

Original Article

Relieving oxidative stress microenvironment and promoting vascularized bone formation to treat femoral head necrosis using 3D-printed scaffold with ultralong-term multienzyme-like activity

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ABSTRACT

Background: Characterized by microcirculatory disorder and oxidative stress microenvironment, the repair of bone defect after hip preservation therapy (such as core decompression) for osteonecrosis of the femoral head (ONFH) remains a clinical challenge. Thus, an ideal bone scaffold for treating ONFH should not only promote bone and vessel formation but also alleviate hypoxia and oxidative stress.

Method: We integrated manganese oxides (MnO_x) nanoparticles (NPs) with a 3D-printed poly(lactic-co-glycolic acid) (PLGA) scaffold to achieve this goal. The MnO_x NPs were synthesized using an oxidation reaction and the scaffold was 3D-printed using a fused deposition modeling method. The characterization and the enzyme-like activity of the scaffold was investigated. The biocompatibility and biofunctions of the scaffold were evaluated both in vitro and in vivo, including the antioxidant capacity, the effects on promoting bone formation and vascularization, and the therapeutic effect in animal model.

Results: The resultant MnO_x-doped PLGA scaffold could catalyze reactive oxygen species into oxygen through its superoxide dismutase (SOD)-like and catalase (CAT)-like activities. In vitro studies revealed that this multienzyme-like activity of the scaffold could be maintained for more than 30 days, thereby improving cell viability under oxidative stress. The underlying mechanism was shown to involve regulation of the antioxidant activity of cells via PI3K/AKT signaling pathway. The scaffold also significantly improved capabilities of osteogenesis and angiogenesis compared to pure PLGA scaffold. In vivo studies further demonstrated that the combination therapy of core decompression and scaffold implantation efficiently reduced osteoblast necrosis and enhanced vascularized bone formation in a clinically relevant ONFH rabbit model.

Conclusion: The 3D-printed MnO_x-doped PLGA scaffold not only relieve oxidative stress to protect osteocytes under ONFH microenvironment but also promote vascularized bone formation, showing the potential for treating ONFH. **Translational potential of this article:** PLGA has been already applied in clinical bone implants. Mn is an essential trace element for the human body and MnO_x NPs offer the advantage of biocompatibility, ease of large-scale preparation, and low cost. Hence, this scaffold has the potential for clinical translation in the treatment of ONFH.

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1. Introduction

Osteonecrosis of the femoral head (ONFH) is a common disease with high morbidity and disability. There are approximately 10,000 new cases of osteonecrosis every year in the United States, and 200,000 to 300,000 new cases every year in China [1,2]. ONFH often leads to the collapse and deformation of the femoral head, and ultimately, only hip replacement can be performed, seriously threatening people's health and quality of life. Thus, early intervention is crucial in treating ONFH. Current hip preservation surgeries for ONFH include core decompression, bone grafting (vascularized and nonvascularized), and rotational osteotomy between femoral rotors [3,4]. Among them, core decompression combined with bone grafting can reduce pain and promote bone regeneration by relieving the pressure and improving the blood supply within the femoral head. However, approximately 30 % of patients still experience further necrotic collapse of the femoral head 5–10 years after hip preservation surgery [5]. The main challenges are as follows: first, core decompression by drilling destroys a large number of normal trabecular structures, which causes local bone defects and reduces the mechanical properties in the femoral head and neck, thereby increasing the difficulty of bone repair [6]; second, the microcirculatory disorder in the femoral head prevents the delivery of oxygen (O_2) and nutrients through the blood vessels to support bone repair [7]; third, the formation of a hypoxic microenvironment and the accumulation of reactive oxygen species (ROS) [8], which in turn cause tissue peroxidation, oxidative stress damage to the mitochondrial DNA of bone cells, and osteoblast apoptosis, thereby hindering bone repair [9].

To address these challenges, here we developed a composite bone scaffold by integrating manganese oxides (MnO_x) nanoparticles (NPs) with poly(lactic-co-glycolic acid) (PLGA) using a 3D printing method. PLGA and Mn were chosen because they are both biocompatible components that have been used in U.S. Food and Drug Administration (FDA) approved biomedical products [10,11]. More importantly, Mn is an essential element in the human body and functions as a cofactor for various enzymes that participates in many biological processes such as defense against ROS (Mn superoxide dismutase) and bone homeostasis (glycosyltransferases) [12]. We showed that the as-prepared 3D-printed MnO_x -doped PLGA scaffold has good mechanical properties comparable to natural cancellous bone and it could support cell adhesion and proliferation. Notably, the MnO_x NPs exhibited both superoxide dismutase (SOD)-like and catalase (CAT)-like activities [13–15]; this enabled the scaffold to catalyze the overexpressed ROS and release of O_2 for more than 30 days to alleviate the oxidative stress microenvironment and prevent cell apoptosis. Moreover, the scaffold presented dual promoting effects of osteogenesis and vascularization. In a steroids-induced ONFH rabbit model, core decompression combined with the implantation of the MnO_x -doped PLGA scaffold significantly decreased the number of empty lacunas in femoral head and increased the formation of new bone and vessel tissues (Fig. 1a). The integration of NPs with 3D-printed scaffold could not only enable the localized sustained release of MnO_x NPs to avoid systemic adverse effects, but also provide biomechanical support, establishing a synergistic mechanobiological interplay. This dual-functional design facilitates minimally invasive core decompression surgery, potentially delaying or obviating the necessity for late-stage joint replacement surgery through sustained osteogenic microenvironment modulation. This work provides a strategy that integrate appropriate biomechanical properties and biological attributes including osteoinduction, pro-vascularization, and improvement of the oxidative stress and hypoxic microenvironment in one bone repair material for treating ONFH.

2. Results

2.1. Preparation and characterization of the scaffolds

We first synthesized MnO_x NPs using a simple oxidation reaction

(Fig. 1a). Transmission electron microscopy (TEM) images (Fig. 1b and Fig. S1) showed that the as-prepared MnO_x NPs were well dispersed with a size of ~ 10 nm. Mn and O elements were evenly distributed on the NPs (as determined by mapping; Fig. S2). This facile method could be scaled up for the large-scale production of good-quality MnO_x NPs in one reaction (Fig. S1). The X-ray diffraction patterns of the MnO_x NPs were indexed to MnO (Mn^{2+} ; PDF#04–0326), Mn_3O_4 (Mn^{2+} and Mn^{3+} ; PDF#24–0734), and MnO_2 (Mn^{4+} ; PDF#44–0142). X-ray photoelectron spectroscopy (XPS; Fig. 1c) further revealed that the Mn 2p $^{3/2}$ spectrum of the MnO_x NPs could be divided into three main peaks at 652.8, 641.9, and 640.5 eV, corresponding to Mn^{2+} , $Mn^{3+/4+}$, and Mn^{4+} , respectively. The proportion of Mn^{2+} , $Mn^{3+/4+}$, and Mn^{4+} was 27.2 %, 27.1 %, and 45.7 %, respectively. Hence, MnO_x NPs exhibited mixed valence states of Mn, which is conducive to enzyme-catalyzed reactions [13,14].

Then, MnO_x NPs were doped into PLGA, and 3D-printed scaffolds were prepared via a fused deposition modeling (FDM) method (Fig. 1a). To observe the influence of MnO_x on the performance of the PLGA scaffolds, we doped MnO_x NPs at various concentrations (1, 2.5, and 5 w/w%) into PLGA, and the resulting scaffolds were designated as PLGA/Mn1, PLGA/Mn2.5, and PLGA/Mn5, respectively. The color of the scaffolds became darker with increasing NP concentration. Scanning electron microscopy (SEM) images revealed that all the scaffolds had a regular and interconnected porous structure, while the surface of the PLGA/Mn5 scaffolds was rough (Fig. 1e). As designed, the pore size of the scaffolds was ~ 400 μm because large pores (250–425 μm) can promote the exchange of nutrients, the ingrowth of vessels, and the osteogenic differentiation of stem cells. Energy dispersive spectroscopy (EDS) elemental mapping (Fig. 1f) indicated that the elemental composition of the PLGA/Mn scaffolds consisted of C, O, and Mn.

