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Original article

Hydroxyapatite based biocomposite scaffold: A highly biocompatible material for bone regeneration

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ABSTRACT

The conventional approaches for treating bone defects such as autografts donor tissue shortages and allografts transmission of diseases pose many shortcomings. The objective of this study was to design a nano strontium/magnesium doped hydroxyapatite (Sr/Mg-HA) with chitosan (CTS) and multi-walled carbon nanotubes (MWCNT) (Sr/Mg-HA/MWCNT/CTS) biocomposite was created to support the growth of osteoblasts using a solvent evaporation method. To help the growth of osteoblasts, a solvent evaporation technique was used to design a nano strontium/magnesium doped hydroxyapatite with chitosan and multi-walled carbon nanotubes biocomposite. We studied the biocompatibility and efficiency in vitro of biocomposite following physicochemical analyzes. Tests of biocompatibility, cell proliferation, mineralization, and osteogenic differentiation have shown that in-vitro safety and effectiveness of biocomposite are good. The performance of biocomposite was more efficient in in-vitro as well as in vivo experiments than in Sr/Mg-HA nanoparticles. Briefly, the Sr/Mg-HA/MWCNT/CTS biocomposite is an ideal candidate for effective bone repair in clinics with excellent mechanical properties with durable multi-biofunctional antibacterial properties and osteoinductivity.

1. Introduction

Skeletal disorders and trauma have become a global, functional problem and a clinical challenge for bones and fractures (Lems and Raterman, 2017; Xie et al., 2019). A bone graft autologously is known as the good scaffolds for fixing bone defects and fractures, which has an exceptional bone fusion therapeutic effect (Nandi et al., 2010). The drawbacks of autologous bone graft, for instance, the morbidities of the donor site, its restricted availability, trauma and pain, however, limit its broad use. Although the allograft and xenograft could defeat the downsides of the autologous bone join, they may bring different worries up in the potential spreading of sickness and invulnerable dismissal (Oryan et al., 2014; Offner et al., 2019). The advancement of bone tissue engineering has proposed promising ways to bone regeneration in recent decades. Perfect bone fix composite should have comparative properties to regular bones, incorporating high porosity, magnificent cytocompatibility, great degradability, reasonable mechanical quality, and the upgraded capacities for mineralization and bone cell proliferation (Ibrahim et al., 2016; Chen et al., 2016).

Chitosan (CTS) is a natural polymer and has extensively been utilized as a platform in bone tissue engineering and other biomedical applications because of its cytocompatibility, degradability, pore development conduct, appropriateness for cell ingrowths and inborn bactericidal nature (Ramya et al., 2012; Escárcega-Galaz et al., 2018; Ahmad et al., 2020). Though, CTS based biocomposite materials have ideal mechanical quality and low interconnected porosity for cell connection, which are deprived to be improved further (Wang et al., 2005; Spinks et al., 2006). Poor mechanical characteristics nevertheless restrict the medical applications of CTS in hard tissue engineering

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(Souza et al., 2017; Sánchez et al., 2019). Different methods to boost the mechanical properties of CTS based biocomposite have been suggested (Javaid et al., 2019; Price et al., 2019).

A further strategy for the enhancement of the compressive strength of the CTS based implants is the nano-reinforcement (Li et al., 2013; Wang et al., 2019; Cao et al., 2020). The long-term effort is made to use MWCNTs notable electrical, thermal, mechanical and chemical characteristics, to improve the properties of polymer biocomposites. The introduction of MWCNT as refurbishing materials into the polymer medium leads to substantial changes in the mechanical characteristics resulting in better properties for biocomposite than pristine polymer (Limón-Martínez et al., 2019).

The induction of osteoinduction is one of the most significant aspects of bone repair (Mehrotra et al., 2019). While CTS can heal bones, there can be no reconstruction of vast areas of bone defects. Therefore, a variety of bioceramics were used in hard tissue repair by combining CTS. Hydroxyapatite (HA) is one of BTE's most widely used bioceramics that is well biocompatible, has a high osteoinductivity and is capable of bonding to bone tissue. Numerous investigations have discovered that bone recovery can be fundamentally advanced by joining HA into a biopolymer.

