DMOG attenuates steroid-associated endothelial progenitor cell impairment and osteonecrosis of the femoral head via regulating HIF-1α signaling pathway in rabbit

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Research Article

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Abstract

Background: Steroid-associated osteonecrosis of the femoral head (SONFH), generally caused by chronic or/and high-dose use of Glucocorticoids (GCs), has long been puzzling mankind for a long time as the most essential etiology for non-traumatic ONFH. Hypoxia inducible factor-1α (HIF-1α) is an essential transcription factor that plays a significant role in maintaining bone homeostasis. By repressing prolyl hydroxylase domain (PHD) enzymic activity, Dimethyloxalylglycine (DMOG) was reported to inhibit the transcription and activation of HIF-1α. Recently, the impairment and dysfunction of endothelium has been closely related to the pathogenesis of SONFH. Our previous studies have shown the compromised number and function of endothelial progenitor cells (EPCs) which are the major source of restoring the endothelium during reendothelialization in SONFH patients. Nevertheless, whether activated HIF-1α by DMOG could alleviate GCs-related damaged biological function of EPCs remains unclear.

Methods: We investigated that regulating HIF-1α signaling pathway of EPCs could be the therapeutic target for SONFH in vitro and vivo. Western blot analysis was used to assess protein expression. Transwell, tube formation, senescence-associated β-galactosidase and ELISA assays were carried out in vitro. SONFH was induced by methylprednisolone combined with lipopolysaccharide in rabbits. Histological staining of sections were conducted to evaluate the expression of HIF-1α which is of significance for osteogenesis and angiogenesis. Immunohistochemical staining of local femoral head slices and flow cytometry analysis of peripheral blood were used to quantify the number of EPCs.

Results: High dose of GCs remarkably inhibited HIF-1α expression and subsequently compromised the bioactivity in EPCs. DMOG treatment was able to reverse the impairment effects in a concentration-dependent manner in vitro, while inhibition of HIF-1α by KC7F2 essentially attenuated these outcomes. Our in vivo experiments also showed that DMOG obviously increased blood supply and alleviated osteonecrosis owing to the increased HIF-1α expression of EPCs.

Conclusion: The importance of HIF-1α to the regulation of EPCs is emphasized due to its reendothelialization in SONFH. Our results indicated that DMOG not only promoted the activation of HIF-1α signaling pathway in EPCs, but also boosted endogenous EPCs targeting and homing to damaged endothelium beneficial to reendothelialization in femoral head.

Introduction

A wide variety of diseases could be treated with Glucocorticoids (GCs), including chronic illness, acute inflammation, and autoimmune disorders[1, 2]. Importantly, remarkable clinical efficacy improvements have been observed owing to the widespread clinical application of GCs against the epidemic of viral outbreak at the height of coronavirus disease 2019 (COVID-19)[3]. Up to nowadays, osteonecrosis of the femoral head remains a conundrum that has puzzled over 20 million of people all over the world [4]. Unfortunately, chronic or high-dose GCs may induce steroid-associated osteonecrosis of the femoral head (SONFH), which has become one of the most dominant etiologies for non-traumatic ONFH. However, the

pathogenesis of SONFH is still less well understood, and is considerate to be the multiple complex mechanisms, such as osteocytes apoptosis, homeostasis dysfunction of osteoblasts and osteoclasts, and differentiation imbalance of Mesenchymal stem cells (MSCs)[5–7]. Amid the existing mechanisms, it seems that vascular hypothesis, which presumes that impaired micro-vessels and reduced blood flow are responsible for the occurrence and development of SONFH, is the most persuasive one[8]. GCs could impact the number of micro-vessels by inducing the apoptosis of endothelial cells (ECs) and inhibiting the angiogenesis and repair capability of precursor cells of ECs, resulting in the impaired and reduced local blood supply, thereby eventually causing osteonecrosis[9, 10].

Among the precursor cells of endothelial cells, endothelial progenitor cells (EPCs), one of the precursor cells of endothelial cells, are essential to maintaining the integrity of vascular structure and function [11, 12]. Firstly, it is reported that EPCs could significantly increase the density of micro-vessels, promote endothelial regeneration, and improve the patency rates of vessels [13]. Besides, several studies have demonstrated that EPCs not only take part in the development of vascular endothelium in the embryonic stage, but also the neovascularization and the maintenance of vascular homeostasis during the adulthood[14, 15]. Nevertheless, high doses of GCs might disrupt the normal biological function of EPCs. Essentially, our previous studies have shown that the decreased number and function of EPCs in patients with SONFH[16, 17]. Thus, a better therapeutic strategy could be taken into consideration through targeting EPCs.

HIF-1, a heterodimeric protein consisting of two subunits, α and β, is the master transcriptional regulator of cellular response to hypoxic conditions[18]. HIF-1α could be activated to improve the expression of downstream essential target genes under hypoxia, including VEGF, SDF-1, TGF-β, PDGF-BB, and Ang-1, etc.[19, 20]. HIF-1α is constitutively regulated by the prolyl hydroxylase domain (PHD) proteins and von Hippel-Lindau (VHL) proteins. Under normal oxygen levels, hydroxylated HIF-1α through PHD is recognized by VHL, and then contributing to the degradation of HIF-1α[21]. However, in hypoxia, PHI can promote HIF-1α stabilization via inhibition of PHD activity, hence participating in the regulation of repair of ischemia-related diseases[22].