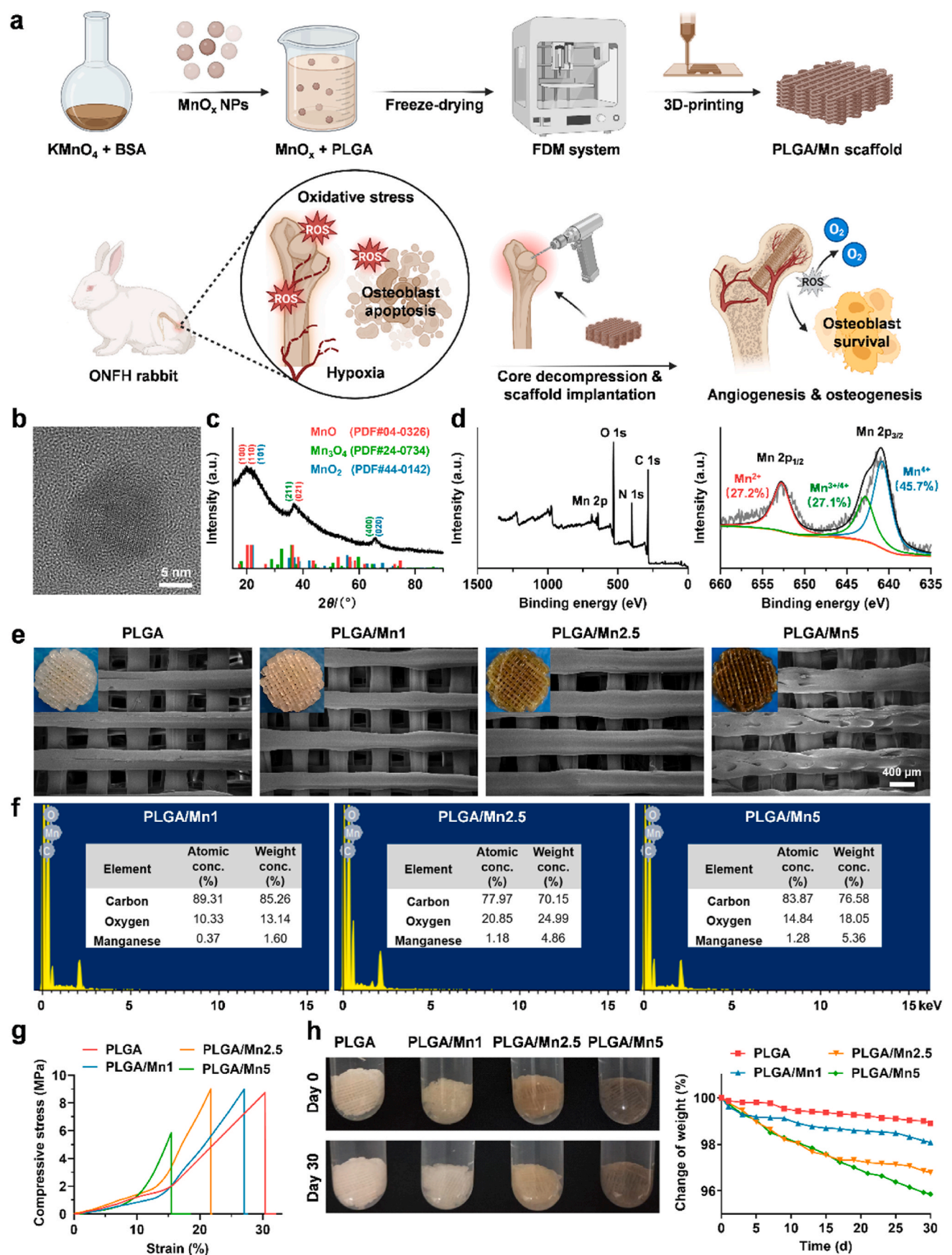
The stress–strain curve (Fig. 1g) confirmed that the PLGA, PLGA/Mn1, and PLGA/Mn2.5 scaffolds exhibited similar mechanical strengths (~ 9 MPa), while the compression strength of the PLGA/Mn5 scaffold decreased to ~ 6 MPa but was still comparable to that of natural cancellous bone (2–12 MPa). Moreover, in vitro degradation tests revealed that all the scaffolds were stable during incubation in simulated body fluid (SBF) at 37 °C for 30 days. Changes in the weight of the scaffolds indicated that the doping of MnO_x NPs slightly accelerated the degradation of the scaffolds (Fig. 1h). In addition, ICP-MS results showed that MnO_x NPs could be gradually released from both PLGA/Mn1 and PLGA/Mn2.5 scaffolds (Fig. S3).

To investigate whether the scaffolds could scavenge the overexpressed ROS and RNS within the microenvironment of ONFH, we first incubated hydrogen peroxide (H_2O_2) with the MnO_x NPs at various concentrations. With increasing incubation time and NP concentration, more bubbles were generated in the H_2O_2 solution (Fig. 2a), suggesting the reduction of H_2O_2 and the production of O_2 . The absorption at 240 nm in H_2O_2 + MnO_x decreased over time, indicating the reduction of H_2O_2 (Fig. 2b). The generation of a large amount of O_2 in the H_2O_2 solution was determined using a dissolved oxygen meter (Fig. 2c).

Moreover, we incubated the scaffolds in H_2O_2 solution and revealed that all the MnO_x -doped PLGA scaffolds could efficiently produce O_2 for 30 days (the maximum measurement range is 15 mg/L; Fig. 2d). The 2-Phenyl-4,4,5,5-tetramethylimidazoline-3-oxide-1-oxyl (PTIO) test was conducted to detect the scavenging ability of $O\cdot$ such as superoxide anion radicals ($O_2^{\cdot -}$) and hydroxyl radicals ($\cdot OH$). The scaffolds reduced the amount of $O\cdot$ to half the original level (Fig. 2e). The total antioxidant capacity (T-AOC) test also revealed that the scaffolds exhibited good antioxidant ability (Fig. 2f). Hence, the scaffolds exhibited both SOD-like activity and CAT-like activity (Fig. 2g). These results indicated that the MnO_x -doped PLGA scaffolds not only scavenged ROS but also retained ultralong-term multienzyme-like activity.

2.2. Biocompatibility of the scaffolds in vitro

To investigate the optimal concentration of MnO_x used in the preparation of the scaffolds, we cultured bone marrow mesenchymal stem



(caption on next page)

Fig. 1. Preparation and characterization of the scaffolds. (a) Schematic illustration of the preparation and application of MnO_x -doped PLGA scaffold in ONFH. MnO_x NPs were synthesized through large-scale preparation and integrated with PLGA, and then the scaffold was 3D-printed via a FDM method. After core decompression and implantation of the resultant PLGA/Mn scaffold in a steroid-induced ONFH rabbit model, the scaffold catalyzed toxic ROS into O_2 to alleviate the oxidative stress and hypoxic microenvironment, thereby reducing osteoblast necrosis. Moreover, the osteoinductive and angiogenic effects of the scaffold promoted vascularized bone formation. (b) TEM image of MnO_x NPs. (c) XRD pattern of MnO_x NPs corresponds with the standard MnO , Mn_3O_4 , and MnO_2 crystal phases. (d) XPS spectra of MnO_x NPs index to Mn^{2+} , Mn^{3+} , and Mn^{4+} . (e) SEM images of PLGA and PLGA/Mn prepared with different MnO_x concentrations. Inserted: photos of the scaffolds. (f) EDS spectra of scaffolds. (g) Stress–strain curve of the scaffolds. (h) In vitro degradation of the scaffolds.

cells (BMSCs) with MnO_x NPs at various concentrations (0–500 $\mu\text{g/mL}$). The cell counting kit-8 (CCK-8) assay confirmed that the cell viability of the BMSCs exceeded 100 % in all the NP groups without significant differences after coculture for 24 h, indicating the good biocompatibility of the MnO_x NPs (Fig. S4). Then, we cocultured the MnO_x -doped PLGA scaffolds with BMSCs and human umbilical vein endothelial cells (HUVECs) for 7 days to study their long-term biocompatibility. The OD values of the MnO_x -doped PLGA scaffold groups were slightly lower than that of the PLGA group after 24 h of coculture (Fig. 3a). However, the OD values of PLGA/Mn5 on day 3 and day 7 decreased and were significantly lower than those of the other groups.

Live/dead staining indicated that both BMSCs (Fig. 3b) and HUVECs (Fig. 3c) were alive (green fluorescence) and proliferated rapidly in the PLGA, PLGA/Mn1, and PLGA/Mn2.5 groups during 7 days of coculture. In contrast, the cells began to die (red fluorescence) on day 3 when they were cocultured with the PLGA/Mn5 scaffolds. Moreover, the scaffolds were cocultured with blood cells to study hemocompatibility (Fig. S5).

The supernatant of the PBS (negative control) and scaffold groups was transparent, but the supernatant was red in the H_2O group (positive control) due to hemolysis. In contrast, the supernatants of the scaffold groups and negative control group (PBS) were transparent. The hemolysis rate of all the scaffolds was calculated to be approximately 0.04 %. Thus, the scaffolds were hemocompatible.

We observed the adhesion of BMSCs by SEM (Fig. 3d). In the PLGA, PLGA/Mn1, and PLGA/Mn2.5 groups, BMSCs were firmly adhered and well spread on the surface of the scaffolds with the typical long shuttle morphology after 24 h of coculture. However, the number of cells decreased, and the cell morphology was disrupted by the PLGA/Mn5 scaffolds. Therefore, 2.5 w/w% was regarded as the optimal concentration of MnO_x NPs for the preparation of the scaffolds in this study.

