Effects of graphene oxide and graphene oxide quantum dots on the osteogenic differentiation of stem cells from human exfoliated deciduous teeth

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ABSTRACT
Graphene and its derivatives, graphene oxide (GO) and graphene oxide quantum dots (GOQDs), have recently attracted much attention as bioactive factors in differentiating stem cells towards osteoblastic lineage. The stem cells from human exfoliated deciduous teeth (SHEDs) possess the properties of self-renewal, extensive proliferation, and multiple differentiation potential, and have gradually become one of the most promising mesenchymal stem cells (MSCs) in bone tissue engineering. The purpose of this study was to explore the effects of GO and GOQDs on the osteogenic differentiation of SHEDs. In this study, GO and GOQDs facilitated SHED proliferation up to 7 days \textit{in vitro} at the concentration of 1 \(\mu\text{g/ml}\). Because of their excellent fluorescent properties, GOQD uptake by SHEDs was confirmed and distributed in the SHED cytoplasm. Calcium nodules formation, alkaline phosphatase (ALP) activity, and RNA and protein expression increased significantly in SHEDs treated with osteogenic induction medium containing GOQDs but decreased with osteogenic induction medium containing GO. Interestingly, the Wnt/\(\beta\)-catenin signaling pathway appeared to be involved in osteogenic differentiation of SHEDs induced with GOQDs. In summary, GO and GOQDs at the concentration of 1 \(\mu\text{g/ml}\) promoted SHED proliferation. GOQDs induced the osteogenic differentiation of SHEDs, whilst GO slightly inhibited it.

Introduction
Bone defects seriously affect the physical and psychological well-being of patients. In the repair of bone defects, targeting bone regeneration in the sites is a research highlight in bone tissue engineering [1–3].

Bone tissue engineering consists of stem cells [2], bioactive factors [1], and scaffolds [3,4]. According to numerous researchers, the stem cells from human exfoliated deciduous teeth (SHEDs) possess the properties of self-renewal, extensive proliferation, and multiple differentiation potential and conform to ethical concerns, thus gradually becoming one of the most promising mesenchymal stem cells (MSCs) in bone tissue engineering [5–7]. In fact, not only can SHEDs be isolated from primary teeth that are routinely extracted in childhood and generally discarded without any ethical concerns, but also they can be isolated from deciduous caries-affected teeth [7,8]. The main advantages of using SHEDs are that they can be easily obtained and present higher proliferation rates and higher levels of osteocalcin production than dental pulp stem cells (DPSCs) during osteogenic differentiation [9–11]. Numerous studies [12–14] have reported that SHEDs were capable of osteogenic differentiation both \textit{in vitro} and \textit{in vivo}. Osteogenic-related RNA and protein expression increased when SHEDs were treated with osteogenic induction medium \textit{in vivo} [12]. Additionally, SHEDs effectively induced robust bone formation \textit{in vivo} when they were transplanted into immunocompromised mice [13] or swine [14].

Graphene, a typical sp\(^2\) carbon-based material, has drawn attention since 2004 [15]. Due to its potential as a delivery protein or drug molecules [16,17], antibacterial agent [18], or cancer-targeting therapy [19], graphene and its derivative, graphene oxide (GO), have recently attracted attention in the biomedical field. GO is a single-atom-thick and two-dimen-sional carbon material prepared by the oxidation of graphite from a highly oxidized form of graphite [20]. Moreover, GO has many excellent properties, such as large specific surface area, amphiphility and rich oxygen-containing functional groups [20]. GO, the amphiphilic compound, has abundant functional groups, such as hydroxyl and epoxy groups on the planes and carboxylic acid groups at the edges [20,21], which enhances its interactions with molecules and proteins in osteogenic induction medium through hydrophobic and electrostatic interactions [22], then potentially enhance stem cell osteogenic differentiation [23–25]. Published works have
suggested that GO has good biocompatibility. Rosa V et al. showed that DPSCs were anchored to the rough surface provided by GO, as assessed by scanning electron microscopy (SEM) [26]. Moreover, GO at the concentration of 0.1 µg/ml allowed for BMSC attachment and promoted proliferation, whilst GO at 10 µg/ml was cytotoxic [27]. In a review by Mohammadrezaei D et al., up to 50 µg/ml for GO seems to be safest for most cell lines [25]. In addition, GO could induce BMSC and DPSC osteogenic differentiation in vitro [26,27]. GO-coated β-tricalcium phosphate scaffold increased the rate of new bone formation in vivo [28]. Hence, dental materials and bone tissue engineering materials may benefit from these appealing properties of GO.

