retained; one part was placed in ribonucleic acid (RNA) lysing solution, and the other part was placed in a 1.5-ml test tube, which was sealed and stored in liquid nitrogen. Both were stored in -80°C refrigerators for reverse transcription polymerase chain reaction (RT-PCR) and western blotting (WB).

Radiological evaluation

The X-ray film of the right knee joint was irradiated to observe bone formation. Micro-computed tomography (CT) was performed to reconstruct the osteogenesis around the titanium rod, and bone tissue volume/total volume of bone tissue and prosthesis (BV/TV) was calculated[15].

Bone and synovial tissue staining and analysis

The synovial tissue of the knee joint was treated with paraffin sections and stained with haematoxylin and eosin (HE). Bone containing titanium rods was processed by non-decalcified hard tissue sections. The non-decalcified bone tissue retained the structure of the trabecular bone and accurately reflected bone growth, which was convenient for the study of bone growth and absorption. Bone tissue was cut into 50-micron sheet structures and cut along the long axis of the femur using a thin slicer. The hard sections were subjected to Van Gieson (VG) and Von Kossa staining. Based on the VG staining, the bone-prosthesis contact rate(B-PCR, the length of the circumference of the bone in contact with the prosthesis/total circumference of the prosthesis[16]) and bone volume percentage (BVP, squares of the bone tissue in a1 mmarea around the prosthesis/total squares of the 1mmarea around the prosthesis[17]) were analysed. Von Kossa staining can be used to measure the number and area of calcium salt deposit islands (CSDIs) around the prosthesis[18].

Western blotting

The tissue stored in liquid nitrogen was moved to anEppendorftube, and lysis buffer was added to lyse the cells. The protein concentration was quantified using a bicinchoninic acid (BCA) protein concentration determination kit. A 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)gel was prepared, the protein sample was loaded, electrophoresis was performed, the proteins were transferred to a membrane, andthe membrane was blocked and incubated with the indicated antibodies, namely,mouse monoclonal antibody matrix metalloproteinase-9 precursor (MMP9) and osteocalcin (OCN), rabbit polyclonal antibody OPG and RANKL, and rabbit polyclonal β-actin, at 4°C. After 24 h, membranes were rinsed and subsequently incubated with horseradish peroxidase (HRP)-labelled goat anti-mouse and goat anti-rabbit secondary antibodies for 2 h at room temperature. Membranes were developed in a dark room with the electrochemiluminescence (ECL) light-emitting kit. The Bio-Rad Gel Imaging System was used to collect the images and to perform greyscale scanning analysis of the images.

Real-time polymerase chain reaction

Total RNA was extracted using TRIzol reagent, and 2μg of total RNA was used for the reverse transcription reaction. The reverse transcription conditions were as follows: 25°C for 5 min,50°C for 15 min,85°C for 5 min, and 4°C for 10 min. Two microlitres of the above reaction solution was used for PCR,and the following PCR conditions were used: 50°C for 2 min, 95°C for 10 min, 95°C for 30 s, and

60℃ for 30 s, with a total of 40 cycles. The primers were designed using Primer Premier 5.0 software and verified by Blast. The primer sequences are listed in Table 1.Five microlitres of the amplified product was used for analysis by 1% agarose gel electrophoresis, pictures were taken under an ultraviolet light, and the greyscale value was determined using ImageJ 1.45s software. The relative expression level of the target messenger RNA (mRNA) is expressed as the ratio of the greyscale values of the target band and the internal reference, namely, glyceraldehyde 3-phosphate dehydrogenase.

Statistical analysis

Image-Pro Plus (IPP) 6.0 was used for graphics and data processing. Data analysis was performed using GraphPad Prism 6.03 data results for standard deviation analysis. The data model used one-way analysis of variance, with a p value of 0.05 considered statistically significant.

Results

CCK8 test

Chitosan can promote cell proliferation and increase cell viability. In the range of 0-1 mg/ml, as the concentration of carboxymethyl chitosan increased, the cell proliferation rate increased. At 25 mg/ml, chitosan inhibited cell proliferation and had obvious cytotoxicity. We chose 1mg/ml for subsequent experiments (FIGURE 2).

Radiological osteogenesis

After 12 weeks of feeding, two groups of animals were irradiated with the right knee joints. Compared with that in the control group,osteoporosis around the prosthesis in the chitosan group was lower, manifesting as a better amount of bone deposits (FIGURE 3). Three-dimensional reconstruction of micro-CT showed that there was significantly more bone formation around the prosthesis in the chitosan group than in the control group (FIGURE 4).

CMC reduces inflammation

HE staining showed a significant inflammatory response around the prosthesis in the control group, with synovial hyperplasia and infiltration by a large number of inflammatory cells. In the chitosan group, the levels of inflammatory cell infiltration and synovial hyperplasia were lower than those in the control group (FIGURE 5).

Does CMC promote bone deposition?