Dimethyloxalylglycine (DMOG) is a permeable oxoglutarate analogue which could suppress the enzymic activity of PHD, contributing to the transcription and activation of HIF-1α, which is significant to induce downstream gene expression and then be applied to treatment strategies such as bone-associated disorders and bone tissue regeneration [23, 24]. For one thing, DMOG can importantly promote the angiogenic potency of bone marrow-derived and adipose-derived MSCs via activating HIF-1α signaling pathway, thereby increasing the number of MSCs of peripheral blood[25, 26]. For another thing, MSCs-derived exosomes is induced to indirectly stimulate angiogenesis and bone regeneration by DMOG treatment[27]. And thirdly, coupling of angiogenesis and osteogenesis partially alleviated the bone loss caused by OVX-induced osteoporosis after DMOG injection in mouse[28]. Hence, we are extremely interested in whether immediate activation of HIF-1α with DOMG could potentially protect against high doses GCs-related injure for EPCs.

The purpose of our present study aims to investigate whether DMOG treatment strategy could relieve the osteonecrosis, enhance bone formation and neovasculization in SONFH rabbit models caused by MPS. Furthermore, we presume that DMOG could improve the biological function of EPCs partially via HIF-1a signaling pathway. In conclusion, our research could shed light on the prevention and development of SONFH in a promising vascular endothelium-based strategy.

Materials And Methods

Culture and characteristic of endothelial progenitor cells（EPCs）

Isolation and culture of rabbit EPCs were conducted as described previously[29]. In brief, young New Zealand white rabbits were anesthetized and about 20ml of blood was withdrawn from the bone marrow using an aspiration needle containing 0.2ml heparin(3000U/ml). The mixture was added to an equal volume of lymphocyte separation solution and centrifuged at 2000rpm for 18 minutes. Intermediate white flocculent cells are the mononuclear cells（MNCs）that we need. Then cells were rinsed twice and recirculated with M199 medium containing 10% fetal bovine serum（FBS）, VEGF(10ug/ml), and bFGF (2ug/l).Cell were seeded at 1×10⁶ cells per wells in a fibronectin-coated 6-well and the culture liquid were changed every 48 hours. On day 2, the nonadherent cells were collected and seeded at 1×10⁶ cells per wells and cultured in M199 culture medium supplemented with 500μM DMOG and 2% fetal bovine serum (FBS) in a 24-wells so as to analyze the numbers of colonies. Control cells were treated with M199 containing 2% FBS alone. EPCs colonies were identified as elongated sprouting cells radiating from a central cluster of round cells and enumerated blindly by two independent investigators on day 7 using an inverted microscope.

Flow cytometry analysis was conducted to evaluate the phenotype of EPCs. Rinsed with PBS three times, cells were digested and resuspended at a frequency of 1×10⁶ cells/ml. EPCs were incubated at room temperature for 1 h with the following antibodies: anti-CD34 antibody (ab81289, Abcam), anti-VEGF Receptor 2 (VEGFR2) antibody (Santa Cruz Biotechnology, sc-6251). EPCs were then treated in the dark for 30 mins with FITC-conjugated mouse anti-rabbit IgG or PE-conjugated Donkey anti-mouse IgG antibody, rinsed three times with PBS, and resuspended in 200μl PBS. The fluorescence intensity of the cells was evaluated using a FACS Calibur flow cytometer (BD, Franklin Lakes, USA). The negative controls were EPCs incubated with isotype R-PE or IgG-FITC staining.

Cell viability assay

EPCs were seeded at an initial density of 2×10^3/well in 96-well plates cultured with M199 medium containing 10% FBS in 37 °C incubator for 48 h. Cell counting kit-8(CCK-8) assay was carried out to estimate the cell viability after MPS treatment with different concentration (0, 1, 10, 100, 1000 μM) for various time points (24, 48, 72h), or 100 μM MPS treated with diverse concentration of DMOG (0, 50, 100, 200, 500, 1000) for 48h. Then, 10μl CCK-8 reagent was added to each well incubated at 37 °C for 2h. The absorption spectrum at 450 nm was determined by a microplate reader.

Senescence-associated β-galactosidase (SA-β-Gal) assay

The senescence-associated β-galactosidase (SA-β-Gal) staining Kit was conducted to assess β-galactosidase activity of DMOG to EPCs based on manufacturer protocol. After treated with 7 days, they were fixed for 15 mins with 4% paraformaldehyde at room temperature and then stained with working solution overnight in a 37℃ incubator.

Colony-Forming unit assay

For CFU assays, EPCs derived from bone marrow cells were placed in 25 cm^2 culture flask with culture medium containing 500μM DMOG, M199 supplement with 2% fetal bovine serum (FBS). The control group was cultured in medium consisting only of M199 supplement with 2% FBS. CFUs of EPCs were counted about day 7. A colony (>50 cells) was marked as one unit.

Matrigel tube formation assay

Tube formation assay was performed according to the Matrigel protocol (BD Biosciences). In brief, EPCs treated with DMOG or cells from control group at a density of 1×10^4 were seeded on Matrigel for 18h of coincubation, live cell were stained with calcian; then the tube formation ability of EPCs was assessed with a microscope (Olympus, Japan), with total line length quantified by Image J software.

Transwell assay

To assess the chemotaxis of the stimulated EPCs, 5×10^4 EPCs treated with 500μM DMOG were seeded into a Transwell upper chamber (Corning Co, 8 μm pores). Medium with VEGF (5 ng/ml) was added to the lower chamber. Cells were cultivated for 24h at 37℃ and nucleus were stained with DAPI.

Enzyme-linked immunosorbent assay (ELISA) and detection of NO secretion

EPCs were cultured with 500μM DMOG or 500μM DMOG with HIF-1α inhibitor KC7F2 (10μM) for 24 h before the cell culture medium was obtained. Control group was incubated only with M199 supplement containing 2% FBS. The VEGF and SDF-1 concentration was measured with a rabbit VEGF ELISA kit (SEA143Rb, Cloud-Clone). The supernatant of cell culture medium was collected to detect NO level according to the kit instructions.