2.3. Antioxidant capacity of the scaffolds and the underlying mechanism

To study whether MnO_x -doped PLGA scaffolds could protect cells

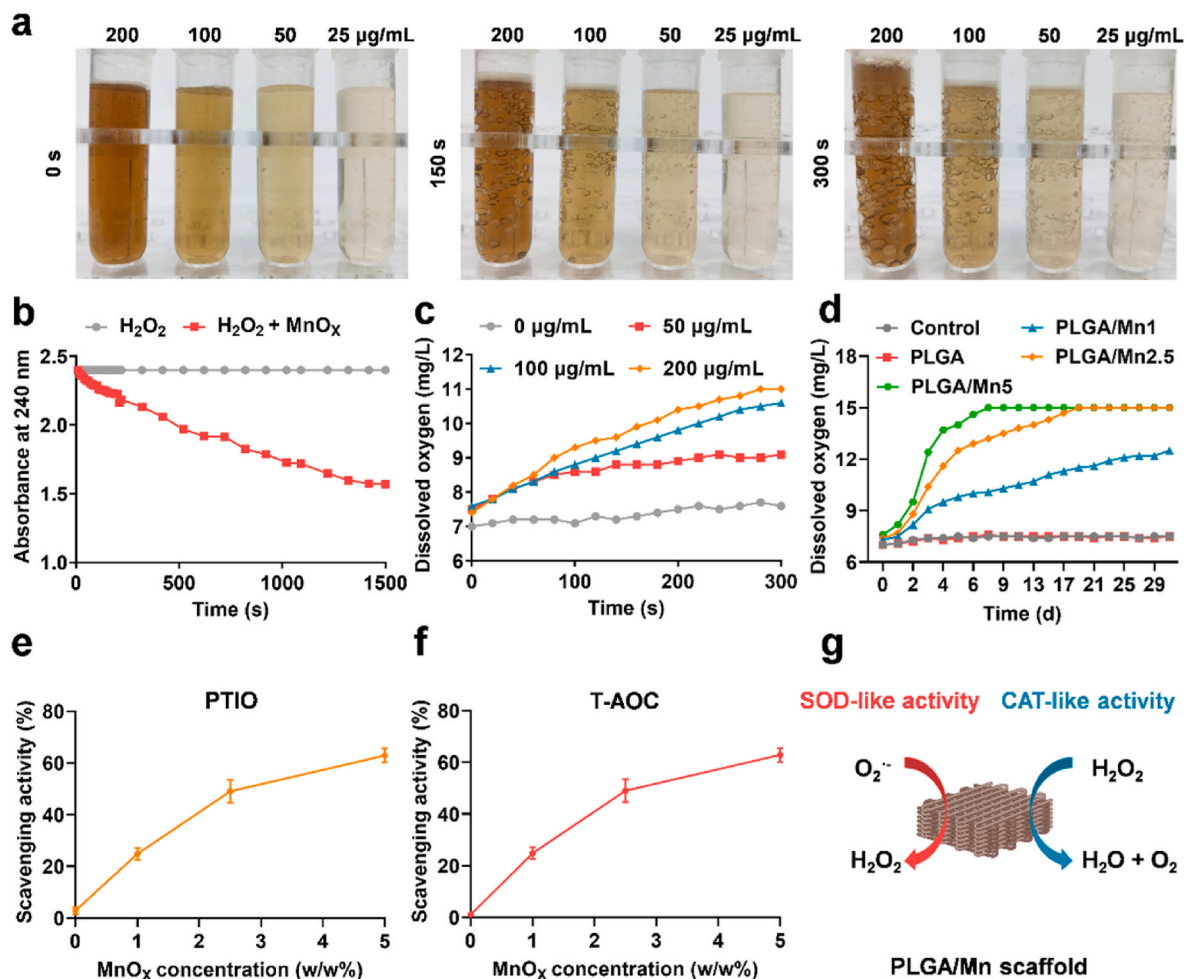


Fig. 2. Multienzyme-like activity of the NPs and scaffolds. (a) Photos of MnO_x NPs incubated in H_2O_2 solution. The reduction of H_2O_2 (b) and generation of O_2 (c) in H_2O_2 solution with or without MnO_x NPs. (d) Long-term detection of O_2 of in H_2O_2 solution incubated with the scaffolds. It should be noted that the maximum measurement range of the dissolved oxygen meter is 15 mg/L. Free radical scavenging ability (e) and antioxidant ability (f) of the scaffolds. (g) Schematic illustration of the CAT-like and SOD-like activities of the MnO_x -doped PLGA scaffold. Data are presented as mean $\pm$ SD ($n = 3$).

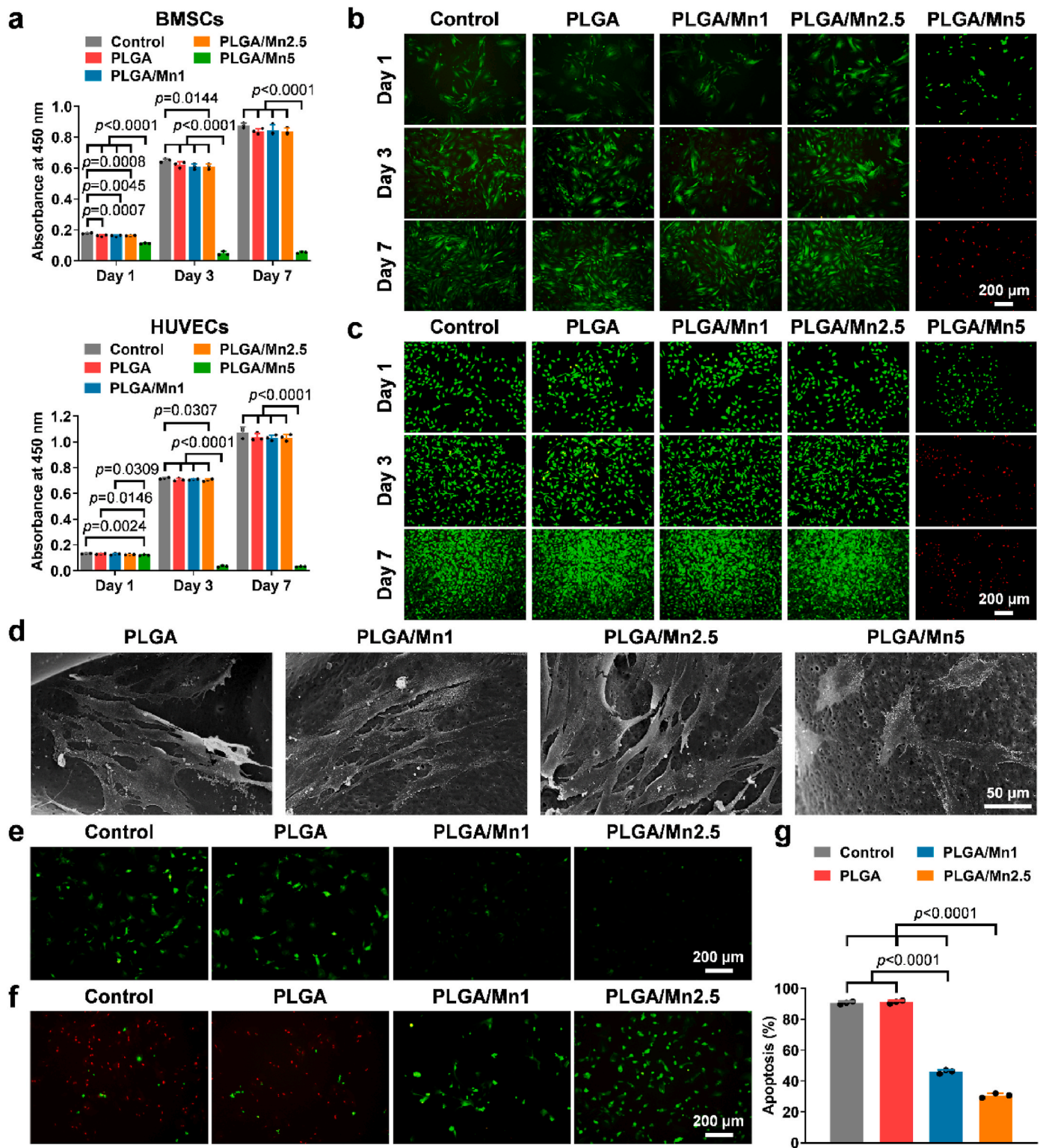


Fig. 3. In vitro biocompatibility and cell viability under oxidative stress. (a) Cell proliferation of BMSCs (up) and HUVECs (down) cocultured with the scaffolds for 1, 3, and 5 days. Live/dead staining images of BMSCs (b) and HUVECs (c) at different time intervals. (d) BMSC adhesion on the scaffolds. (e) Intracellular ROS generation in BMSCs after incubated with H_2O_2 and the scaffolds. In the control and PLGA scaffold groups, intense green fluorescence was detected, indicating large amounts of ROS were produced within BMSCs when they were exposed to elevated oxidative stress. (f) Live/dead staining images of BMSCs after treated with H_2O_2 . More than half of BMSCs were dead (red fluorescence) in the control and PLGA scaffold groups. In contrast, all cells were alive (green fluorescence) in the PLGA/Mn1 and PLGA/Mn2.5 scaffold groups. (g) Quantitative analysis of necrotic BMSCs after treated with H_2O_2 in different groups (as determined by flow cytometry). Data are presented as mean $\pm$ SD ($n = 3$).

under oxidative stress in the microenvironment of ONFH, we cocultured BMSCs with the scaffolds in the presence of H₂O₂. The production of intracellular ROS was detected using DCFH-DA staining (Fig. 3e). Strong green fluorescence intensity was observed in the control and PLGA groups. In contrast, the green fluorescence became weaker in the PLGA/Mn1 and PLGA/Mn2.5 groups. Semi-quantification of the green

fluorescence intensity indicated that ROS generation was lowest in the PLGA/Mn2.5 group (Fig. S6). These results confirmed that MnO_x-doped PLGA scaffolds could indeed scavenge ROS in the microenvironment due to their CAT- and SOD-like activity. Therefore, most BMSCs were alive, and dead cells were negligible in both the PLGA/Mn1 and PLGA/Mn2.5 groups, whereas a large number of dead cells were found in the control