Hard tissue sections containing titanium rods received special staining. The VG staining results showed that there was more bone deposition in the chitosan group than in the control group, suggesting that the prosthesis was better in the chitosan group than in the control group. According to the staining results and the analysis of the image software IPP 6.0, B-PCR and BVP were significantly higher in the experimental group than in the control group (FIGURE 6).

Based on the Von Kossa staining results, we used IPP 6.0 to calculate CSDIsaround the prosthesis in both groups. The CSDIsin the control group were mainly composed of a small amount of stellate. In contrast, the CSDIsin the chitosan group were linear and abundant. The results showed that the control group was significantly worse than the chitosan group in terms of bone mineralization and deposition (FIGURE 7).

Effects of CMC on the metabolism of osteoblasts and osteoclasts

RT-PCR and WB were used to study specific mRNAs and proteins in the periprosthetic tissue. Two pairs of specimens were processed simultaneously. The appropriate exposure time and developer based on β-actin were chosen. Compared with that of the control group, the osteoblast-specific marker OCN protein content of the chitosan group was higher, while the osteoclast-specific marker MMP9 protein content was lower. Simultaneously, the content of OPG protein increased, while the content of RANKL protein decreased in the chitosan group, resulting in an increased OPG/RANKL ratio. The OPG/RANKL balance plays an important role in maintaining bone metabolism. The increase in the OPG/RANKL ratio enhanced the activity of osteoblasts, weakened the activity of osteoclasts, promoted osteogenesis, and suppressed osteolysis. Considering the above results, we conclude that CMC around the titanium rod can promote bone formation and inhibit osteolysis (FIGURE 8).

Discussion

Aseptic loosening is one of the most common complications of surgical joint replacement[19]. It is a chronic and continuous pathological process accompanied by infiltration of inflammatory cells[20]. The inflammatory factors secreted by inflammatory cells activate the OPG/RANKL/RANK signalling pathway and promote osteolysis, eventually leading to aseptic loosening[21]. Therefore, it is possible to intervene in the loosening of the prosthesis through anti-inflammatory and anti-osteoporosis treatment.

Our experiments demonstrated that CMC has a positive effect on osteogenesis around prostheses. In this experiment, non-decalcified hard tissue sections were obtained from the prosthesis and its surrounding femur, and subsequently, special staining was performed. It demonstrated the actual condition of the contact between the prosthesis and bone tissue. The positive effects of CMC on osteogenesis and its beneficial anti-inflammatory effects were confirmed. Although there was a relatively slight difference in osteogenesis between the two groups during X-ray examination, micro-CT and VG staining of hard tissue sections showed that the BV/TV, B-PCR, and BVP levels of the chitosan group were higher than those of the control group. Von Kossa staining showed that the average area and number of CSDIs in the chitosan group were significantly higher than those in the control group, and most of them were continuous. The CSDIs in the control group were mostly networked and intermittent. These results suggest that the osteogenesis of the chitosan group is significantly better than that of the control group. We detected the synovial inflammatory response by HE staining, and there were significantly fewer inflammatory cells in the chitosan group than in the control group.

There are four advantages of CMC applied around the knee prosthesis. First, chitosan and its various compounds are widely used due to their biodegradability and biocompatibility, which does not affect the implantation of joint prostheses, can degrade itself and will not cause damage to surrounding tissues[22, 23]. Second, CMC has a broad-spectrum antibacterial effect, which interferes with the synthesis and metabolism of the bacterial outer wall, thereby causing damage to the structure of bacteria and inhibiting bacterial growth. At the same time, CMC reduces local inflammation by increasing lysozyme activity in macrophage responses and increases the body's immune function[24]. Third, the biocompatibility of CMC makes bone cells easy to grow. Klokkevold et al. studied the differentiation of osteoblasts and bone formation in vitro, and the experimental results showed that chitosan can enhance the differentiation of bone progenitor cells and promote osteogenesis[25]. Finally, multiple studies have proven that chitosan and its derivatives have various antioxidant properties[26]. Yang et al. placed wear particles obtained from the interface membrane around a patient's sterile loosening prosthesis into the primary osteoblasts of rabbits and found that the increased production of reactive oxygen species can induce osteoblast apoptosis[27]. The specific mechanism is that active oxygen mediates osteoblast apoptosis through the mitochondrial apoptotic protease-dependent pathway and the endoplasmic reticulum stress pathway, and this apoptosis can be attenuated by antioxidant antagonistic effects.