Recently, nanomaterials, another promising material, have attracted an increasing number of studies focusing on their relationship with MSCs. Nanoparticles can be ingested by cells and then influence cell differentiation. Yi C et al. [29] showed that gold nanoparticles (AuNPs) exhibited a positive influence on MSC osteogenic differentiation by interacting with the cell membrane and binding with proteins in the cytoplasm, whilst Liu D et al. [30] demonstrated that carbon nanotubes (CNTs) inhibited MSC osteogenic differentiation. Moreover, a new type of carbon-based quantum dots, graphene oxide quantum dots (GOQDs), has attracted increasing attention due to their quantum confinement and edge effects [31]. As zero-dimensional stable materials converted from two-dimensional GO sheets, GOQDs not only exhibit the excellent properties of GO but also other new properties, such as optimal stability and good photoluminescence performance [31,32]. Unlike the ability of GO to absorb small molecules and some proteins from an osteogenic induction medium, which further proved its bioactivity, GOQDs can be ingested by cells and then influence cell behaviour [33]. Actually, most reports about GOQDs have been focused on their biomedical applications, for instance, biosensing [34], bioimaging [35], drug delivery [36] and photodynamic therapy [37]. Meanwhile, correlated research found graphene quantum dots’ (GOQs) uptake by MSCs and effectively induced MSC osteogenic differentiation [38]. However, until now, there have been no studies about the interaction between GOQDs and SHEDs.

It is essential to explore better materials for surface modification of scaffolds and guided bone regeneration in bone tissue engineering. As shown previously [26,27,33,34,36,39], both GO and GOQDs have good biocompatibility and the potential of differentiating stem cells towards osteoblastic lineage. However, whether GO or GOQDs could promote SHED osteogenic differentiation, and the different abilities of GO and GOQDs in inducing osteogenic differentiation of SHEDs remain unknown.

In this study, we aimed to explore the effects of GO and GOQDs on the osteogenic differentiation of SHEDs. Initially, we evaluated the proliferation of SHEDs incubated with GO or GOQDs. Then, mineralization, ALP activity, osteogenic-related RNA and proteins were investigated for assessment of the ability of GO and GOQDs in inducing SHED osteogenic differentiation. The hypothesis was that 1 µg/ml GO and GOQDs could promote SHEDs proliferation and that GOQDs would have better ability than GO in inducing SHED osteogenic differentiation.

Materials and methods

Preparation and characterization of the GO and GOQDs

GO was prepared according to the modified Hummer’s method [40]. In detail, 180 ml H2SO4 and 20 ml H3PO4 were mixed, then 2 g of graphite and 9 g of KMnO4 were subsequently added to the mix. The suspension was stirred at 50 °C in a thermostatic water bath for 2 h whilst 40 ml of deionized water was added. After the suspension was cooled to room temperature, it was centrifuged to yield solids. Next, the solids were dissolved in deionized water whilst 4 ml of H2O2 were added. Finally, the suspension was centrifuged, washed with deionized water and hydrochloric acid, and freeze-dried to yield GO.

GOQDs were purchased from Nanjing XFNANO Materials Tech Co., Ltd (Nanjing, China). GOQDs with the concentration of 1 mg/ml were sterilized through a 0.22 µm filter membrane (EMD Millipore, Billerica, MA, USA).

The compositions of GO were characterized by a Laser Micro-Raman Spectrometer (Renishaw InVia, Gloucestershire, UK) with 532 nm laser excitation and Fourier transformation infra-red spectroscopy (FTIR; Bruker, Karlsruhe, Germany).

The morphology of GO and GOQDs was characterized by transmission electron microscopy (TEM; FEI Tecnai G2 Spirit, Hillsboro, OR, USA) at an acceleration voltage of 300 KV.