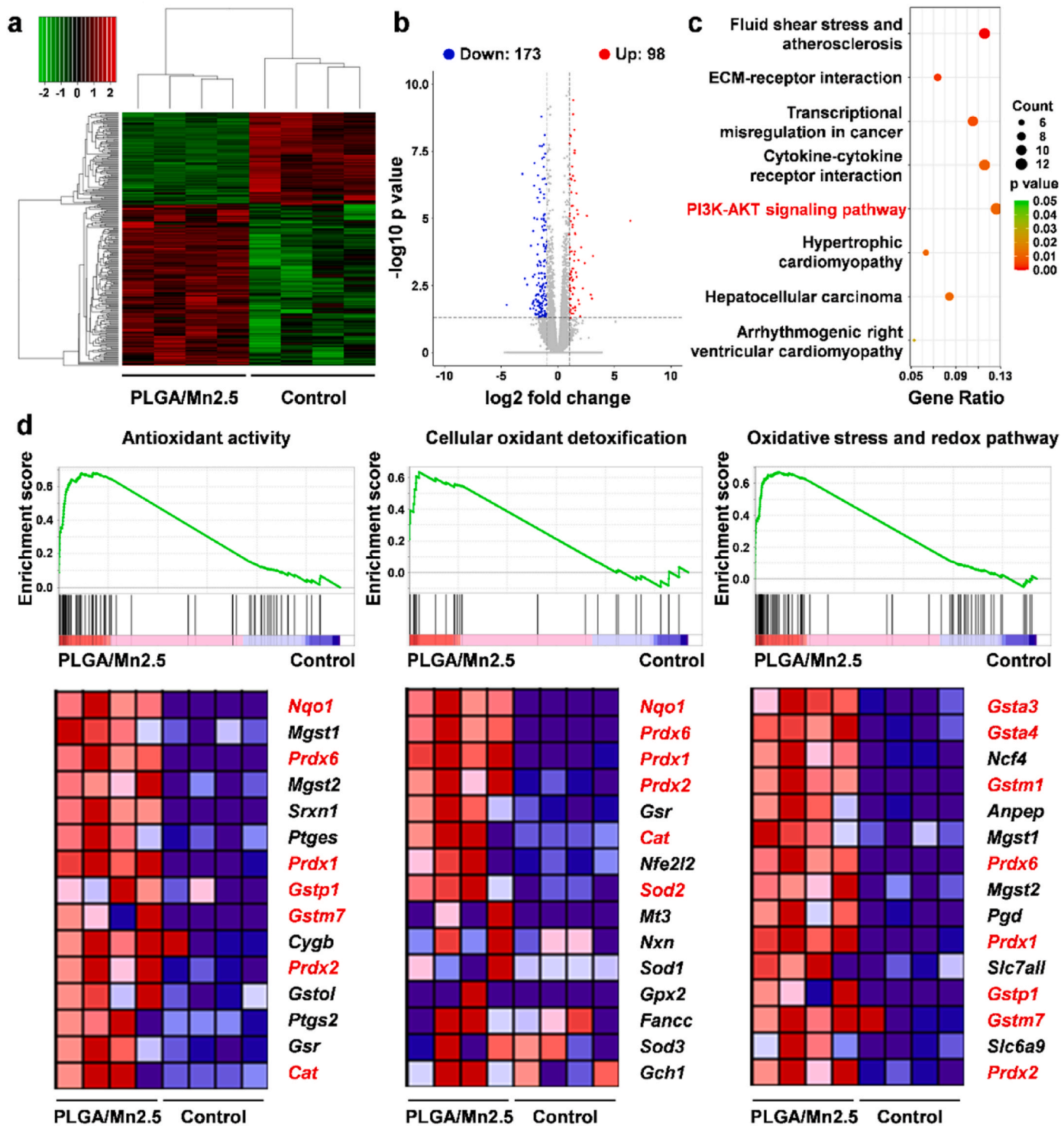


Fig. 4. Antioxidative mechanisms of the scaffold. (a) Heatmap of differentially expressed genes in BMSCs in the PLGA/Mn2.5 scaffold group and control group ($n = 4$). (b) Volcano plots. There were 98 up-regulated genes and 173 down-regulated genes in the comparison of the PLGA/Mn2.5 scaffold group with control group. (c) KEGG pathway enrichment analysis. The PI3K/AKT signaling pathway is the highly correlated pathways. (d) GSEA analysis and the corresponding DEGs for the antioxidant activity, cellular oxidant detoxification, and oxidative stress and redox pathway. The results revealed that these pathways were enhanced antioxidant genes, including *Nqo1*, *Cat*, *Sod2*, GST family, and PRDX family, were up-regulated in the PLGA/Mn2.5 scaffold group.

and PLGA groups (as determined by live/dead staining; Fig. 3f). The flow cytometry results (Fig. 3g and Fig. S7) demonstrated that the presence of H_2O_2 caused a very high percentage of apoptotic BMSCs (approximately 90 %). Remarkably, the percentages of apoptotic BMSCs in the PLGA/Mn1 and PLGA/Mn2.5 groups were approximately 40 % and 30 %, respectively. Thus, PLGA/Mn2.5 scaffolds could efficiently improve cell viability under ONFH-related oxidative stress.

We further performed transcriptome sequencing analysis to explore the mechanism underlying the antioxidant effect of the scaffolds on cells. The heatmaps (Fig. 4a) and volcano plots (Fig. 4b) revealed that the genes in BMSCs were differentially expressed between the PLGA/Mn2.5 scaffold and control groups. There were 98 significantly upregulated genes and 173 down-regulated genes in the PLGA/Mn2.5 scaffold group compared to the control group. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis suggested that these differentially expressed genes were enriched in the phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) signaling pathway (Fig. 4c). Western blotting (Fig. S8) confirmed that the protein expression levels of

both phosphorylated PI3K (pPI3K) and pAKT were enhanced in PLGA/Mn2.5 group as compared to the control and PLGA groups, indicated that the PLGA/Mn2.5 scaffold could activate the PI3K/AKT pathway. Moreover, gene set enrichment analysis (GSEA; Fig. 4d) indicated that the PLGA/Mn2.5 scaffold group presented a marked up-regulation transcripts associated with antioxidant activity, cellular oxidant detoxification, and the oxidative stress and redox pathway. The mainly up-regulated genes in BMSCs cocultured with the PLGA/Mn2.5 scaffolds were important antioxidant genes, including NADPH quinone oxidoreductase-1 (*Nqo1*), *Cat*, *Sod2*, glutathione S-transferase (GST) family (e.g., *Gsta3*, *Gsta4*, *Gsta3*, *Gstm1*, and *Gstp1*), and peroxiredoxin (PRDX) family (*Prdx1*, *Prdx2*, and *Prdx6*). These results were consistent with the multienzyme-like activities of the PLGA/Mn2.5 scaffold.

2.4. Osteoinductivity and angiogenesis of the scaffolds in vitro

Core decompression is a routine treatment for ONFH, but drilling causes bone defects and requires materials to prevent pathological

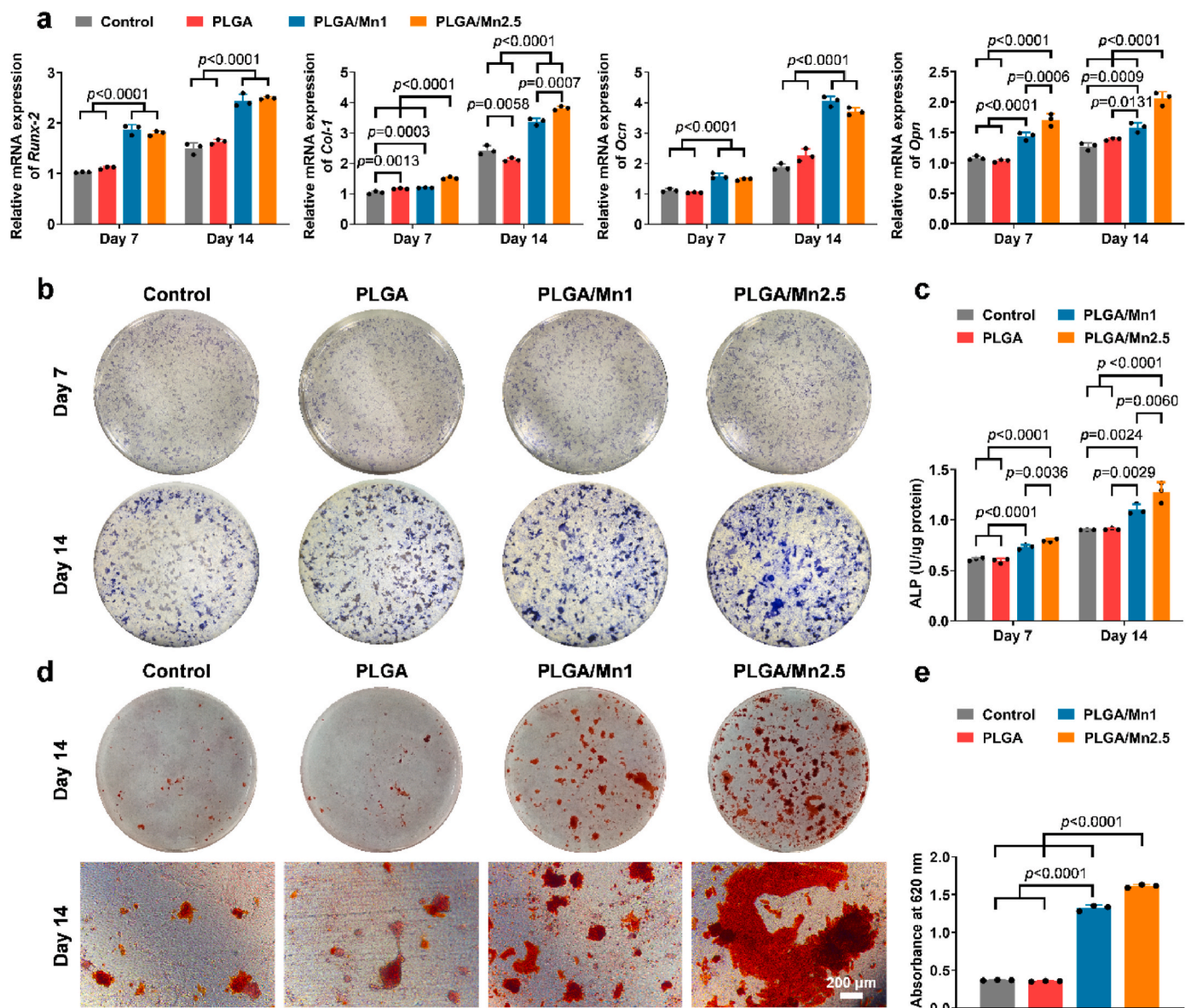


Fig. 5. In vitro osteoinductivity of the scaffolds. (a) Relative expression levels of osteogenic genes in BMSCs cocultured with different scaffolds for 7 and 14 days. (b) ALP staining images of BMSCs on days 7 and 14. (c) Quantitative analysis of ALP activity of BMSCs. (d) Alizarin red S staining images of BMSCs on day 14. (e) Semi quantitative analysis of alizarin red S staining. Data are presented as mean $\pm$ SD ($n = 3$).

fractures; thus, these scaffolds are expected to promote bone repair [16, 17]. We cocultured BMSCs with the scaffolds, and the expression levels of osteogenic genes were detected at days 7 and 14 by real-time quantitative polymerase chain reaction (RT–qPCR; Fig. 5a). We found that the expression levels of all osteogenic genes, including runt-related transcription factor 2 (*Runx-2*), type I collagen (*Col-1*), osteopontin (*Opn*), and osteocalcin (*Ocn*), were greater in the PLGA/Mn1 and PLGA/Mn2.5 scaffold groups relative to the PLGA and control groups. The early transcription factor *Runx-2* was significantly upregulated in the BMSCs cocultured with the MnO_x-doped PLGA scaffolds on day 7. Subsequently, the late-stage mineralization markers *Col-1*, *Opn*, and *Ocn* were significantly upregulated in the PLGA/Mn1 and PLGA/Mn2.5 scaffold groups compared to those in the PLGA and control groups on day 14.