OPG/RANKL/RANK signalling is the most important pathway affecting osteogenesis. RANKL binds to its membrane-bound receptor nuclear factor receptor activation factor RANK, which stimulates osteoclast nuclear differentiation and maturation[21]. The soluble protein OPG is mainly produced in osteoblasts and acts as a RANKL-soluble bait receptor to inhibit the effect of RANKL[28].The imbalance between RANKL and OPG is the key to osteoclast differentiation, osteolysis, and loss of bone mass. A review by Kobayashi et al. noted that the oxidative stress response can be accompanied by an increase in the expression of RANKL, while the OPG/RNAKL ratio decreases[29]. This means that oxidative stress can stimulate the RANKL/RANK interaction and inhibit the OPG/RANKL/RANK signalling pathway. The study of XiaoFeng He et al. found that oxidative stress can induce osteoporosis through OPG/RANKL/RANK, and antioxidants can alleviate this bone loss[30]. The WB and real-time PCR results of the chitosan group in this study showed that the OPG/RANKL ratio increased, the expression of osteoblast-specific marker OCN increased, and the expression of osteoclast-specific marker MMP9 decreased, suggesting that CMC could promote bone formation by interfering with this signal.

Conclusion

This study investigated the effects of CMC in a TKA model. It has been preliminarilyshown that CMC can reduce the inflammatory response around rabbit knee prostheses, affect the OPG/RANKL/RANK signalling pathway, and promote osteogenesis. This is of significant importancein preventing aseptic loosening of the knee joint prosthesis. However, our study has some limitations. The titanium rods used in this studywere different from clinical prostheses, and they could not simulate human biomechanical mechanisms. Additionally, the experimental period was short, and the metabolism of CMC in each stage was not investigated. Hence, further large-scale studies are required in the future.

Abbreviations

TKA total knee arthroplasty

CMC carboxymethyl chitosan

OPG osteoprotegerin

RANKL receptor activator of nuclear factor kappa-B ligand

RNA ribonucleic acid

RT-PCR reverse transcription polymerase chain reaction

WB western blotting

CT computed tomography

BV/TV bone tissue volume/total volume of bone tissue and prosthesis

HE haematoxylin and eosin

VG Van Gieson

B-PCR bone-prosthesis contact rate

BVP bone volume percentage

CSDIs calcium salt deposit islands

BCA bicinchoninic acid

SDS-PAGEsodium dodecyl sulfate polyacrylamide gel electrophoresis

MMP9 matrix metalloproteinase-9 precursor

OCN osteocalcin

HRP horseradish peroxidase

ECL electrochemiluminescence

mRNA messenger RNA

IPP Image-Pro Plus

Declarations

Ethics approval and consent to participate

The protocol of this clinical study was reviewed and approved by the research Ethics Committee of Wuhan Fourth Hospital (Approval Number:20191947).

Consent for publication

Not Applicable.

Availability of data and material

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request

Competing interests

The authors declare no competing financial interests.

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Authors' contributions

HX and YZW was responsible for experiment design, conceptualization and supervision, HNZ contributed lab experiment, data collection, and manuscript writing; FL and ZW conducted data collection, data entry, and contributed to manuscript writing. HYL made the statistical data analysis. The author(s) read and approved the final manuscript.

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Tables

Table 1 Primer sequence

Gene	Upstream Primer	Downstream Primer
MMP9	GTACTCGACCTGTACCAGCG	TTCAGGGCGAGGACCATAGA
OCN	AGCAAAGGTGCAGCCTTTGT	GCGCCTGGGTCTCTTCACT
OPG	GGAACCCCAGAGCGAAATACA	CCTGAAGAATGCCTCCTCACA
RANKL	CAGAAGATGGCACTCACTGCA	CACCATCGCTTTCTCTGCTCT
GAPDH	ATGGGGAAGGTGAAGGTCG	GGGGTCATTGATGGCAACAATA

