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Original Article

Human umbilical cord mesenchymal stem cells secretome and nanoemulsion propolis combination ameliorate osteoclastogenesis in lipopolysaccharide-induced osteolysis in hyperglycemia rats

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Abstract *Background/purpose:* Human umbilical cord mesenchymal stem cell secretome (HUCMSCS) and nanoemulsion propolis (NEP) from Tetragonula biroi may be beneficial regenerative medicine to reduce LPS-induced osteolysis with hyperglycemia. This study aimed to investigate the HUCMSCS and NEP effect on nuclear factor associated T-cell-1 (NFATc1), sclerostin, receptor activator kappa beta (RANK), its ligand (RANKL), the osteoprotegerin (OPG) expression, tartrate-resistant acid phosphatase (TRAPase), and cathepsin K (Ctsk) serum level in LPS-induced osteolysis in hyperglycemic Wistar rats (*Rattus novergicus*).
Materials and methods: Twenty-eight healthy male Wistar rats, 1—2 months old and 250—300 g body weight, were divided into seven groups, namely K1: control group; K2: 100 μl LPS; K3: hyperglycemia (≥230 mg/dL); K4: 100 μl LPS with hyperglycemia; K5: LPS, hyperglycemia, and 100 μl NEP; K6: LPS, hyperglycemia, and 100 μl HUCMSCS; and K7: LPS, hyperglycemia, and 100 μl HUCMSCS and NEP. *Escherichia coli's* LPS was used to induce osteolysis on the calvaria. NEP and HUCMSCS were formulated and collected, then injected subcutaneously on the calvaria. Hyperglycemia-induced streptozotocin 30 mg/kg injection for one week, intraperitoneally. All samples were sacrificed on day 8. Blood samples were collected to examine TRAP and Ctsk levels with enzyme-linked immunosorbent assay. NFATc1, sclerostin, and RANK-RANKL-OPG expression investigated with immunohistochemistry.
Results: HUCMSCS and NEP administration inhibit TRAP and Ctsk levels, reduce NFATc1, sclerostin, and RANK-RANKL, but enhance OPG expression in LPS-induced osteolysis with hyperglycemia with significant differences between groups ($P \leq 0.05$).
Conclusion: HUCMSCS and NEP post-administration reduced osteoclastogenesis in LPS-induced osteolysis calvaria with hyperglycemic *in rats* in the early phase of bone remodelling.

Introduction

Lipopolysaccharide (LPS) is a potent endotoxin known to stimulate the production of proinflammatory cytokines, thereby contributing to pathological bone resorption, a condition referred to as osteolysis.[1] In macrophages, hyperglycemic conditions combined with LPS stimulation significantly increase tumor necrosis factor-alpha (TNF-α) production.[2,3] Furthermore, this impaired macrophage function may contribute to increased susceptibility to infection in hyperglycemic conditions.[2] Moreover, in recent studies, *Escherichia coli's* LPS negatively impacted trabecular bone structure but not cortical bone structure, and an upregulation in bone resorption demonstrated by bone cell staining and serum biomarkers was reported.[4]

Propolis is a resinous natural product collected by bees from plant exudates and bud secretions, exhibiting complex biochemical properties. Propolis derived from stingless bees (*Tetragonula biroi*) native to Sulawesi Island has traditionally been utilized for its anti-inflammatory, antioxidant, and immunomodulatory activities, and is increasingly recognized for its potential in biomedical application.[5] Propolis, with its multipotent activities, can be enhanced through encapsulation in nanoemulsion. Nanoemulsions are colloidal dispersions with droplet sizes not beyond 200 nm, formed via droplets of one liquid dispersed in an immiscible liquid, which are then stabilized using a surfactant layer.[6]

Human umbilical cord mesenchymal stem cell secretome (HUCMSCS), also known as conditioned medium, has a variety of bioactive molecules that are secreted by the mesenchymal stem cells (MSCs), including proteins, growth factors, antioxidants, and proteasomes.[7] HUCMSCS contains low levels of proteins, nucleic acids, and lipids, which do not induce inflammation.[8] The combination of NPE and HUCMSCS may potentially be anti-inflammatory and pro-regeneration for LPS-induced inflammatory osteolysis.