The biological and physicochemical characteristics of hydroxyapatite may be further improved by doping HA with mono-mineral and multi-minerals. By adding, for example, a certain amount of strontium (Sr) or magnesium (Mg) the mechanical characteristics of brittle HA will greatly improve. Research has demonstrated that Sr and Mg can advance bone recovery and development by empowering osseous separation while lessening bone ingestion, by restraining osteoclasts arrangement (Jiang et al., 2019; Arcos and Vallet-Regí, 2020; Chen et al., 2020; Li et al., 2020).

For this research, Sr/Mg-HA and Sr/Mg-HA/MWCNT/CTS biocomposite were prepared for bone repair and rejuvenation. Such Sr/Mg-HA and biocomposite have been tested with their structural, physical and biological characteristics.

2. Materials and method

2.1. Preparation of minerals doped hydroxyapatite and biocomposite

To prepared minerals doped hydroxyapatite, 0.08 M of calcium chloride, 0.01 M of magnesium chloride, 0.01 M of strontium chloride and 0.06 M of phosphoric acid were joined in a 1:6 M proportion (natural bone ratio). Mineral (Ca + Mg + Sr) arrangement was sonicated in 50 mL of twofold refined water for 10 min until a homogeneous suspension was acquired. Phosphoric acid (diluted with 50 mL of twofold refined water) was added drop wise to this arrangement for more than 30 min, with nonstop blending, with the pH kept up over 10 by the option of sodium hydroxide arrangement, as important. The arrangement was sonicated for a further 1 h, and afterward permitted to stand medium-term, after which the item was sifted under suction and dried in encompassing conditions. The powders were warmed in a high-temperature heater. Tests were warmed from 100 – 1200 °C, holding at 900 °C for 5 h, trailed by cooling the items from 900 °C to 100 °C and afterward cooling to room temperature.

In the wake of sonicating MWCNT in the CH_3COOH, Sr/Mg-HA nanoparticles were added to the blend and sonicated to scatter the nanoparticles. The homogenous blend was then placed into a CTS arrangement. The biocomposite scaffold were prepared after casting the obtained mixture into a Petri glass 60 mm in diameter and evaporating the solvent at 50 °C for 48 h in an oven.

3. Characterization of minerals doped hydroxyapatite and biocomposite

The application of XRD was used for the main phases of Sr/Mg-HA nanoparticles and biocomposite (X'Pert Pro, PANalytical, Netherlands). Sr/Mg-HA nanoparticles and biocomposite morphology were examined using TEM (JEOL, EM-2100F). A universal testing machine (Instron 5567) tested the compressive and mechanical strength of the Sr/Mg-HA nanoparticles and biocomposite (Han et al., 2019).

3.1. Antibacterial test

In-vitro antibacterial tests were used to determine the antibacterial characteristics of Sr/Mg-HA nanoparticles and biocomposite. On three sample groups including control and Sr/Mg-HA nanoparticles and biocomposite, *E. coli* and *S. aureus* were grown. The optical density of the microbial solution has been determined after one day of culture (Kim et al., 2011).

3.2. In vitro MTT assay

After three and seven days of culture, cell proliferation was assessed by the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenylte trazolium Bromide) analysis. The cultivation medium was first replaced with the solution MTT in the 96-well pot. The MTT solution was replaced by the DMSO after four hours of incubation and dissolved for 20 min at RT. 100 μl reagents have finally been carefully distributed to 96- well plates. The optics densities were tested with a microplate reader at 570 nm (Song et al., 2019).