Figures

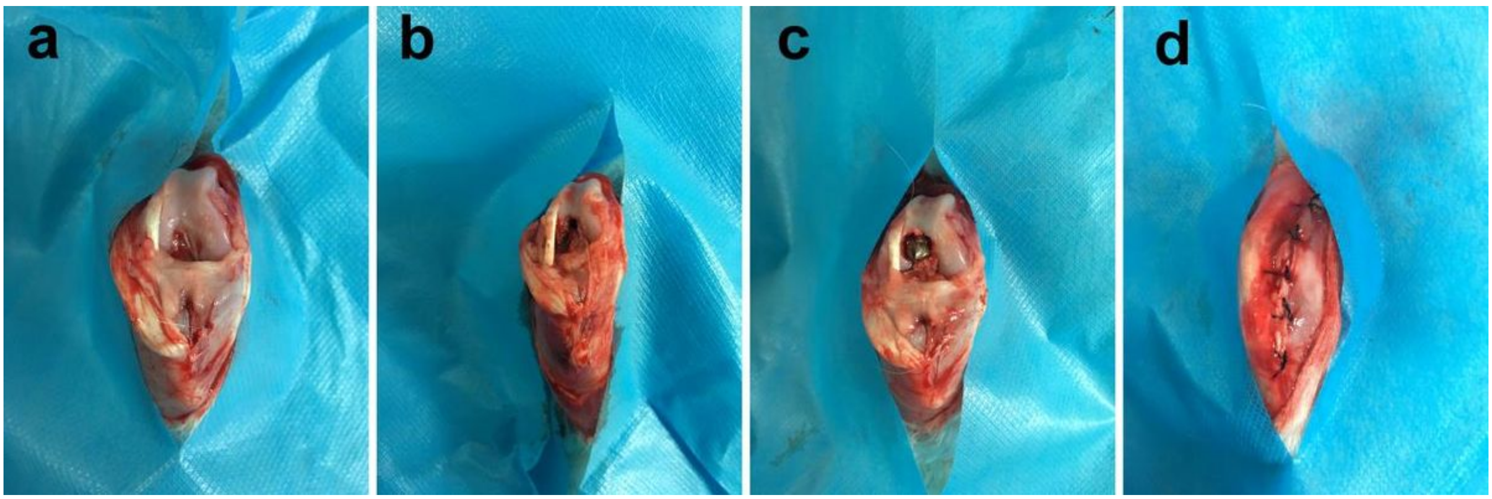
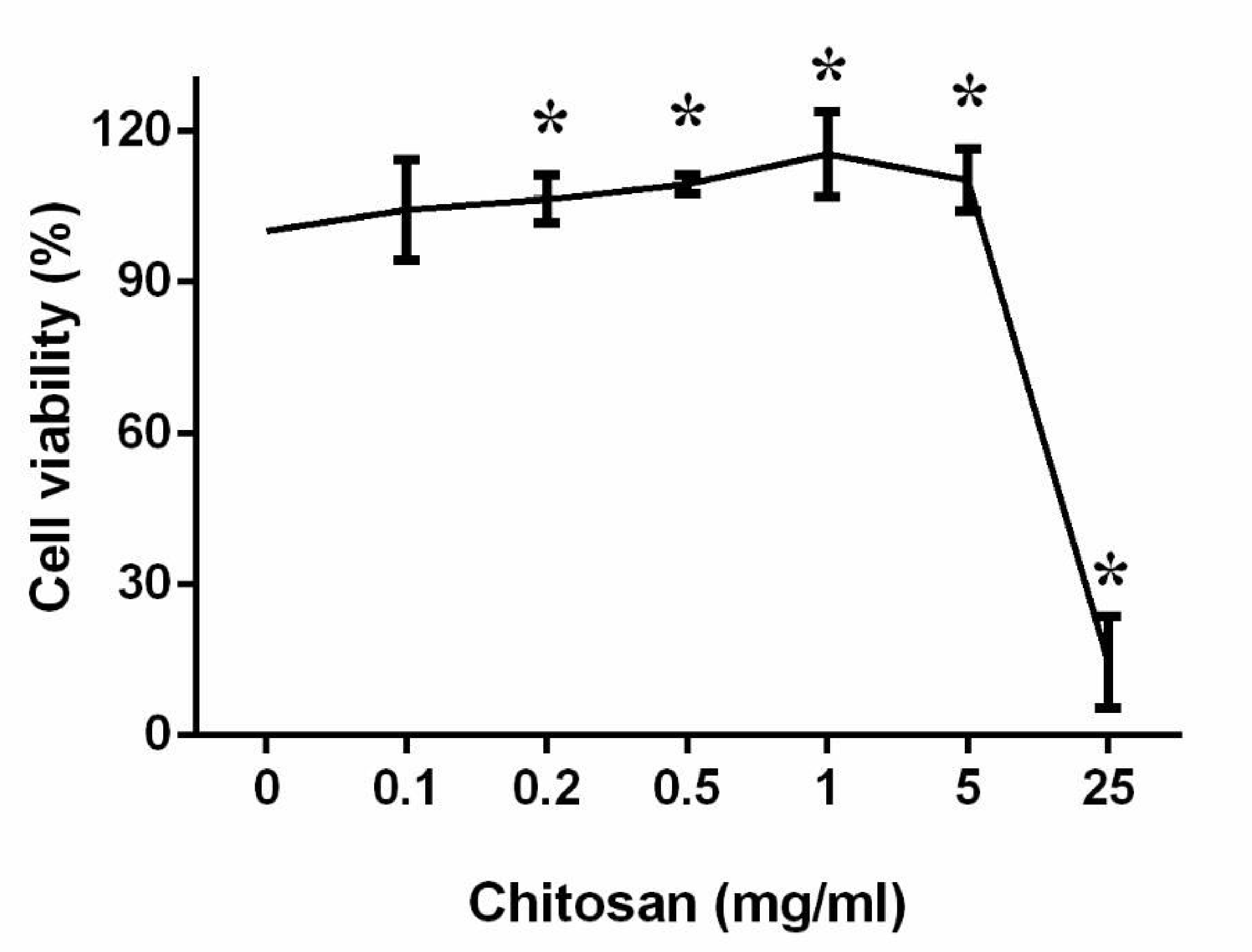


