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Gastrodin improves osteoblast function and adhesion to titanium surface in a high glucose environment

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ABSTRACT

Objective: To investigate the effects of gastrodin on the biological behavior of osteoblasts and osseointegration on the surface of the titanium plate in a high glucose environment, and to explore the possible regulatory mechanisms involved.

Methods: A high glucose-induced oxidative damage model of MC3T3-E1 cells was established *in vitro* to observe the effects of gastrodin on cellular oxidative stress, cell viability, osteogenic differentiation, mineralization, migration, and adhesion ability on the titanium surface.

Results: High glucose environment can cause oxidative stress damage to MC3T3-E1 cells, leading to a decrease in cell viability, osteogenesis, migration, adhesion and other functions. Gastrodin can upregulate the expression of antioxidant enzymes (Nrf2 and HO-1) and osteogenic differentiation related proteins (RUNX2 and BMP2) in MC3T3-E1 cells in high glucose environment, thereby inhibiting the excessive production of intracellular reactive oxygen species (ROS), reversing the decrease in cell viability, and improving the osteogenic differentiation and mineralization ability of osteoblasts. And gastrodin alleviated the decline in cell migration ability, improved the morphology of the cytoskeleton and increased the adhesion ability of osteoblasts on the surface of titanium plates in high glucose environment. However, gastrodin itself did not affect the cell viability, osteogenic differentiation and mineralization ability of osteoblasts in normal environment.

Conclusions: Gastrodin may protect MC3T3-E1 cells osteogenesis and osseointegration on the surface of the titanium plate *in vitro* by upregulating antioxidant enzymes expression, and attenuating high glucose-induced oxidative stress. Therefore, gastrodin may be a potential drug to address the problem of poor implant osseointegration in patients with diabetes.

1. Introduction

Diabetes is becoming a global epidemic [1]. Type 2 diabetes mellitus (T2DM) is the most common form of diabetes, accounting for 90–95% of all cases and is expected to increase to 439 million cases globally by 2030 [2,3]. Diabetic patients are often susceptible to osteoporosis [4]. In addition, they have a higher incidence of periodontitis and tooth loss than healthy people [5]. And oral implants are becoming the increasingly popular choice for restoration of tooth loss [6]. Therefore, the clinical demand for implant restoration in diabetic patients with tooth loss is increasing rapidly [3]. However, as a relative contraindication of oral implant restoration, patients with T2DM are often accompanied with poor bone quality and volume, high incidence of postoperative complications and low long-term survival rate of implants [3,5].

More and more studies showed that oxidative stress plays a key role in the development of diabetes. The hyperglycemia causes mitochondrial dysfunction, resulting in excessive reactive ROS production [7]. In diabetic patients, ROS levels and oxidative stress levels are significantly increased, and oxidative stress is also considered as a major marker for the development and progression of T2DM [8]. ROS inhibits proliferation and differentiation of osteoblast, and induces autophagy and apoptosis, ultimately leading to a decrease in osteogenesis [7,8]. In addition, ROS inhibits cell migration and adhesion by damaging the function of mitochondria and filaments in the cytoskeleton [9]. In the face of oxidative stress damage, the defense within the organism mainly includes antioxidant enzymes, supply of their substrates and repair of injury [10]. In fact, most of the antioxidant defense within cells is achieved by reducing the production of oxidants through antioxidant

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enzymes and their substrates, thereby inhibiting the oxidative damage caused by oxidants to biological macromolecules such as proteins, carbohydrates, lipids, and DNA [11]. At present, antioxidant treatment strategies include clearing oxygen free radicals, increasing the synthesis of antioxidant enzymes, especially by activating Nrf2, enhancing mitochondrial antioxidant defense, supplementing dietary antioxidants, and so on [12].

Gastrodin is extracted from the dried tubers of *gastrodia elata*, a Chinese herb, which has antioxidant, anti-inflammatory and anti-apoptosis effects [13]. Its main active ingredient is 4-hydroxybenzyl alcohol-4-*O*-*β*-D-glucopyranoside, with the molecular formula C13H18O7 and a molecular weight of 286 Da. Gastrodin has the advantages of multi-site action, multi-targeted action, high safety, large dosing space, less toxic side effects, systemic conditioning and low acquisition cost [14]. At present, gastrodin is mainly used to treat nervous system diseases. In recent years, studies have found that gastrodin can reverse the osteoblast oxidative damage induced by glucocorticoids [15] and the human bone marrow mesenchymal stem cell oxidative damage induced by hydrogen peroxide, and improve osteoporosis in ovariectomized mice [16]. However, the effect of gastrodin on osteoblast in high glucose environment, is not clear. Under the condition of simulated hyperglycemia, to explore the effect of gastrodin on osteoblast osseointegration has important guiding significance for implant restoration in diabetic patients.

Therefore, in this study, a model of oxidative damage of MC3T3-E1 osteoblast induced by high glucose was established. The effects and possible mechanism of gastrodin on the function of MC3T3-E1 cells and its osseointegration on the surface of titanium plate were studied *in vitro*, in order to provide a new therapeutic basis for solving the problem of poor implant osseointegration in diabetic patients. To our knowledge, this is the first experimental study to explore effects of gastrodin on osteogenesis and osseointegration of osteoblast in an *in vitro* diabetic model.

2. Materials and methods

2.1. Materials

Gastrodin (IG0580) was purchased from Solaibao (Beijing, China). Glucose (47,249) was purchased from Sigma-Aldrich (USA). The custom-made round titanium plates were provided by the Northwest Institute of Nonferrous Metals, China. Antibodies of nuclear factor erythroid 2-related factor 2 (NRF2) (12,721) and runt-related transcription factor 2 (RUNX2) (12,556) were purchased from Cell Signaling Technology (USA). Antibodies of heme oxygenase 1 (HO-1) (ab68477) and bone morphogenetic protein 2 (BMP2) (ab214821) were purchased from Abcam (UK). Antibodies of β-actin was purchased from Abways (Shanghai, China). Reactive oxygen determination kit (E004-1-1) was purchased from Nanjing Jianchengbio (Najing, China). Cell counting kit-8 (CCK-8) (CA1210), Alizarin Red S solution (G1450), cetylpyridinium chloride monohydrate (IC0280), alkaline phosphatase (ALP) activity assay kit (BC2145), BCA protein assay kit (PC0020), 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (C0065) tetramethyl isothiocyanate rhodamine (TRITC) phalloidin (CA1610) and modified hematoxylin-eosin (H&E) stain kit (G1121) were purchased from Solaibao (Beijing, China). Experimental instruments include microplate reader (BioTek, ELX5018, USA), flow cytometry NovoCyte™1R2D (Aceabio, Hangzhou, China), ChemiDOC^{TM}XRS + imaging system (Bio-Rad, USA), inverted microscope (Leica, DM IL LED, Germany), Confocal microscope (Leica, TCP SP8 X, Germany) and stereomicroscope (Nikon, SMZ745T, Japan).

