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FABRICATION AND PHYSIOCHEMICAL CHARACTERISATION OF MEDIUM MOLECULAR WEIGHT HYALURONIC ACID COPPER OXIDE BASED GBR MEMBRANE

Type of study: Original Research
Running Title: Fabrication and physiochemical characterisation of medium molecular weight hyaluronic acid copper oxide based GBR membrane

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ABSTRACT:

Introduction: Hyaluronic acid, as a natural linear polysaccharide, has attracted researchers' attention from its initial detection and isolation from tissues in 1934 until the present day. Hyaluronic acid has a wide range of uses in bioengineering and biomedicine because of its biocompatibility and high biodegradation rate, from polymeric scaffolds and wound dressings to endoprostheses for joint fluid and biorevitalizing skin cosmetics. The aim of the study was to find about fabrication and Physicochemical characterization of medium molecular weight hyaluronic acid /CuO GBR membrane.

Materials and methods: Fabrication of Medium molecular hyaluronic acid, gelatin and Carrageenan and preparation of scaffold of control and test group. Micrographs of all scaffolds were taken at 100X.The expected pendant functionalities of scaffolds were confirmed by the FTIR spectrum. To determine the hydrophilicity of the scaffolds, water contact angles of the scaffolds were measured by goniometer software. Three measurements at different positions of each scaffold were conducted. Swelling/shrinkage studies were performed to calculate the water content (%) of the scaffolds. After obtaining informed consent and ethical approval from SIMATS ethics committee, the Dental Pulp stem cells were isolated from molars. The reaction product was transferred to a 96 well ELISA plate and A590 was measured with ELISA plate reader. All values are expressed as the mean ± standard error of the mean (SEM) of at least three independent experiments. A one-way ANOVA followed by Scheffe's method. Statistical significance was set at $p < 0.05$.

Results: The contact angle shows hydrophilic nature in addition with CuO nanoparticles and also elevation of swelling indicates it can be used in the chemo static based membrane or socket preservation procedures. The addition in CuO decrease in porosity is highly evident that CuO based particles have higher cell adherence, increased cell migration, increased cell penetrating efficiency and a high flow of nutrition.

Conclusion: In this study, it has been concluded that a new material made by a medium molecular weight/CuO hybrid was fabricated and tested using Dental Pulp stem cells. We found that M-HLA exhibited a stronger osteoregenerative and better osteoconductive properties and significant intracellular responses for bone grafts, making it reasonable to suggest this M-HLA/CuO hybrid as a potential novel dental material for guided bone regeneration surgery.

Keywords: biocompatibility, degradability, hyaluronic acid, molecular weight, structure, receptors, viscosity, water polymer solution

INTRODUCTION:

Hyaluronic acid (HA), a member of the hyaluronan family, was discovered in 1934 by K. Meyer and John W. Palmer and is still being studied by chemists, biochemists, bioengineers, and other researchers from other scientific fields(1). HA is a necessary component of the extracellular and pericellular matrices, as well as within cells.(2,3) Human combs contain 7.50 mg/mL of hyaluronic acid, human navel cords (gelatin of Wharton)—4.10 mg/mL, human joint synovial fluid—1.50-3.60 mg/mL, vitreous humor—0.14-0.34 mg/g, and human dermis and epidermis—0.20-0.50 and 0.10 mg/g, respectively(2). The average turnover of hyaluronic acid in vertebrate tissues is 5 g per day, which is given via production and enzymatic breakdown. Meanwhile, hyaluronic acid turnover in the bloodstream approaches 30-100 mg each day.(4) The source clearly influences the molecular weight of hyaluronic acid(5). As a result, hyaluronic acid derived from animal sources has a very high molecular weight (up to 20,000 kDa). In comparison, bacterial hyaluronic acid has a molecular weight range of 1000 to 4000 kDa; however, the

enzymatic approach allows for the production of polysaccharides with a molecular weight range of 550 to 2500 kDa.(6) The molecular weight of hyaluronic acid is also affected by other factors: for example, in normal human synovial fluid, it is equal to 6000-7000 kDa, whereas in rheumatoid fluid, it is equal to 3000-5000 kDa. (7)The biological effects of hyaluronic acid are largely influenced by its molecular weight. Hyaluronic acid with molecular weights ranging from 0.4 to 4.0 kDa works as a heat shock protein inducer and has a non-apoptotic characteristic. Polysaccharides having molecular weights ranging from 6 to 20 kDa have immunostimulatory, angiogenic, and phagocytic properties(7,8). Hyaluronic acid, which has a molecular weight of 20-200 kDa, is involved in biological processes such embryonic development, wound healing, and ovulation. High molecular weight hyaluronic acid (>500 kDa) on the other hand possesses anti-angiogenic activity and can operate as a space filler and natural immunosuppressant(9).