One important signaling mechanism that regulates osteoclastogenesis—the formation and activation of osteoclasts responsible for bone resorption—is the receptor

activator of nuclear factor kappa-B (RANK), its ligand (RANKL), and the osteoprotegerin (OPG) system. The RANK receptor on osteoclasts is activated by RANKL, which promotes osteoclast differentiation and activation. Osteoclastogenesis is inhibited when OPG, a decoy receptor, binds to RANKL and prevents its interaction with RANK. The balance between bone formation and resorption is determined by the RANKL/OPG ratio.[9] When osteoclasts differentiate, RANKL initiates a prolonged nuclear factor associated T-cell-1 (NFATc1)-dependent transcriptional program by inducing calcium oscillations that result in calcineurin-mediated activation of NFATc1. According to previous studies, NFATc1-deficient stem cells fail to differentiate into osteoclasts upon RANKL stimulation, whereas ectopic expression of NFATc1 can induce effective differentiation of precursor cells even in the absence of RANKL signaling. These findings suggest that NFATc1 functions as a master regulator of osteoclast terminal differentiation by acting downstream of the RANKL pathway.[10] The sclerostin (SOST) gene encodes sclerostin, which is mostly secreted by mature osteocytes. Because it is an antagonist of the canonical Wnt signaling pathway (WNT), it prevents the production of bones. Interleukin (IL)-6 and RANKL/OPG are also signaling molecules that are important in inflammatory processes. Sclerostin has been shown to control bone remodeling through a number of possible pathways, including the Extracellular signal-regulated kinase $^{1}/_{2}$ (ERK1/2)- runt related transcription factor-2 (Runx2) pathway, WNT, and OPG/Mitogen-Activated Protein Kinase (MAPK).[11] Previous studies also mentioned that tumor necrosis factor-alpha (TNF-α) increased osteocytes' expression of sclerostin and osteocytes' RANKL expression, which was stimulated by sclerostin, increased osteoclastogenesis during OTM. As a result, controlling sclerostin may be a useful therapeutic strategy to counteract bone resorption.[12]

Tartrate-resistant acid phosphatase (TRAP) is a glycoprotein produced by mature osteoclasts, activated dendritic cells, and macrophages.[13] Elevated TRAP levels in gingival crevicular fluid and serum have been observed in a Gaucher's disease and chronic periodontitis making it a potential predictor of bone pathology.[14,15] Therefore, RANK-RANKL-OPG, NFATc1, and sclerostin expression, TRAP, and cathepsin-K (ctsk) serum level may act as an excellent indicator for osteoclastogenesis and osteoclast activity. To date, there is a limited study that focuses on the potential effects of HUCMSCS and NEP on inflammatory osteolysis induced by *E. coli's* LPS and hyperglycemic conditions. The hypothesis is that HUCMSCS and NEP are able to reduce TRAPase and Ctsk serum levels and decrease RANK-RANKL-OPG, NFATc1, and sclerostin expression in osteoclasts in hyperglycemia rats with LPS-induced inflammatory osteolysis. Thus, the aim of this study was to investigate the HUCMSCS and NEP effect on TRAPase and Ctsk serum levels and decrease RANK-RANKL-OPG, NFATc1, and sclerostin expression related to osteoclastogenesis in hyperglycemia with LPS-induced inflammatory osteolysis of Wistar rats (*Rattus novergicus*).

Materials and methods

Experiment setting and design in animals

Wistar rats (*R. novergicus*) aged 2—3 months were obtained from the Stem Cells and Research Development Centre, Universitas Airlangga. The animal experiments were acclimatized and kept in a controlled environment with a 12-h light/dark cycle and a temperature range of 21—24 °C in the animal facility. Every animal experiment was carried out in compliance with Universitas Airlangga regulations and followed the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines checklist.[16] This study was approved by the Ethical Committee of Health Research with number: 0856/HRECC.FODM/V/2025. This study was a true experimental study with a post-test-only control group design. The sample was selected with simple random sampling.

