GTKO rabbit: A novel animal model for preclinical assessment of decellularized xenogeneic grafts via in situ implantation

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ABSTRACT

Wild type (WT) animals cannot be used to objectively assess the immunogenicity of animal tissue-derived biomaterials when used as recipients due to difference with human in α-Gal expression. The purpose of this study is to compare the differences of immunological responses between the GGTA1 gene-knockout (GTKO) rabbits and WT rabbits after implantation with animal tissue-derived biomaterials. The porcine-derived decellularized bone matrix (natural bone material, NBM) and fresh porcine cancellous bone (PCB) were implanted in GTKO rabbits and WT rabbits, respectively, and sham operation was used as control (Con). At 2- and 6-week post-implantation, the related immunological items including antibody levels, serum-mediated cell lysis, cytokines, lymphocyte subtypes, and histopathological changes were assessed.

GTKO rabbits exhibited more sensitive immune responses than WT rabbits after PCB implantation, resulted from a significant increase of antibodies (except total antibodies) and cytokines levels, cell lysis ratios, CD4/CD8 proportions, and inflammatory cells infiltration. Immunological factors and inflammatory cells infiltrate in GTKO rabbits after NBM implantation were significantly lower than those in the PCB group. Among the three groups, the NBM group showed the highest contents of new bone formation elements.

In conclusion, the GTKO rabbit is a more sensitive alternative model than WT rabbit for preclinical study of xenografts via in situ implantation. Studies on multiple gene-edited animals are also necessary for more comprehensively evaluating xenoinmunogen risks of animal tissue-derived biomaterials in the future. Additionally, the immunogenicity of NBM was remarkably decreased compared to PCB.

1. Introduction

Residual xenoantigens in animal tissue-derived biomaterials are important factors causing immune rejection or graft failure [1–3]. Galactose-α1,3-galactose (α-1,3-galactosyl, α-Gal) is the main antigen contributing to xenograft rejection [4]. Because of base mutations in the α-1,3-galactosyltransferase (GGTA1) gene, humans cannot synthesize α-Gal but produce high levels of anti-Gal antibodies due to bacteria stimuli in the intestinal flora [5–7]. After implantation of animal-derived tissues or organs, anti-Gal antibodies and other antibodies induced by xenotransplantation cooperate with the complement system to activate immune responses, leading to fibrous encapsulation or necrosis of the implant, ultimately causing implant failure [8,9]. Therefore, preclinical animal studies are important for evaluating the biocompatibility and tissue repair effects of xeno biomaterials. However, commonly used experimental animals express α-Gal, and xenogenous α-Gal do not induce anti-Gal antibodies in these animals [6]. There are innate differences in the in vivo immune environment of humans and commonly used experimental animals, resulting in the inability to objectively and comprehensively evaluate the potential risks of animal tissue-derived biomaterials to humans by using WT experimental animals as receptors [10].

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Owing to the development of gene-editing technology, GTKO mice have been established and used to study the specific mechanism of xenograft rejection [11,12]. We have successfully established a GTKO mouse model using gene targeting techniques (homologous arm recombination) [10,12–14] and revealed that anti-Gal antibody levels increased significantly after implantation of xenogeneic bone matrix or xenogeneic decellularized cornea in GTKO mice. However, due to the small size of GTKO mice, it is difficult to assess the biocompatibility and effectiveness of xenografts via in situ implantation.

Rabbits are an invaluable animal model in tissue engineering and could offer insights into the graft-specific immune responses of biomaterials [15,16]. They are the “largest” small animal experimental species available, easy to house, with short reproduction cycles, and organ systems similar to those of humans [16]. These advantages make them an excellent experimental model for biomaterial assessment [17]. We have previously developed a GTKO rabbit using the CRISPR/Cas9 technique by editing exon 8 of GOTA1 gene in rabbit, which successfully induced a 21bp base deletion and inhibited α-Gal expression in GTKO rabbits [18]. Additionally, the anti-Gal antibody level in GTKO rabbits increased with age, peaking at 5 months.