2.2. Cell culture and osteogenic differentiation

MC3T3-E1 cells (subclonal 14 cell line of mouse anterior cranial bone cells) were purchased from Saiye Biology, Guangzhou, China. And the handling and disposal of the cells were correctly performed, to avoid hazardous events that may result from improper handling and disposal. MC3T3-E1 cells were cultured at 37 °C in a 5% CO2 incubator (Thermo, USA) using α-minimum essential medium (α-MEM) (Hyclone, USA) containing 10% heat-inactivated fetal bovine serum (Cellmax, Beijing, China) and 1% penicillin and streptomycin (Solaibao, Beijing, China). And the glucose concentration is 5.5 mM in α-MEM. Osteogenic induction medium was supplemented with 10 mM sodium β-glycerophosphate, 50 μg/mL ascorbic acid, and 100 μM dexamethasone, which were all purchased from Solaibao, Beijing, China, for the induction of osteogenic differentiation of MC3T3-E1 cells [17].

25 mMD-glucose concentration is usually used to simulate blood glucose levels in patients with diabetes, which was often used as the high glucose condition for the culture of MC3T3-E1 cells [15,18,19], while 5 μM concentration of gastrodin was mostly used as an intervention condition [15]. In addition, in our previous study [20] gastrodin improved redox status in type 2 diabetic rats model and microstructural indices of peri-implant bone trabeculae. Based on the above studies, we used 25 mM D-glucose as a high glucose condition to induce oxidative stress damage in MC3T3-E1, and 1 μM and 5 μM concentrations of gastrodin were used to intervene in the cells to confirm the effect of gastrodin on function and osteointegration of osteoblast in a high glucose environment.

2.3. Preparation and handling of titanium plates

The custom-made round titanium plates were provided by the Northwest Institute of Nonferrous Metals, China. The titanium plates were 0.8 mm thick and 12 mm in diameter, polished in the order of 120, 400, 600, 800, 1000, and 1200 mesh using silicon carbide sandpaper (Maike, Hubei, China). And then titanium plates were ultrasonically cleaned with acetone (Zhi Yuan Chemical Reagents, Tianjing, China), anhydrous ethanol (purity: >99.5%) (Zhi Yuan Chemical Reagents, Tianjing, China), and ultra pure water in turn. Then they were autoclaved and dried in preparation for subsequent experiments [21].

2.4. CCK-8 assay

CCK-8 (Solaibao, Beijing, China) was used to estimate cell viability. To evaluate the effect of gastrodin (Solaibao, Beijing, China) alone on the viability of MC3T3-E1 cells, we seeded the cells in 96-well plates at 1 × 10^3 cells/well overnight. The experimental groups were treated with 1, 5, 10 and 20 μM of gastrodin for 1 and 3 days [15,22,23]. And the glucose concentration is 5.5 mM in α-MEM. The absorbance of wells at 450 nm was measured by microplate reader (BioTek, ELX5018, USA), after incubation with CCK-8 solution at 37 °C for 1 h.

Cells were then seeded in the same way as above, except that cells were treated with 25 mM D-glucose and gastrodin for 1 and 3 days, to investigate the effect of gastrodin on the viability of MC3T3-E1 cells in a high glucose environment.

2.5. ROS detection

MC3T3-E cells were seeded in 6-well plates (1 × 10^5 cells/well), incubated overnigt. Gastrodin and high glucose was added for 24 h. The cells were collected, loaded with 2′,7′-dichlorofluorescin diacetate, which is the main component in reactive oxygen determination kit (Nanjing Jianchengbio, Najing, China), washed with phosphate buffered saline (PBS) (Meilunbio, Dalian, China), and detected by flow cytometry NovoCyte™1R2D (Aceabio, Hangzhou, China) for intracellular reactive oxygen content [15].

2.6. Western blot analysis

Western blot analysis was performed as previously described [15]. Briefly, MC3T3-E1 cells were treated with 5 μM gastrodin or 25 mM

D-glucose in osteogenic induction medium for 3 days, and the cells were rinsed twice in ice-cold PBS. With lysis solution (Solaibao, Beijing, China) and protease inhibitor (MCE, USA) added, the cells were scraped from 6-well plates and left on ice for 30 min. The supernatant was collected by centrifugation for 20 min at 4 °C, 12,000 rpm. The BCA protein assay kit (Solaibao, Beijing, China) was performed to quantify the protein. Specifically, protein samples were gradient diluted and measured the absorbance value at 562 nm. Then the protein concentration was calculated based on the plotted standard curve. Loading buffer was added to the protein samples, mixed well, denatured at 100 °C for 10 min. Then the protein samples were frozen at −80 °C, or directly used for experiments. Protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (8%–10%) (BOSTER, Wuhan, China) and electrophoresistically transferred by wet to a polyvinylidene fluoride membrane (Millipore, USA). After blocked by protein dry powder (BOSTER, Wuhan, China) for 2 h, the membranes were incubated with the specific primary antibodies, including NRF2, RUNX2 (Cell Signaling Technology, USA), BMP2, HO-1 (Abcam, UK) and β-actin (Abways, Shanghai, China) overnight at 4 °C, followed by HRP conjugated affiniPure goat anti-rabbit IgG (BOSTER, Wuhan, China) for 1 h at room temperature. The signals were visualized after exposure to the hypersensitive ECL chemiluminescence substrate (BOSTER, Wuhan, China) with the ChemiDOC^{TM}XRS + imaging system (Bio-Rad, USA). The levels of protein expression were analyzed with ImageJ software (National Institutes of Health, USA).