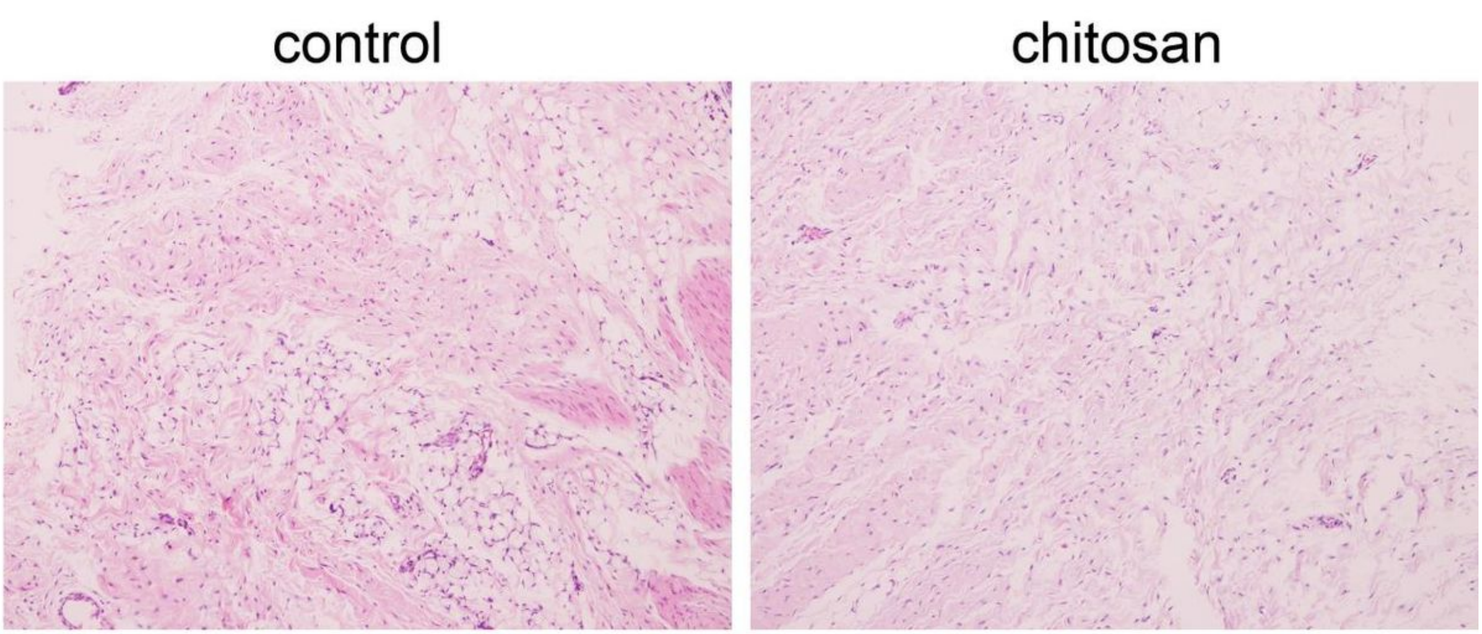
Figure 1

Operative process of titanium rod prosthesis implantation surgery.(a) The distal femur of the knee prosthesis was exposed. (b) We opened and expanded the femoral medullary cavity with drills of increasing size. (c)The titanium rod prosthesis was implantedinto the femoral medullary cavity. (d) The incision was closed after thorough rinsing.



120
90
60
30
0
Cell viability (%)
*
*
*
*
*
0
0.1
0.2
0.5
1
5
25
Chitosan (mg/ml)