In this study, we aimed to investigate whether GTKO rabbits are beneficial for evaluating the immunogenicity and host local effects of xenografts via in situ implantation.

2. Materials and methods

2.1. Experimental rabbits

A total of 18 GTKO rabbits and 18 WT rabbits (9 months old, body weight: 2.8 kg–3.7 kg) were provided by the Institute of Laboratory Animal Resources, National Institutes for Food and Drug Control (NIFDC), China. All animal experiments were approved by the Experimental Animal Welfare Ethical Review Committee of NIFDC (approval number: Zhongjiandong (Fu) No. 2020 (B) 013).

Animal management was carried out in accordance with the standard operating regulations established by the Laboratory Animal Protection and Use Regulations. SPF rabbit feed (Beijing Keyao Xieli Feed Co., Ltd.) was provided daily, and purified water was provided in a drinking bottle. Each rabbit was bred separately, and the label information for each cage indicated gender, animal number, and implantation date. The room temperature and relative humidity were controlled at 20 °C–25 °C and 40%–70%, respectively.

Polymerase chain reaction (PCR) and Sanger sequencing were used to validate that the target gene sequence was successfully edited using genomic DNA extracted from RBCs. The primers used for PCR and sequencing were forward primer: CAGAATCCCTTTGATAGAGC; reverse primer: TTATGGCTTGTGTTGACGG. Rabbit red blood cells (RBCs) were collected before the implantation experiments to validate the deletion of α-Gal epitopes by a standardized method [19].

2.2. Preparation of xenografts

Xenografts were provided by Zhejiang Decellmatrix Biotech Co., Ltd., China. Briefly, the porcine cancellous bone (PCB) harvested from porcine femurs (raw material without decellularization) was cut into round pieces with a diameter of 6 mm and a thickness of approximately 2 mm, washed with isotonic saline, and used as the positive control. The decellularized bone matrix (natural bone repair material, NBM) made from the porcine femur (cancellous bone) was manufactured using specific decellularization processes (patent No. PCT/CN2019/077754) with polyethylene glycol octyl phenyl ether (Triton-100) and sodium dodecyl sulphonate (SDS). Both PCB and NBM were freeze-dried and sterilized with ethylene oxide for standby use.

2.3. Characterization of NBM and PCB

The NBM was sputter-coated with Au–Pd (Gold and Palladium) and observed by scanning electron microscopy (SEM).

The remnant α-Gal antigens of PCB and NBM were determined in accordance with the Chinese industry standards “Tissue engineering medical device products—Remnant α-Gal antigen determination in scaffold materials utilizing animal tissues and their derivatives” (YY/T 1561–2017) via ELISA [20,21] using a commercial α-Gal antigen detection kit (Meitan 70101; Beijing Sanyao Co., Beijing, China). The remnant DNA/s of NBM and PCB were determined according to the Chinese industry-standard “Tissue-engineered medical products- Part 25: Determination of DNA residues in animal tissue-derived biomaterials: Fluorescence staining method” (YY/T 0606.25). Briefly, DNA was released from ECM materials by proteinase K digestion, and the released DNA was subsequently purified using a nucleic acid extraction kit (4400793, 4400795, 4400675, ABI) and determined by fluorescence assay with Pico Green dsDNA reagent (P7589, Invitrogen, Waltham, MA, USA).

Liquid chromatography–mass spectrometry (LC-MS/MS) was used to identify peptide sequences of NBM and PCB. Briefly, samples were homogenized in a mixture of 4% SDS and 100 mM dithiothreitol and subjected to a multi-enzyme digestion protocol, as previously described [22]. The peptide solutions were collected, desalted, and cleaned using a ZIPTIP C18 (MilliporeSigma, Burlington, MA, USA). High-resolution LC-MS/MS was used to identify the protein components of each sample. Protein annotation, functional classification, and functional enrichment analyses were also performed. Proteins were classified by Gene Ontology (GO) annotation based on the categories “biological processes”, “cellular components”, and “molecular functions”.