2.7. Alizarin red S staining and quantitative detection

1×10^5 MC3T3-E1 cells/well were seeded in 6-well plates and cultured overnight. Then the culture medium was replaced with osteogenic induction medium. Then cells were treated with gastrodin and 25 mM D-glucose. After 14 days of osteogenic induction, the cells were observed for osteogenic mineralization using Alizarin Red S staining (Solaibao, Beijing, China). Paraformaldehyde was used to fix the cells, followed by staining with alizarin red solution for 10 min. After washing 3 times, the cells were observed under an inverted microscope (Leica, DM IL LED, Germany) and photographed for documentation. A cetylpyridinium chloride monohydrate (Solaibao, Beijing, China) 10% solution was added to decolorize the calcified nodules for 30 min, and the absorbance was recorded by microplate reader (BioTek, ELX5018, USA) at a wavelength of 562 nm [24].

The cells were seeded as above. Then the cells were cultured in normal glucose concentration and treated with 5 μM gastrodin. Alizarin Red S staining and semi-quantitative analysis were used to evaluate the effect of gastrodin itself on the mineralization function of osteoblasts.

2.8. ALP activity assay

Each group of cells was seeded in 6-well plates at a density of 1×10^5 cells/well overnight. α-MEM medium was replaced with osteogenic induction medium, and the cells were treated for 7 days by adding gastrodin and high glucose. Thereafter, the cells were fragmented using ultrasound, and the supernatant was collected; After ALP activity assay kit (Solaibao, Beijing, China) processing, the absorbance values were measured at 510 nm on the microplate reader (BioTek, ELX5018, USA); The BCA protein assay kit (Solaibao, Beijing, China) was used to determine the total protein concentration. Intracellular ALP activity was calculated according to protein concentration, thus evaluating the osteogenic differentiation level of osteoblasts [25].

The cells were seeded as above. Then the cells were cultured in normal glucose concentration and treated with 5 μM gastrodin. The effect of gastrodin itself on osteogenic differentiation of osteoblasts was evaluated according to the intracellular ALP activity.

2.9. Cell migration assay

MC3T3-E1 cells were seeded in a 6-well plate. Scratch assay was carried out when the cell growed to confluence and reached the growth inhibition [26]. Three straight lines per well were scratched vertically by 200 μl plastic tip. PBS washed the cells three times to remove the detached cells. Then the cells were treated with gastrodin and 25 mM D-glucose for 24 h. After PBS washing, the gap closure of the scratched area was observed under microscope (Leica, DM IL LED, Germany). And the gap closure area of each group at 0 h and 24 h was analyzed by ImageJ software (National Institutes of Health, USA) to quantify the level of cell migration.

2.10. Cell adhesion assay on titanium surface

Titanium plates prepared in advance were placed at the bottom of 24-well plates and seeded with cells at a density of 1×10^4 cells/dish. The cell culture medium contained 5% serum. After treatment with gastrodin and high glucose for 24 h, the cells were fixed and treated with Triton X-100 (Solaibao, Beijing, China) for 10 min. Next, DAPI (Solaibao, Beijing, China) was added, and the mixture was incubated for 5 min in the dark. The titanium plates were then removed. The cells were observed and photographed under a Confocal microscope (Leica, TCP SP8 X,Germany) with 3 fields of view per well. The ImageJ software (National Institutes of Health, USA) was used to count the cells [21].

The cells were seeded and treated with drugs for 24 h as described above. After incubated with TRITC phalloidin (Solaibao, Beijing, China) for 30 min in the dark, the cells were incubated with DAPI for 5 min. The cytoskeletal morphology was observed under a confocal microscope (Leica, TCP SP8 X,Germany). Then the average number of pseudopods per cell was quantified [27].

The cells were seeded and treated with drugs for 24 h as described above. The cells were then fixed with paraformaldehyde. The cells were stained using a modified H&E stain kit (Solaibao, Beijing, China) and observed under a stereomicroscope (Nikon, SMZ745T, Japan); 3 randomly selected fields of view were photographed in each well. And the H&E staining area was calculated by ImageJ software. (National Institutes of Health, USA) [27].

2.11. Statistical analysis

Data are expressed as the mean ± SD of the three experiments. The Shapiro-Wilk method tested data conforming to the normal distribution, and data were compared using one-way ANOVA and multiple comparisons were performed using Dunnett's method. Statistical significance was set at $p < 0.05$. Graphpad Prism software (version 9.0) was used to plot the data.

3. Results

3.1. Effect of gastrodin on the viability of MC3T3-E1 cells

MC3T3-E1 cells were cultured for 1 and 3 days in normal glucose concentrations with the addition of gastrodin in each experimental group, and there was no significant effect on osteoblast survival in the set concentration range ($P > 0.05$) (Fig. 1(a) and (b)). Furthermore, MC3T3-E1 cell viability was significantly reduced after 1 and 3 days of culture in a high glucose environment ($P < 0.05$); whereas the intervention of gastrodin (5 μM) resulted in increased cell survival on days 3 ($P < 0.05$) (Fig. 1(c) and (d)), suggesting that gastrodin attenuated the cytotoxicity of high glucose on MC3T3-E1.