Copper has been hypothesized to have a similar mode of action to silver. However, the specific mechanism through which copper nanoparticles exert antimicrobial action remains unknown(10)(11)(12,13). Copper, like silver, is considered to work by interacting with the -SH groups of important microbial enzymes.(14) Yoon et al. found that copper nanoparticles outperformed silver nanoparticles in antibacterial activity against *E. coli* and spore-forming *Bacillus subtilis*. Other research, however, shows that silver outperforms copper against a wide spectrum of various species and strains(15). Considering the scarcity of evidence regarding the fabrication and Physicochemical characterization of medium molecular weight hyaluronic acid /CuO GBR membrane, this experimental study aimed on fabrication and Physicochemical characterization of medium molecular weight hyaluronic acid /CuO GBR membrane.

MATERIALS AND METHOD:

Fabrication of Scaffolds:

The stock solution of 0.75% of Medium molecular weight Hyaluronic acid 0.5% carrageenan and 1% gelatin was prepared. To fabricate the scaffold the materials were blended in the ratio 6:1:3 respectively. Then, 10mg of Copper oxide nanoparticles was added to the solution for the test group. 3ml of the homogeneous mixture was transferred to six well plates. 100 ul of the crosslinking agents TPP (15%) was added to each well. The plates were stored in -20 C for 12 hrs and followed by -80 C overnight. The samples were then lyophilized for 24hrs and stored in dry condition.

SEM Analysis:

The morphological characteristics of scaffolds were observed using scanning electron microscopy (SEM, JEOL, Tokyo, Japan) after freeze drying. The cross-sections of freeze-dried samples were coated with platinum via a sputter-coater at ambient temperature. Micrographs of all scaffolds were taken at 100X.

Fourier transform infrared (FTIR) analysis:

Attenuated total reflectance fourier transform infrared spectroscopy (ATR-FTIR) is a powerful technique to determine any possible chemical interaction ATR-FTIR spectroscopic analysis was performed using Bruker ATR infrared spectrometer (model). The expected pendant functionalities of scaffolds were confirmed by the FT-IR spectrum.

Contact Angle:

To determine the hydrophilicity of the scaffolds, water contact angles of the scaffolds were measured by goniometer software. During the measurements, the scaffolds were cut into square specimens with the size of 1 cm × 1 cm, and further they were placed on the testing plate. Subsequently, 50 μL distilled water was carefully dropped onto the prepared specimens. The contact angles between water droplets and the scaffolds were measured by taking photos immediately (within 2s) when the droplets touched the surface of the scaffolds. Three measurements at different positions of each scaffold were conducted.

Swelling ratio (%) of scaffolds:
Swelling/shrinkage studies were performed to calculate the water content (%) of the scaffolds, wherein 10 mg of freeze-dried scaffolds were placed in 500 µl of PBS at 37 °C. After 24 hours, these scaffolds were removed from the PBS, dabbed with a Kimwipe to remove any excess water on the surface, weighed and placed back into the buffer. The swelling ratio and shrinkage ratio (%) were calculated using the following equations. All experiments were performed 6 times.
Swelling ratio (SR)= ((Ww−W0)/W0)×100%
W0 and Ww are the initial dry weight and the wet weight, respectively.

Dental Pulp stem cells (hDPSC) Cell Culture:
After obtaining informed consent and ethical approval from SIMATS ethics committee, the Dental Pulp stem cells were isolated from molars. The cells were cultured in DMEM low glucose/10% FBS/1%Penicillin;streptomycin. After two passages, 10000 cells per well were seeded in 48 well plates for cell viability and compatibility assays.