The other characterization of PCB has been described in a previous study [23].

2.4. Experimental grouping, implantation and sampling

Rabbits (GTKO and WT) were divided into three experimental groups at two time points (2 and 6 weeks): control (Con, sham operation), NBM, and PCB groups (positive control), as shown in Table 1 (see Table 2).

Xenografts were surgically implanted into the rabbit’s femoral condyles. Briefly, after the rabbits were anesthetized, their femoral condyles were depepilated and disinfected with iodophor, lateral femoral condyles were exposed, and an 8 mm–10 mm depth full-thickness bone defect was then induced using a low-speed drill with an outer diameter of 5 mm. To avoid tissue damage, the wound surface was rinsed and cooled during drilling with isotonic saline. The NBM and PCB groups were implanted with the corresponding materials, and the Con group only had defects without filling any materials. A total of 160,000 units of penicillin combined with 100,000 units of streptomycin were administered intra-muscularly on the day before and two days after the operation to avoid acute infection in experimental animals.

Two and six weeks after implantation, the GTKO and WT rabbits were
humanely euthanized, and blood was collected for immunological and inflammatory factor assessment and cell lysis test. The femoral condyles of the rabbits were removed and fixed in neutral formaldehyde for histopathological evaluation.

2.5. Antibodies level detection

At the intended experimental endpoint, the peripheral blood of rabbits was collected, incubated at 37 °C for 1 h, and overnight at 4 °C. After centrifugation at 900g for 10 min, the upper serum was collected and stored at −80 °C for follow-up test.

Total IgG and IgM levels were determined using commercial kits (Rabbit IgG ELISA Kit, 6520 and 6580; Alpha Diagnostic, San Antonio, TX, USA) according to the kit’s instructions.

Anti-Gal IgG and IgM levels were detected by ELISA [12]. First, Gal-o-3Gal-BSA (Gal-BSA, NPG0203; Dextra Laboratories Ltd., Thames Valley Science Park, UK) was used as a solid-phase antigen coating in a 96-well plate (0.2 μg per well), incubated at 37 °C for 2 h, and then transferred to 4 °C overnight. Human serum albumin (1.5%) was added (150 μL/well) to block nonspecific antigens. After washing, diluted rabbit serum (1:1000) was added and incubated for at least 60 min, followed by a 30 min incubation with HRP-labeled goat anti-rabbit secondary IgG or IgM antibodies (1:10000; ab97195 and ab6721; Abcam, Cambridge, UK). Finally, 3,3'-5,5'-tetramethylbenzidine (TMB) was added for color development and incubated for 15 min. A stop solution (10% H2SO4) was then added, and absorbance was measured at 450 nm (A450) using a microplate reader (Spectramax M5; Molecular Devices, San Jose, CA, USA).

Graft-specific IgG and IgM levels were detected using indirect ELISA [24-28]. The PCB homogenate contains various immunogenic substances that may be contained in the material, including proteins, polysaccharides, and even small molecular substances such as chemical reagent residues. The antibodies produced by these substances are collectively referred to as “graft-specific antibodies”. The homogenate of PCB was coated on an ELISA plate to capture antibodies expressed in serum of xenograft implanted experimental animals. Briefly, the homogenate supernatant made from fresh PCB was used as the solid-phase antigen coated in a 96-well plate for graft-specific antibody detection. According to the pre-experiment, the homogenate supernatant was coated in a 96-well plate at 1 μg per well in sodium carbonate buffer (pH 9.0), and 1% bovine serum albumin was used to block nonspecific antigens. After washing, diluted rabbit serum (1:2000 for IgG and 1:400 for IgM) was added to the wells and incubated overnight at 4 °C. After washing, HRP-labeled goat anti-rabbit IgG or IgM antibodies (1:10000; ab97195 and ab6721, Abcam) were added for 30 min. Finally, TMB was used for color development. After adding the stop solution (10% H2SO4), the absorbance value was measured at 450 nm.