3.2. Effect of gastrodin on oxidative stress in MC3T3-E1 cells in a high glucose environment

To elucidate whether the effects of gastrodin on cells under high

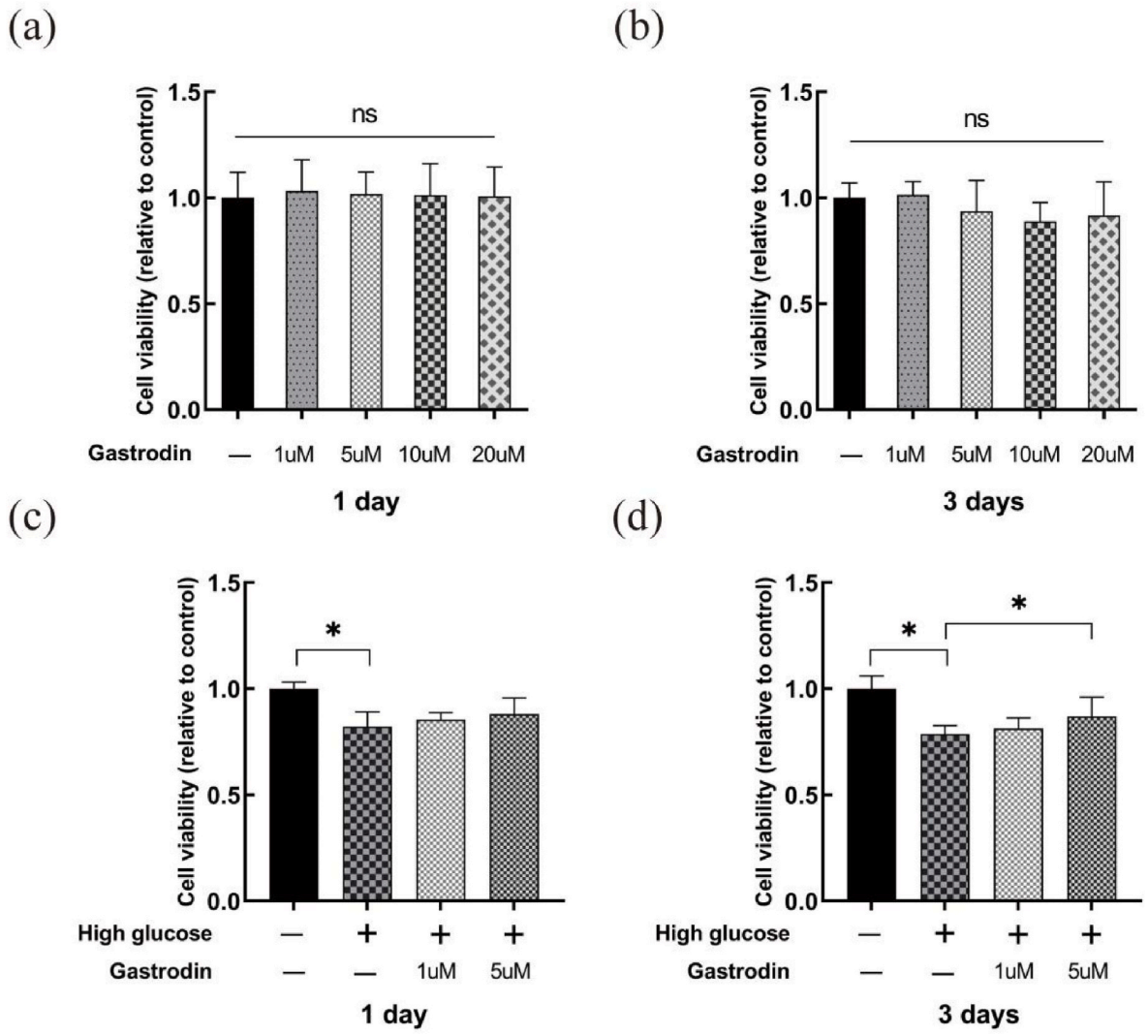

Fig. 1. Effect of Gastrodin on toxicity of MC3T3-E1 cells in high glucose environment. (a, b) In the 5.5 mM glucose environment, MC3T3-E1 cells were cultured with different concentrations of gastrodin alone. (c, d) MC3T3-E1 cells were pretreated gastrodin for 2 h before treatment with 25 mM glucose. Each value is reported as the mean ± standard deviation of three independent experiments. $n = 9$, $^{*}P < 0.05$.

glucose induction were related to its antioxidant properties, intracellular ROS production was assayed. The flow cytometry results showed that the intracellular ROS content increased and the peak shifted to the right in the high glucose group compared to the normal group ($P < 0.05$), indicating that oxidative stress occurred in cells induced by high glucose. Gastrodin reduced intracellular ROS levels and showed antioxidant effects ($P < 0.05$) (Fig. 2(a) and (b)). Meanwhile, Western blot analysis showed that, in high glucose environment, 5 μM gastrodin upregulated the expression of NRF2 and HO-1 in MC3T3-E1 cells and enhanced cellular antioxidant defense mechanism ($P < 0.05$) (Fig. 2(c), (d), and 2(e)).

3.3. Effect of gastrodin on the osteogenic differentiation and mineralization of MC3T3-E1 cells in high glucose and normal environment

MC3T3-E1 cells were treated with 25 mM D-glucose for 3 days, and showed a decrease in the expression of BMP2 ($P < 0.05$) and an insignificant decrease of RUNX2 ($P = 0.16$). With the increased expression of antioxidant proteins (NRF2 and HO-1) ($P < 0.05$),5 μM gastrodin reversed this inhibition of BMP2 and RUNX2 ($P < 0.05$) (Fig. 2(c), (f), 2(g)). Furthermore, The Alizarin Red S staining and ALP activity assay kit were used to assess osteogenic differentiation and mineralization, respectively, and the presence of high glucose markedly reduced the osteogenic and mineralized potential of MC3T3-E1 cells ($P < 0.05$). However, Alizarin red S staining showed that cells in the gastrodin-treated group formed large and an increased number of connected mineralized nodules compared to the high glucose group, increasing the osteogenic mineralization capacity of the cells ($P < 0.05$) (Fig. 3(a) and (b), 3(c)). And gastrodin reversed the reduction of ALP activity in the high glucose environment ($P < 0.05$) (Fig. 3(d)). However, under the normal glucose concentration condition, gastrodin itself did not affect the osteogenic mineralization and differentiation of cells ($P = 0.58$; $P = 0.70$) (Fig. 3(e), (f), 3(g), 3(h)).

3.4. Effect of gastrodin on the migratory capacity of MC3T3-E1 cells in a high glucose environment

The level of cell migration was detected by scratch assay, and the results showed that most of the gap area in the normal group was covered by migrated cells after 24 h. Compared with the normal group, the gap closure rate of high glucose group decreased significantly ($P < 0.05$). However, the treatment of 5 μM gastrodin increased the gap closure rate and improved the cell migration ability ($P < 0.05$) (Fig. 4(a) and (b) and 4(c)).

