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Analysis of Risedronate Analog on Extraction Socket Healing in Mice

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Abstract

Background/Aim: Medication-related osteonecrosis of the jaw (MRONJ) is a severe adverse effect associated with anti-resorptive medications like nitrogen-containing bisphosphonates (N-BPs), particularly zoledronate (ZOL). This study aimed to investigate whether a BP analog with high bone affinity but minimal anti-resorptive activity, NE-58051, could induce MRONJ-like lesions in a mouse model.

Materials and Methods: Female C57BL/6J mice (n=6 per group) were administered ZOL (250 μg/kg intravenously, twice weekly) for one or two weeks, or NE-58051 (250 μg/kg intravenously, twice weekly) for two weeks. Two weeks after initiation of study, the bilateral first molars were extracted. Mice were euthanized after a total duration of four weeks. Histological assessments evaluated necrotic bone area and osteoclast activity at extraction sites. Serum tartrate-resistant acid phosphatase isoform 5b (TRAcP-5b) levels were measured.

Results: Mice treated with ZOL for two weeks exhibited significant increases in empty osteocytic lacunae and necrotic bone area compared to the saline group, indicating the development of MRONJ-like lesions. NE-58051-treated mice did not show significant differences in necrotic bone area or osteoclast activity compared to controls. No significant differences were observed in serum TRAcP-5b levels among all groups.

Conclusion: High bone affinity without potent inhibition of bone resorption does not induce MRONJ-like lesions in mice. These findings suggest that the potent anti-resorptive activity of N-BPs is a key factor in MRONJ development, highlighting the importance of bone turnover suppression in the pathogenesis of this condition.

Keywords: Osteonecrosis of the jaw (ONJ), bisphosphonate analog, mouse model of ONJ.

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Introduction

Medication-related osteonecrosis of the jaw (MRONJ) is a severe adverse event characterized by exposed necrotic bone in the maxillofacial region that persists for more than eight weeks (1). This condition is primarily associated with anti-resorptive medications, particularly nitrogen-containing bisphosphonates (N-BPs) used to treat osteoporosis and bone metastases (2, 3). Accurately estimating the incidence of MRONJ remains challenging due to its multifactorial etiology. Among patients receiving N-BPs, the condition is reported more frequently in those treated for cancer compared to those treated for osteoporosis (1).

BPs have unique mechanisms of action based on their high affinity for bone mineral. The P-C-P structure of BPs allows them to bind strongly to hydroxyapatite crystals through chelation with calcium ions (2, 3). This distinctive property leads to selective accumulation in skeletal tissue, where BPs can persist for extended periods. Among BPs, zoledronate (ZOL) demonstrates particularly high bone affinity and potency in inhibiting bone resorption (2, 3).

The primary mechanism underlying MRONJ development appears to be profound suppression of bone remodeling through direct and indirect inhibition of osteoclast function (2, 4). N-BPs inhibit farnesyl pyrophosphate synthase (FPPS) in the mevalonate pathway, leading to disrupted osteoclast function and accelerated apoptosis (4). This extensive suppression of bone turnover, particularly when combined with local risk factors such as tooth extraction or infection, creates conditions favorable for osteonecrosis development (2, 4).

Multiple mouse models have been developed to study MRONJ pathophysiology, typically combining high-dose N-BP treatment with various local risk factors (5-7). These models include tooth extraction alone (8), extraction with concurrent periodontal disease (9), or periapical disease (10). While these models have successfully replicated clinical, radiographic, and histological features of MRONJ, they have primarily focused on commercially available N-BPs at doses equivalent to or exceeding those used in cancer treatment (5-7).

The extent to which N-BPs alone contribute to MRONJ development remains unclear. Some studies suggest that other mechanisms, such as anti-angiogenic effects, may contribute to MRONJ pathogenesis (11, 12). Therefore, the purpose of this preliminary study was to investigate whether a BP analog, which has similar bone affinity to BPs but less inhibitory effect on bone resorption, could induce MRONJ-like lesions in a mouse model and potentially provide new insights into the fundamental mechanisms underlying this disease.