2.6. Serum antibody-mediated cell toxicity

Porcine kidney cells (PK15) homologous to the implanted NBM and PCB were selected as target cells to assess serum antibody-mediated cell toxicity. PK15 cells were cultured at 1 × 10^6/tube, and the serum (3% diluted with MEM) was added to each tube. Samples were incubated for 2 h at 37 °C and 5% CO2. PK15 cells were then washed to stop the cell lysis process, and were resuspended in 0.5 mL PBS, stained with propidium iodide (PI), incubated in the dark for 60 min, and assayed by flow cytometry. Positive PI staining indicated a change in cell membrane permeability, which was identified as lytic cells.

2.7. Rabbit serum cytokines levels

Cytokine levels in rabbit sera, including complement 3A (Rabbit C3A ELISA Kit, ab273249, Abcam), IL-6 (Rabbit IL-6 ELISA Kit, ab277389, Abcam), TNF-alpha (Rabbit TNF-alpha DuoSet ELISA, DY5670; R&D Systems, Minneapolis, MO, United States), and IFN-γ (Rabbit IFN-gamma ELISA Kit, ab273238, Abcam), were detected using commercially available kits.

2.8. Analysis of lymphocytes in PBMCs

Peripheral blood mononuclear cells (PBMCs) were separated from heparinized peripheral blood using density gradient separation and were cryopreserved. CD4 (Anti-Rabbit CD4 Purified, MRB4020; Antigenix America Inc., Melville, NY, USA), CD8 (Anti-Rabbit CD8 Purified, MRB8020, Antigenix America Inc.) purified antibodies coupled with goat anti-mouse fluorescent secondary antibody (Alexa Fluor® 647, ab150115, Abcam), and B-PE (Anti-Rabbit B Cells-PE, MRB9997; Antigenix America Inc.) were used to identify lymphoid subsets in PBMCs. Flow cytometry was performed using a flow cytometer (CANTO II; BD Biosciences, Franklin Lakes, NJ, USA) and analyzed using the Diva software package (BD Biosciences).

2.9. Histopathological analysis

At 2- and 6-week after implantation, the GTKO and WT rabbits were euthanized, and their femoral condyles were removed and fixed in neutral formaldehyde for at least one week. EDTA solution was used to decalcify the bone tissue samples at 37 °C, the EDTA decalcification solution was changed every week for at least 1 month. After completely decalcification, samples were embedded in paraffin, sliced to 5 μm thickness, stained with hematoxylin and eosin (H&E) and Masson, mounted with a neutral resin, and observed under a microscope. Additionally, the new bone formation element contents of the graft materials were calculated according to the Chinese industry standards “Allogeneic grafts - In vivo evaluation of osteoinductive potential for materials containing demineralized bone” (YY/T 1680-2020).

2.10. Statistical analyses

Data analysis was performed using a two-way analysis of variance (ANOVA) with Tukey’s post hoc test. For comparisons between two groups, a two-tailed Student’s t-test was used. Data are presented as mean ± standard deviation (n = 3). Statistical significance was set as P < 0.05 and P < 0.01 (extremely significant).

3. Results

3.1. Characterization of GTKO rabbits

GGTA1 and Gal epitope were sequenced (Sanger) to confirm α-Gal deletion in GTKO rabbits. The base deletion in GTKO rabbits is shown in Fig. 1. GTKO rabbits had a 21bp deletion in exon 8 (LOC100348435) of the GGTA1 gene (Gene ID:100348435).

The content of α-Gal epitope which is regulated by GGTA1 protein and expressed on the surface of RBCs in GTKO rabbits was (3.54 ± 1.25) × 10^6 epitopes/cell, which was 96.48% decreased than that in WT rabbits (1.01 ± 0.63) × 10^6 epitopes/cell. For more detailed information on the development and characterization of GTKO rabbits, please refer to our previous studies [18,29].