Isolation and characterization of SHEDs

The cells from human exfoliated deciduous teeth were taken from 6- to 10-year-old children whose caries-free deciduous teeth required extraction. Ethics Committee approval was provided by the School of Stomatolgy, Sun Yat-sen University. Briefly, the teeth were placed in pre-cooled phosphate-buffered saline (PBS, Hyclone, Logan, UT, USA) with 1% penicillin/streptomycin (P/S; Gibco, Thermo Fisher Scientific, Inc., Grand Island, NY, USA) and taken to the laboratory within 8 h of extraction. Pulp tissue was extracted with a barbed broach, cut into pieces and digested with 1:1 3 g/l collagenase type I (Sigma-Aldrich, St. Louis, MO, USA) and 4 g/l dispase (Roche, Basel, Switzerland) at 37 °C for 30 min. The pulp cells were then suspended in the primary culture medium, which included Dulbecco’s modified Eagle’s medium (DMEM; Gibco), 20% foetal bovine serum (FBS; Gibco), and 2% penicillin/streptomycin, then cultured in 37 °C and 5% CO2 incubator.

Morphology of primary SHEDs and third-passage (P3) SHEDs was observed by an inverted microscope (Zeiss, Oberkochen, Germany). SHEDs were characterized by flow cytometric analysis for CD34, CD44, CD45, CD73, CD90, and CD105 (BD Pharmingen, San Diego, CA, USA). Flow cytometry was performed with a Beckman Coulter CytoFlex system (Beckman Coulter, Fullerton, CA, USA).

Adipogenic induction medium (DMEM with 10% FBS, 1% P/S, 1 µM dexamethasone (Sigma), 0.2 mM indomethacin (Sigma-Aldrich), 10 µg/ml insulin (Sigma-Aldrich), and 0.5 mM methylisobutylxanthine (Sigma-Aldrich)) was applied to induce the adipogenic differentiation of SHEDs. Twenty-one days later, the cells were fixed with 4% paraformaldehyde for
30 min at room temperature and washed with PBS, after which Oil red O solution was added and incubated for 15 min and then washed thoroughly with PBS for evaluation of the formation of lipid droplets.

SHEDs were passaged for 3 times, and each passage was determined when cells reached 80–90% confluence. Only the third and fourth passages of SHEDs were used for the in vitro experiments. SHEDs were cultured in the culture medium that included DMEM, 10% FBS and 1% P/S.

### Proliferation assay

SHEDs were plated on 96-well plates at a concentration of 5 x 10^4 cells/well. GO (1 and 10 μg/ml) or GOQDs (1 and 10 μg/ml) were added and supplied with culture medium. The control group was placed in SHED-treated culture medium. SHED proliferation was tested by means of Cell Counting Kit-8 (CCK-8; Beyotime Institute of Biotechnology, Haimen, China). After the indicated time points for 1, 3, 5, 7 days of incubation, the culture medium of each well was replaced with 10 μl of CCK-8 solution and 100 μl DMEM, after which the plates were incubated for 1 h at 37°C in 5% CO2. The OD value of each well was measured by means of a microplate reader (Tecan, Männedorf, Switzerland) at a wavelength of 450 nm.

Confocal laser scanning microscopy (Zeiss) was used to observe the morphology of SHEDs treated with GO or GOQDs. Initially, SHEDs were plated on a laser-scanning confocal petri dish at a concentration of 1 x 10^4 cells/dish, after SHEDs were attached, then treated with culture-medium-coated GO or GOQDs at the concentration of 1 μg/ml. After 48 h, SHEDs were washed three times with PBS and then imaged.

### Alizarin red staining

After cell adhesion, SHEDs were seeded in 6-well plates at a density of 2 x 10^5 per well, then treated with GO/OIM or GOQDs/OIM for 14 days. The control group was SHEDs treated with OIM. Total RNA was extracted by Trizol (Invitrogen, Thermo Fisher Scientific, Inc., Waltham, MA, USA), and the concentration was determined by spectrophotometry. cDNA was prepared through reverse transcription (Takara Bio Inc, Shiga, Japan). Gene expression was quantified by qPCR with an SYBR Green kit (Roche, Basel, Switzerland) and gene-specific primers. The primers were synthesized by the Beijing Genomics Institute, China. qRT-PCR primer sequences are shown in Table 1. GAPDH was set as the internal control, after which the qRT-PCR, OCN, Runx2, and β-catenin RNA levels were calculated and compared among the different groups.