Western blot analysis

The EPCs were lysed in RIPA buffer containing a protease inhibitor cocktail before being used in the experiments. A 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate the proteins, which were then transferred to PVDF membranes (0.45 μm, Millipore, USA) to be analyzed. After being blocked with 5% non-fat milk, the membranes were treated with primary antibodies HIF-1α (ab179483, Abcam, 1:1000) and β-actin (BM0627, Boster, 1:2000) overnight at 4℃ and incubated for 1h at room temperature with secondary antibodies. The membranes were exposed by a Bio-Rad scanner after being prepared with an ECL Substrate Kit (Thermo Pierce, USA).

Animal experiment

The Experimental Animal Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China, authorized all animal experimental procedures. Thirty-two New Zealand white rabbits (4 months old, body weight 3-4kg) were used in this study. Free access to normal food and water as well as 12:12-hour light/dark cycles were provided to all animals in hygienic plastic cages maintained at 24°C in a clean, well-ventilated environment. The rabbits were randomly divided into three groups: MPS group (n=10), MPS+DMOG group (n=10), and MPS+DMOG+KC7F2 group (n=10). Rabbits were intravenously injected with lipopolysaccharide (LPS, Sigma, 10 μg/kg) via auricular vein for 2 consecutive days, three injections of 20 mg/kg body weight of methylprednisolone (MPS; Pfizer, USA) were given intramuscularly at a time interval of 24 h within each group. Rabbits in the MPS+DMOG group received intramuscularly injection of DMOG (Caymen, 20 mg/kg) on 1, 3, 5, and 7 days after 4 intramuscularly injected with the KC7F2(Selleck, 10mg/kg) on 1, 3, 5, and 7 days after 4 weeks of injection of MPS, where KC7F2 was pretreatment 2h before DMOG injection. The KC7F2 dose instruction wase based on the previous article[30].

Histological and immunohistochemical staining

All animals were sacrificed, and the femurs were fixed in 4% paraformaldehyde (pH 7.4) for 3 days. Then the samples were decalcified with 10% EDTA (pH 7.4) for about 30 days. The specimens were embedded in paraffin, cut along the coronal plane into 5μm thick sections. For one thing, osteonecrosis (ON) was detected by staining sections with hematoxylin and eosin (H&E). ON is characterized by the presence of lacunae or pyknotic nuclei between the bone trabecular and marrow, as well as hypertrophy of the adipocytes and vascular thrombosis. All sections were classified as ON if they contained at least one or more these features above. Moreover, toluidine blue staining was performed to detect the newly generated bone tissue around the ON area. For another, immunohistochemical staining was performed to assess HIF-1α (Santa Cruz Biotechnology, USA) expression. HIF-1α, and CD34(Santa Cruz Biotechnology, USA) of immunohistochemical staining was performed to analyze the situation of the migration of bone marrow-derived EPCs into peripheral blood. All positive staining of sections was captured by a microscope (IX71, Olympus Corporation, Tokyo, Japan).

Immunofluorescence staining

To assess the neo-vasculization of sections around the ON, the deparaffinized sections of femur were processed by 0.25% trypsin antigen retrieval and were blocked with 10% FBS for 1h at room temperature. The sections were incubated with primary antibody anti-□ (MAXIM, USA) at 4℃ overnight, and then incubated with secondly antibody (Boster, Wuhan, China) for 1h. Images were captured under means of fluorescence microscope (IX71, Olympus Corporation, Tokyo, Japan).

Statistical analysis

There were three times of tests in all. All of the data were represented as mean ± standard deviation (SD). The Student's two-tailed t test was performed to evaluate whether there were differences in numerical data between the two groups. One-way ANOVA was used to determine differences among groups. A p value of less than 0.05 was defined statistically significant.

Results

DMOG essentially attenuates the cytotoxicity of MPS on EPCs

EPCs are widely used to treat vascular disorders due to functions of repairing vascular injury and promoting angiogenesis[31]. Cells in early EPCs colonies were observed to be predominantly spindle-shaped, with a few spherical cells in the center after a week of culture (Fig. 1A). The results of cytometry analysis showed that EPCs positively expressed CD34 and VEGFR2, which demonstrated that we had successfully obtained EPCs with higher purity for further studies (Fig.1B).

To investigate the proliferation of MPS on EPCs, and to evaluate whether DMOG could attenuate the cytotoxicity of MPS, CCK-8 assay was examined by the effect of different concentrations of MPS (0, 1, 10, 100, 1000 μM) on EPC viability at various times (24, 48, 72h) of treatment. The results demonstrated that MPS had a dose-dependent effect on EPC viability with the importantly effect at a concentration of 100 μM after 48h treatment (Fig.1C). Next EPCs were pretreatment different concentrations of DMOG (0, 50, 100, 200, 500, 1000μM) for 24h, prior to incubation with MPS (100 μM) for 48h. The results showed that DMOG decreased the cytotoxicity of MPS on EPCs dose dependently below the concentrations of 1000 μM, and 500 μM was the optimum concentration to it (Fig.1D).

DMOG significantly attenuates the inhibitory effect of MPS on the HIF-1α in EPCs

It is important for mobilization, migration and homing of EPCs to the site of vascular injury at early process to facilitate tissue repair[32]. HIF-1α pathway could effectively couple angiogenesis and osteogenesis during bone development and bone repair after injury by its downstream target genes[33]. The results showed that MPS at 1 μM promoted HIF-1α protein expression, while MPS (10, 100, 1000 μM) significantly downregulated the expression of HIF-1α at 48h (Fig.1E, F). To explore the effect of DMOG on the HIF-1α pathway, the expression of HIF-1α in EPCs was detected by western blotting after the treatment with 100μM DMOG for 6h, 12h, 24h and 48h. The results demonstrated that HIF-1α protein expression was increased in a time-dependent manner up until 6h, while a slightly dropped occurred at 24h (Fig. 2A, B). Then different concentrations of DMOG were used for evaluating the optimal concentration to EPCs. The results showed that DMOG at 500 μM significantly promoted the expression of HIF-1α at the same 24h point (Fig. 2C, D).