3.2. Characterization of NBM and PCB

The surface morphology of NBM is shown in Fig. 2. The pores with a diameter of (591.94 ± 78.81) μm were evenly distributed in the NBM (Fig. 2A), showing a loose spongy structure, which was

| Table 2 | Residual α-Gal antigen and DNA contents of implants (n = 3). |
|---------|----------------------------------------------------------|
| Sample  | Residual α-Gal content (epitope/mg) | Residual DNA content (ng/mg) |
| PCB     | 15.65 ± 0.49 × 10^{12} | 71.234 ± 30.28 |
| NBM     | 0.31 ± 0.01 × 10^{11} | 2.67 ± 0.13 |

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conducive to cell ingrowth (Fig. 3A). Under high magnification (Fig. 2B–D), the pore walls were composed of several neatly arranged collagen fibers, indicating that after demineralization treatment, the collagen fibers were fully exposed, thereby providing more adhesion sites for cells.

Proteomic analysis of NBM is shown in Fig. 3, bioinformatics analysis revealed that the proteins in NBM were related to extracellular constituents, not nuclear components (Fig. 3A). Biological process analysis indicated that NBM proteins are involved in collagen biosynthesis, blood vessel development, cell adhesion, and ossification, which are closely related to bone repair processes (Fig. 3B, F). Biological function analysis showed that these proteins are involved in calcium ion binding and extracellular matrix formation, which may promote bone repair (Fig. 3D). Moreover, after the decellularization processes, the inflammatory agents were not detected in NBM compared to the PCB, whereas proteins that mediate M2-polarization, such as collagen VI and fibronectin, were detected (Fig. 3C, E).

The remnant α-Gal and DNA contents of the implanted materials are shown in Table 1. After the decellularization process, the α-Gal contents in the NBM and PCB were 0.31 (±0.01) × 10^{11} and 15.65 (±0.49) × 10^{12} epitopes/mg, respectively, and the Gal antigen clearance rate of NBM was 97.96%. The residual DNA contents of NBM and PCB were 0.23 ± 0.013 and 713.24 ± 30.28 ng/mg (dry weight), respectively, and the DNA clearance rate of NBM was 99.97%. The remarkably low levels of residual α-Gal and DNA in the NBM suggested that it was well decellularized.

Detailed PCB and NBM characterization have been shown in previous study [23].

3.3. Antibodies level analysis

The total IgG and IgM levels in the NBM and PCB of both GTKO and WT rabbits were not significantly different than those in the Con group as shown in Fig. 4A and B (P > 0.05).

Changes in anti-Gal IgG and IgM levels are shown in Fig. 4C and D. In GTKO rabbits, the anti-Gal IgG levels in PCB groups at 2- and 6-week after implantation were 3.98-fold and 5.81-fold higher than those in the Con group, respectively. Anti-Gal IgM levels in PCB groups at 2- and 6-week were 2.49-fold and 2.15-fold higher than those in the Con group, respectively. But, anti-Gal IgG and anti-Gal IgM in the serum of WT rabbits at both 2- and 6-week after implantation were not detected. These results suggested that under xenografts stimulation, GTKO rabbits, but
not WT rabbits, showed a prominent immune response, that resembling that of humans, due to they have high sensitivity on α-Gal related xeno-immunogenic responses.

The anti-Gal IgG and IgM levels in the NBM group were significantly higher than those in the Con group at 2-week after implantation. But there was no significant difference at 6-week compared to Con groups. Meanwhile, the anti-Gal IgG and anti-Gal IgM levels in the NBM groups were significantly lower than those in the PCB groups both at 2- and 6-week. These results suggested that, after decellularization, the residual α-Gal content of the NBM was very low and therefore α-Gal-related xeno-immunologic responses were decreased.

The graft-specific IgG and IgM levels are shown in Fig. 4E and F. In GTKO rabbits, the graft-specific IgG antibody levels in the PCB group at 2- and 6-week after implantation were 32.39-fold and 49.62-fold higher than those in the Con group, respectively. Graft-specific IgM antibody levels in the PCB group at 2- and 6-week after implantation were 9.98-fold and 5.17-fold higher than those in the Con group, respectively. At the same time, although a significant increase in graft-specific antibodies was also detected in the PCB group of WT rabbits, the changes in antibody titers were lower than those of GTKO rabbits in the same group. These results suggested that GTKO rabbits had more sensitive immune response compared to WT rabbits under the same degree of immune stimulation.

