氏　　名	KAUNG HTIKE
授与した学位	博　士
専攻分野の名称	歯　学
学位授与番号	博甲第７２５１号
学位授与の日付	令和７年３月２５日
学位授与の要件	医歯薬学総合研究科生体制御科学専攻 （学位規則第４条第１項該当）
学位論文の題目	Herbal medicine Ninjinyoeito inhibits RANKL-induced osteoclast differentiation and bone resorption activity by regulating NF-κB and MAPK pathway. （人参養栄湯による破骨細胞分化と骨吸収においての抑制効果）
論文審査委員	上岡　寛　教授　　美藤　純弘　准教授　　大森　一弘　准教授

学位論文内容の要旨

Objectives: Osteoporosis is a systemic bone metabolism disorder characterized by decreased bone mass and strength. Osteoclasts (OCs) are multinucleated giant cells that regulate bone homeostasis by degrading bone matrix. Excessive OC differentiation and activity can lead to serious bone metabolic disorders including osteoporosis. Current treatments, including antiresorptive drugs, exert considerable adverse effects, including osteonecrosis of the jaw. Herbal medicines, such as Ninjinyoeito (NYT), may also offer efficacy, but with fewer adverse effects. In this study, we investigated NYT's effects on osteoclastogenesis.

Introduction: OCs are multinucleated cells that derived from macrophage/monocyte lineage cells and are responsible for bone homeostasis. Specifically, the binding of receptor activator of nuclear factor-κB ligand (RANKL) to its corresponding receptor (RANK) and macrophage colony-stimulating factor (M-CSF) to its receptor, colony-stimulating factor 1 receptor (c-fms), induce OC differentiation. These bindings activate downstream signaling cascades, leading to the nuclear translocation of two core transcription factors: (1) nuclear factor of activated T-cell cytoplasmic 1 (NFATc1) and (2) c-fos. Subsequently, OC-related genes, such as tartrate-resistant acid phosphatase (*TRAP*), Cathepsin K (*CTSK*), and matrix metalloproteinase 9 (*MMP9*), are involved. For OC-induced bone resorption, OCs are required to adhere to the bone surface to create a sealing zone. Following the adhesion of OCs to the bone surface, activated OCs form the ruffled border within the sealed zone, and secrete the acidic ions such as H^+ and Cl^- into the bone extracellular matrix (ECM) to establish the acidic milieu of the resorption lacuna and dissolve the mineralized ECM. Destruction of the mineralized ECM exposes organic elements, which are subsequently degraded by several secreted lysosomal enzymes, such as TRAP, CTSK, and MMP9, thereby triggering bone resorption. Ninjinyoeito (NYT), a traditional Japanese Kampo medicine known for its pharmaceutical effects, such as anti-inflammatory action, is useful for treating anemia and enhancing immunity. NYT is composed of various herbal extracts including *Ginseng*, *Glycyrrhiza*, *Japanese angelica root*, *Cinnamon bark*, *Polygala root*, *Citrus unshiu peel*, *Astragalus root*, *Schisandra fruit*, *Peony root* and *Rehmannia root*. NYT exerts anti-depressant action and reduces β-

amyloid-induced axonal damage by enhancing the central noradrenergic system and neurotrophic factors. Moreover, it suppressed the onset of arthritis, pain, and muscle atrophy in a rheumatoid arthritis mouse model.

A recent study reported that NYT suppresses OC differentiation in RAW264.7 cells, however, this study emphasized the effect of Unkeito in osteoclastogenesis and the detailed mechanism of the NYT has not yet been elucidated. Therefore, we investigated the effect of NYT on osteoclastogenesis in this study.