Figure 2



control
chitosan

Figure 3

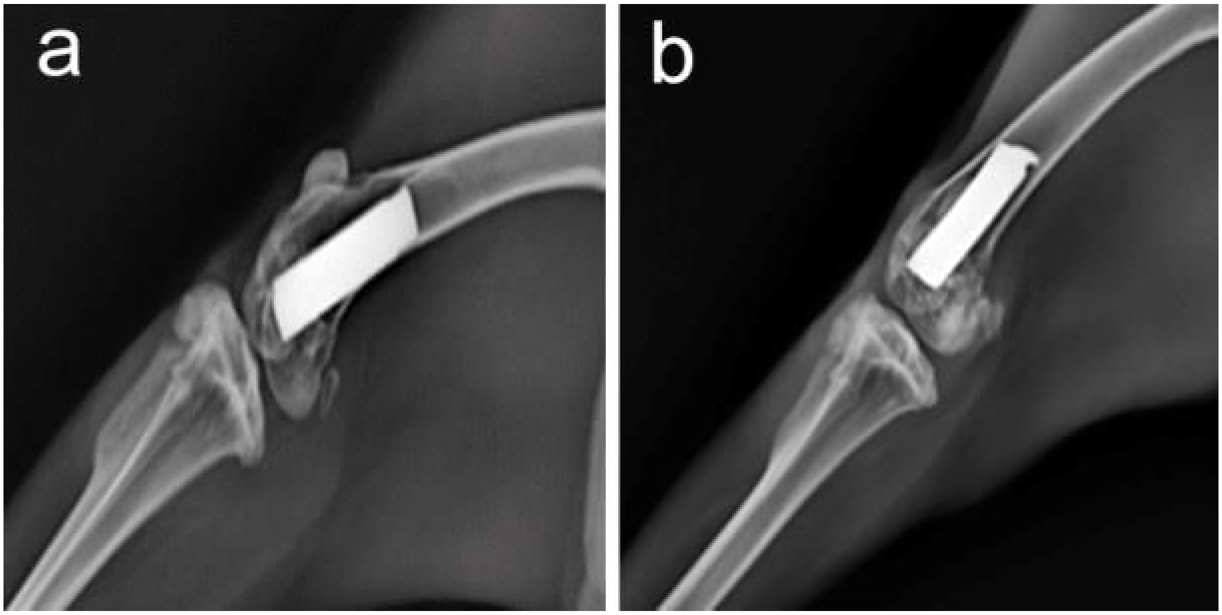


Figure 4

Lateral X-ray of the right femur (a) Osteoporosis around the prosthesis in the control group (b) Small amount of bone deposit in front of prosthesis in the chitosan group.

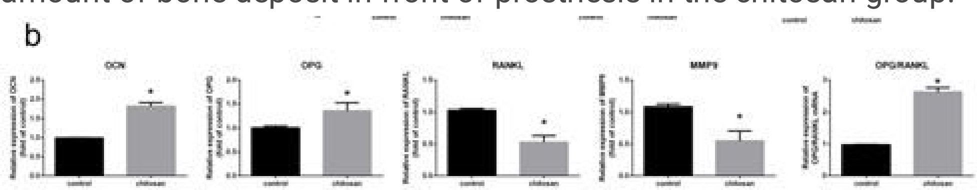


Figure 5

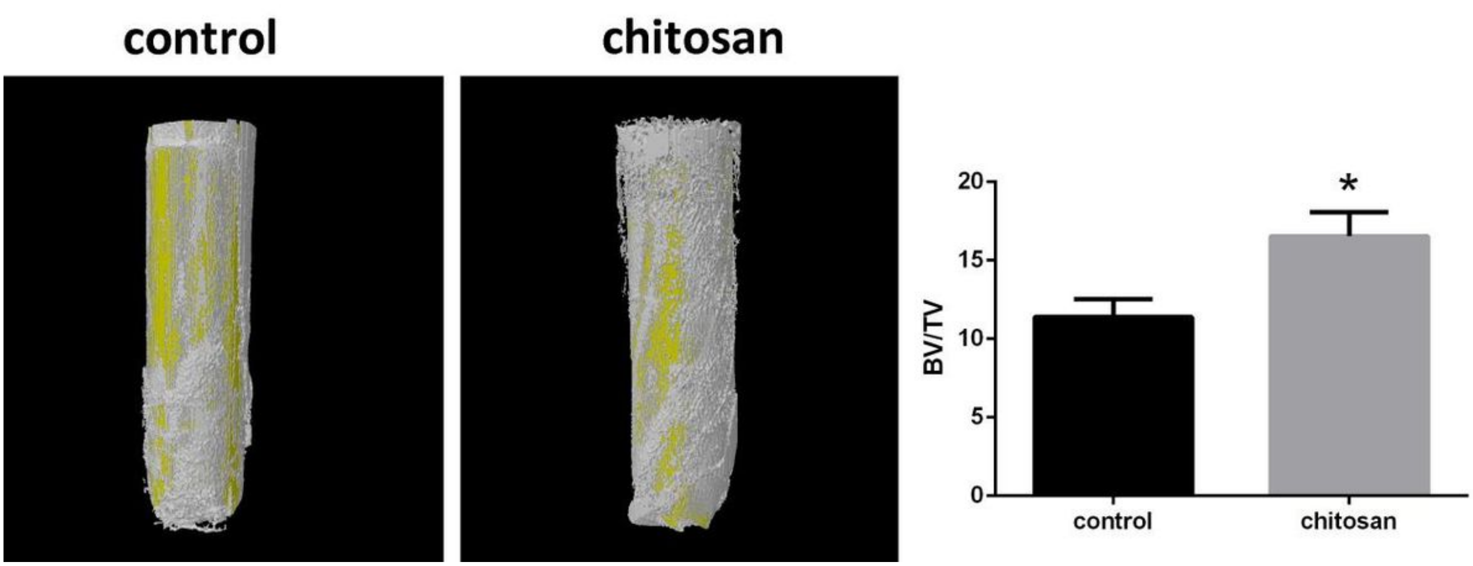


Figure 6

Micro-computed tomography three-dimensional reconstruction of osteogenesis around the prosthesis. (a) Bone deposition scattered around the prosthesis in the control group. (b) Bone deposition on the surface of the prosthesis in the chitosan group. (c) Bone tissue volume/total volume difference was statistically significant.