Materials and Methods

Reagents. ZOL was purchased from Novartis AG (Zometa; Basel, Switzerland). NE-58051, a risedronate analog, was synthesized by Fuji Molecular Planning (Yokohama, Japan). NE-58051 had a bone mineral affinity similar to that of risedronate, but a 3000-fold lower bone anti-resorptive activity *in vivo* (13-16).

Animals and drug administration. All animal care and experimental procedures were conducted in compliance with the ARRIVE guidelines and approved by the Ethics Committee for Animal Experiments at Hiroshima University (approval number: A23-121). Eight-week-old female C57BL/6J mice (n=6 per experimental group; body weight, 17-20 g) were obtained from Charles River Laboratories Japan (Yokohama, Japan). Mice were housed in standardized laboratory enclosures under controlled environmental conditions with a 12-h light/dark cycle. Standard diet and tap water were provided ad libitum throughout the experimental period. Twenty-six of these mice were randomly distributed into four groups: saline, ZOL 1 week treatment (1w), ZOL 2 weeks treatment (2w), and NE-58051. The established mouse models of MRONJ were used as previously described (5-7). Mice were administered ZOL (250 μg/kg intravenously, twice weekly) or NE-58051 (250 μg/kg intravenously, twice weekly) for 1 or 2 weeks. Two weeks after initiation of the study, the bilateral first molar were extracted in the manner previously reported (17). The mice were euthanized after a total duration of four weeks (Figure 1).

Histological analysis. The preparation and histological analysis of maxilla samples were performed as previously described (17). Briefly, fixed maxilla samples were decalcified, paraffin-embedded, and sectioned. Hematoxylin and eosin staining was performed for general histological observation, while TRAP staining was used to identify osteoclasts in the extraction sockets.

Histological analysis was performed using digital images captured with a BZ-X710 microscope (KEYENCE), following our previously described methods (17, 18). Briefly, necrotic bone was identified by the presence of at least five consecutive empty or pyknotic osteocytic lacunae, and quantified within the region of interest (ROI) surrounding the extraction socket. The number of empty osteocytic lacunae, percentage of necrotic bone area, and distribution of osteoclasts were analyzed using ImageJ software (version 1.54, National Institutes of Health, Bethesda, MD, USA) as previously described (17, 18).

Murine serum TRAP isoform 5b (TRAcP-5b) measurement. Blood samples were immediately collected *via* cardiac puncture at the time of euthanasia. Each blood sample was centrifuged at 1,300 g for 20 min and the serum supernatant was harvested. Serum TRAcP-5b was measured by an enzyme-linked immunosorbent assay (ELISA) kit (Elabscience, Houston, TX, USA). The absorbance at 450 nm in the Mouse ELISA kit was recorded using a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis. Statistical analysis was performed using R software (version 4.4.1, R Foundation for Statistical Computing, Vienna, Austria). The normality of data distribution was assessed using the Shapiro-Wilk test. For datasets meeting the assumption of normality, one-way analysis of variance was conducted, followed by Tukey's honest significant difference test for multiple comparisons. In cases where the normality assumption was not met, the Kruskal-Wallis test was employed as a non-parametric alternative, followed by Dunn's test for pairwise comparisons. These analyses were used to evaluate differences among four independent groups. The values are expressed as means±standard deviations. Significance was set at $p<0.05$.

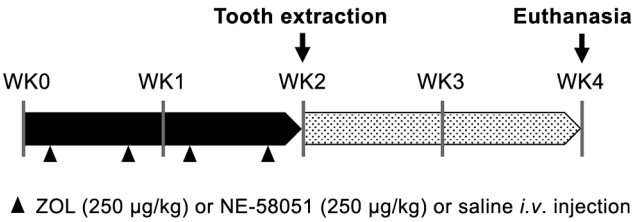

Figure 1. *Schematic of drug treatment and tooth extraction. Mice were administered zoledronate (ZOL, 250 μg/kg intravenously, twice weekly) or NE-58051 (250 μg/kg intravenously, twice weekly) for 1 or 2 weeks. Two weeks after initiation of the study, the bilateral first molars were atraumatically extracted under anesthesia.*

Results

Clinical appearance of tooth extraction socket healing. To evaluate the effects of ZOL and NE-58051 on the healing of the tooth extraction socket, clinical appearance was evaluated in all experimental groups. Intraoral photographic analysis showed complete mucosal closure with no open wounds in the saline group. Similarly, complete wound closure was observed in the ZOL 1w, ZOL 2w, and NE-58051 treatment groups (Figure 2).