3.4. Complement-mediated cell lysis in rabbit sera

When PK15 cells are exposed to the serum of graft-implanted rabbits, graft (porcine tissue-derived)-specific (anti-porcine) antibodies quickly bind to antigens contained in the graft material, which activates the complement system and forming membrane attack complexes (MAC) and resulting in target cell lysis [30,31]. The results of complement-mediated PK15 cell lysis ratios in rabbit sera are shown in Fig. 5. The flow cytometry analysis graphs of serum-mediate PK15 cell lysis in different groups as showed in Fig. 5A. The serum complement-mediated PK15 cell lysis ratios in the PCB group of GTKO rabbits, were 2.96-fold and 2.59-fold higher than those in the Con group at 2- and 6-week after implantation, respectively. These results suggested that under PCB stimulation, which contained xenoantigens, GTKO serum complement-mediated PK15 cell lysis ratios were significantly higher in the PCB group than in the Con group, indicating a more pronounced immunological response. However, in WT rabbits, the serum-mediated PK15 cell lysis ratios in the PCB group were lower than those in the Con group, indicating that WT rabbits were less sensitive than GTKO rabbits in rabbit serum-mediated cell lysis tests.

In GTKO rabbits, rabbit serum-mediated PK15 cell lysis ratios in the NBM group at 2- and 6-week after implantation were 1.13-fold and 1.04-fold higher than those in the Con group, respectively, and were significantly lower than those in the PCB group, indicating a weak immunological response after NBM implantation.

3.5. Changes in cytokines in rabbit sera

As shown in Fig. 6, compared to the Con group, the serum C3a (complement component 3), TNF-α (tumor necrosis factor-α), IL-6 (interleukin-6), and IFN-γ (interferon-γ) levels at 2-week after implantation were 3.08-, 7.55-, 3.24-, and 2.74-fold higher in the GTKO-PCB group, and 1.34-, 2.33-, 2.26-, and 2.01-fold higher in the WT-PCB group, respectively, and at 6-week, the cytokine levels in the GTKO-PCB group were similar to those in the Con group, except for TNF-α. These results indicate that in the early stages of implantation, GTKO rabbits had a better immunological response than WT rabbits, and could better analyze the dynamic changes of the inflammatory responses as the implantation time increased.

In the GTKO-NBM group, serum C3a, TNF-α, IL-6, and IFN-γ levels were higher than those in the Con group at 2-week after implantation, but they were significantly lower than those in the PCB group. At 6-week, the levels of these cytokines (except TNF-α), have no significant differences compared to the Con group, indicating a weak inflammatory response at the early implantation stage and returned to normal at later stages.
3.6. Changes in the proportion of lymphocytes in PBMCs

Acquired immunity in organisms is mediated by numerous immunological cells. Early studies [32–34] have proposed that B and T cells play important roles in both humoral and cellular immune-mediated xenotransplant rejection. The proportions of CD4⁺ T cells, CD8⁺ T cells, and B cells in rabbit PBMCs are shown in Fig. 7. There was no significant difference in the proportions of B cells, CD4⁺ T cells, and CD8⁺ T cells in the three groups at 2- and 6-week after implantation in both GTKO and WT rabbits. However, the CD4⁺/CD8⁺ proportions in GTKO-PCB rabbits were 1.42-fold and 1.31-fold higher than those in the Con group at 2- and 6-week after implantation, respectively. In WT rabbits, there were no significant differences in the proportions of CD4⁺/CD8⁺ between the PCB and Con groups at 2- and 6-week after implantation.