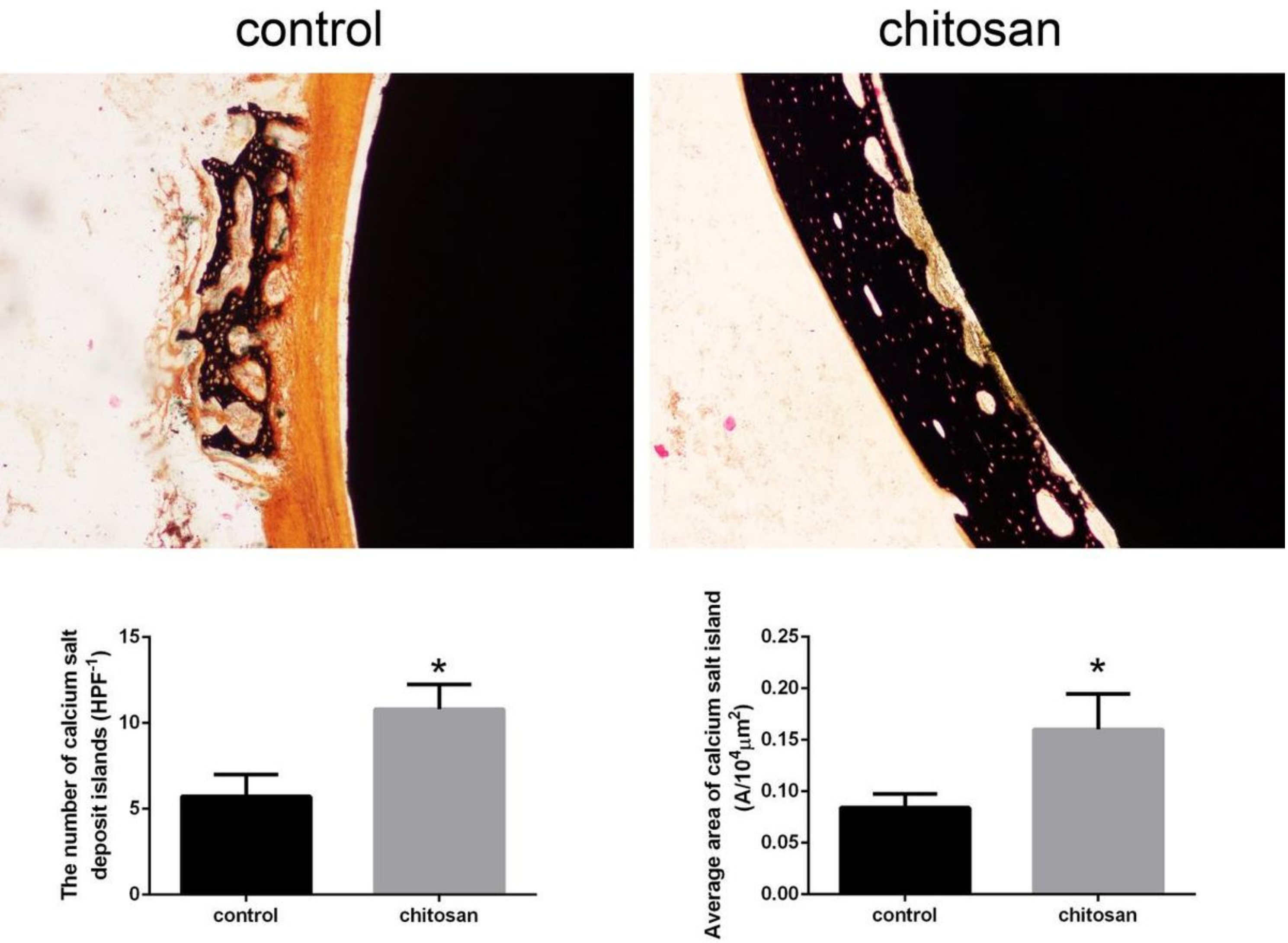


Figure 7

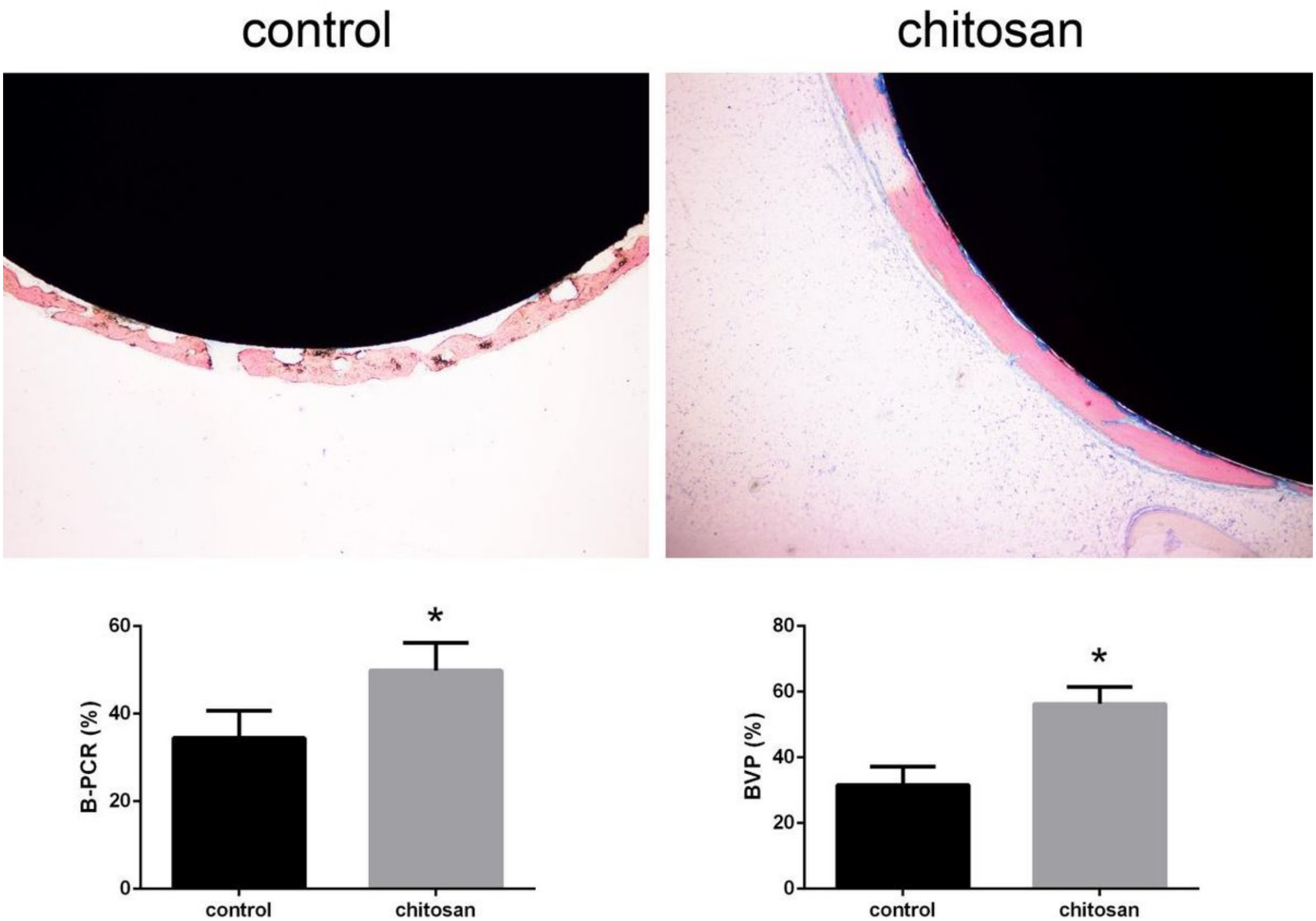


Figure 8

Van Gieson staining of bone tissue and hard tissue sections of the prosthesis (40x, bar=250 μm). (a) The control group showed less bone formation around the prosthesis and intermittent bone formation. (b) The chitosan group showed more bone formation around the prosthesis and continuous bone formation. (c) The difference between the two groups' bone-prosthesis contact rate and bone volume percentage was statistically significant.

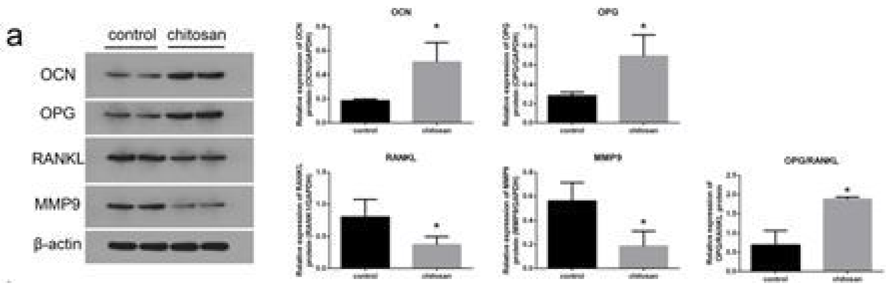


Figure 9

Results of western blotting of protein. Compared with the control group, protein content of osteocalcin and osteoprotegerin (OPG) in the chitosan group significantly increased ($p<0.05$). In contrast, protein content of the receptor activator of nuclear factor kappa-B ligand (RANKL) and matrix metalloproteinase-9 precursor in the chitosan group significantly decreased. The OPG/RANKL ratio significantly increased in the chitosan group ($p<0.05$)

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Westernblotting.docx
- NC3RsARRIVEGuidelinesChecklistfillable.pdf