Human umbilical cord mesenchymal stem cell secretome and nanoemulsion propolis preparation

Preparation of HUCMSCs begins with the collection of plain culture media after passing the fourth HUCMSCs culture. The culture media was collected after the fourth HUCMSCs culture passage, then centrifuged at 1500 rpm for 3 min to remove debris and dead cells. Furthermore, the media was filtered through a 0.45 μm syringe filter (Merck Millipore, Burlington, USA) to isolate the bioactive substances released by HUCMSCs during growth. Then, the HUCMSCS from the culture media was purified using a dialysis method to remove residual secretome products, resulting in isolated soluble bioactive factors released by HUCMSCS during culture, followed by characterization, protein concentration quantification, microfiltration for the filtered purification as mentioned in a previous study.[17] Formulation of NEP derived from stingless bee (*T. biroi*) was shown in a previous study. 5 In 2.5 L jars, 250 g of propolis (*T. biroi*) was macerated with 96 % ethanol. The mixture was filtered, and the ethanol was then evaporated at 40—50 °C using a rotary evaporator (Sigma—Aldrich, Wuxi, China). Once the thick extract had dried in the oven, ethanol was added in a volume equal to that of the extract. A previous study found that the 100 % propolis extract was diluted to 1 % concentration in dimethyl sulfoxide (DMSO) (Merck Millipore) and phosphate-buffered saline (PBS) (Merck Millipore) with nanoparticles ranging from 151.28 to 182.2 d nm.[5] In addition, A phytochemical test revealed that NEP included phenol, flavonoids, and alkaloids. The typical value is ±30 mV, but the zeta potential test revealed a value of 32.76 mV.[5] Formulation of HUCMSCS and NEP was done in a 1:1 ratio.

Hyperglycemia and lipopolysaccharide-induced osteolysis animal model

Twenty-eight healthy male Wistar rats (Rattus Novergicus, 1—2 months old, 250—300 g body weight) were divided into

five groups, namely K1: control group; K2: 100 μl LPS; K3: hyperglycemia (>230 mg/dL); K4: 100 μl LPS with hyperglycemia; K5: LPS, hyperglycemia, and 100 μl NEP; K6: LPS, hyperglycemia, and 100 μl HUCMSCS; and K7: LPS, hyperglycemia, and 100 μl HUCMSCS and NEP. Animal model of Wistar rat calvarial osteolysis (*R. novergicus*) induced by lipopolysaccharide (LPS) *E. coli*. Rats were given an intraperitoneal injection of 30 mg of streptozotocin (Sigma Aldrich Inc., MO, USA) dissolved in citrate buffer (CV. Gamma Scientific Biolab, Malang, Indonesia). 30 mg/mL (pH 4.5) injected intraperitoneally in the area beside the midline between two nipples or in the right/left mouse umbilication. Induction was done once; the mouse was held, and the part to be injected was rubbed with 70 % alcohol. After the needle was inserted perpendicular to the peritoneal cavity's right and left umbilical, the STZ was injected gradually for seven day.[18] The dose of phosphate-buffered saline (PBS), HUCMSCS, and LPS given was 100 μL once a day for 7 days.[19] All samples were terminated on day 8 with anesthetization and cervical dissection. The animal experiments' calvaria were isolated for further analysis by means of immunohistochemistry. In addition, blood from the sample for each group was collected and processed for plasma for further analysis by means of enzyme-linked immunosorbent assay (ELISA) with sandwich technique.

Enzyme-linked immunosorbent assay

The sandwich ELISA method was used to detect the presence and concentration of TRAPase and Ctsk in the serum. The steps of the sandwich ELISA method begin with preparation, sample incubation, washing, target antibody incubation, sample washing, analysis, and interpretation according to the instructions of the rat TRAP and Ctsk ELISA kit (Shanghai Korain Biotech Co., Ltd., Shanghai, China).

Immunohistochemistry

Immunohistochemistry was used to evaluate the positive expression of osteoclastogenesis markers in rats from various groups, including sclerostin, RANK, RANKL, NFATc1, and OPG. To put it simply, tissue samples embedded in paraffin were cut at a thickness of 4 μm, dewaxed in xylene, and then dehydrated using graded ethanol. The slices were incubated in 3 % H_2O_2 for 15 min and blocked in 5 % goat serum for 15 min following a 15-min microwave irradiation in sodium citrate (Sigma–Aldrich) at 95 °C. Following a 12-h incubation period at 4 °C with a particular primary antibody (anti-NFATc1, -Sclerostin, -RANK, -RANKL, -OPG (Abcam, Cambridge, MA, USA), the sections were rinsed with phosphate buffer saline and then incubated with a secondary antibody coupled with horseradish peroxidase (Abcam) for 30 min at 37 °C. The slices were then photographed under a light microscope (Olympus, Tokyo, Japan), counterstained with hematoxylin (Santa Cruz Biotechnology, Dallas, Texas, USA), and visualized using a diaminobenzidine kit (Sigma–Aldrich). The positive expression of NFATc1, sclerostin, and RANK expression was observed in the osteoclast, while RANKL and OPG positive expression were observed in osteoblasts examined by two observers across five different fields under a microscope at 100 ×, 400 ×, and 1000 × magnification, identified by brown precipitate.