Biocompatibility Analysis (MTT Assay):
100 mg of 5 mm cylindrical blocks were prepared. The prepared blocks were immersed in DMEM- low glucose media formulated with 10 % FBS and 1% Penicillin/streptomycin. The media were collected after 24 hrs and 7 days of immersion and treated with cells to test the compatibility. After 24hrs of culture, add the 10uL/100mL of MTT reagent (5 mg/mL stock) to cultured cells and then incubate for 4 h to allow formation of the formazan dye at 37°C. The medium is exchanged to DMSO (200 µL) and stands for 10min. The reaction product was transferred to a 96 well ELISA plate and A590 was measured with ELISA plate reader.

Statistical analysis:
All values are expressed as the mean ± standard error of the mean (SEM) of at least three independent experiments. A one-way ANOVA (analysis of variance) was used to test for significant differences, and multiple comparisons were performed using Scheffe's method. Statistical significance was set at $p < 0.05$.

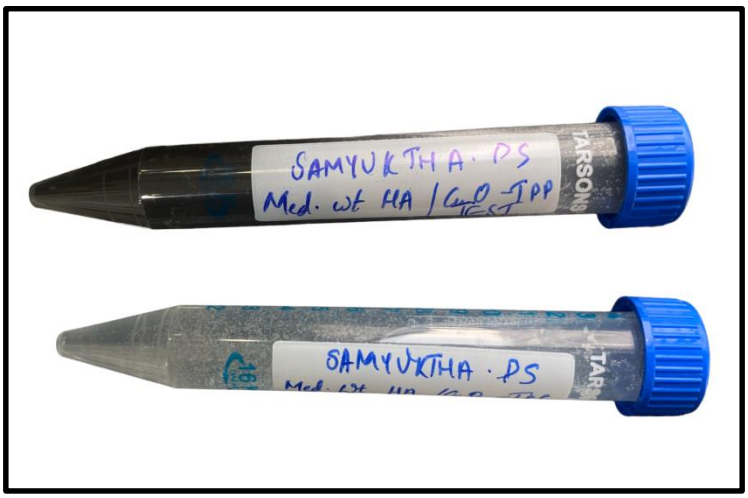

Figure 1: Image depicting the Fabrication of the material of both Control (HA) and Test (HA+CuO) group

FIGURE:2: Image depicting the Scaffold preparation of Control (HA) and Test (HA+CuO) group

RESULTS:

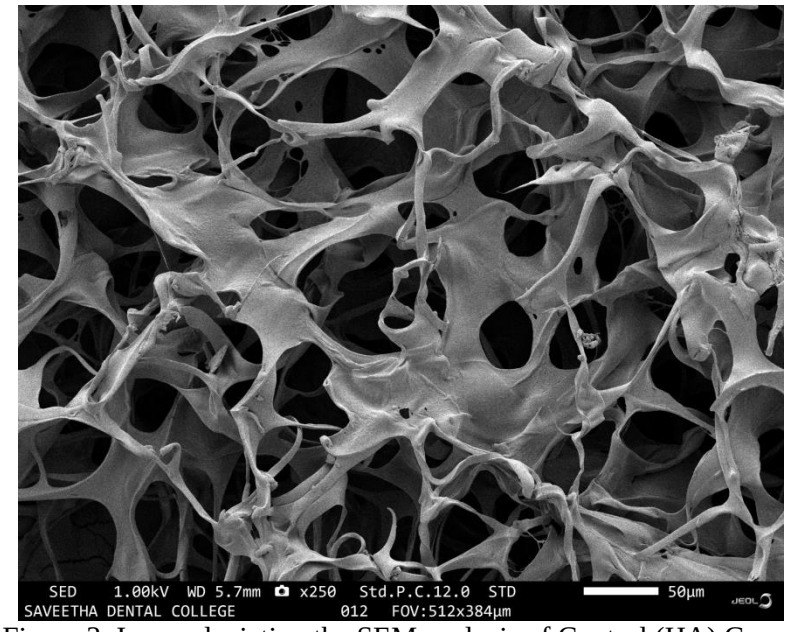

Figure 3: Image depicting the SEM analysis of Control (HA) Group

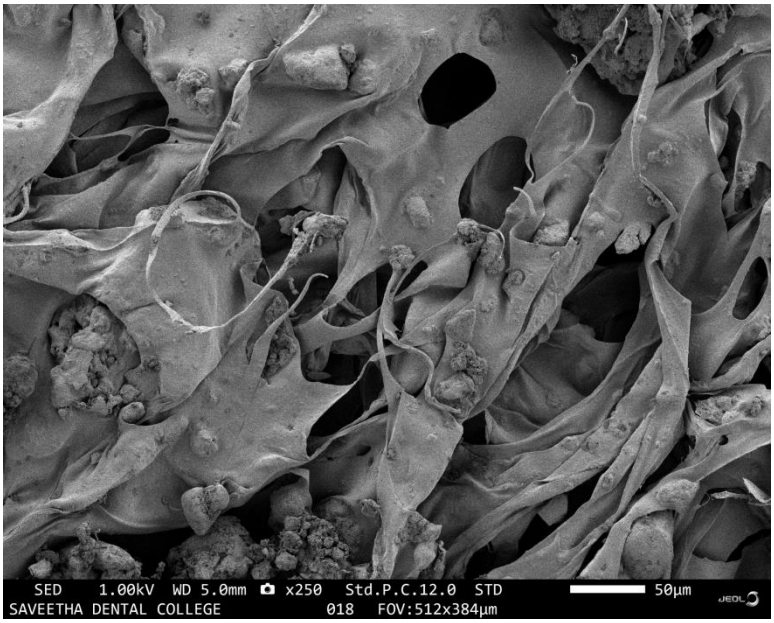

Figure 4: Image depicting the SEM analysis of the Test (HA+CuO) Group

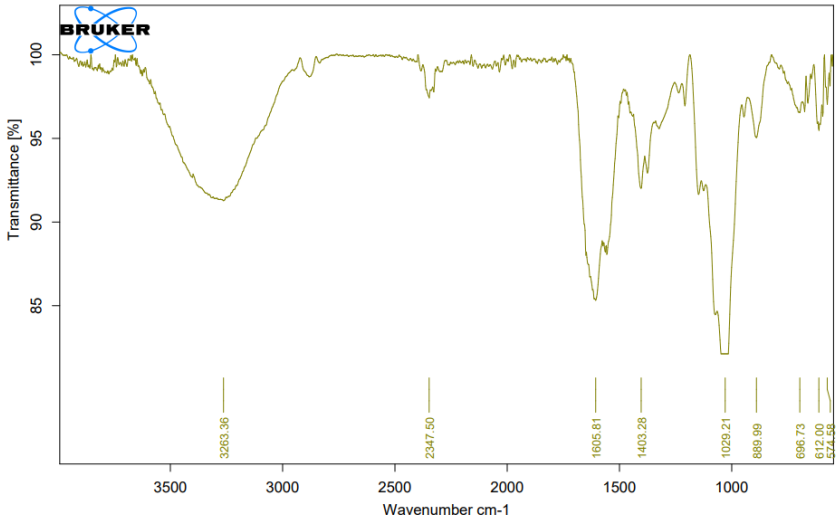

Figure 5: Graph representing the FTIR of the Control (HA) group.