3.3. In vitro Live/dead cell assay

Fluorescence stain has been used to determine the structure of osteoblast cells. The medium has been removed from the pools after three and seven days of rising, and phosphate buffer solution washed twice on the cells of Sr/Mg-HA and biocomposite. Also, the cells were recolored utilizing the Live/dead cell measure packs for four hours. The recolored tests were washed twice with phosphate buffer solution and were seen under a fluorescent magnifying instrument (Wu et al., 2017).

4. Results and discussion

4.1. X-ray diffraction analysis

The X-ray diffraction patterns of Sr/Mg-HA nanoparticles and biocomposite are shown in Fig. 1. The XRD peaks of Sr/Mg-HA nanoparticles could be listed to hexagon-staged pure hydroxyapatite with impressive planes at 002, 211, 112, 300, 130 and 213 (JCPDS card no. 09-0432) (Venkatesan and Kim, 2010; Fahami and Nasiri-Tabrizi, 2013). The diffraction patterns of Sr/Mg-HA nanoparticles relative to those of biocomposite are significantly different, suggesting that the Sr/Mg-HA nanoparticles were successfully incorporated in the MWCNT-CTS matrices. Nevertheless, it is worth pointing out that the MWCNT diffraction patterns were hard to detect in the biocomposite patterns, due to the convergence of the main peaks of MWCNT and hydroxyapatite by 26° (Hooshmand et al., 2014; Rodrigues et al., 2016). In addition, the characteristic plane of MWCNT cannot be identified with diffraction patterns of the biocomposite even though MWCNT was wrapped directly in the prepared hydroxyapatite. This also may explain the active biocomposite fabricated. Furthermore, the broad diffraction planes indicate a high biocomposite consistency.

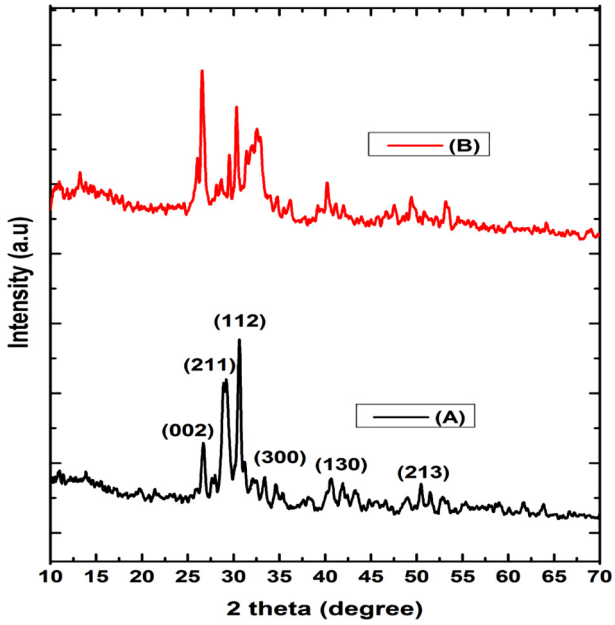

Fig. 1. XRD patterns of Sr/Mg-HA nanoparticles (A), and biocomposite (B).

4.2. Morphological analysis

The structure and the morphology of the prepared samples were seen under TEM (Fig. 2). The pictures with comparing chosen zone electron diffraction examples and high-goals TEM pictures of the prepared samples were recorded. The prepared Sr/Mg-HA nanoparticles show a rod-like surface. Fig. 2 (A, B) showed the morphology of biocomposite: an interlaced system of MWCNT covered with Sr/Mg-HA, the particles were tied down onto the out mass of the MWCNT (Park et al., 2019). The SAED images of composite demonstrate the trademark ring example of rod structure, and they affirm the higher level of crystallinity of nanoparticles.