Statistical analysis

The data's mean standard deviation (SD) from each biological replicate is shown. For statistical analyses, t-tests and one-way analysis of variance (ANOVA) continued with post-hoc test using Tukey Honest Significat Different (HSD) were employed, with significance set at $P \leq 0.05$.

Results

NEP and HUCMSCS combination inhibits osteoclastogenesis-related markers (NFATc1, Sclerostin, RANK, RANKL, and OPG) in LPS-induced osteolysis with hyperglycemic as documented immunohistochemistry, *in vivo*. NFATc1 (11.6 ± 2.51), Sclerostin (12.4 ± 0.79), RANK (10.6 ± 0.46), and RANKL (11.2 ± 2.37) had the highest expression in the osteoclast in the LPS-induced osteolysis with hyperglycemic group (Fig. 1A–D). In the LPS-induced osteolysis with hyperglycemic treatment with NEP and HUCMSCS combination found that NFATc1, Sclerostin, RANK, and RANKL were significantly reduced ($P \leq 0.05$). On the other hand, the greatest OPG expression (11.2 ± 2.1) in the osteoblast was shown in LPS-induced osteolysis treated with an NEP and HUCMSCS combination (Fig. 1E). There was a significant difference between groups in NFATc1, Sclerostin, RANK, RANKL, and OPG ($P \leq 0.05$). ELISA investigation showed that HUCMSCS in conjunction with NEP can considerably lower the TRAP and Ctsk serum level of LPS-associated inflammatory osteolysis calvaria in hyperglycemia condition (Fig. 2A and B) ($P \leq 0.05$). This result showed that HUCMSCS and NEP act synergistically in reducing TRAPase and Ctsk serum levels.

Discussion

To date, this is the first *in vivo* study to investigate the effects of HUCMSCs and NEP on osteoclastogenesis-related markers—including NFATc1, sclerostin, RANK, RANKL, and OPG—as well as serum levels of TRAP and Ctsk in LPS-induced osteolysis under hyperglycemic conditions. The result obtained from this study indicates that HUCMSCS and NEP can significantly reduce TRAP and Ctsk serum levels, NFATc1, Sclerostin, and RANK-RANKL but increase OPG expression in inflammatory osteolysis in the calvaria in hyperglycemic rats.

LPS is known to activate the nuclear factor kappa beta (NF-κB) pathway, which plays a vital role in inflammation, and increased pro-inflammatory cytokines may promote RANKL expression, which then causes osteoclastogenesis, resulting in bone resorption, thus inhibiting bone formation.[20] Our previous studies also mentioned the same result.[1,19] LPS and hyperglycemia can synergistically exacerbate inflammatory responses, potentially leading to more severe osteolysis. In osteoclasts, hyperglycemia activates the reactive oxygen species (ROS)/MAPK)/NF-κB/family