### Western blotting

After cell adhesion, SHEDs were seeded in 6-well plates at a density of 2 x 10^5 per well, then treated with GO/OIM or GOQDs/OIM for 14 days. The control group was SHEDs treated with OIM. Cells underwent lysis in RIPA buffer (KeyGen BioTECH, Nanjing, China) containing 1% protease inhibitor cocktail (CWBio, Beijing, China) and protein concentration were measured by means of the BCA assay kit (CWBio). A 40 μg quantity of protein was applied to each lane, separated on Tris-Glycine SDS-PAGE (CWBio, Beijing, China), and then transferred to a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA). Subsequently, the membranes were blocked for 1 h at room temperature by incubation in TBST (10 mM Tris-HCl, 50 mM NaCl, 0.25% Tween 20) containing 5% nonfat milk, then incubated with the primary antibodies against osteocalcin (OCN; 1:500, Abcam, Cambridge, MA, USA), runt-related transcription factor-2 (Runx2; 1:1000, Cell Signaling Technology, CST, Danvers, MA, USA), collagen 1 (COL I; 1:500, Abcam), β-catenin (1:1000; CST), and GAPDH (1:1000; CST) for 18 h at 4°C. Subsequently, after 1 h of incubation with secondary antibody (1:2000; CST) and analysis with an enhanced chemiluminescent (ECL) detection system (EMD Millipore).

### ALP activity assay

After cell adhesion, SHEDs were seeded in 24-well plates at a density of 5 x 10^4 per well, and the culture medium was placed with OIM including 1 μg/ml GO or GOQDs for 3 and 7 days. The control group was SHEDs treated with OIM. SHEDs were subjected to alkaline phosphatase (ALP) activity assay by means of the ALP assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer’s instructions.

### Quantitative real-time reverse transcription polymerase-chain-reaction (qRT-PCR)

After cell adhesion, SHEDs were seeded in 6-well plates at a density of 2 x 10^5 per well, then treated with GO/OIM or GOQDs/OIM for 14 days. The control group was SHEDs treated with OIM. Total RNA was extracted by Trizol (Invitrogen, Thermo Fisher Scientific, Inc., Waltham, MA, USA), and the concentration was determined by spectrophotometry. cDNA was prepared through reverse transcription (Takara Bio Inc, Shiga, Japan). Gene expression was quantified by qPCR with an SYBR Green kit (Roche, Basel, Switzerland) and gene-specific primers. The primers were synthesized by the Beijing Genomics Institute, China. qRT-PCR primer sequences are shown in Table 1. GAPDH was set as the internal control, after which the qRT-PCR, OCN, Runx2, and β-catenin RNA levels were calculated and compared among the different groups.

### Table 1. Primer sequences used in qRT-PCR.

| Gene          | Forward                          | Reverse                          | Sequence                  |
|---------------|----------------------------------|----------------------------------|---------------------------|
| Osteocalcin (OCN) | 5'-AGCAAGGAGTGCAGCCTTTGT-3'    | 5'-GCCCTGCGTCTCCTACT-3'         |
| Runt-related transcription factor-2 (Runx2) | 5'-CCGGAACACATGCAACGCCG-3'     | 5'-CTGAGTTCCAGTTCTATGCAACA-3' |
| Collagen I (COL I) | 5'-TGTTCCGGGCTGTGATGATG-3'     | 5'-AGCAGACCTGCAACACCA-3'        |
| GAPDH         | 5'-TTTCCCTGACTCACAAGGCACA-3'   | 5'-CCGTGTTGCTTGAGCAAAATTGC-3'  |

**Table 1.** Primer sequences used in qRT-PCR.
Statistical analysis

All data were expressed as the mean ± standard deviation (mean ± SD), of at least, triplicate determinations. The SPSS 20.0 software package was used for statistical analysis of the data. Results were performed by one-way analysis of variance (ANOVA). Tukey’s multiple comparisons test was used for comparison among groups. \( p < 0.05 \) was considered statistically significant.

Results

Characterization of GO and GOQDs

From the Raman spectrum [Figure 1(A)(a)] of GO, the G and D bands were found to be the most prominent, with the G band (~1605 cm\(^{-1}\)) an ordered sp\(^2\) structure, whereas the D (~1359 cm\(^{-1}\)) band was associated with the defects and amorphous structures on the graphene edge. The ID/IG ratio (0.85) exhibited the typical response of GO.