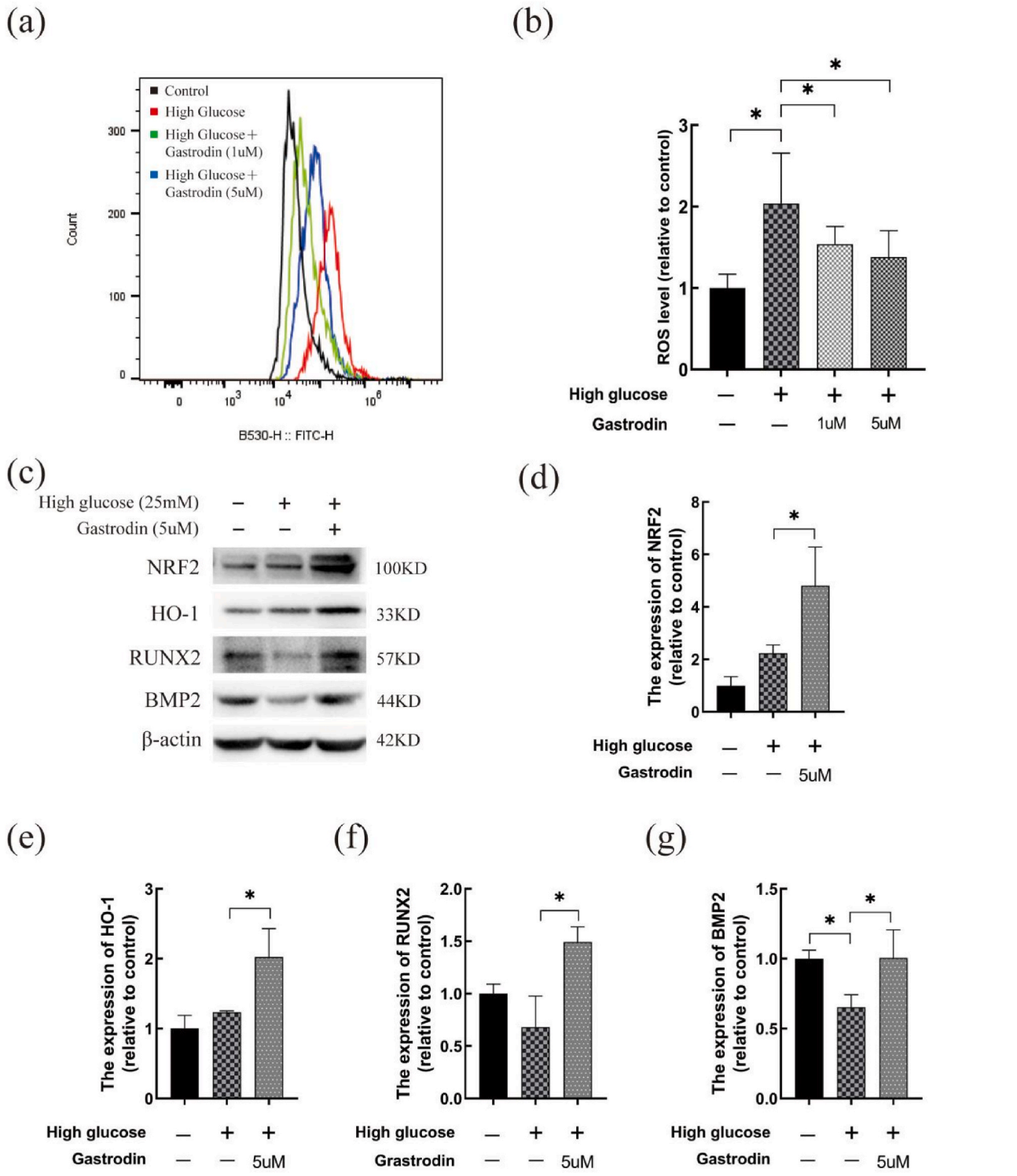

Fig. 2. Effect of gastrodin on oxidative stress and osteogenic differentiation of MC3T3-E1 cells in high glucose environment. (a, b) Detection of ROS Levels in MC3T3 E1 cells by flow cytometry, after treatment with gastrodin and high glucose for 24 h (c, d, e, f, g) Effects of gastrodin in expression of antioxidant enzymes (NRF2, HO-1) and osteogenic differentiation-related proteins (BMP2, RNNX2) in MC3T3-E1 cells during high glucose environment. Each value is reported as the mean ± standard deviation of three independent experiments. $N = 9$, $^*P < 0.05$.

3.5. Effect of gastrodin on the adhesion capacity of MC3T3-E1 cells on titanium surfaces in a high glucose environment

After drug treatment to MC3T3-E1 cell for 24 h, DAPI staining was performed, and the number of adherent cells to the titanium surface decreased in the high glucose group ($P < 0.05$), whereas it increased in the gastrodin (5 μM) treated group compared to that in the high glucose group ($P < 0.05$) (Fig. 5(a) and (d)). TRITC phalloidin staining was performed 24 h after drug treatment. Most cells in the high glucose group were round in morphology and smaller in size, and only a few spindle-shaped cells extended shorter pseudopods; in contrast, most cells in the treatment group were dendritic and spindle-shaped, larger in