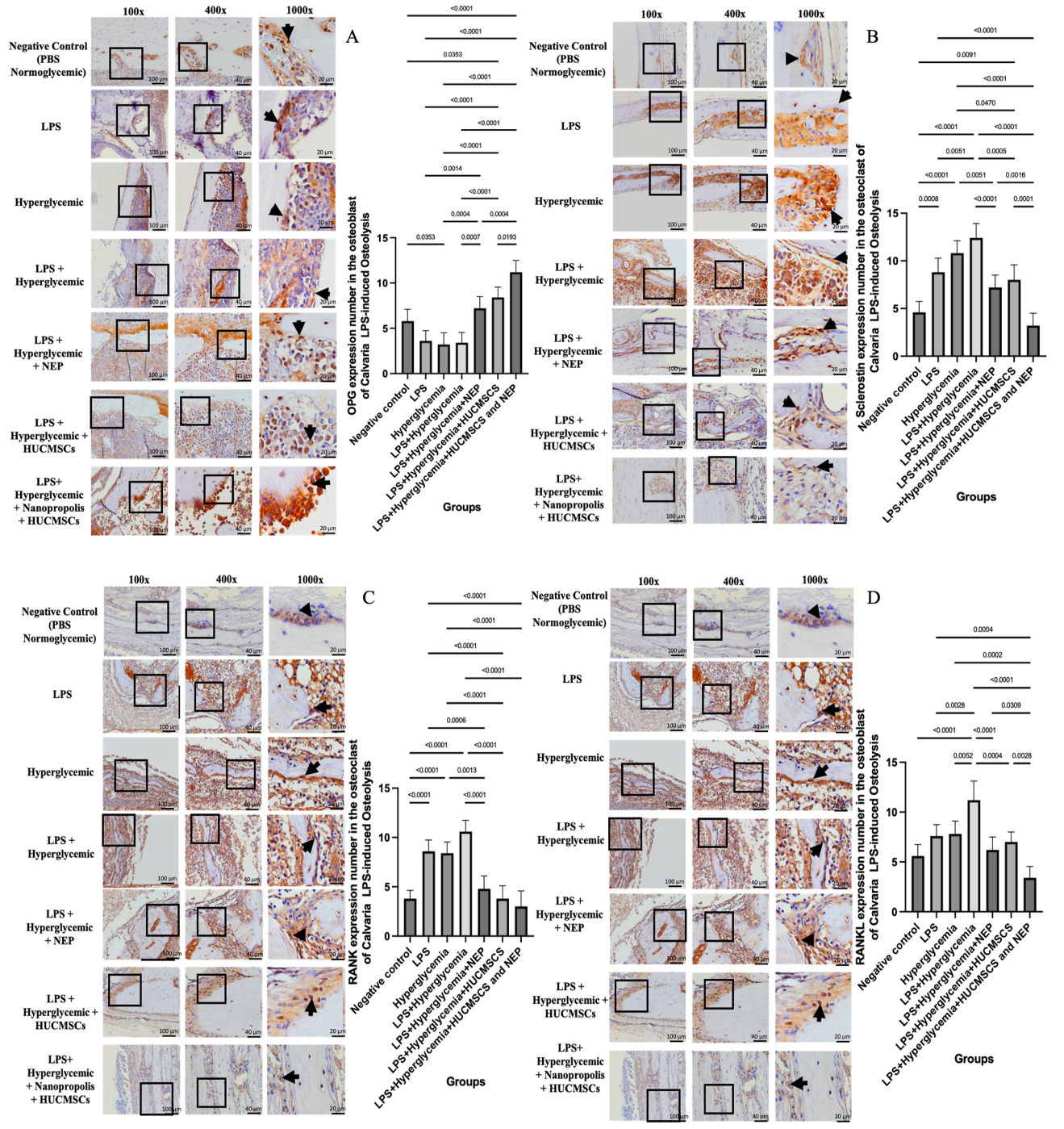

Figure 1 Effect of NEP and HUCMSCS combination on LPS-induced osteolysis in hyperglycemic condition. Image of the NFATc-1 (A), sclerostin (B), RANK (C), RANKL (D), and OPG (E) immunohistochemistry analysis with DAB staining and counterstained with hematoxylin. NFATc-1, sclerostin, RANK, RANKL, and OPG-positive cells were stained brown at 100x, 400x, and 1000× magnification. Results are expressed as the mean ± SD. The statistical significance of differences was determined by t-tests and one-way analysis of variance (ANOVA) continued by Tukey Honest Significant Different (HSD) with significance set at $P \leq 0.05$. n = 4 for each group. (Scale bar = 100 μm, 40 μm, and 20 μm, respectively). DAB, 3,3′-Diaminobenzidine Tetrahydrochloride; HUCMSCS, human umbilical cord mesenchymal stem cells secretome; LPS, lipopolysaccharide; NEP, nanoemulsion propolis extract; NFATc1, nuclear factor associated T-cell-1; OPG, osteoprotegrin; PBS, phosphate-buffered saline; RANK, receptor activator nuclear kappa beta; RANKL, receptor activator nuclear kappa beta ligand; SD, standart deviation.

pyrin domain containing 3 pathway (NLRP3), increasing bone resorption and inhibiting efferocytosis.[21] This activation leads to increased expression of inflammatory cytokines such as IL-1β, IL-18, and TNF-α.[22] The ROS-dependent activation of endothelial NLRP3 inflammasomes contributes to endothelial dysfunction in diabetes.[23]