The FTIR of GO is shown in Figure 1(A)(b). As for GO, the absorption band at 3340 cm\(^{-1}\) was observed due to \(-\text{OH}\) stretching, 1670 cm\(^{-1}\) (stretching vibrations of C=O), 1610 cm\(^{-1}\) (hydroxyl stretching), and in the region of 1040–1120 cm\(^{-1}\) due to C–O–C and C–O stretching vibrations. Thus, the GO which we prepared showed hydrophilicity.

The morphology of GO and GOQDs was observed by TEM [Figure 1(B)]. The morphology of GO showed two-dimensional irregularly shaped flakes with lateral size over 50 nm whereas GOQDs showed dot sizes with a lateral dimension of less than 10 nm.

Characterization of SHEDs

The major morphology of SHEDs was typically spindle-like or fibroblast-like [Figure 2(A)]. As for the analysis of cell-surface markers, 98.4% of the cells were CD44-positive, 96.1% were CD73-positive, 96.8% were CD90-positive, and 95.4% were...
CD105-positive, only 0.42% of the cells were CD34-positive and 0.32% were CD45-positive [Figure 2(B)]. This indicated that cells were positive for the expression of mesenchymal stem-cells-related markers and negative for the expression of hematopoietic markers. In addition, after the induction of adipogenic and osteogenic differentiation of SHEDs, clear, distinct calcium deposits and lipid droplets were observed [Figure 2(C)]. The results demonstrated that cells from human exfoliated deciduous teeth were mesenchymal stem cells, which had multiple differentiation potential.

Figure 2. Characterization of SHEDs. (A) (a) 4 days of primary SHEDs (b) 3 days of SHEDs at P3, x50. (B) Flow cytometry analysis of cell surface marker CD34, CD45, CD44, CD73, CD90, CD105. (C) (a) Cells were treated with osteogenic induction medium after 14 days and stained with Alizarin red, x50. (b) Cells were treated with adipogenic induction medium after 21 days and stained with oil red O, x50.
Proliferation of SHEDs

To choose a material with low cytotoxicity, we investigated the proliferation ability of SHEDs by a CCK-8 assay. From the results (Figure 3), GO and GOQDs showed low cytotoxicity at the concentrations of 1 μg/ml and 10 μg/ml. Moreover, 1 μg/ml GO and GOQDs significantly promoted the proliferation of SHEDs, so we chose 1 μg/ml of GO and GOQDs in the subsequent assays.

Confocal laser scanning microscopy was used to show the morphology of SHEDs cultured with GO or GOQDs. As shown in Figure 4, over a period of 48 h, SHEDs exhibited normal morphology in both the GO and GOQD culture media compared with the control cells. Interestingly, after incubation for 48 h, the cells treated with GOQDs emitted blue fluorescence under 405 nm excitation wavelength, whilst there was no fluorescence from the cells of the control group and the cells treated with GO. This indicated that GOQDs can easily penetrate the SHED membrane and enter the cells distributed in the cytoplasm, which then might influence the function and behaviour of SHEDs.

Mineralized matrix nodule formation

The results of Alizarin red S staining after 14 days of osteogenic-induced culturing are shown in Figure 5(B). Obviously, there was calcium nodule formation in the three groups with a higher level of mineralization in the GOQDs/OIM group.

ALP activity

ALP activity is showed in Figure 5(A). During the 7 days, GOQDs/OIM showed a stronger SHED-induced osteogenic differentiation ability, as compared with the control group, in which ALP activity increased significantly, whilst during the first 3 days, compared with the control group, GO/OIM significantly inhibited the osteogenic differentiation of SHEDs. However, five days later, there were no significant differences in ALP activity between the GO/OIM group and the control group, indicating that GO could neither affect the ALP generation nor further induce osteogenic differentiation in the early days.

qRT-PCR analysis of osteogenic-related mRNA

To investigate the osteogenic differentiation of SHEDs induced by GO or GOQDs, we studied osteogenic-related gene expression. Moreover, we analyzed Wnt signaling pathway-related RNA. From the results shown in Figure 6, the expression of OCN, Runx2, COL I, and β-catenin was distinctly higher in the GOQDs/OIM group than in the control group after both 7 and 14 days of osteogenic-induced culturing. However, the expression of those RNAs was lower in the GO/OIM group than in the control group, but had no statistically significant difference.