We detected alkaline phosphatase (ALP) expression to confirm the RT–qPCR results. ALP is one of the most important markers of osteogenesis. As determined by ALP staining, BMSCs cocultured with PLGA/Mn2.5 scaffolds expressed the highest ALP level relative to that of the other groups on days 7 and 14 (Fig. 5b). This result was further confirmed by an ALP detection kit (Fig. 5c). Moreover, alizarin red S staining was performed to visualize the mineralization of the extracellular matrix (Fig. 5d). In the late stage of osteogenic differentiation, the extracellular matrix produces calcium nodules, which can form dark-red substances after binding to alizarin red S. After coculture with the scaffolds for 14 days, many more calcium nodules were found in the extracellular matrix of the BMSCs in the MnO_x-doped PLGA scaffold groups than in the PLGA and control groups. Specifically, the mineralization of the extracellular matrix was most significant in the BMSCs cocultured with the PLGA/Mn2.5 scaffold (as determined by semi-quantitative analysis; Fig. 5e). These results demonstrated that the PLGA/Mn2.5 scaffold exhibited the strongest osteoinductivity.

Angiogenesis plays an important role not only in bone repair but also in alleviating ONFH [18,19]. We first conducted the scratch test and transwell assay to observe the effect of the scaffold on cell migration. As shown in Fig. 6a, the scratches in the PLGA/Mn1 and PLGA/Mn2.5 groups were obviously smaller than those of the control and PLGA group. The wound healing rate was 44.08 %, 42.55 %, 68.96 %, and 84.50 % in the control, PLGA, PLGA/Mn1 and PLGA/Mn2.5 groups, respectively. Similarly, in transwell assay (Fig. 6b), the number of migrated HUVECs after 24 h were also significantly greater in the PLGA/Mn1 and PLGA/Mn2.5 groups relative to the PLGA and control groups. The migrated cell number was approximately 50, 55, 167, and 392 in the control, PLGA, PLGA/Mn1 and PLGA/Mn2.5 groups, respectively. These results indicated that the MnO_x NP-dope scaffolds could promote the migration of HUVECs.

We cocultured HUVECs with the scaffolds and investigated the gene expression levels of angiogenic markers in the cells (Fig. 6c). We found that both the PLGA/Mn1 and PLGA/Mn2.5 scaffolds promoted the expression of vascular endothelial growth factor (*VEGF*; a key neo-vascularization factor) and kinase-insert domain containing receptor (*KDR*; also known as vascular endothelial growth factor receptor 2) compared to those in the PLGA and control groups. Moreover, the expression of *CD31* (a main vascular endothelial marker) was significantly upregulated in the PLGA/Mn2.5 group. Fluorescence staining revealed that *CD31* expression was low in the control, PLGA, and PLGA/Mn1 groups (Fig. 6d). Notably, the fluorescence intensity of HUVECs cocultured with PLGA/Mn2.5 scaffolds increased (Fig. 6h), which was consistent with the RT–qPCR results. In addition, typical tube formation by HUVECs was observed in the PLGA/Mn1 and PLGA/Mn2.5 groups, while tube formation was negligible in the control and PLGA groups (Fig. 6e). The number of junctions and the tube length were greatest in the PLGA/Mn2.5 group among all the groups (Fig. 6f and g). Hence, PLGA/Mn2.5 scaffolds have the potential to promote angiogenesis.

2.5. Therapeutic effects of the scaffolds in a rabbit ONFH model

The long-term and extensive use of steroids is one of the main causes of ONFH in the clinic, especially in young patients [20]. Hence, we established a clinically relevant rabbit model of ONFH [21] through the administration of methylprednisolone (MPS) and lipopolysaccharide (LPS) for 6 weeks, followed by core decompression surgery and scaffold implantation (Fig. 7a). The PLGA/Mn2.5 scaffold was chosen for the in vivo study because it was better than the PLGA/Mn1 scaffold in terms of antioxidation, osteogenesis, and angiogenesis. The bone repair ability of the scaffolds was investigated by micro-CT. The 2D images of micro-CT scanning showed that the bone tunnel and scaffolds were within the femoral head and neck (Fig. 7a), which was consistent with clinical core decompression surgery. As shown in the 3D reconstruction images (Fig. 7b), new bone formation was negligible in the control groups, and the PLGA scaffolds only slightly promoted bone formation after 12 weeks of surgery because the oxidative stress microenvironment hampers bone repair. Impressively, a large amount of newly formed bone tissue was observed in the PLGA/Mn2.5 group. The bone volume/total volume (BV/TV) at 12 weeks postsurgery was 8.80 %, 10.29 %, and 19.96 % in the core decompression area of the control, PLGA, and PLGA/Mn2.5 groups, respectively. The bone mineral density (BMD) at 12 weeks postsurgery was 0.11, 0.13, and 0.23 g/cm³ in the control, PLGA, and PLGA/Mn2.5 groups, respectively (Fig. 7c). Hence, new bone formation was significantly enhanced in the PLGA/Mn2.5 group.

Hematoxylin and eosin (H&E) staining revealed that many empty lacunae formed in the femoral head in the control group (only core decompression without scaffold implantation) at 6 weeks postsurgery, and the number of empty lacunae increased at 12 weeks postsurgery; thus, only core decompression failed to alleviate ONFH in the rabbit model (Fig. 8a). This is in accordance with the fact that the effectiveness of core decompression is limited in the clinical treatment of ONFH. Similarly, the implantation of PLGA scaffolds did not reduce the number of empty lacunae. By comparison, the ratio of empty lacunae in the PLGA/Mn2.5 group was significantly lower than that in the control and PLGA groups at all time intervals (Fig. 8b). The trabecular bone area was also largest in the PLGA/Mn2.5 scaffold group (Fig. 8c). These results indicated that the PLGA/Mn2.5 scaffold efficiently prevented osteonecrosis and improved osteonecrosis. Moreover, the number of vessels were significantly increased in the PLGA/Mn2.5 scaffold group (Fig. 8d). Angiogenesis within the femoral head after 12 weeks of surgery was further studied by immunohistochemical staining for CD31 and VEGF (Fig. 8e). Both CD31 and VEGF were highly expressed in the femoral heads of rabbits in the PLGA/Mn2.5 group. More new vessels were also observed after PLGA/Mn2.5 treatment. However, CD31 and VEGF expression was low in the PLGA and control groups. Finally, the main organs of the rabbits after treatment were collected to study the histocompatibility of the scaffolds in vivo. H&E-stained sections (Fig. S9) of the main organs showed normal morphology, suggesting that the scaffolds had no systemic toxicity. These results demonstrated that the implantation of PLGA/Mn2.5 scaffolds successfully reduced osteonecrosis and enhanced bone repair and vessel formation without causing any undesired side effects in the rabbit ONFH model.

3. Discussion

In this work, we demonstrated the 3D-printed MnO_x-doped PLGA scaffold exhibited dual SOD- and CAT-like activities, enabling sustained ROS scavenging and oxygen release to mitigate oxidative stress. The scaffold combined with core decompression significantly reduced empty lacunae while enhancing new bone and vessel formation, showing its dual osteogenic and angiogenic therapeutic potential. Core decompression combined with autogenous bone transplantation is currently considered as the most effective hip preservation surgery for treating ONFH. However, this strategy has drawbacks, including limited bone supply, additional trauma in bone-supply area, and transplant failure.

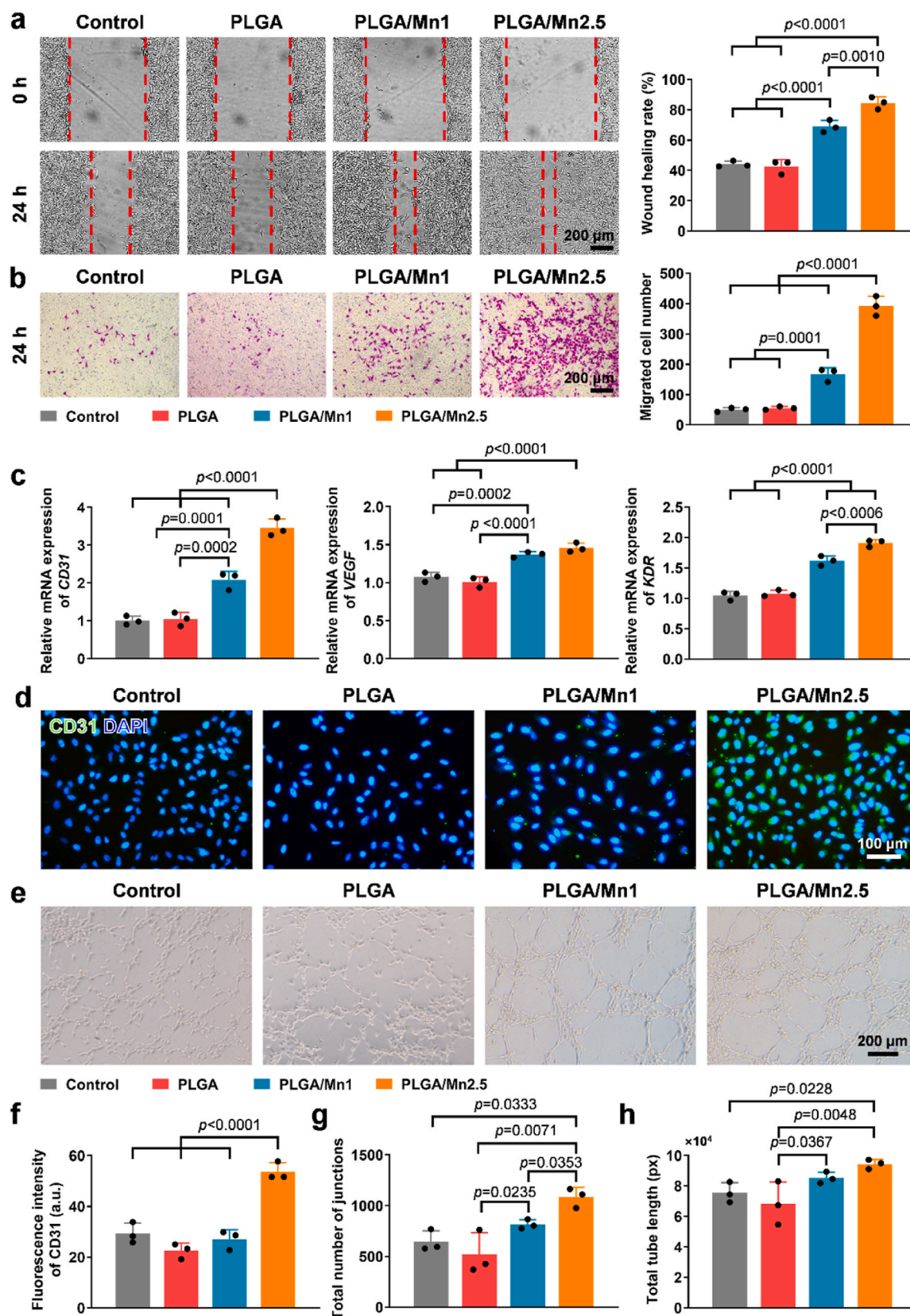


Fig. 6. In vitro angiogenesis effect of the scaffolds. (a) Representative images and statistical result of scratch test of HUVECs for 0 h and 24 h. (b) Representative images and statistical result of transwell assay of HUVECs for 24 h. (c) Relative expression levels of angiogenic genes in HUVECs cocultured with different scaffolds. (d) Immunofluorescence staining of CD31 expression in HUVECs in different groups. (e) Tube formation of HUVECs. (f) Semi-quantitative analysis of CD31 expression derived from panel (d). The degree of tube formation, including number of junctions (g) and tube length (h) derived from panel (e). Data are presented as mean $\pm$ SD ($n = 3$).