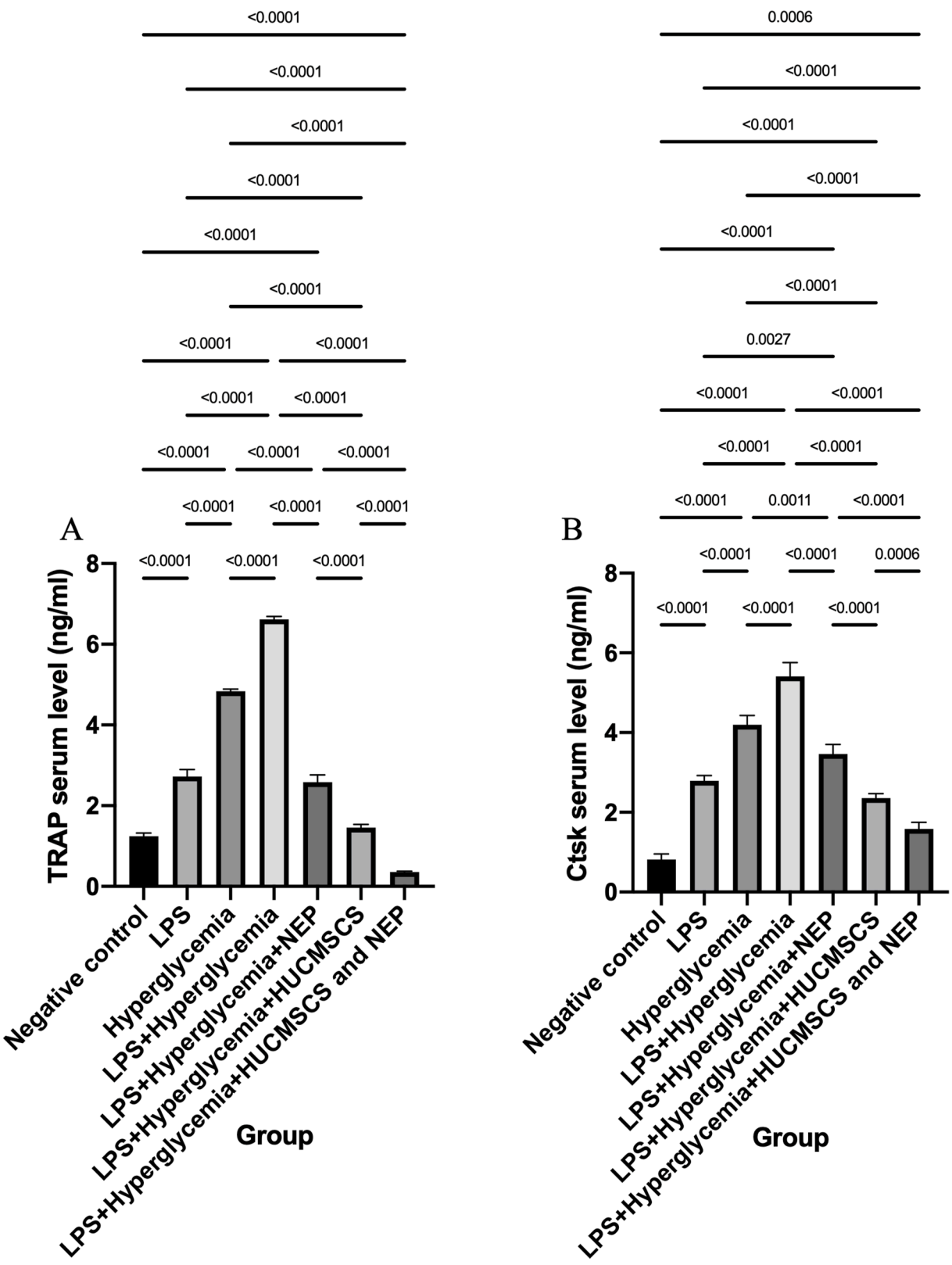

Figure 2 Effect of NEP and HUCMSCS combination on LPS-induced osteolysis in hyperglycemic conditions in TRAPase and Ctsk serum levels. (A) Concentration of TRAP in serum was measured by ELISA. (B) Concentration of Ctsk in serum was measured by ELISA. A comparison of (A) TRAPase and (B) Ctsk serum levels in all experimental groups. Results are expressed as the mean ± SD. The statistical significance of differences was determined by t-tests and one-way analysis of variance (ANOVA) by Tukey Honest Significant Different (HSD) with significance set at $P \leq 0.05$. n = 4 for each group. CTSK, Cathepsin K; HUCMSCS, human umbilical cord mesenchymal stem cells secretome; LPS, lipopolysaccharide; NEP, nanoemulsion propolis extract; PBS, phosphate-buffered saline; SD, standart deviation; TRAP, tartrate-resistant acid phosphatase.