Histological analysis of tooth extraction socket healing. Histological examination showed new bone formation within the extraction sockets across all experimental groups (Figure 3A-D). Morphometric analysis revealed a significantly higher density of empty osteocytic lacunae in the ZOL 2w treatment group compared to the other experimental groups (Figure 4A). While areas of necrotic bone characterized by empty lacunae were present in all groups, quantitative assessment of necrotic bone revealed that the ZOL 2w group had significantly greater areas of necrotic bone compared to the other experimental groups (Figure 4B). The NE-58051 group had a similar number of empty osteocytic lacunae and necrotic bone area as the saline group (Figure 4B).

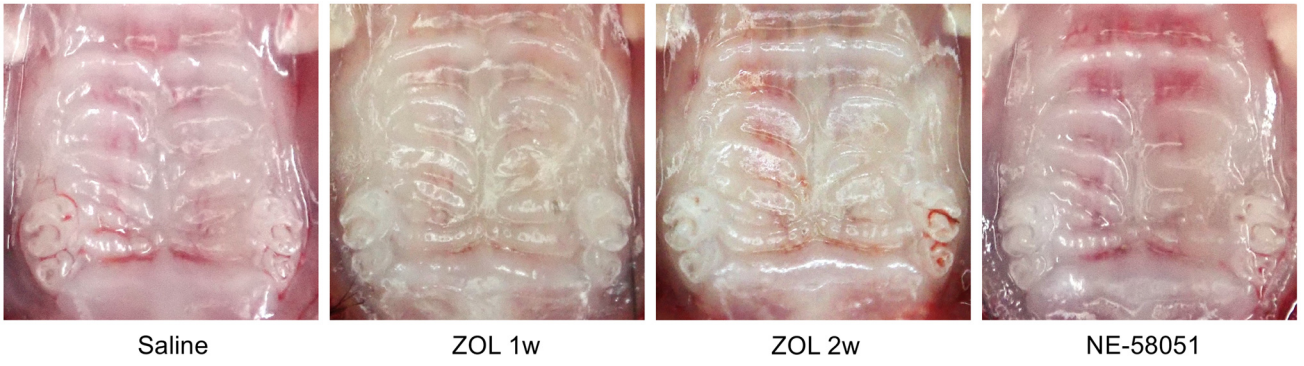

Figure 2. *Representative intraoral photographs showing clinical appearance of tooth extraction sockets.*