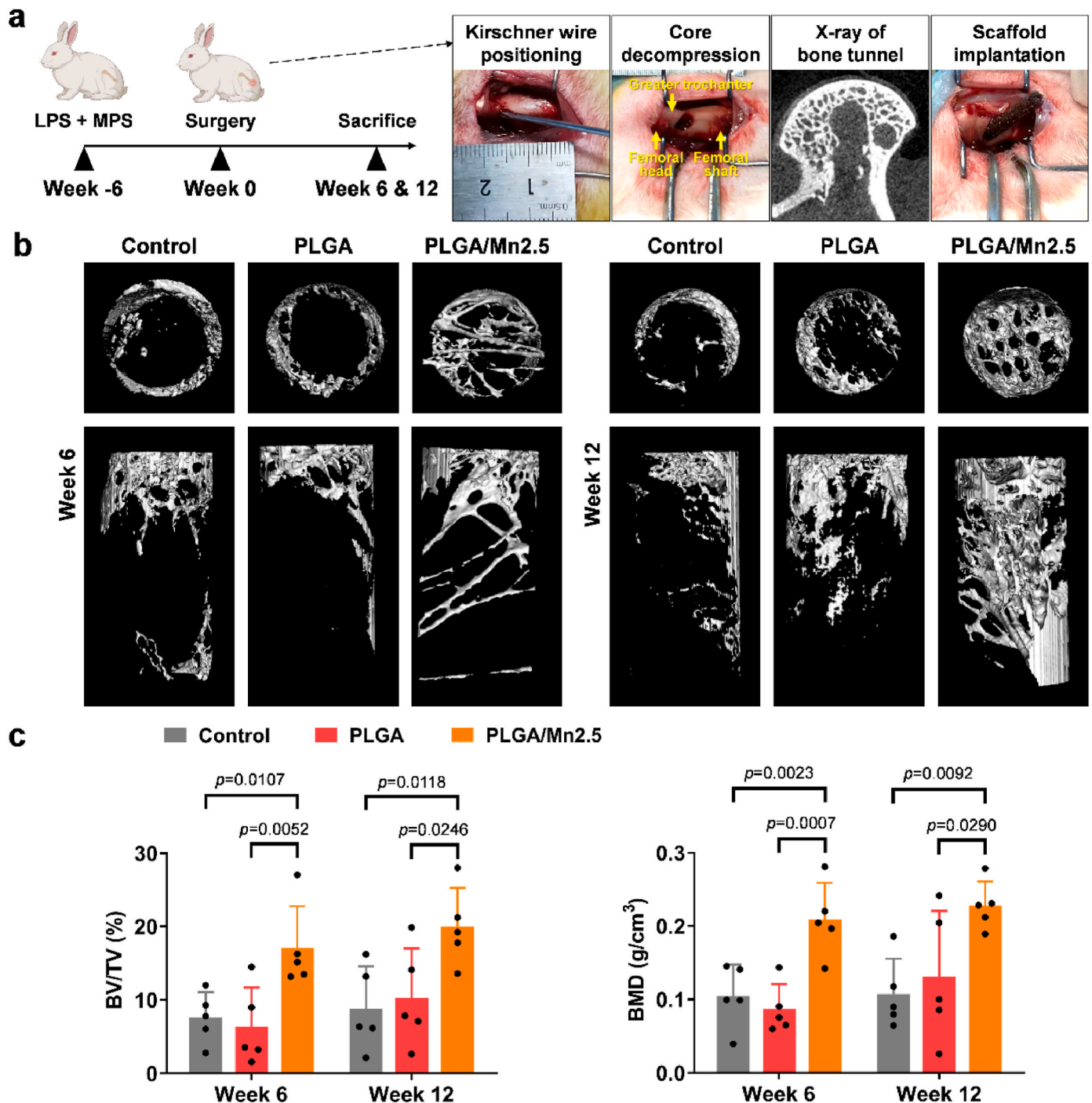


Fig. 7. Therapeutic effect of the scaffolds in a rabbit ONFH model. (a) Schematic illustration and surgery photographs of the establishment of the rabbit ONFH model and surgery. LPS and MPS were used to induced ONFH. After 6 weeks, core decompression and scaffold implantation were performed. (b) Micro-CT scanning of new bone formation in the core decompression area. (c) The bone volume/total volume (BV/TV) and bone mineral density (BMD) of the newly formed bone derived from panel (b). Data are presented as mean $\pm$ SD ($n = 5$).

Moreover, the ONFH-related oxidative stress and hypoxic microenvironment hampers bone formation and revascularization after surgery. In fact, the elevated ROS and hypoxia directly causes cell apoptosis during ONFH. The proliferation and osteogenic abilities of osteoblasts also decrease significantly under high ROS and hypoxia. Previous in vitro studies reveal that the differentiation and bone nodule formation of osteoblasts cultured under 0.2 % O_2 are significantly inhibited [22] and osteoblasts become apoptosis when O_2 concentration below 0.1 % [23]. Hypoxia can also stimulate the differentiation of mononuclear precursor cells into osteoclasts and enhance their bone resorption activity [24,25].

Thus, the abnormal microenvironment becomes one of the main obstacles of vascularized bone formation after hip preservation surgery in ONFH. Efforts have been made in the fields of O_2 release materials in recently years [26]. Through doping calcium dioxide, Abudula et al. [27] prepared nanofibers to release O_2 for one week; Erdem et al. [28] prepared hydrogel with oxygen release for 10 days. that oxygen releasing tissue engineering materials can improve cell survival in tissue engineering materials. Previously, 3D-printed scaffold also modified with calcium dioxide NPs doped-hydrogel, which can release O_2 for 19 days [29]. However, most of these materials cannot meet the

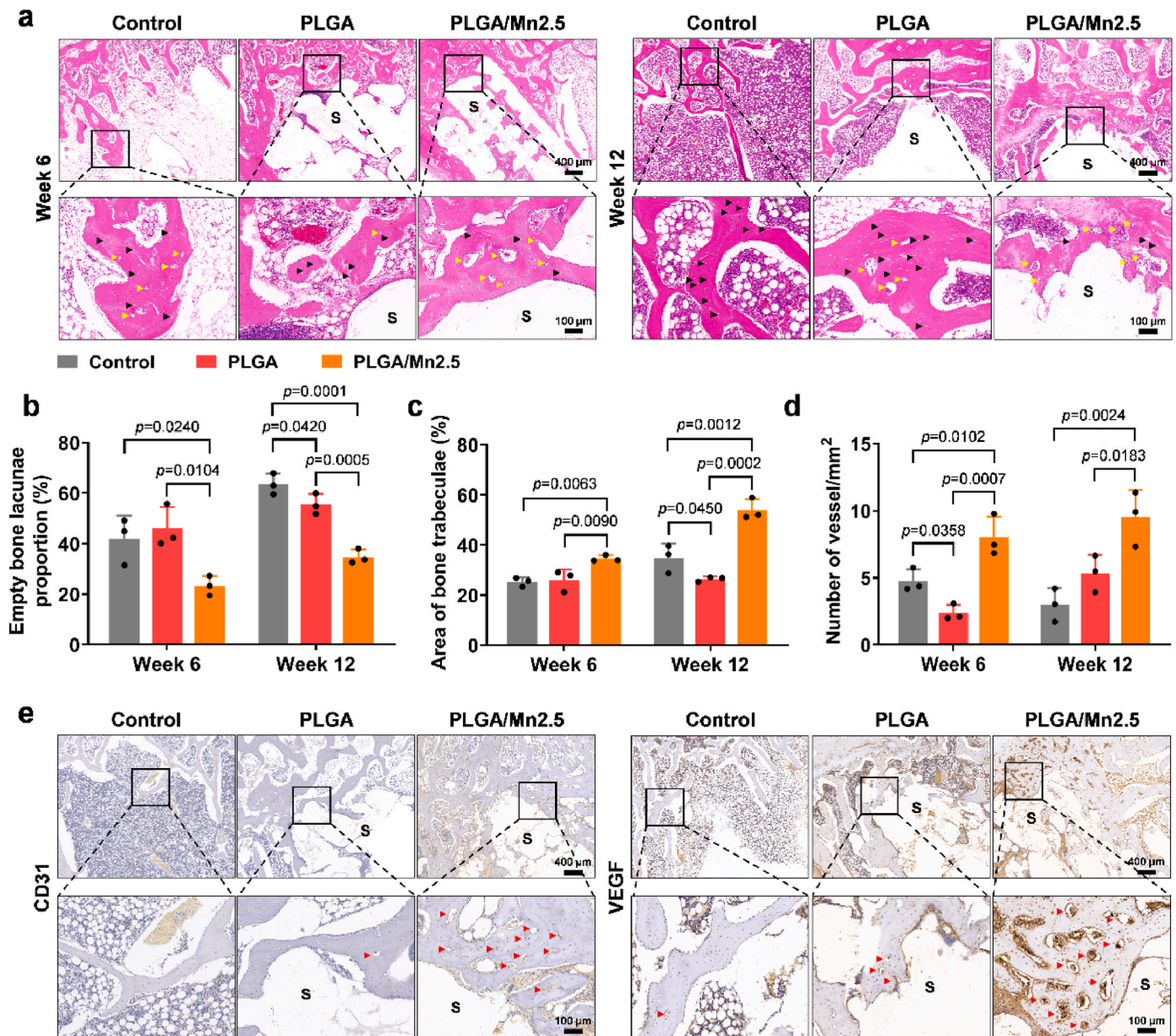


Fig. 8. Histological analysis of the femoral head after treatment. (a) Representative H&E staining images of the femoral head after treatment. Many empty lacunae (indicated by black arrows) were observed in the control (only core decompression without scaffold implantation) and PLGA groups at 6 and 12 weeks postsurgery. More vessel formation (indicated by yellow and red arrows) was observed in the PLGA/Mn2.5 group relative to other groups. The proportion of empty lacunae (b), the trabecular bone area (c), and the number of vessels (d) within femoral head derived from the H&E staining images ($n = 3$). (e) Immunohistochemical staining of CD31 and VEGF expression within femoral head at 12 weeks postsurgery. S represents the area of the scaffold.

requirement of O_2 supply in the revascularization and bone formation process (exceeding two weeks [30]) of femoral head; these materials cannot alleviate the oxidative stress microenvironment either.