Hyperglycemia caused tissue destruction, delayed wound healing, and bone remodeling, as shown in our previous studies.[18,24,25]

The HUCMSCS represents a novel approach in wound healing management by releasing bioactive factors instead of directly replacing damaged cells.[26] Various bioactive factors that are released from the MSC secretome consist of indoleamine 2,3-dioxygenase (IDO), prostaglandin-E2 (PGE2), IL-4, IL-10, IL-12, and hepatocyte growth factor (HGF) that can be harvested by isolating their culture medium contained in the MSCs and have the capacity to target and modulate various pathways in the wound healing process. These factors act via a paracrine mechanism to downregulate the pro-inflammatory gene expression and cytokine production of immune cells, potentially accelerating the resolution of the inflammatory phase.[27] Previous studies showed gingival mesenchymal stem cell metabolites ameliorate LPS-induced osteolysis in the calvaria by reducing TRAP, NFATc1, and sclerostin which enlight that secretome derived MSCs has potential regenerative capability.[19] Meanwhile, in recent studies, propolis nanoparticles exhibited anti-inflammatory and anti-apoptotic effects in liver and kidney tissues, reducing transforming growth factor-beta (TGF-β) and Caspase-9 expression while increasing B-cell lymphoma 2 (Bcl-2) levels.[28] Therefore, nanoformulations of propolis can enhance its bioavailability and therapeutic potential for various diseases, including oral conditions.[29] This study finding was supported by several previous studies that mentioned the propolis treated the bone resorption due to inflammation.[29—31] NPE has shown potential in treating osteoporosis by specifically modulating gut microbiota dysbiosis. In a randomized clinical trial, subgingival administration of NPE demonstrated promising results when used as an adjunct to scaling and root planing in periodontitis patients with periodontal pockets.[31]

Regenerative potential of HUCMSCS for bone inflammatory disease is also mentioned in several studies.[32—35] Over a 5-week period, HUCMSCS intra-articular injections demonstrated no side effects, biomarker alterations, and improved clinical improvement when compared to hyaluronic acid.[32] In comparison to patients receiving conventional care, HUCMSCS percutaneous injections into bone grafts every three days for 21 days promote bone repair, as evidenced by increased collagen density and osteoblast cell formation.[33] When it comes to treating early-stage osteoarthritis in the animal model, intra-articular injection of secretome is just as effective as hyaluronic acid and comparable toMSC injection.[34] An earlier study using an animal model with calvarial bone defects treated with 2-dimension (2D) HUCMSCS showed superior bone regeneration capacity in comparison to the 3-dimension (3D) secretome supported by a number of important molecules influencing bone regeneration capability.[35]

In conclusion, NEP and HUCMSCS treatment effectively reduced osteoclastogenesis in LPS-induced osteolysis with hyperglycemia by decreasing the expression of NFATc1, sclerostin, RANK-RANKL, TRAP, and Ctsk serum levels while increasing the expression of OPG in the early bone remodeling process. These results demonstrate that NEP and HUCMSCS together may be useful as an herbal regeneration therapy for inflammatory bone disease. In order to mitigate pathological bone resorption and potentially help patients, more research is needed to understand the variables driving osteoclast differentiation, bone resorption and investigate the potential uses of NEP and HUCMSCs. Furthermore, only limited molecular markers related to osteoclastogenesis were assessed as markers, which may limit insights into the broader mechanisms involved. Nonetheless, further research is necessary to build upon these findings, as this study has certain limitations. The analysis primarily relied on ELISA and immunohistochemistry analysis, which, while useful, may not capture the full spectrum of biochemical interactions at play. Additionally, the observation period was relatively short—only an 8-day observation period for a long-term process like bone remodeling—potentially overlooking longer-term effects of HUCMSCs and NEP administration. Furthermore, the study did not include detailed quantification of protein concentrations or thorough characterization of the HUCMSCs, which constitutes another limitation. Future research should consider extending the observation period, incorporating a broader range of molecular markers, employing diverse methodological approaches, and performing protein quantification and stem cell characterization. Moreover, studies should include optimized single-agent control groups to better validate the combined effects of HUCMSCs and NEP, thereby providing a more comprehensive understanding of their roles in inflammatory osteolysis under hyperglycemic conditions.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

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