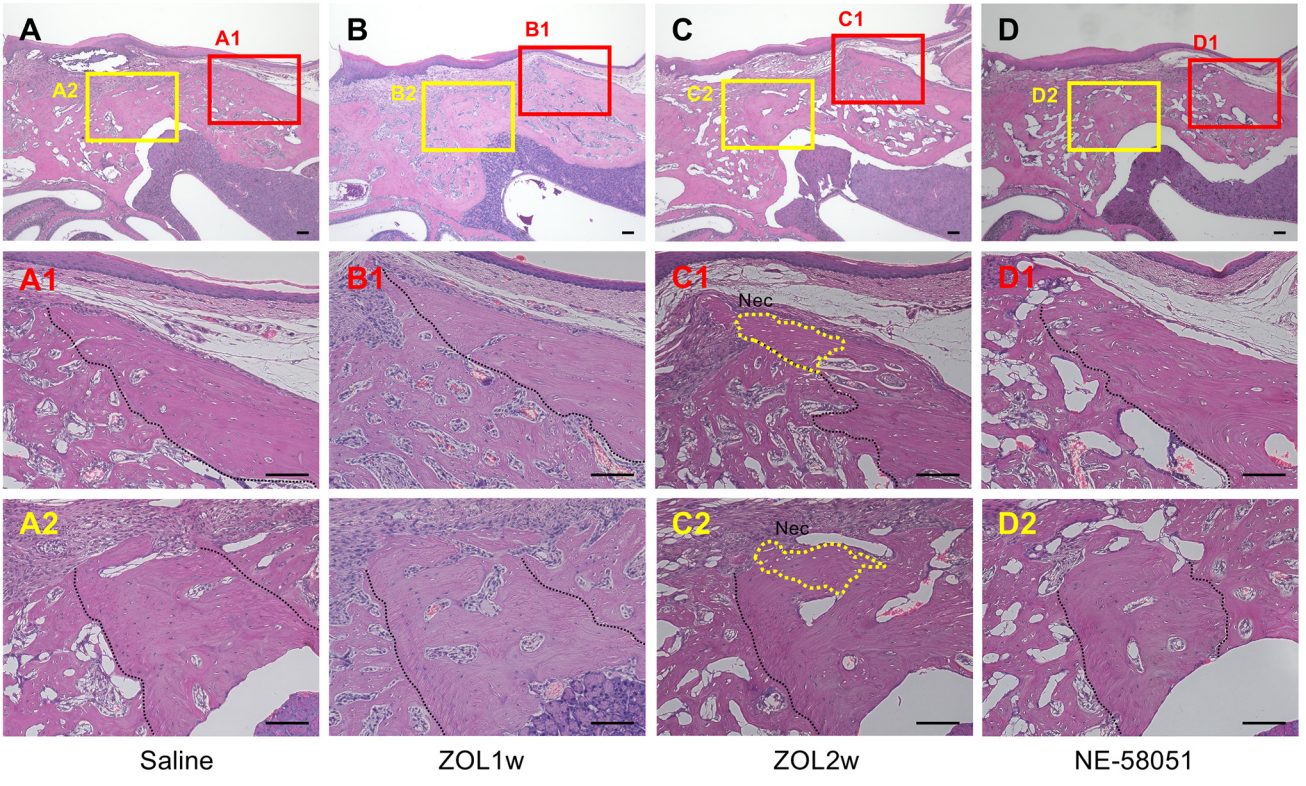

Figure 3. *Histological assessment of the alveolar bone area. Maxillae from mice that had been subjected to (A) saline, (B) zoledronate (ZOL) 1 week, (C) ZOL 2 weeks, and (D) NE-58051. Black dotted line indicates extraction socket boundary. Nec: Necrotic bone; Bars, 100 μm.*

Assessment of TRAP-positive cells in tooth extraction sockets and TRAcP-5b levels in serum. To examine the presence of osteoclasts at tooth extraction sockets, TRAP staining was performed (Figure 5). There were no significant differences in total TRAP-positive cell counts, TRAP-positive cells adhering to bone surfaces, or detached TRAP-positive cells in all groups, but there was a trend toward an increase in detached TRAP-positive cells in the ZOL 2w group (Figure 6A). There were no significant differences in serum TRAcP-5b levels in all groups (Figure 6B).