The CD4⁺/CD8⁺ proportions in the NBM-GTKO group were 1.08-fold...
Fig. 5. Rabbit serum-mediated cell lysis. A) Flow cytometry analysis of serum-mediated PK15 lysis in different groups; B) rabbit serum-mediated cell lysis. The dotted line represents the cut-off value (7%), below which cytotoxicity is considered negative. Experiments were performed in triplicate. WT, wild type; GTKO, α1,3-galactosyltransferase gene-knockout; Con, control; NBM, natural bone material; PCB, porcine cancellous bone; N.S., not significant.
higher than those in the Con group at 2- and 6-week after transplantation. However, the difference was not significant, indicating that the decellular process effectively reduces the immunogenic response.

3.7. Histopathological analysis

3.7.1. Hematoxylin and eosin (HE) staining

As shown in Fig. 8, at 2-week after implantation, a large amount of fibrous tissue ingrowth was observed in the defect area, accompanied by hemorrhage, a small amount of inflammatory cell infiltration, and no obvious osteogenesis in the Con groups of WT and GTKO rabbits. In the PCB groups, a large amount of fibrous tissue ingrowth and hemorrhage were observed in both WT and GTKO rabbits, and the degree of inflammatory cell infiltration was markedly higher in GTKO rabbits than in WT rabbits, suggesting that GTKO rabbits were more sensitive on local effects after implantation of PCB.

As shown in Fig. 9, a large amount of fibrous tissue ingrowth, accumulation of adipocytes, and a small amount of inflammatory cell infiltration was observed in the defect area in the Con groups of WT and GTKO rabbits at 6-week. In the PCB groups, similar results were observed with that at 2-week, including a large amount of fibrous tissue ingrowth and hemorrhage in both WT and GTKO rabbits. However, the degree of inflammatory cell infiltration and necrosis was markedly higher in the GTKO rabbits than in WT rabbits, suggesting that GTKO rabbits were more sensitive on local effects after implantation of PCB.

The histopathological observations in the NBM groups of both GTKO and WT rabbits were similar to that in the Con groups at 2- and 6-week after implantation, suggesting that the immunogenicity of NBM was acceptable following implantation.

3.7.2. Masson’s staining

In Masson’s staining, the mature mineralized tissue was stained red, and the immature mineralized tissue and collagen fibers were stained blue. The results showed in Fig. 10A and B. No obvious collagen fibers were formed in the defect area in the Con group at 2-week. In the NBM group, a small number of collagen fibers was observed in the gap of the implant material, but no obvious collagen fiber mineralization was observed, and in the PCB group, the number of collagen fibers was significantly lower than that in the NBM group, with no evident collagen fiber mineralization. Some changes occurred at the sixth week after implantation. Small number of collagen fiber mineralization and osteogenesis were observed in the defect area of the Con group at 6-week. In the NBM group, the implanted graft was partially degraded, a large number of collagen fibers were formed and mineralized in the gap of the graft, and a massive new bone was formed. While, collagen fibers and new bone formation were significantly lower in the PCB group than those in the NBM group, with a large number of infiltrated inflammatory cells.

These results suggested that NBM has good bone regeneration and
repair effect evidenced by collagen fiber formation and mineralization in the gap of the graft, and a massive new bone formation.

4. Discussion

Preclinical animal studies are the gold standard for evaluating the biosafety and function of implantable biomaterials. However, WT animals cannot sensitively and effectively evaluate the immunological risks caused by animal tissue-derived materials after human implantation. Rabbits are best choice for in vivo evaluation on immunological responses and local effects after in situ implantation, due to they are relatively large, have a short breeding cycle, and are easy to care for. Therefore, we have developed a GTKO rabbit in previous study. In this study, we comparatively evaluated the immunological responses, local effects after implantation and biological functions of NBM with a raw material control (PCB) via GTKO and WT rabbits.