Western blot analysis of osteogenic-related protein

In addition to assessing the ability of GO and GOQDs to induce SHED osteogenic differentiation, we analyzed the protein expression after 14 days of differentiated cells. Notably, the expression of OCN, Runx2 and COL I was distinctly higher in the GOQDs/OIM group as compared with the control group, whilst the expression of those proteins was lower in the GO/OIM group. In addition, the results showed that the expression of β-catenin was the same as that of the osteogenic-related protein (Figure 7).

Discussion

In recent years, the exploration of promising materials for surface modification of scaffolds or guided bone regeneration...
has become a major research focus in the field of bone tissue engineering. In this study, we proposed to gain new insights by studying the cellular behaviours of SHEDs towards GO and GOQDs to explore better biomaterials to induce stem cell osteogenic differentiation.

GO can be obtained by chemical exfoliation of graphite, which introduces oxygen functional groups [20,40]. The compositions of GO were characterized by Raman spectroscopy [Figure 1(A)]. The D peak was due to the defects and amorphous structures on the graphene edge, whilst the G peak was an ordered sp² structure [41]. Moreover, the 0.85 ID/IG ratio is consistent with the values reported in the literature for graphene oxide [42]. The FTIR of GO results [Figure 1(A) (b)] suggests that graphite became part of GO containing oxygen-containing functional groups, such as –OH, C=O, C=O, and C–O–C, so that GO can readily form colloidal suspensions in triple-distilled water. The hydrophilicity of GO could connect with many osteogenic-related factors that may influence stem cell differentiation [21–23]. The TEM images [Figure 1(C)] showed that GO and GOQDs contained a good crystal graphene structure and that GO showed two-dimensional irregularly shaped flakes with lateral size over 50 nm, whereas GOQDs showed dots with sizes under 10 nm. Different sizes might have different effects of GO and GOQDs on the osteogenesis of SHEDs.

Currently, autologous stem cell transplantation [43], a new regenerative treatment in bone tissue engineering techniques, requires promising therapeutic stem cells. SHEDs, compared with BMSCs and DPSCs, possess the properties of being easily obtained, harmless to the human body, having a low risk of immune rejection and cross-infection, and having higher osteogenic and neurogenic differentiation capacity [11,44–46]. Further, they have been recognized as promising stem cells for regenerative medicine. In this study, we successfully separated and cultured SHEDs and observed their proliferation and differentiation towards GO and GOQDs.

SHEDs were capable of proliferating and maintaining normal cell morphology on GO and GOQDs (Figure 3 and Figure 4). As shown in Figure 3, GO and GOQDs at the concentration of up to 10 μg/ml had no effect on cell viability. As for the proliferation of SHEDs, cells cultured with 1 μg/ml of GO or GOQDs were significantly more numerous after 7 days.
compared with the control group. These results indicated that 1 μg/ml GO and GOQDs was beneficial for proliferation, because SHED growth was improved. However, there were some differences from early work, Wei C et al. [27] demonstrated that 1 μg/ml of GO could not promote BMSC proliferation as compared with the control group, possibly because the SHEDs had a higher proliferative capability than BMSCs.

Moreover, the photoluminescence performance of GOQDs allowed for their uptake by SHEDs, being monitored with

Figure 6. SHEDs were cultured in osteogenic induction medium containing GO or GOQDs. (A) RNA was extracted at 7 days. (B) RNA was extracted at 14 days. *p < .05, **p < .01, ***p < .001, ****p < .001.
fluorescence microscopy. As shown in Figure 4, after SHEDs were incubated with GOQDs for 48 h, the cells were labeled in blue, whilst SHEDs incubated with GO or in the absence of both did not show any fluorescence. It was shown that GOQDs could distribute homogeneously throughout the cell body whilst GO could not. Nanoparticles could be ingested by cells and then influence cell differentiation.