HIF-1α activation is required for DMOG-mediated anti-MPS effects on EPCs

Our results have demonstrated that DMOG promoted the HIF-1α protein expression. Nevertheless, EPCs chemotactic migration to injury tissue and vessel have to be mediated by chemokines and other secreted

factors. To further elucidate whether these effects of HIF-1α activation are a result of DMOG-mediated anti-MPS in EPCs, we used HIF-1α inhibitor to suppress its protein translation. The western blot results showed that KC7F2 at 40 μM importantly repress HIF-1α protein level activated by DMOG (Fig. 2E, F). Next ELISA assay was performed to detect the secretion levels of VEGF, SDF-1 and NO, which are downstream of HIF-1α and play the significant roles in promoting both the mobilization and the homing of EPCs. There is no doubt that pretreatment of EPCs with DMOG largely attenuated the inhibitory effects of VEGF, SDF-1 and NO secretion by MPS, while the addition of KC7F2 obviously rescued all the promotions of these factors. (Fig. 2G-I).

DMOG alleviates MPS-induced biological disfunctions via HIF-1α in EPCs.

EPCs are the essential basis of promoting vascular repairing and regeneration due to stable biological functions[34]. To assess whether the effect of MPS and DMOG is mediated via the HIF-1α pathway, we first tested the colony formation ability of EPCs. The results showed that DMOG-treated group at 500 μM remarkably enhanced colony-forming units (CFU) of EPCs than MPS group, while inhibitor of HIF-1α significantly suppressed the capability to maintain CFU in EPCs (Figure 3A, B). The migration ability of the DMOG to EPCs was detected by transwell assay and nuclear staining. The results showed that MPS essentially inhibited migration of EPCs, while DMOG attenuated the poor outcome of MPS to EPCs. Loss of expression of HIF-1α by KC7F2 (10 μM) disrupted these effects (Figure 3C, D). To further investigate the angiogenesis potential of DMOG to EPCs, a Matrigel tube formation assay was performed in vitro. In our experiments, it could be observed that the tube structure of DMOG group was richer than that of MPS group that had no loop formation. The total line of tubes, which simulated the start of sprouting of vascular, was calculated more in DMOG group($P<0.001$), while HIF-1α inhibitor KC7F2 compromised structural conformation of normal tube (Figure 3E, F). The development of vascular endothelial cell senescence impairs normal vascular function and contributes to aging-related vascular diseases[35, 36]. In our results, though EPCs pretreated with DMOG showed a remarkably lower senescence rate than the MPS group in β-galactosidase-positive cells, KC7F2 significantly exhibited more senescence-associated cells (Fig. 3F, G). Consequently, based on these results, we could be concluded that activation of HIF-1α have a better capability against biological disfunction by MPS in EPCs.

DMOG reversed osteonecrosis in vivo

The success rates of femoral head osteonecrosis was 83.3% (15/18) at 6 weeks after injection of MPS in rabbits, while the incidence of steroid-induced osteonecrosis dropped to 50% (9/18) in the MPS+DMOG group. Significantly, more osteonecrosis changes (12/15) were found in group treated by KC7F2 injection before initiation of DMOG and MPS treatments. H&E staining showed the diffuse presence of empty lacunae in the trabeculae bone that accompanied and paralleled the complete microvascular embolization, which was replaced by large number of proliferated fibrous tissues (Fig. 4). The MPS+DMOG group showed more trabeculae bone with less empty lacunae surrounded by adipocytes with homogeneous shape. However, In KC7F2 group, microvascular embolism could be seen compared with MPS+DMOG group (Fig. 4).

DMOG promoted osteogenesis and angiogenesis

In this study, toluidine blue staining was performed to detect osteogenesis in the rabbit femoral head. The results showed that obviously enlarged adipocytes accompanied by bone marrow cells with cytolysis were found in MPS group, while the formation of neonatal bone around necrotic bone trabecular appeared, which demonstrated part of the repair process after DMOG treatment, and this therapy potential was collapsed when pretreatment with KC7F2 (Fig. 5A, C). Furthermore, immunofluorescence staining was conducted to test angiogenesis in necrotic area. The results demonstrated that DMOG importantly enhanced number of □-positive new vessels compared with MPS group (Fig. 5B, D), while injection of HIF-1α inhibitor attenuated osteogenic response by DMOG. In a nutshell, HIF-1α pathway activation is required to alleviate MPS-induced osteonecrosis partially through its potential of angiogenesis and osteogenesis in vivo.

DMOG increased the numbers of EPCs directional migration partially via activating HIF-1α in rabbit

To further explore the potential role between DMOG and HIF-1α activation in steroid-induced osteonecrosis of the femoral head, immunohistochemistry staining for HIF-1α was conducted. Obviously, compared to the model group, DMOG significantly decreased expression of the HIF-1α in necrotic region. Nevertheless, a weaker HIF-1α signal surrounded by microvascular embolization area was observed in KC7F2 pre-treated group than DMOG therapy group (Fig. 6A, C). In view of our findings that DMOG significantly upregulated HIF-1α of EPCs in vitro, we next sought to further explore the effects of these results on the mobilization of bone marrow-derived EPCs to a site of circular peripheral blood, then targeting and homing to the ischemic necrotic tissue, which contributed to remodeling of injury blood vessels. Flow cytometric analysis was performed to test number of the circular EPCs (CD34□/VEGFR□ positive cells). The results revealed that rate of the circular EPCs significantly increased after DMOG treatment group compared with HIF-1α inhibitor group in rabbit (Fig. 6E). Besides, compared to MPS group, immunohistochemistry of CD34□ also revealed that the abundance of CD34 positive cells were visible around the necrotic tissue in DMOG group, while KC7F2 addition remarkably repress the number of CD34-positive cells in femoral head (Fig. 6B, D). Together, these results demonstrated that directional migration of EPCs could be activated partially through HIF-1α activation after DMOG treatment, thereby helping to remedy and reverse the progression of steroid-induced osteonecrosis of the femoral head.