Our results revealed no significant differences in total antibody levels after the implantation of xeno graft materials in all groups, suggesting that the total antibodies are not specific sensitive factors. The PCB positive control group in GTKO rabbits, but not WT rabbits, caused higher levels of anti-Gal IgG and anti-Gal IgM antibodies, suggesting GTKO rabbits are more sensitive to α-Gal stimulation. Additionally, graft-specific antibody detection is another commonly used method for evaluating the immunogenicity of animal tissue-derived biomaterials at the antibody level. The results from this study showed that, graft-specific IgG and IgM levels were significantly higher in the PCB-GTKO group than in PCB-WT group, which further proves that GTKO rabbits are more sensitive to xeno immunogenic materials than WT rabbits.

In this study, the complement-mediated cell lysis ratio in the PCB-GTKO group was more than two-fold significantly higher than the Con group at 2- and 6-week after implantation. But in WT rabbits, the ratios of complement-mediated cell lysis in the PCB-WT groups did not significantly differ from those in the Con group at 2-week after implantation.

Serum factors play an important role in cellular immunity and are commonly used as important factors to evaluate the degree of cellular immunity. In this study, four inflammatory factors (serum C3a, TNF-α, IL-6, and IFN-γ) in the PCB-GTKO group were significantly higher than those in Con-GTKO group at 2-week after implantation. But at 6-week, except TNF-α, the levels of the other three factors in GTKO rabbits recovered to normal levels, were not significantly different from those in the Con group. These results suggested that in the early post-implantation period, GTKO rabbits revealed more serious inflammatory responses than WT rabbits, and with prolonged implantation time, the levels of inflammatory factors in GTKO rabbits gradually returned to normal levels, which was consistent with the local effects after implantation showed in histopathological observations.

Lymphocyte analysis is often used to evaluate the immune rejection response after the implantation of materials. Compared to the Con groups at 2- and 6-week after implantation, the proportions of CD4+/CD8+ were significantly higher in PCB-GTKO, but not in PCB-WT group. CD4 is a marker on the surface of helper T cells. CD8 is a marker of suppressive T cells, and the proportion of CD4+/CD8+ increase indicates that helper T
cells are higher than suppressor T cells, indicating higher immunity. Therefore, GTKO rabbits were shown to be more sensitive to immune rejection after xenograft implantation than WT rabbits.

In addition, at the histopathological level, in PCB-GTKO group showed strong local effects at 2- and 6-week after implantation, such as a large number of inflammatory cell infiltrations and necrosis, which was
considered a moderate stimulatory response. However, in the PCB-WT group, no obvious local effects after implantation was observed, which was considered a mild stimulatory response. These results suggested that GTKO rabbits revealed stronger local effects than WT rabbits after the implantation of xeno immunogenic grafts.

In the biological function evaluation, H&E and Masson’s staining revealed a large number of blood vessels and new bone formation in the defect site in the NBM group compared to the PCB group, and the immune rejection in the NBM group was slightly than that in the PCB group.

In conclusion, the results from this study showed that GTKO rabbits were more sensitive than WT rabbits in evaluating the xeno-immunogenic responses of animal tissue-derived biomaterials. But xenogeneic rejection is not only driven by α-Gal, as have been reported that there are antigens expressed by genes such as CMAH and β4GalNT2 [35,36], they can also mediate xenogeneic rejection. In the future, studies on multiple gene-edited animals are also necessary for more comprehensively evaluating xeno immunological risks of animal tissue-derived biomaterials. Additionally, the results demonstrated that the NBM has a reasonable decellularized degree, and gained low residual immunogenicity and good osteogenic properties.

Credit author statement

Yufeng Mu: Data curation, Writing – original draft, Investigation, Formal analysis, Validation, Methodology, Conceptualization. Yu Zhang: Writing – review & editing, Data curation, Formal analysis, Investigation. Lina Wei: Funding acquisition, Conceptualization, Methodology. Liang Chen: Project administration, Supervision. Feng Hao: Resources. Anliang Shao: Methodology, Supervision. Shuxin Qu: Project administration, Supervision. Liming Xu: Funding acquisition, Project administration, Supervision, Writing – review & editing, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence
the work reported in this paper.

Data availability

Data will be made available on request.

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