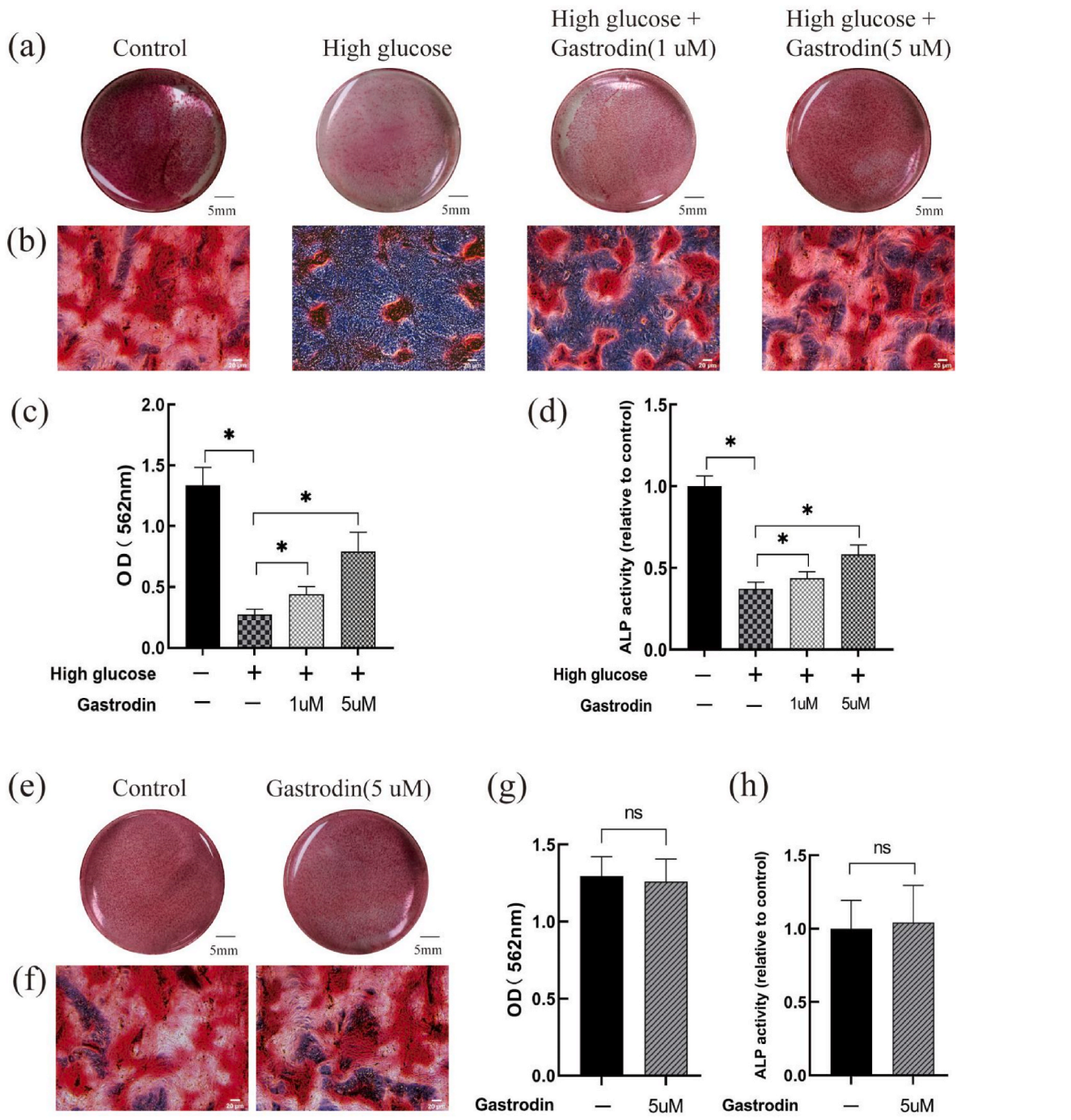

Fig. 3. Effect of gastrodin on osteogenic differentiation and mineralization of MC3T3-E1 cells in high glucose and normal environment. (a, b) The Alizarin Red S staining of MC3T3-E1 cells which were treated with gastrodin and high glucose for 14 days. Scale bar = 20 μm. (e, f) The Alizarin Red S staining of MC3T3-E1 cells which were treated with gastrodin alone for 14 days. Scale bar = 20 μm. (c, g) Semi-quantitative assay of the calcium nodules. (d, h) Analysis of ALP activity in MC3T3-E1 cells at day 7. Each value is reported as the mean ± standard deviation of three independent experiments. $N = 9$, $^*P < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

size. And the average number of pseudopods per cell in gastrodin (5 μM) treated group was higher than that in high glucose group. ($P < 0.05$) (Fig. 5(b) and (e)). H&E staining showed that the staining area increased in the gastrodin (5 μM) treated group compared to that in the high glucose group ($P < 0.05$) (Fig. 5(c) and (f)).

4. Discussion

The results of this study revealed that gastrodin alone did not affect the cell viability and osteogenic differentiation and mineralization ability of MC3T3-E1 cells in normal environment. While high glucose environment induced oxidative stress in osteoblasts and inhibited cell viability and osteogenic differentiation and mineralization. Gastrodin up-regulated the expression of antioxidant enzymes (NRF2 and HO-1), reduced the ROS content in osteoblasts, increased the expression of osteogenic-related biomarkers (BMP2 and RUNX2), and improved cell viability and osteogenic function in high glucose environment. This suggests that gastrodin may protect osteoblast function through anti-oxidant effects in high glucose environment. In this study, oxidative stress induced by high glucose inhibited cell viability and osteogenic