In our study, we indicated that most BMSCs were dead in the control and PLGA scaffold groups when incubated with H_2O_2 (to stimulate high ROS environment). In contrast, cell apoptosis of BMSCs significantly decreased in MnO_x -doped PLGA scaffold groups. Moreover, our in vitro study confirmed that the scaffolds maintained their multienzyme-like activity for a least 30 days, thereby catalyzing ROS into O_2 to overcome the oxidative stress and hypoxic microenvironment. Furthermore, transcriptome sequencing analysis revealed that the antioxidant genes were significantly up-regulated in cells cocultured with the PLGA/Mn2.5 scaffolds. These antioxidant genes included *Nqo1*, *Cat*, *Sod2*, GST family [31] and PRDX family [32], and the mechanism was highly correlated with the PI3K/AKT signaling pathway. The PI3K/AKT

signaling pathway is a critical signaling network in cells that can be activated by various stimuli or toxic damage, and plays an important role in regulating basic cellular functions such as transcription, translation, proliferation, growth, and survival. The activation of the PI3K/AKT pathway can efficiently regulate downstream nuclear factor erythroid 2-related factor 2 (*Nrf2*) [33], thereby promoting the expression of antioxidant genes such as *Nqo1* [34]. Therefore, the possible mechanism of the antioxidant effect may be that the PLGA/Mn2.5 scaffold promotes the synthesis of antioxidant enzymes through the PI3K/AKT pathway to protect cells from the oxidative stress microenvironment.

About 40 % of Mn is stored in bone, which plays a crucial role in maintaining bone metabolism. As a cofactor for several enzymes, Mn is needed for the synthesis and calcification of bone matrix [35–37]. Mn deficiency can lead to various bone deformities, including stunted bone

growth, osteoporosis, impaired motor coordination, and congenital diseases of the skeletal system [38,39]. Mn can enhance the expression levels of osteogenic genes and proteins of osteoblasts. Like other osteotropic metal ions (such as copper and zinc), Mn also accelerates bone growth via activation of insulin-like growth factor 1 (IGF-1) [36,40]. Moreover, a recent study reveals that Mn-based materials can recruit CD4⁺ T cells to bone defect site, enhance T helper (Th) 2 cell polarization, and inhibit Th1 cell polarization [41]. This creates an anti-inflammatory microenvironment via the MnSOD/AMPK pathway, favoring bone repair. However, Mn overload impairs bone development and presents neurotoxicity. Wu et al. [42] indicate that the cell proliferation of BMSCs is significantly inhibited when the concentration of Mn²⁺ was greater than 28.21 µg/L. They determine the effective concentration of Mn²⁺ for promoting cell proliferation is lower than 7.17 µg/L. In addition, Mn can also promote cell adhesion at appropriate concentrations. Integrins are a large family of receptors that dominate cell adhesion and cellular interactions [43]. Mn can increase the ligand-binding affinity of integrins because integrins require divalent cations when they bind to ligands [44]. With many empty orbitals to accept electrons from ligands, Mn can enhance the integrin-ligand interactions and activate integrin α5β1 into a high affinity state [45]. In our study, we showed that the PLGA/Mn scaffolds can enhance cell proliferation, cell adhesion, and osteogenesis of BMSCs, consistent with these above-mentioned findings. In addition, the angiogenesis-osteogenesis coupling is essential for bone regeneration, which is regulated by the dynamic microenvironment involving biomaterial cues, hypoxic signaling, and cellular metabolic crosstalk [46]. Engineered scaffolds with porous structure and bioactive components can enhance vascular network maturation and osteoblast activity through coordinated biochemical and structural cues [46]. Reciprocal metabolic interactions between endothelial cells and osteoblasts establish a mechanosensitive regulatory network that sustains the bone regeneration process [47]. Mn²⁺ can promote angiogenesis by enhancing endothelial cell migration and stabilizing HIF-1α to upregulate VEGF expression. Mn²⁺ also synergizes with CO to polarize macrophages toward the M2 phenotype, further stimulating vascular growth [48]. Our results are consistent with previous reports. The doping of MnO_x NPs significantly enhanced the proangiogenic effect of the scaffolds, and thus PLGA/Mn scaffolds improved bone repair of ONFH after core decompression in vivo.

There are some limitations in this work. First, the antioxidative mechanism of the scaffold were only evaluated in the control and the PLGA/Mn2.5 groups in vitro. More comprehensive in vivo mechanism study that includes PLGA scaffold is needed. Second, the in vivo angiogenesis was only evaluated by histological studies. Other advances technologies such as can be applied to further confirm the in vivo proangiogenic effect of the scaffold. Third, the exact ROS level in ONFH remains unclear and the oxygen level play a complex role in osteogenesis because hypoxia may stimulate angiogenesis. Hence, the balance of oxygen level and hypoxia in ONFH needs to be explored. Finally, although PLGA and MnO_x NPs presented very low cytotoxicity, there may be residual BSA and dichloromethane in the final product, which will raise considerations for the approval for future clinical translation. Hence, the preparation process of the scaffold still needs continuous improvement.

In conclusion, we developed a new 3D-printed scaffold by integrating MnO_x NPs with PLGA using a FDM method. The in vitro and in vivo experiments revealed that the scaffold exhibited an ultralong-term multienzyme-like activity that catalyzed ROS to alleviate oxidative stress and simultaneously slowly released O₂ to improve the hypoxic microenvironment. The MnO_x NPs within the scaffold further promoted osteogenesis and angiogenesis to synergistically improve ONFH. The combination of biocompatibility, mechanical strength, microenvironment improvement, accelerated repair, ease of large-scale preparation, and low cost makes this scaffold promising for the treatment of ONFH.

4. Materials and methods

4.1. Preparation of MnO_x NPs and 3D-printed MnO_x-doped PLGA scaffolds

We use a previous reported BSA-assisted method with modifications to synthesize MnO_x NPs [49,50]. In a typical process, 5 g of BSA was dissolved in 1 L of deionized (DI) water and stirred it for homogenization. Next, KMnO₄ solution (3 g) was added and stirred for 2 h at room temperature. Then, the solution was dialyzed against DI water for 72 h and freeze-dried to obtain the MnO_x NPs.

4.2. Preparation of 3D-printed scaffolds

The 3D-printed scaffolds were prepared according to a previous reported FDM method [51,52]. PLGA (LA: GA = 75:25, Mw = 106000–127000) was purchased from Jinan Daigang Biomaterial Co., Ltd (Jinan, China). PLGA powder (100 g) was added to the dichloromethane solvent and stirred until completely dissolved. Then, 1, 2.5, and 5 g of MnO_x NPs were added and stirred to obtain homogeneous mixtures. After evaporating at room temperature (25 °C) for 24 h in a fume hood, dichloromethane was removed and the composited was freeze-dried. The mixtures were melted and extruded to obtain a standard print filament (450 µm in diameter) using a twin-screw extruder (SHJ-30, Nanjing Jiesite Electromechanical Company, China). The scaffolds were designed as cylinders with 400 µm between printed filaments by using 3D structure design software (SolidWorks, Dassault Systemes S.A.). Then, all the scaffolds were 3D-printed into cylinders with 400 µm between printed filaments using a FDM system (Prusa i3, China). The control PLGA scaffolds were prepared following the same method but without doping MnO_x NPs.

4.3. Characterization of the NPs and scaffolds

The shape and size of MnO_x NPs was observed using a high-resolution TEM. The distribution of element on MnO_x NPs were observed by elemental mapping using TEM. The crystal structure and the valence states of Mn of MnO_x NPs was verified using an X-ray diffractometer and X-ray photoelectron spectroscopy, respectively. The surface morphology, pore size and elemental analysis of the scaffolds were studied by using a SEM and EDS. The mechanical properties of the scaffold were evaluated by a universal machine at a constant speed of 1 mm/min. To explore the in vitro degradation, the scaffolds were incubated in SBF (BestBio, China) at 37 °C for 30 days. At each time intervals, the scaffolds dried with absorbent paper and weighed. To investigate the release of MnO_x NPs, PLGA/Mn1 and PLGA/Mn2.5 scaffolds were incubated in SBF at 37 °C for 2 weeks. The supernatant was collected, treated with nitric acid, and the content of Mn ions were detected by ICP-MS.

4.4. Enzyme-like activity of the NPs and scaffolds

The enzyme-like activity of the MnO_x NPs was first detecting the reduction of H₂O₂ by measuring the ODs of the mixture of H₂O₂ (100 µM) and the NPs (25 µg/mL) at 240 nm. The generation of O₂ in the mixture was determined using a dissolved oxygen probe [53]. To study the long-term enzyme-like activity, the scaffolds were incubated in simulated body fluids containing an H₂O₂ (100 µM) under dark condition. The dissolved O₂ production was continuously measured over a 30-day period. The ability of the scaffolds to scavenge O[•] was evaluated by a PTIO test. The antioxidant ability of the scaffold was detected by a T-AOC test. The PTIO and T-AOC tests were performed according to the manufacturer's instructions using commercial kits (Nanjing Jiancheng Bio-Technology Co., China).