Discussion

Glucocorticoids (GCs) are a widely used drug for auto-immune ailments due to its significantly anti-inflammatory activity[37]. Nevertheless, more than 50% of GCs users could make progress obvious bone loss, and about 40% of GCs users could be faced with various degrees of osteonecrosis. The issue of long-term or excessive usage of GCs, resulting in an increasingly rates of steroid-induced osteonecrosis of the femoral head (SONFH), has puzzled humanity for many years. Nevertheless, the etiological hypothesis of SONFH has not been fully elucidated.

Currently, vascular hypothesis seems to be the most convincing one which presumes that impaired micro-vessels and reduced blood flow exist in the occurrence and development of SONFH[38]. The EPCs play an important role in post-natal neovascularization and the functions of repairing injury blood vessels. For instance, transplantation of autologous EPCs could obviously improve neovascularization and tissue repair[39]. Moreover, exosomes secreted from EPCs could significantly stimulate coupling of angiogenesis and osteogenesis. Besides, inhibition of PTEN could protect against steroid induced EPCs apoptosis by targeting mitochondrial apoptosis pathway, and thereby alleviate the development of SONFH[10]. Importantly, our previous findings also show that the number and function of EPCs are damaged in SONFH patients[16, 17]. Therefore, EPCs may serve as a promising therapeutic target for the prevention and therapy of SONFH.

HIF-1α is considered the most essential transcriptional regulator that mediates endothelial energy metabolism, cell proliferation and survival in response to hypoxia [40, 41]. Recent research has shown that the important role in development of SONFH, while other study reported that HIF-1α appears to be less substantial for the prevention and therapy of SONFH[42, 43]. It is possible, even likely, that high expression level of HIF-1α occurred, yet relatively decreased HIF-1α expression at the late phase of osteonecrosis[44]. Therefore, the relationship between HIF-1α and SONFH remains incompletely understood, especially in EPCs.

Under normoxia conditions, HIF-1α is hydroxylated by PHD and then rapidly degraded through the ubiquitin-proteasome pathway, while in low oxygen conditions, PHD block hydroxylation owing to its decreased activity, thus resulting the stabilization and activation of HIF-1α. Interestingly, DMOG, inhibitor of PHD, could stabilize HIF-1α and mimic true hypoxic conditions, which in turn participates in repair of the ischemic associated diseases[45]. Previous studies have shown the therapeutic potential of DMOG in osteoporosis and osteoarthritis[28, 46]. Furthermore, DMOG has been widely used in bone tissue regeneration engineering for its excellent bone repair capability, thereby coupling angiogenesis and osteogenesis[47]. Nevertheless, the effect of DMOG in SONFH has rarely been studied, especially in EPCs.

In our current results, we have shown that the biological functions of EPCs is increased profoundly by treatment with DMOG. Although DMOG at 1000 μM presented a certain degree of restriction for EPCs, the cell viability and proliferative capability of EPCs were obviously enhanced in DMOG treatment at 50–500 μM in a time- and concentration-dependent manner. SDF-1 could improve endothelial cells mobilization, migration, homing, and angiogenesis partially via SDF-1/CXCR4 axis under ischemic conditions[48]. In our present results, we found that DMOG induced the protein expression of HIF-1α accompanied by increased downstream targets secretion of VEGF, SDF-1 and NO, which may promote the migration of EPCs through the bone marrow to the sites of ischemic femoral head, thereby directly or indirectly contributing to the formation of new blood vessels. However, we proved these effects could be attenuated by HIF-1α inhibitor KC7F2. Besides, DMOG treatment not only promoted the chemotactic motility and the colony-forming ability of EPCs, which was the direct indicators of reendothelialization and neovasculization, but also delayed EPCs senescence induced by MPS. Altogether, we proved that activation of HIF-1α by DMOG is

indispensable against MPS-induced impairment for EPCs through repressing HIF-1α signaling pathway by KC7F2 in vitro.

The damaged function of vascular endothelial cell caused by glucocorticoids is believed the essential causes of SONFH[16]. Our data in vivo was consistent with previous studies, which revealed that MPS treatment significantly promoted the osteonecrosis rate in rabbit as evaluated by H&E staining. Nevertheless, the damaged micro-vessels could be alleviated after DMOG injection therapy in rabbit SONFH models. Immunofluorescence also found DMOG induced the significantly increased number of neo-vessels. Toluidine blue staining showed that DMOG obviously increased the number of osteoblasts in the femoral head necrosis area. This finding is in sharp contrast to a great deal of empty bone lacuna by MPS treatment. Furthermore, the enhanced number of EPCs were detected by flowcytometry and immunohistochemistry staining, whether in necrosis areas or circulating blood. Nevertheless, rabbit pre-treatment with HIF-1α inhibitor significantly disrupted DMOG protective effects on MPS-induced ischemic osteonecrosis. In conclusion, our findings confirmed that targeting the activation of HIF-1α in EPCs by DMOG might be a promising therapy for SONFH.

In the present research, we illustrated the protective effects of DMOG in MPS-induced ONFH in rabbit. Our studies in vitro and vivo confirmed that HIF-1α activated by DMOG may play a highly significant role in SONFH. Inevitably, there are still some limitations to our present study. For instance, mechanisms about HIF-1α activation are limited in its downstream signaling molecule SDF-1, VEGF and NO caused by DMOG. Further experiments of these potential mechanisms targeting HIF-1α signaling pathway might be of great value. In addition, owing to the incompletely consistent mechanisms between rabbit-derived and human-derived EPCs, further sufficient clinical samples of EPCs should be conducted to better account for DMOG effects in our studies.