Next, we used Alizarin red S staining to evaluate the mineralized nodules formed by osteoblasts derived from SHEDs. As shown in Figure 5(B), the mineralized matrix nodule formation was increased after GOQDs were added to the OIM whilst it was decreased in the GO/OIM group. However, it requires confirmation whether more cells enhanced mineralization in the GOQDs/OIM group or GOQDs indeed upregulated the osteogenic-related RNA and proteins and then promoted mineralized matrix formation. The ALP activity assay was used to evaluate the impact of GO and GOQDs exposure on the differentiation of SHEDs. ALP is a phenotypic marker for the early osteogenic differentiation stage [47]. As shown in Figure 5(A), the ALP activity of SHEDs cultured in GO/OIM or GOQDs/OIM increased from day 3 to day 7. Moreover, there was a significant difference between the two biomaterials whereby ALP activity increased significantly in the GOQDs/OIM group compared with the GO/OIM group, suggesting that SHEDs incubated in GOQDs might have greater osteogenic ability than GO. However, this finding differed from those of early established studies (Rosa V et al. [26] and Wei C et al. [27]), which reported that GO could induce MSC and BMSC osteogenic differentiation. Some reasons that might cause the differences in GO characterization include concentration [27], synthetic method [26] and oxidation state [25,48]. In this study, because of the differences in synthesis, the ID/IG ratio of GO we prepared was 0.85, which was lower than that of the micro-sized GO published by Kang et al. [49], indicating different degrees of oxidation that may cause the differences in ability to induce osteogenic differentiation of stem cells. Moreover, Wei C et al. [27] reported that 0.1 μg/mL GO promoted BMSC osteogenesis. However, in this study, we used 1 μg/mL GO, which was a higher concentration, to induce SHED osteogenic differentiation. The difference of concentrations might cause different results.

To further confirm this conclusion, we studied several gene expressions. As we know, Runx2 is a key transcription factor regulating genes that encode for proteins involved in the osteogenic differentiation and bone formation [50,51]. In addition, bone extracellular matrix proteins such as OCN and specific collagen such as COL I, which is synthesized and secreted by osteoblasts, play an essential role in the mineralization of bone tissue [51,52]. Usually, COL I is a marker expressed in early stages of osteoblastic differentiation, whilst Runx2 and OCN are markers expressed in late stages [50–53]. According to the results shown in Figure 6, the aforementioned conclusion—that, compared with GO, GOQDs significantly improved the osteogenic differentiation of SHEDs—was confirmed. The expression of OCN, Runx2 and COL I RNA in SHEDs cultured with GOQDs significantly increased on day 7 and day 14, especially Runx2 RNA on day 7, whilst those RNAs in SHEDs cultured with GO slightly decreased on day 7 and day 14 but without a statistically significant difference, except OCN RNA on day 7. Interestingly, the expression of β-catenin RNA, one of the key components of the Wnt/β-catenin signaling pathway, was up-regulated after SHEDs were treated with GOQDs. It is usually known that the Wnt/β-catenin pathway plays an important role in homeostasis and osteogenesis [54,55]. Jiang S et al. [56] reported that SHEDs could differentiate into osteoblasts and odontoblasts and suppress the adipogenic differentiation through Wnt signaling pathways. Results from Western blot were in agreement.
with the results from qRT-PCR. The protein expressions of OCN, Runx2, COL I and β-catenin were distinctly higher in the GOQDs/OIM group as compared with the GO/OIM group. It seems that GOQDs up-regulated the expression of bone formation RNA and protein and appeared to be related to the Wnt/β-catenin signaling pathway, which needs further study.

Hence, our in vitro results have demonstrated that GO and GOQDs at the concentration of 1 μg/ml promoted SHED proliferation and GOQDs showed advantages over GO in inducing SHED osteogenesis. However, more research is required to confirm whether this occurs through the mechanism whereby GOQDs are taken up by SHEDs so that the protein level of β-catenin in the cytoplasm was up-regulated and the Wnt/β-catenin signaling pathway was activated, then induced SHED osteogenic differentiation with a stronger ability to induce osteogenesis than GO.

Conclusions

In this study, we compared GO with GOQDs to study their effects on the osteogenic differentiation of SHEDs. Both GO and GOQDs facilitated SHED proliferation up to 7 days in vitro at the concentration of 1 μg/ml. Due to their photoluminescence properties, GOQDs were distributed homogeneously in the cytoplasm of SHEDs. In inducing SHEDs osteogenesis, GOQDs showed advantages over GO. Mineralization and ALP activity increased significantly in SHEDs treated with osteogenic induction medium containing GOQDs, whilst they decreased in the group treated with osteogenic induction medium containing GO. Moreover, the DNA and protein expression of OCN, Runx2, COL I and β-catenin in SHEDs were distinctly higher in the GOQDs group than in the GO group. All these results demonstrate GOQDs as a potential biomaterial for improving bone regeneration.

Disclosure statement

The authors have no competing interests to declare.

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