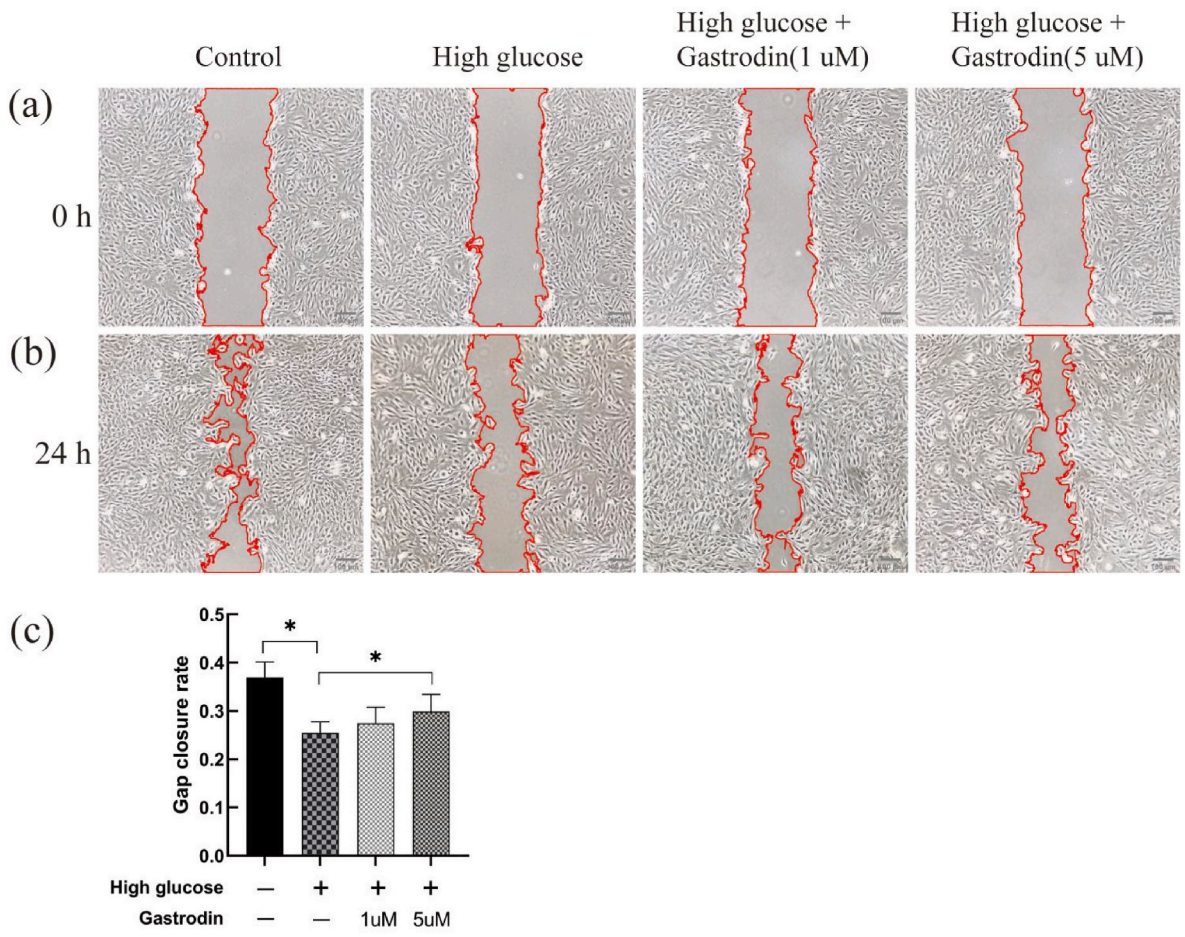

Fig. 4. Effect of gastrodin on migration capacity of MC3T3-E1 cells in high glucose environment. (a, b) Cell migration and gap closure images at 0 h and 24 h. Scale bar = 100 μm. (c) The cell migration ability was quantified by gap closure rate. Each value is reported as the mean ± standard deviation of three independent experiments. $N = 9$, $^*P < 0.05$.

differentiation of osteoblasts, also stimulated the initiation of intracellular antioxidant defense mechanisms [compared to the normal group,a slight increase in the expression of antioxidant enzymes (NRF2 and HO-1) in the high glucose group, but the difference was not statistically significant], which was similar to the findings of Lin [22]. However, in the study of Takanche [17], high glucose condition and glucose oxidase (Glucose oxidase can increase the level of oxidative stress) inhibited the expression of antioxidant enzymes. This difference in results may be attributed to distinctions in drug type, concentration and induction time as well as differences in the degree of oxidative damage in osteoblasts. When the degree of oxidative damage in osteoblasts caused by high glucose is not severe, the high glucose level may not be sufficient to inhibit the expression of antioxidant enzymes within a certain time frame, while the osteoblasts will activate antioxidant defense mechanisms in a stressful manner.

The migration and adhesion of osteocytes on the material surface are depend on the actin filaments [17,28,29]. Actin filaments at the leading edge of the cell forms lamellar and filamentous pseudopods, generating a force to propel cell migration [30]. While, the excess ROS can damage the function of actin filaments [9,31,32]. In this study, we found that the cells extended a large number of pseudopods while gastrodin acted as an antioxidant. Therefore, the cells in the gastrodin-treated group showed better migration ability, which is also consistent with the results of our cell migration assay. In addition, oxidative stress has been found to inhibit the migration ability of cells in previous studies on blood vessels [33], and antioxidants can reverse this process.

Only ATP-bound actin monomers can form actin filaments with normal function [30]. However, oxidative stress damages the function of mitochondrial to produce ATP [29]. In the present study, high glucose-induced oxidative stress was accompanied by a decrease in the number of pseudopods and migration ability of osteoblasts, while gastrodin reversed the overproduction of ROS and prompted cells to extend many pseudopods. Therefore, we hypothesized that the mechanism by which high glucose inhibits pseudopod formation and migration ability of osteoblasts in this experiment may be as follows: high glucose-induced oxidative stress in osteoblasts disrupted mitochondrial homeostasis and function, resulting in reduced ATP production, which in turn inhibited microfilament assembly, affected pseudopod formation, and further inhibited cell migration ability. However, gastrodin improved the migration ability of MC3T3-E1 cells, which is conducive to the osseointegration of osteoblast. Of course, this process needs further research and exploration.

Osteoblast adhesion ability is critical in early osseointegration of implant. Improving the adhesion ability of cells on the surface of biomaterials is also one of the goals of many studies [34]. Oxidative stress inhibits osteoblast adhesion to the material surface [35]. In the present study, we found that gastrodin ameliorated high glucose-mediated oxidative stress and alleviated the decrease in the number of osteoblasts adhering to the titanium surface, prompting more cells to become dendritic and improving cell adhesion ability, which is quite beneficial to osseointegration. In addition, the inhibition rate of cell adhesion number and total adhesion area by high glucose was 17.83% and 23.29%, respectively, compared with the normal group, and the number of cell adhesion and total adhesion areas were enhanced by 14.37% and