4.3. Mechanical test

Carbon nanotubes can promptly be added as building squares to accomplish mechanically stable bony platform networks (Eivazzadeh-Keihan et al., 2019). The impact of CNTs stacking on the mechanical characteristics of certain polymers utilized in bone tissue engineering. For recovery of bone deformities with composite configurations and perplexing shapes, fruitful reconciliation is basic (Pei et al., 2019). Bone tissue engineering framework substances must keep up the most suitable auxiliary geometry as the procedure of recovery continues. In addition, the mechanical test outcomes appear (Fig. 3) that both Sr/Mg-HA and MWCNT could improve the young's modulus of the frameworks. It has been demonstrated that MWCNT and Sr/Mg-HA nanoparticles can improve the mechanical properties of different frameworks. Through the fuse of MWCNT and Sr/Mg-HA nanoparticles in the delicate composite framework, we can strengthen the CTS platform (Fig. 3).

4.4. Antibacterial test

The aftereffects of the antibacterial tests (Fig. 4) demonstrated that the Sr/Mg-HA nanoparticles with the MWCNT-CTS (Sr/Mg-HA/MWCNT-CTS) biocomposite had antibacterial exercises against *E. coli* and *S. epidermidis*. As appeared in Fig. 4A and B, after one day of culture, the antibacterial proportions of the Sr/Mg-HA nanoparticles and biocomposite bunches were fundamentally higher than those of the microbial arrangement on the Sr/Mg-HA nanoparticles (Tsai et al., 2018). Every one of these outcomes showed that the fuse of Sr and Mg-doped HA nanoparticles improved the antibacterial action of the biocomposite against the development of *E. coli* and *S. epidermidis* microscopic organisms (Kolmas et al., 2014). Besides, it was nothing unexpected that the biocomposite additionally had the certain bactericidal capacity, since the CTS and MWCNT itself previously had a specific level of antibacterial action (Goy et al., 2009; Kassem et al., 2019).

4.5. Biocompatibility assay

The BMSCs cell proliferation results utilizing MTT assay appear in Fig. 5A. There is no measurably huge distinction between each group on day one. Though, with the expansion of incubation time, the multiplication of cells demonstrates a kept developing pattern for prepared samples and the control sample. Although the multiplication on the control samples and Sr/Mg-HA nanoparticles is higher than those biocomposites on day four, the expansion of biocomposite is increasingly quick from day seven. The outcome shows that the biocomposite with higher Sr/Mg-HA nanoparticles substance can display better cell attachment and expansion, which ought to be specially picked for future in vivo examination and application.

The biocompatibility of cells is commonly envisioned utilizing a fluorescent live/dead test (Fig. 5B). On day four, live cells layer can

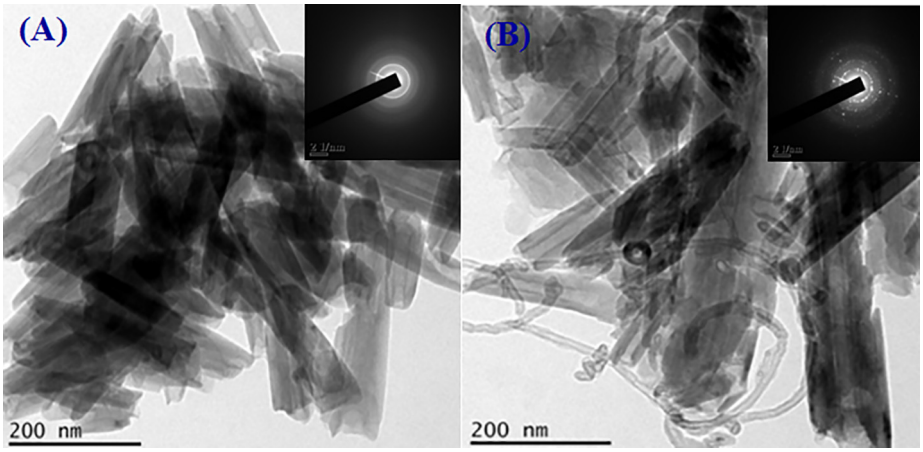

Fig. 2. Morphological characterization of (A) Sr/Mg-HA nanoparticles and (B) biocomposite.