Conclusion

In conclusion, we successfully proved the activation of HIF-1α signaling pathway by DMOG could be a promising EPCs-based therapeutic strategy for the prevention and development of SONFH in rabbit, which is also significant for the drug-based strategy of tissue engineering and regenerative medicine.

Abbreviations

SONFH, steroid-induced osteonecrosis of the femoral head; GCs, Glucocorticoids; HIF-1α, Hypoxia inducible factor-1α; PHD, prolyl hydroxylase domain; DMOG, Dimethyloxalylglycine; EPCs, endothelial progenitor cells; MPS, Methylprednisolone.

Declarations

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Author contributions

YF and XL conceived and designed the study. WS and ZL performed the experiments. BW and SG performed the data analysis. WS wrote the manuscript. LH and ZC helped in designing the figures. XL contributed to manuscript editing. YF supervised the study. The final manuscript reviewed and approved by all the writers and participants.

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Availability of data and materials

All data generated or analysis during this study are included in this published article.

Ethical statement

The present study was reviewed and approved by the Research Ethics Commission of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology.

Consent for publication

The authors have all given their permission for it to be published.

Conflict of interests

No potential conflict of interest was reported by the authors.

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Figures

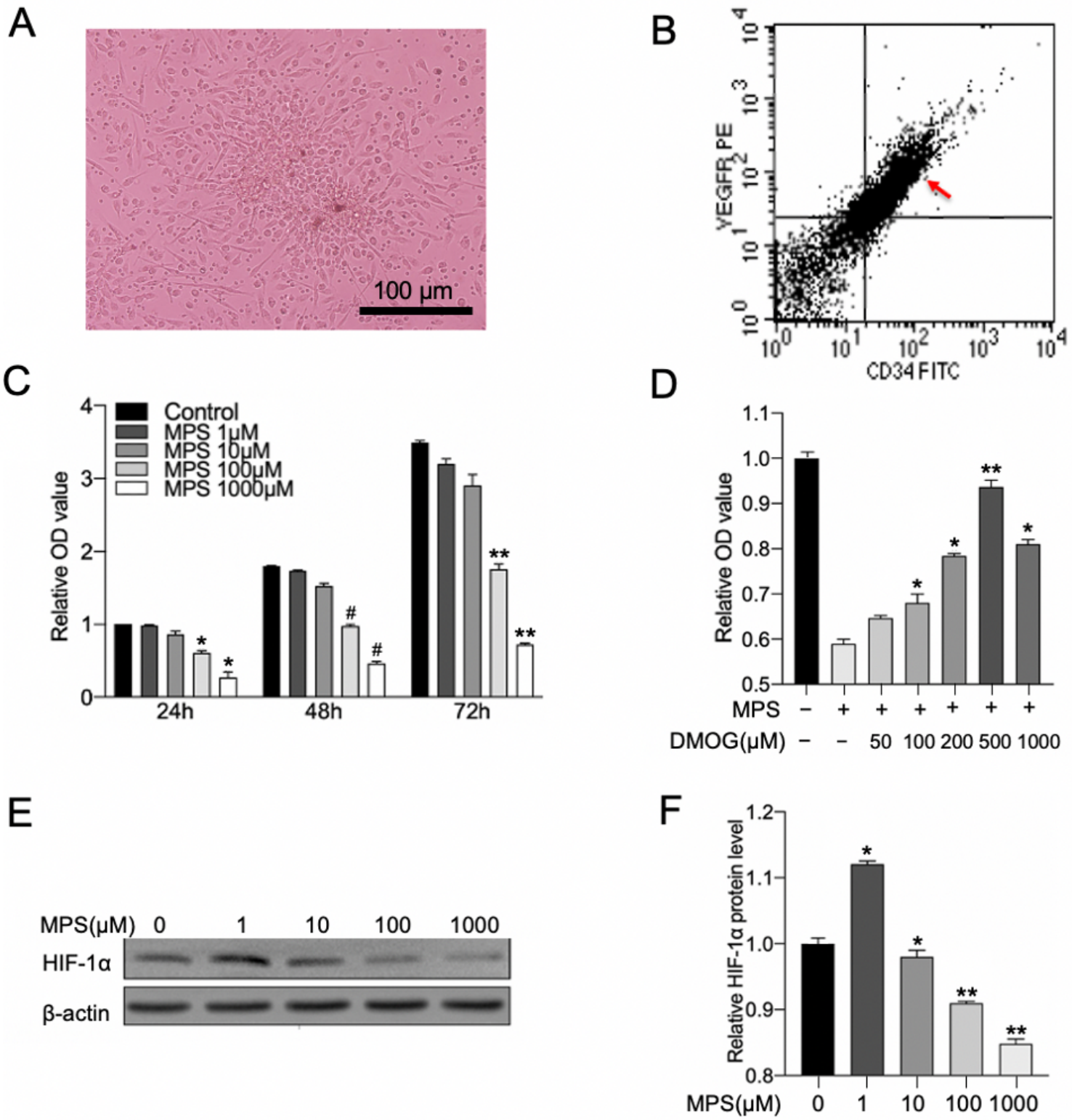

Figure 1

DMOG essentially alleviates the Glucocorticoids-induced cytotoxicity on EPCs. (A) Representative cell morphology of EPCs under the Light source field. (B) The flow cytometry analysis demonstrated that EPCs positively express VEGFR and CD31 marker. (C) The cytotoxicity of methylprednisolone (MPS) was evaluated by CCK-8 assay with various concentrations and different time points (**p < 0.05 versus control group at 24 h; #p <0.05 versus control group at 48 h; **p < 0.05 versus control group at 72 h*)(**p < 0.05; #p*

<0.01 versus control group). (D) DMOG significantly attenuated cytotoxicity of MPS (100 μM) on EPCs at different concentrations (0, 50, 100, 200, 500, 1000 μM) for 48 h(**p < 0.05 versus MPS group; **p <0.01 versus MPS group*). (E-F). HIF-1α protein expression was determined using western blot at 48 h. The band density of HIF-1α was normalized to β-actin (**p < 0.05 versus control group; **p < 0.01 versus control group*). These results were performed at least three biological replicates. Error bar represents SD.