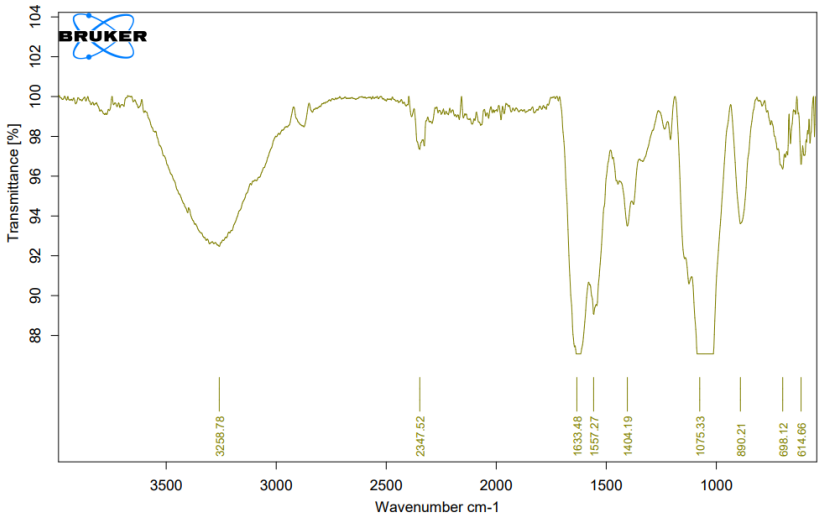

Figure 6: Graph representing the FTIR of the Test (HA+CuO) group

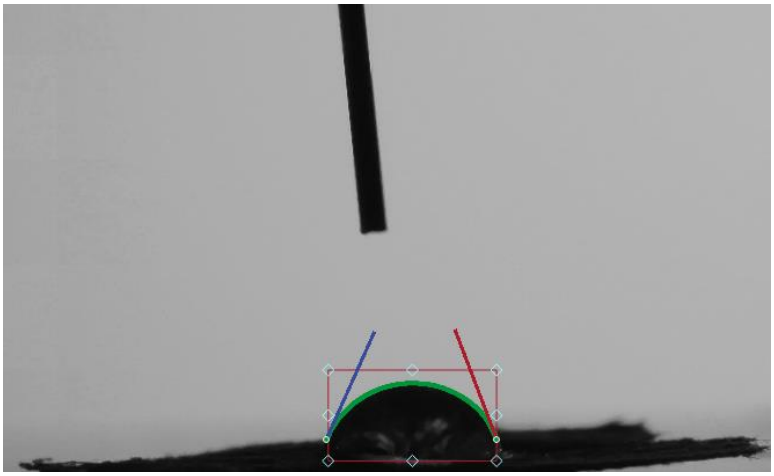

Figure 7: Picture depicting the Contact Angle of the Control (HA) group

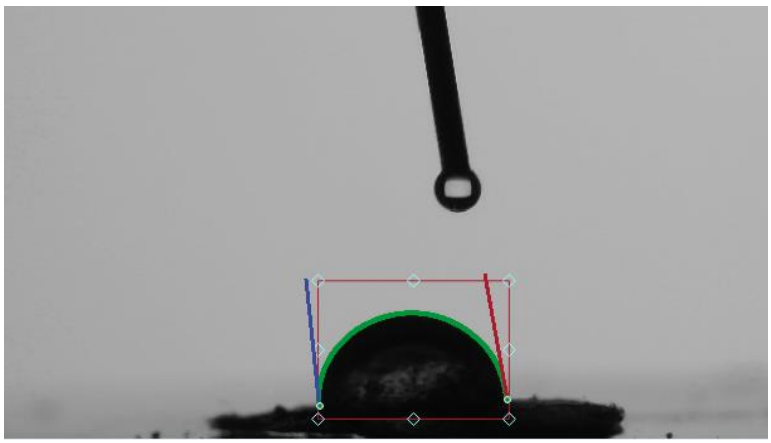

Figure 8: Picture depicting the Contact Angle of the Test (HA+ CuO) group

Table 1: Table depicting the Contact angle of both Control (HA) and Test (HA+CuO) group

Group	CONTACT ANGLE	Average angle
Test	HA (M) + CuO	87.88
Control	HA (M)	67.53

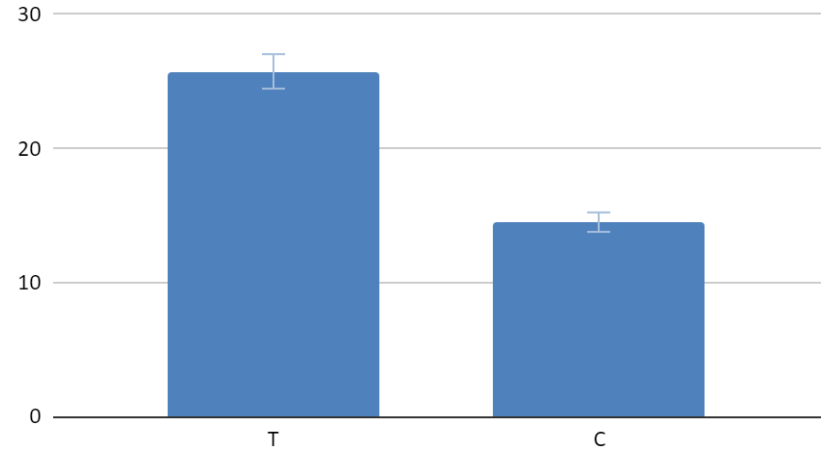

Figure 9: Graph representing the Comparison of Swelling between Control (HA) and Test (HA+CuO) group