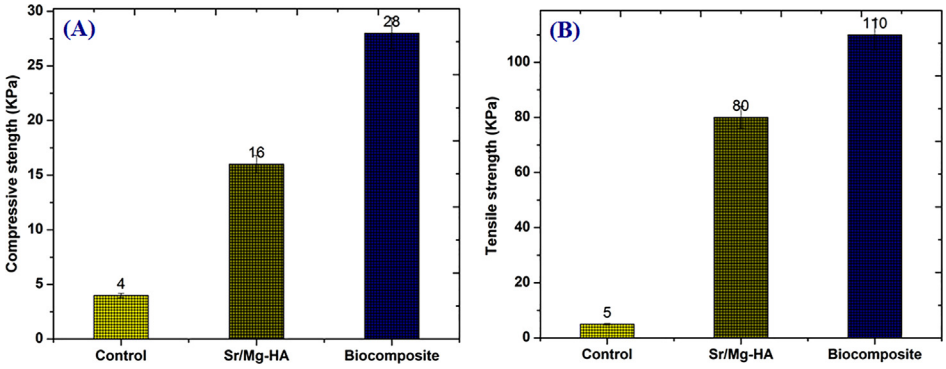

Fig. 3. (A) compressive strength and (B) tensile strength of prepared samples.

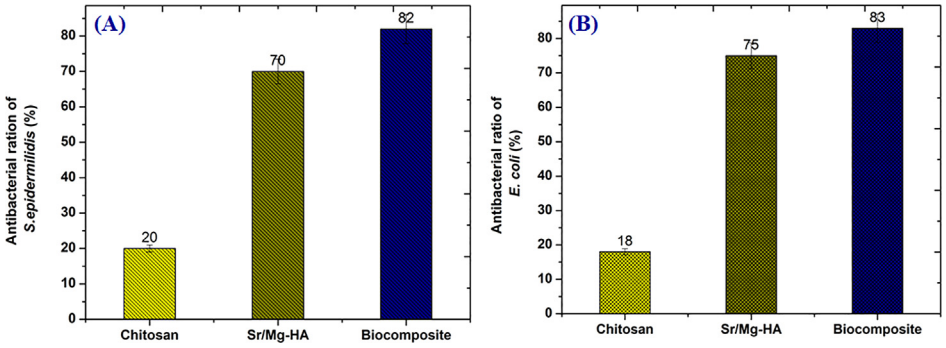

Fig. 4. (A) Antibacterial ratio of *S. epidermidis* and (B) antibacterial ratio of *E. coli* suspension growth on the Sr/Mg-HA and biocomposite for one day.

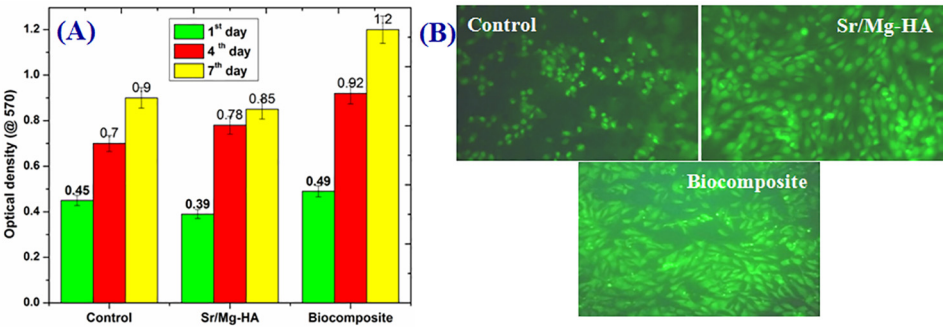

Fig. 5. (A) Proliferation of bone marrow stromal cells after 1, 4 and 7 days of culture on prepared samples. (B) Fluorescence images of bone marrow stromal cells after 4 days of culture prepared samples.

be seen from all culture groups, which shows that the biocomposite and Sr/Mg-HA nanoparticles have no negative impact on biocompatibility. Additionally, the fluorescence pictures likewise display numerous filopodia, exhibiting admirable cells connection on the biocomposite (Mitran et al., 2018).