Methods: As osteoclast precursor cells, a murine monocytic cell line (RAW-D cells) was cultured in minimum essential medium α (MEMα) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (10 mg/mL) in 5% CO_2 and 95% air humidified incubator at 37°C. For OC differentiation from RAW-D cells, RAW-D cells were cultured in MEMα with RANKL (100 ng/mL) for 3 days. For the preparation of NYT, the powder of NYT was dissolved in MEMα and later centrifuged at 12000 rpm for 20 min and filtered by 0.45 μm Cellulose Acetate filter units. The timing of NYT's addition to the medium was simultaneous with RANKL. Tartrate-resistant acid phosphatase (TRAP) staining and bone resorption assays were performed to examine NYT's effects on OC differentiation and function. OC-related gene expression at mRNA and protein levels was investigated using Quantitative Reverse Transcription-Polymerase Chain Reaction (RT-qPCR) and Western blotting (WB) to confirm NYT's inhibitory action against osteoclastogenesis. We conducted time dependent WB study to clarify the involvement of signaling pathways mediated by IκBα and mitogen-activated protein kinases (MAPK) [extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38] following NYT treatment. Immunocytochemistry (ICC) was performed to investigate the nuclear translocation of nuclear factor of activated T-cell cytoplasmic 1 (NFATc1) and nuclear factor kappa B (NF-κB) p65 during osteoclastogenesis.

Results: CCK-8 assay showed that NYT has no significant cytotoxicity until the concentration of 200μg/mL. TRAP staining and bone resorption assays confirmed that NYT significantly inhibited OC differentiation and function. RT-qPCR and WB results showed that NYT mitigated osteoclastogenesis by suppressing protein and mRNA expression of OC-related genes. Moreover, WB and ICC data clarified that NYT abrogated signaling pathways of IκBα and MAPK (ERK, JNK, p38), and nuclear translocation of NFATc1 and NF-κB p65 during OC differentiation.

Conclusion: In this study, NYT significantly inhibited OC differentiation and bone resorption by downregulating the protein and mRNA expression of various OC markers. Furthermore, we elucidated that NYT suppressed osteoclastogenesis by inhibiting the phosphorylation of IκBα and MAPKs (ERK, JNK, p38) and also the nuclear translocation of NFATc1, a master regulator of osteoclastogenesis, and NF-κB p65, an important subunit of IκBα. Additionally, NYT demonstrated minimal cytotoxic effects on OC precursor cells. Thus, NYT has the potential as a therapeutic drug with fewer adverse effects for treating osteoclast-related bone metabolic disorders, such as osteoporosis.

論文審査結果の要旨

【**Introduction**】Osteoclasts (OCs) are multinucleated giant cells derived from macrophage/monocyte linage cells and are responsible for bone remodeling by degrading the bone matrix. Ninjinyoeito (NYT), a traditional Japanese Kampo medicine known for its pharmaceutical effects, such as anti-inflammatory action, is useful for treating anemia and enhancing immunity. In this study, we investigated the effect of NYT on osteoclastogenesis.

【**Methods**】A murine monocytic cell line (RAW-D cells) was used as osteoclast precursor cells. The cytotoxic effect of NYT was confirmed using CCK8 assay. Tartrate-resistant acid phosphatase (TRAP) staining and bone resorption assays were performed to examine OC differentiation and function. OC-related gene expression at protein and mRNA levels was investigated using Western blotting (WB) and RT-qPCR. Time-dependent WB study was conducted to examine signaling pathways mediated by IκBα and mitogen-activated protein kinases (MAPK). Nuclear translocation of NFATc1 and NF-κB p65 was detected by immunocytochemistry (ICC).

【**Results**】CCK-8 assay showed that NYT has no significant cytotoxicity until the concentration of 200 μg/mL. TRAP staining and bone resorption assays confirmed that NYT significantly inhibited OC differentiation and function. Western blot (WB) and RT-PCR results showed that NYT mitigated osteoclastogenesis by suppressing protein and mRNA expression of OC-related genes. Moreover, WB and ICC data clarified that NYT abrogated signaling pathways of IκBα and MAPK (ERK, JNK, p38), and nuclear translocation of NFATc1 and NF-κB p65.

【**Conclusion**】In this study, NYT inhibited OC differentiation and bone resorption by downregulating the protein and mRNA expression of OC markers. NYT suppressed osteoclastogenesis by inhibiting the phosphorylation of IκBα and MAPKs (ERK, JNK, p38) and also the nuclear translocation of NFATc1, a master regulator of osteoclastogenesis, and NF-κB p65. NYT demonstrated minimal cytotoxic effects on OC precursor cells. Thus, NYT has the potential as a therapeutic drug with fewer adverse effects for treating osteoporosis.

This research article has already accepted and published by *Journal of Oral Biosciences*. Therefore, the defense committee hereby accepts this article as a doctoral dissertation in Dentistry/Philosophy.