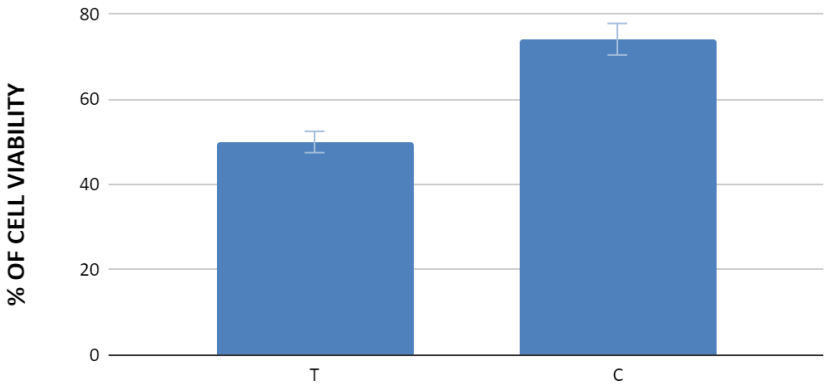

Figure 10: Graph representing the Comparison of Compatibility between Control (HA) and Test (HA+CuO) group

Due to the presence of various sinus cavities, existing bone lacks the mechanical strength required for effective osseointegration with implants(16). As a result, maxillary sinus lift surgery was created to enhance the quantity of bone available for dental implant placement(17). However, due to graft material sagging, this procedure remains difficult to conduct in the posterior maxilla area. Hyaluronic acid was added to bone grafts to enhance bone development and improve handling to solve this issue(18). The mechanism of HLA-induced osteogenesis differed depending on the Molecular weight(19).

Figure 3 and Figure 4 illustrates the SEM analysis of the control group and the test group respectively. The CuO-based group has less porosity and is more evenly distributed, whereas the Hyaluronic acid group has higher porosity and is more scattered. CuO-based particles is thus theorized to have higher cell

adhesion, enhanced cell migration, increased cell penetration efficiency, and a high flow of nutrition as a result of the inclusion of CuO.(20)

Figure 5 and 6 depict the peak values of the Hyaluronic acid and CuO particles, Figure 5 and show the characteristics of amine peak was noticed at 513, The spectral peak at 1053 was observed in the Copper oxide group which reveals the presence of CuO nanoparticle in the Hyaluronic acid membrane.(21)

From figure 7, the contact angle of the Hyaluronic acid is valued to be 67.53 degrees. According to figure 8, the contact angle of the CuO particle with Hyaluronic acid is valued to be 87.88 degrees. Hyaluronic acid is hydrophilic in nature and this infusion of CuO nanoparticles has lead to increase in the contact angle which further lead the substance to be hydrophilic which in turn results in greater efficiency as a guided tissue regeneration membrane(22).

Figure 9 elaborates on the swelling test between the two groups, in which the control group has swelling of about 16% and the addition of copper oxide elevates the swelling up to 25%. This elevation indicates it can be used in the chemo static based membrane or socket
preservation procedures(23).

Figure 10 speaks about the compatibility between the control group and test group where the Control group values were around 70%, and CuO based particles showed around 50% which indicates that the CuO based membrane shows acceptable biocompatibility. Further tests using embryos and live cell cultures should be performed to evaluate the compatibility of the membrane(24).
HLA with MWs ranging from 5 to 20 kDa, for example, demonstrated bone regeneration triggered by cytokine production. However, medium molecular weight hyaluronic acid with molecular weights of 600 and 800 kDa promoted mesenchymal cell development and upregulated osteocalcin mRNA expression(25). Activating stem cells results in the osteogenesis of HLA and CuO nanoparticles with molecular weights ranging from 600 to 800 kDa. Alternatively, 390 kDa HLA increased osteogenesis by enhancing cell adhesion and proliferation(26). The effects of the H-HLA employed in this work on dental pulp stem cell viability were evaluated because its MW was around 600 kDa. Surface markers were employed to identify the properties of the DPSCs used in this study. Furthermore, the presence of calcium deposition after being induced by the osteogenic media proved the cell's osteogenic differentiation potential. These qualities are consistent with the DPSC criteria described in earlier investigations(27).

CONCLUSION:

In this study, a new material made from medium molecular weight/CuO hybrid was fabricated and tested using Dental Pulp stem cells. We found that M-HLA exhibited a stronger osteo regenerative effect. In this study in that all the experiments were performed using a Dental Pulp stem cells were isolated from molars fully represent conditions in the human oral cavity, our data show that the M-HLA/CuO hybrid fabricated in this study provides better osteoconductive properties and significant intracellular responses for bone grafts, making it reasonable to suggest
This M-HLA/CuO hybrid is a potential novel dental material for guided bone regeneration surgery.

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