Figure 2

HIF-1α activation is warranted for DMOG-mediated anti-Glucocorticoids effects on EPCs. (A, D) DMOG (100 μM) effectively promoted HIF-1α protein levels at the time points of 2, 4, 6, 24 h by western blot analysis. (**p < 0.05 versus DMOG group at 2 h*) (B, E) DMOG induced HIF-1α protein expression below the 500 μM in a concentrations-dependent manner by western blot analysis. (**p < 0.05 versus DMOG group at 100 μM*) (C, F) Increased HIF-1α protein content was attenuated by western blot analysis after KC7F2 (40 μM) administration (**p < 0.05 versus control group; #p <0.01 versus MPS group; **p < 0.05 versus MPS + DMOG group*). (G-I) NO, VEGF, and CXCL12 secretion levels in the culture medium supernatant as analyzed by ELISA (**p < 0.05 versus control group; #p <0.01 versus MPS group; **p < 0.05 versus MPS + DMOG group*). These results were performed at least three biological replicates. Error bar represents SD.

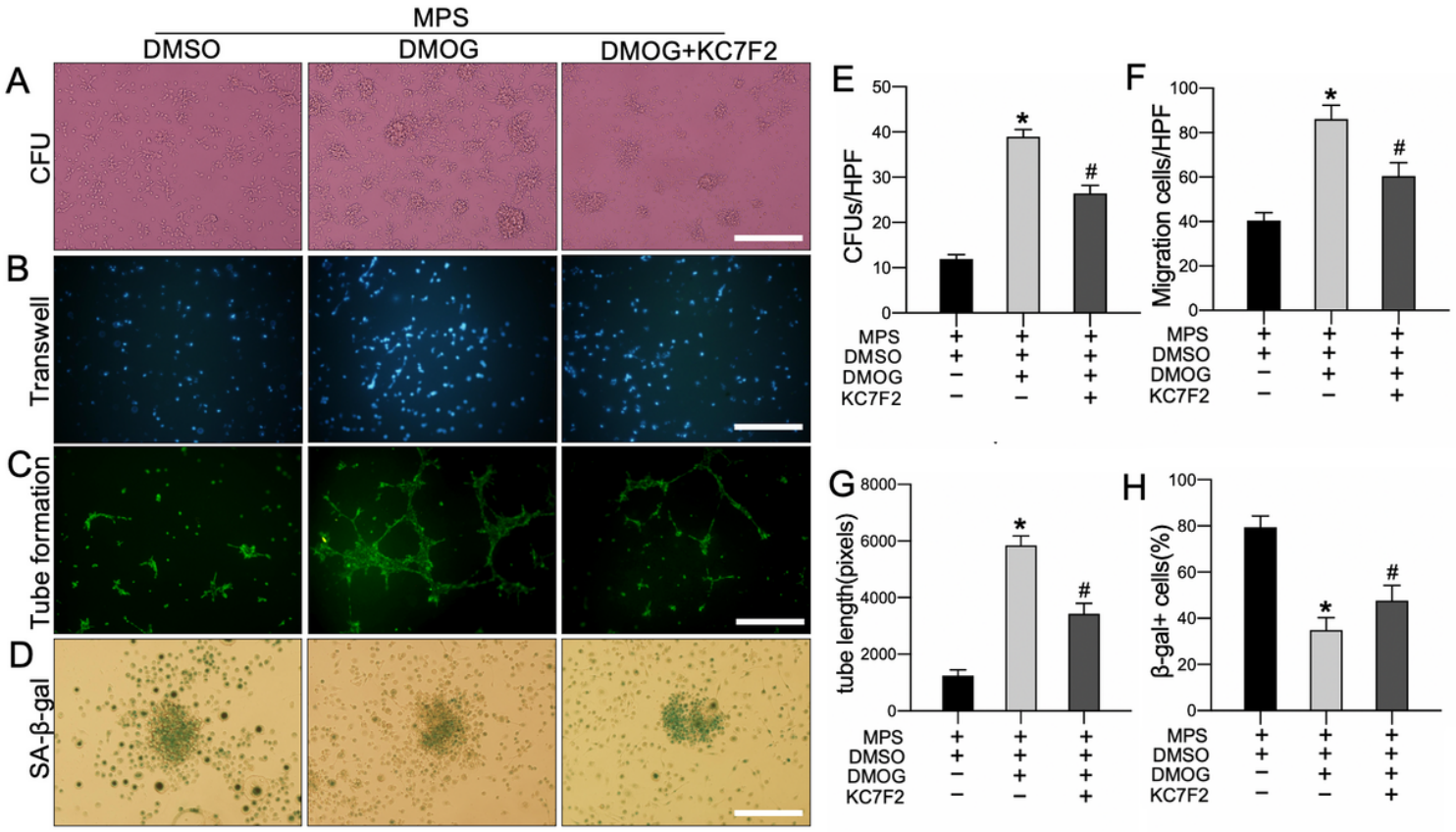

Figure 3

DMOG fulfilling its protective role in MPS-induced biological disfunctions via HIF-1α signaling pathway in EPCs. (A, E) The numbers of EPCs were captured by CFU assay. (B, F) Transwell assay results of EPCs for different treatment groups. (C, G) Tube formation ability of EPCs after DMOG (500 μM, 6h) treatment or DMOG combined with KC7F2 (40 μM) for 24h. (D, H) SA-β-gal assay for detection of EPCs senescence. (**p < 0.05 versus MPS + DMSO group; #p <0.01 versus MPS + DMOG group*). These results were performed at least three biological replicates. Error bar represents SD. Scale bar = 100 μm.