4.5.1. In vitro osteogenic differentiation

In vitro osteogenic properties of bone marrow stromal cells seeded on the Sr/Mg-HA nanoparticles and biocomposite was assessed by the estimation of alkaline phosphatase activity, since alkaline phosphatase was viewed as a beginning time marker of osteogenic properties. As appeared in Fig. 6A, the alkaline phosphatase activity was expanded with the expanding incubation occasion. Following seven days of incubation, the alkaline phosphatase activity in Sr/Mg-HA nanoparticles and biocomposite were essentially higher than that in control. Bone marrow stromal cells seeded on Sr/Mg-HA nanoparticles and biocomposite showed altogether higher alkaline phosphatase activity contrasted with those incubated on chitosan (control). Nonetheless, it was plainly that the biocomposite indicated fundamentally higher alkaline phosphatase activity than that of the Sr/Mg-HA nanoparticles.

Associatively, the mineralized scaffold created by the cells was resolved on day 7. Fig. 6B demonstrates that mineral creation was enormously delivered in cells incubated on Sr/Mg-HA nanoparticles and biocomposite scaffold, and the measure of the mineralized scaffold was fundamentally expanded contrasted with cells on biocomposite. The addition of Sr/Mg-HA nanoparticles in MWCNT-CTS went about as the osteoinductive factor can initiate osteogenic of bone marrow stromal cells without outside expansion. These in vitro assessments recommend that the biocomposite could surprisingly advance osteogenic differentiation, bone cell multiplication, andadhesion.

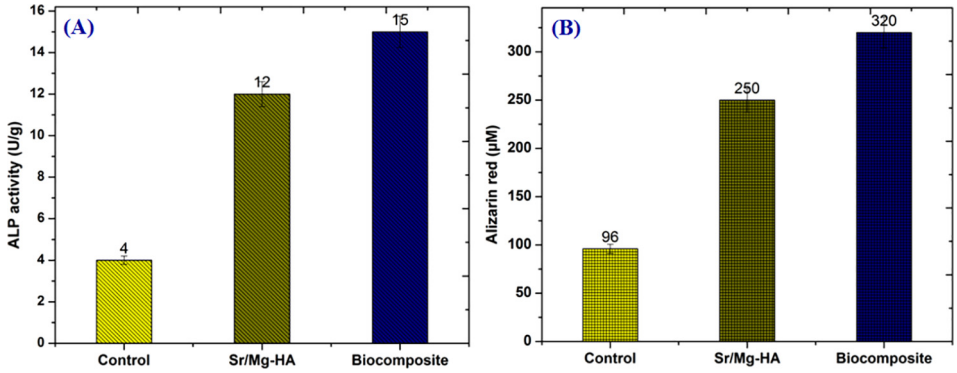

Fig. 6. (A) Alkaline phosphatase activity assay of cells after culturing for 7 days. (B) mineralization formation evaluated by AR staining quantification.

5. Conclusion

In short, the biocomposite displayed impressive mechanical characteristics, antibacterial inductivity, and osteoinductivity. The reinforcement effects of MWCNT also enhanced the biocomposite mechanical strength. The biological characteristics of the biocomposite were improved by dual-ionic doping. Strontium and magnesium were doped into the hydroxyapatite, which can stay away from cytotoxicity, yet can likewise accomplish osteoinductive and antibacterial characteristics for a significant time. Beside, dual-ion doping can successfully eliminate many issues caused by the use, like high prices, growth factors or antibiotics. The in vitro cell proliferation and live/dead experimentations showed that the biocomposite enhanced cell activity and encouraged bone regeneration. In brief, the biocomposite is suitable for potential bone regeneration, with its long-term active multi-biofunction of the osteoinductivity and its antibacterial characteristics.

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