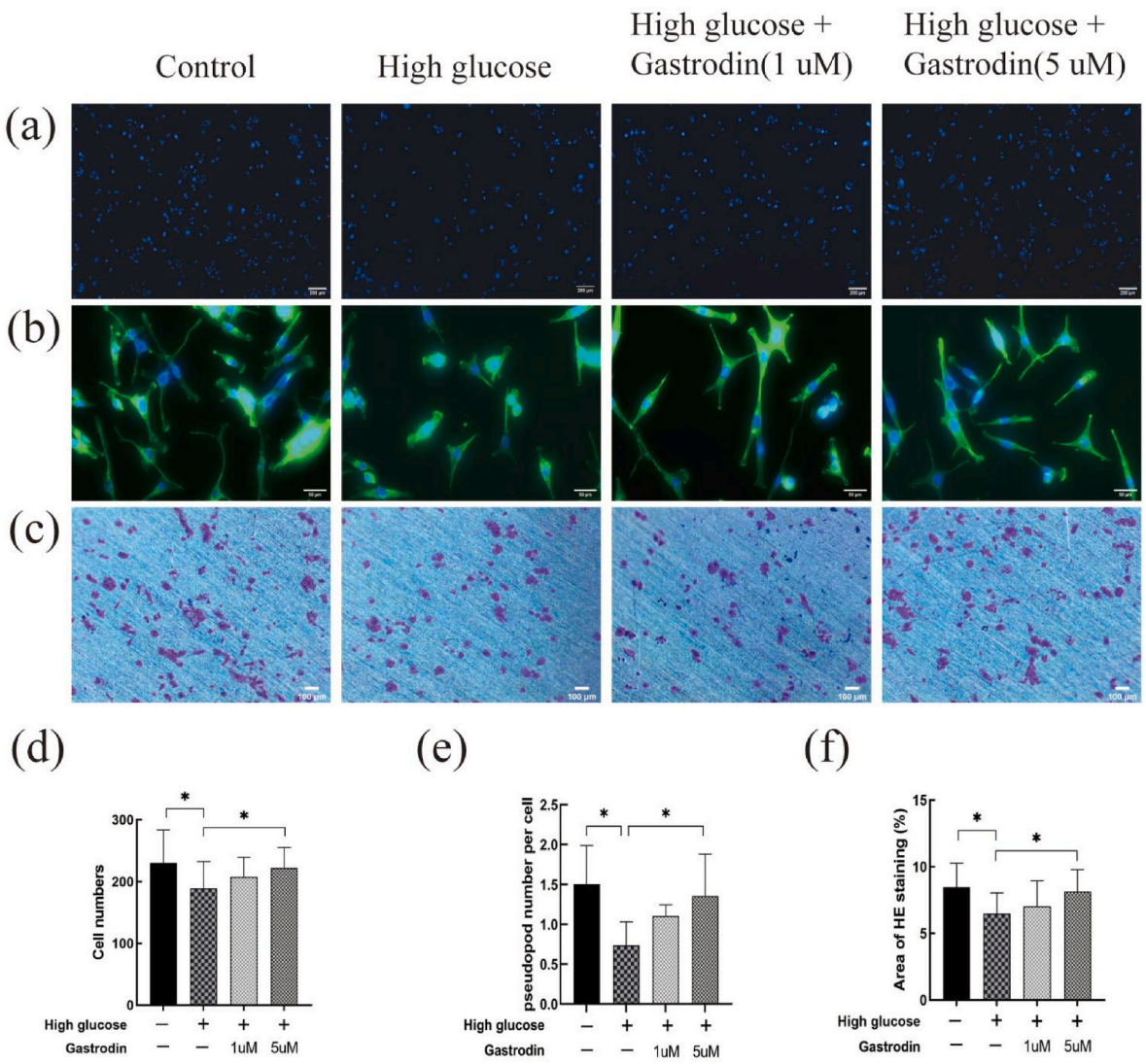

Fig. 5. Effect of gastrodin on adhesion of MC3T3-E1 cells on titanium surface in high glucose environment. (a) Observation of MC3T3-E1 cell nucleus by DAPI staining after cultured for 24 h. Scale bar = 200 μm. (b) Observation of actin cytoskeleton of MC3T3-E1 cells by TRITC phalloidin staining after cultured for 24 h. Scale bar = 50 μm. (c) Observation of MC3T3-E1 cells by HE staining after cultured for 24 h. Scale bar = 50 μm. (d) The number of MC3T3-E1 cell nucleus by DAPI staining. (e) The average number of pseudopods per cell by TRITC phalloidin staining. (f) The H&E staining area of MC3T3-E1 cells adhering on titanium surface. Each value is reported as the mean ± standard deviation of three independent experiments. $N = 9$, $^*P < 0.05$.

19.38% in the high-dose gastrodin treatment group compared with the high glucose group. This may be attributed to the fact that oxidative stress and antioxidant effects impact both the number of cell adhesions on the titanium surface and the morphology and volume of the cells. However, the specific mechanism by which gastrodin promotes osteoblast adhesion through its antioxidant effects requires further investigation.

In combination with our previous study *in vivo* [20] and this experiments *in vitro*, gastrodin showed a good potential to promote osseointegration of implants in diabetic patients. Briefly, this study proved for the first time that gastrodin can protect osteoblast functions including osteogenesis, migration and adhesion on the surface of titanium plate through antioxidation, which are closely related to the occurrence of osseointegration. It preliminarily reveals the potential mechanism and role of gastrodin in protecting osteoblast and osseointegration in high glucose environment. From the cellular level, it provides more theoretical guidance and evidence for the study of gastrodin improving osseointegration of implants in diabetics. It is hoped to provide a new strategy for implant restoration in some diabetics. However, the drug concentration of gastrodin, the more refined and comprehensive mechanism of action, and the loading on the implant surface still need to be explored, which are what we are working hard in the follow-up work.

5. Conclusions

Taken together, these results suggest that gastrodin may alleviate oxidative stress in MC3T3-E1 cells in a high glucose environment and improve the osteogenic differentiation of cells and osseointegration *in vitro* by upregulating the expression of the NRF2 antioxidant pathway. Therefore, gastrodin may be a potentially beneficial drug for implant osseointegration and anti-osteoporosis in patients with diabetes.

Data availability

The data used to support the findings of this study are available from the corresponding author upon request.

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CRediT authorship contribution statement

Yi Li: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Validation, Visualization, Writing – original draft. **Jingyi Zhang:** Conceptualization, Data curation, Formal analysis, Methodology, Software, Visualization, Writing – review & editing. **Fenglan Li:** Conceptualization, Data curation, Funding acquisition, Methodology, Project administration, Resources, Supervision, Visualization, Writing – review & editing.

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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Not applicable.

Appendix ASupplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2023.101623.

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