4.5. Cytotoxicity of the scaffolds *in vitro*

In all *in vitro* experiments, cells were directly contacted with the scaffolds. BMSCs and HUVECs (1×10^4) were seeded in 24-well plates and incubated with the scaffolds. After coculture for 1, 3, and 7 days, 500 μ L of CCK-8 solution (10 %) was added to each well and incubated for an additional 1 h. Then, the optical density (OD) was measured at 450 nm using a microplate reader. Moreover, BMSCs were cocultured with MnO_x NP at various concentrations. After 24 h, cell viability was also detected by CCK-8 method. The cytotoxicity of the scaffolds was also determined by live/dead staining. rBMSCs and HUVECs were cocultured with the scaffolds as described above. On day 1, 3, and 7, each well was washed with PBS, and the cells were stained with calcein-AM/propidium iodide (Beyotime Biotechnology, China) for 30 min. Then, the cells were washed with PBS and imaged using a fluorescence microscope.

4.6. Hemolysis test *in vitro*

Blood from healthy rabbits (4 mL) was collected in anticoagulant tubes and diluted with PBS (5 mL). Then, 0.2 mL of the diluted blood was added to 5 mL of PBS and incubated with the scaffolds. PBS and DI water were used as the negative and positive controls, respectively. After incubating at 37 °C for 30 min, all the samples were centrifuged at 3000 rpm for 10 min, and the ODs of the supernatants were measured at 545 nm. The hemolysis ratio was calculated.

4.7. Cell adhesion

BMSCs were seeded into the scaffolds and cocultured with the scaffolds as described above for 24 h. Then, the cells were fixed with 2.5 % glutaraldehyde overnight at 4 °C. The samples were dehydrated in a graded ethanol series (30 %, 50 %, 70 %, 90 %, and 100 %, respectively), dried in hexamethyldisilazane, coated with gold, and observed using SEM.

4.8. Staining and quantitative analysis of ALP

BMSCs (5×10^3) were incubated with the scaffolds in osteogenic medium (Cyagen Biosciences, USA). After 7 and 14 days of coculture, the cells were washed with PBS and fixed with 4 % paraformaldehyde. Then, all the samples were stained and observed according to the instructions of the ALP staining kit (Solarbio, China).

After being washed with PBS, the cells were cultivated as described above and then lysed with 0.2 % Triton X-100 for 30 min at 4 °C. The ALP level was determined with an ALP detection kit (P0321, Beyotime Biotechnology, China), and the total protein concentration was determined with a BCA protein assay kit (Pierce, USA). The relative ALP activity of each sample was normalized to the corresponding total protein content.

4.9. Expression of osteogenic and angiogenic genes

The osteogenic gene expression level of the BMSCs was evaluated by RT-qPCR. After 7 and 14 days of osteogenic incubation, the total RNA of the cells was extracted using TRIzol reagent (Accurate Biology, China), and the RNA was reverse transcribed into cDNA using a reverse transcription kit (Accurate Biology, China). The gene expression of osteogenesis-related factors, including *RUNX-2*, *OPN*, *OCN*, and *COL-1*, was quantified using a SYBR Green qPCR kit (Accurate Biology, China). β -ACTIN was used as the housekeeping gene. The relative mRNA expression level was calculated by the $2^{-\Delta\Delta CT}$ method [54]. Moreover, after coculturing HUVECs with the scaffolds for 3 days, we evaluated the expression levels of genes related to angiogenesis, including *CD31*, *VEGF*, and *KDR* using the same method.

4.10. Alizarin red S staining

BMSCs were cocultured with the scaffolds for 7 or 14 days as described above. Then, the cells were fixed and stained with alizarin red S solution (Solarbio, China) for 2 h. Then, the dye was removed, and the cells were washed until the supernatant became transparent. The samples were observed using a microscope. For quantitative evaluation, 10 % cetylpyridinium chloride solution was added to dissolve the stained calcified nodules. After incubation for 2 h, the ODs of the samples were measured at 570 nm.

4.11. Tube formation

We evaluated the angiogenic capacity of the scaffolds using classical tube formation assays. Briefly, 20 μ L of growth factor-reduced Matrigel (BD Biosciences) was added to a 96-well plate and incubated for 30 min at 37 °C. After 3 days of coculture with each group of scaffolds, HUVECs were collected and resuspended at a density of 5×10^4 /mL. Then, 100 μ L of cell suspension was added to the prepared 96-well plate and incubated at 37 °C for 12 h. After incubation, tube formation was observed under an inverted microscope. The length and junctions of the tube formations were measured using ImageJ version 1.31 (NIH).

4.12. Scratch test and transwell assay

HUVECs were cocultured with or without the scaffolds for 3 days and then seeded onto a plate. A straight-line scratch was made in the middle of the well and cells were cultured for an additional 24 h. Then the scratch was imaged and the wound healing rate was calculated. For transwell assay, the above-mentioned HUVECs were seeded into the upper chamber of a transwell system and cultured with serum-free medium. The lower chamber of the transwell system was filled with complete medium containing 10 % FBS. After incubating for 24 h, cells in the upper chamber were removed. The migrated cells were stained with crystal violet and imaged using a microscope.

4.13. Immunofluorescence staining

The expression of CD31 was further evaluated by immunofluorescence staining. After coculture for 3 days, the HUVECs were fixed with 4 % paraformaldehyde and blocked with 5 wt% BSA at 37 °C for 30 min. Then, 200 μ L of mouse anti-human CD31 IgG antibody (diluted at 1:200; Abcam, UK) was added, and the mixture was incubated at 4 °C for 12 h. After washing, 200 μ L of rabbit anti-mouse IgG secondary antibody (diluted at 1:500; Abcam, UK) was added, and the mixture was incubated at 25 °C for 1 h. The nuclei were stained with 4,6-diamino-2-phenyl indole (DAPI) for 5 min at 25 °C. Finally, the cells were observed using a fluorescence microscope.

4.14. Intracellular ROS detection

BMSCs were cocultured with different scaffolds and H_2O_2 (100 μ M) for 24 h. To detect intracellular ROS, DCFH-DA (20 μ M) solution was added and incubated at 37 °C for 30 min. All the samples were imaged using a fluorescence microscope. The semi-quantification of DCFH-DA staining (the green fluorescence intensity) was analyzed using ImageJ software.

4.15. Cell apoptosis

BMSCs were seeded in a 24-well plate and cultured for 24 h before being cocultured with different scaffolds and 100 μ M H_2O_2 . Cell viability was then assessed visually using the CCK-8 assay and live/dead staining. Moreover, an Annexin V-FITC/propidium iodide (PI) apoptosis detection kit was used according to the manufacturer's instructions. Flow cytometry and FlowJo 10 software were then used to analyze cell apoptosis.

4.16. Establishment of the ONFH animal model and surgical procedure

All animal procedures were performed in accordance with and approved by the Animal Ethics Committee of Zhujiang Hospital of Southern Medical University (Approval No.: LAEC-2020-241). Male New Zealand rabbits (6–8 weeks old, weighing >2.5 kg) were first injected intravenously with LPS (10 µg/kg). Then, MPS (20 mg/kg) was injected intramuscularly for 3 consecutive days after 24 h, and the ONFH model was continuously induced for 6 weeks. Then, the ONFH rabbits were anesthetized, followed by core decompression by creating a 3.5-mm-diameter bone tunnel in the proximal femur from the greater trochanter through the femoral neck to the femoral head. The 3D-printed PLGA and PLGA/Mn2.5 scaffolds (3.5 mm in diameter and 15 mm in length) were implanted, respectively. No scaffold was implanted in the bone tunnel of the control group. Finally, the tissues were sutured layer by layer and sterilized with iodophor. The rabbits were intramuscularly injected with penicillin sodium (80 U/kg) for three consecutive days after the operation to prevent infection.

4.17. Transcriptome sequencing analysis

We cocultured BMSCs with or without the PLGA/Mn2.5 scaffolds, and then performed transcriptome analysis as previously reported [55]. Total RNA was extracted from cells and the mRNA libraries were generated using Illumina HiSeq X10. Gene expression levels were determined and DEGs (altered expression with $p < 0.05$ and more than 1.5-fold changes) were identified using edgeR software. KEGG pathway enrichment and GSEA analyses were conducted to investigate DEGs enriched in a pathway. To validate the KEGG enrichment, western blotting was conducted. Total protein was extracted from cells in the control, PLGA, and PLGA/Mn2.5 groups, and then the concentration of samples was quantified using a BCA assay kit. Then samples were separated by polyacrylamide gel electrophoresis and transfer it to a PVDF membrane. After blocking, samples were incubated with the primary antibodies (pPI3K and pAKT) overnight, and then incubated with secondary antibodies at room temperature for 2 h. The membranes were visualized using a fluorescence gel documentation system.

4.18. Bone repair analysis

At 6 and 12 weeks after surgery, the rabbits in each group were sacrificed, and the femoral heads were collected and fixed in 4 % paraformaldehyde. Micro-CT was used to evaluate new bone formation in the bone tunnel. After scanning was completed, the tunnel was reconstructed in three dimensions using the accompanying software and analyzed for BMD and BV/TV.

4.19. Histological analysis

The femoral heads were decalcified, sliced, and stained with H&E staining solution. Expression of CD31 (antibody: HUABIO, HA601240, China) and VEGF (antibody: Bioss, bs-1313R, China) in cartilage sections was studied by immunohistological staining. In addition, the main organs, including the heart, liver, spleen, and kidney, were collected, and H&E staining was performed to study the in vivo biocompatibility of the scaffolds. The specimens were observed using an inverted microscope.

4.20. Statistical analysis

All data were represented as mean $\pm$ standard deviation (SD). Group comparisons were made using one-way analysis of variance (ANOVA), followed by multiple post-hoc comparisons (Tukey test). A p -value less than 0.05 was regarded statistically significant.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and the Supporting Information.

Declaration of AI and AI-assisted technologies in the writing process

The authors declare that no AI and AI-assisted technologies are used.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jot.2025.06.010>.

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