Figure 4

Histological staining of the femoral head in rabbits. (A) Representative images of the subchondral bone of the rabbit femoral head in different groups. The fully microvascular embolism could be observed next to the discrete bone trabecular with large number of empty bone lacunae in MPS group. The MPS+DMOG group demonstrated less embolized micro-vessel, while KC7F2 administration further aggravated osteonecrosis. (B) Enlarged local radiographic images were captured to visualize the local preferred orientation. Scale bar = 200 μm.

Figure 5

DMOG promotes osteogenesis and angiogenesis. (A, C) toluidine blue staining of the femoral head in different groups. Comparison of the area of new bone in different groups. (B, D) Immunofluorescence images showed new formed blood vessels. Comparison of the blood vessel density in different group. (**p < 0.05 versus MPS + DMSO group; #p <0.05 versus MPS + DMOG group*). These results were performed at least three biological replicates. Error bar represents SD. Scale bar = 200 μm.

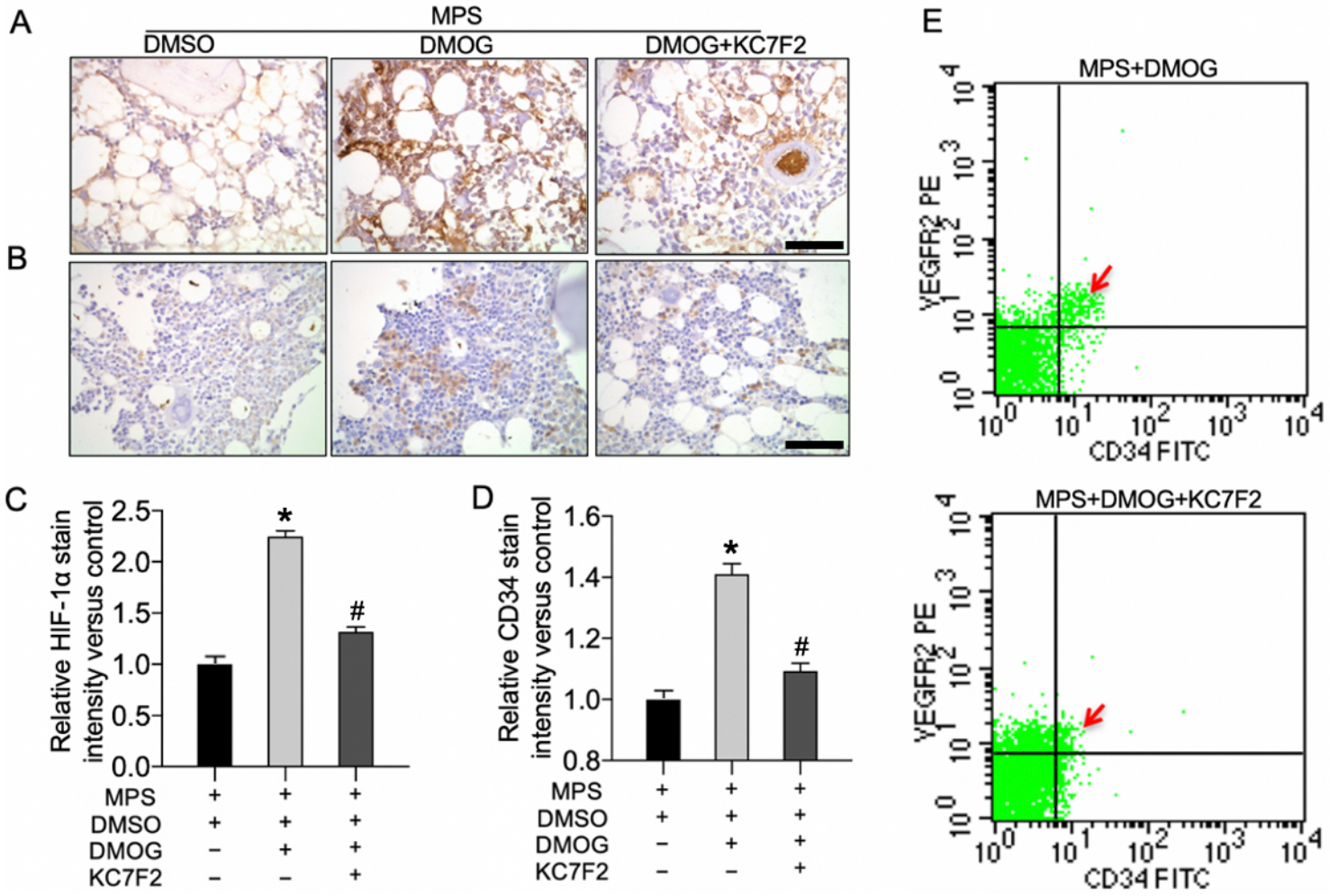

Figure 6

DMOG increased the level of HIF-1α and motived EPCs. (A, C) Immunohistochemistry staining for HIF-1a showed a higher level of HIF-1α in MPS + DMOG than MPS + DMSO group, while MPS + DMOG + KC7F2 group significantly decreased HIF-1α expression. (B, D) Immunohistochemistry detected the number of CD34 positive cells in necrotic tissue in different groups. (E) Flowcytometry detected EPCs in the peripheral blood in different groups. (*$p < 0.05$ versus MPS + DMSO group; #$p < 0.05$ versus MPS + DMOG group*). These results were performed at least three biological replicates. Error bar represents SD. Scale bar = 200 μm