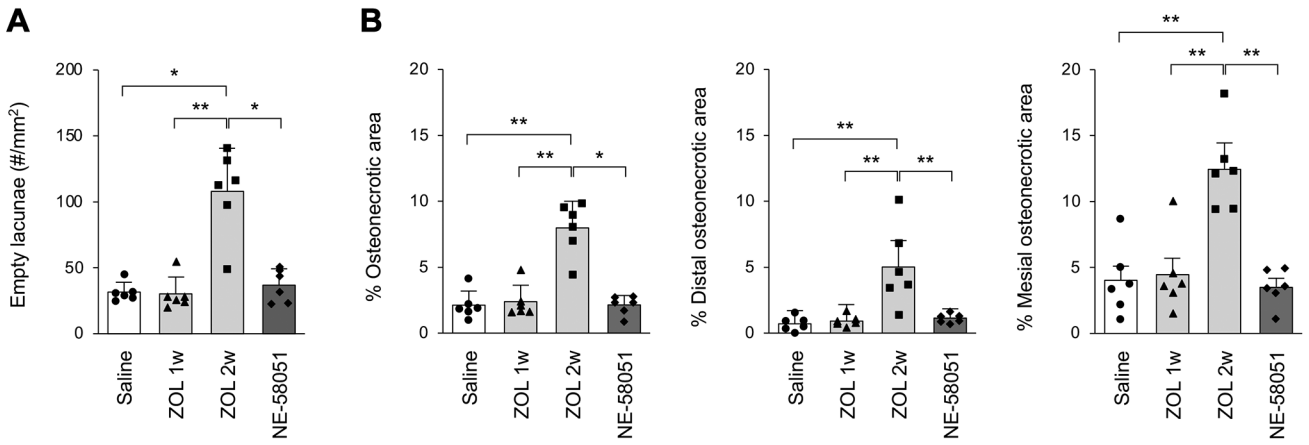

Figure 4. *Quantification of histological assessment in mice that had been subjected to saline, ZOL 1 week, ZOL 2 weeks, and NE-58051. (A) Proportions of empty osteocytic lacunae in the maxilla. (B) Proportions of osteonecrotic area in the maxilla, shown as total (left), distal (middle), and mesial (right) osteonecrotic area. Data represent means±standard deviations. Asterisks indicate statistically significant differences. **p<0.01, *p<0.05.*

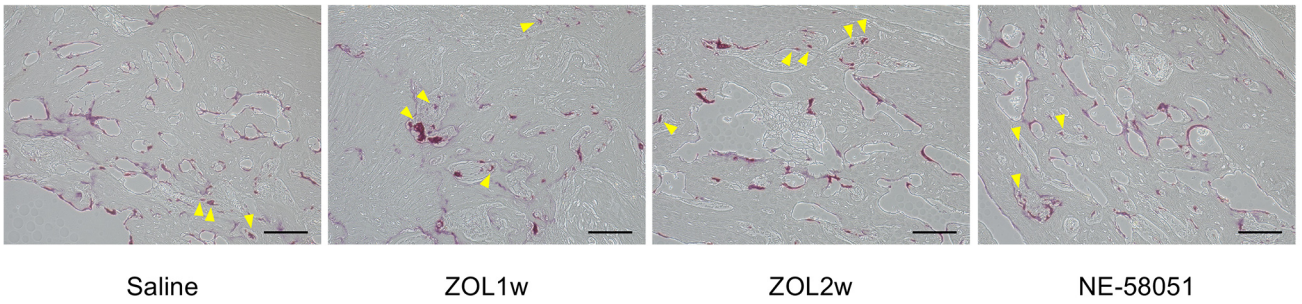

Figure 5. *Representative images of TRAP staining on tooth extraction sockets. Yellow arrowheads indicate detached osteoclasts. Bars, 100 μm.*

Discussion

Clinical observations have led to the reporting of multiple animal models for MRONJ. The majority of these models involve a combination of tooth extraction and the administration of N-BPs. While reports differ regarding the presence or absence of open wounds (8, 18, 19), a common finding is delayed healing of hard tissue-specifically, osteonecrosis characterized by empty lacunae (5-7).

In the present study, we investigated whether a BP analog with high bone affinity, but minimal bone resorption inhibitory activity could induce MRONJ-like lesions in a mouse model. Our results show that NE-58051 did not induce MRONJ-like lesions as evidenced by histological assessments showing similar numbers of empty osteocytic lacunae and necrotic bone areas compared to the saline-treated control group. In contrast, mice treated with ZOL for two weeks showed a significant increase in empty osteocytic lacunae and necrotic bone area, indicating the development of MRONJ-like changes. The lack of MRONJ-like lesions in the NE-58051 group, despite its high bone affinity, indicates that mere accumulation of BPs in bone tissue is insufficient to induce osteonecrosis without significant suppression of bone remodeling. This finding aligns with previous findings that have highlighted the importance of osteoclast inhibition and reduced bone turnover in MRONJ development (2, 5).

The dose-dependent effect observed with ZOL treatment further supports the role of bone resorption inhibition in MRONJ. Mice treated with ZOL for two weeks showed more

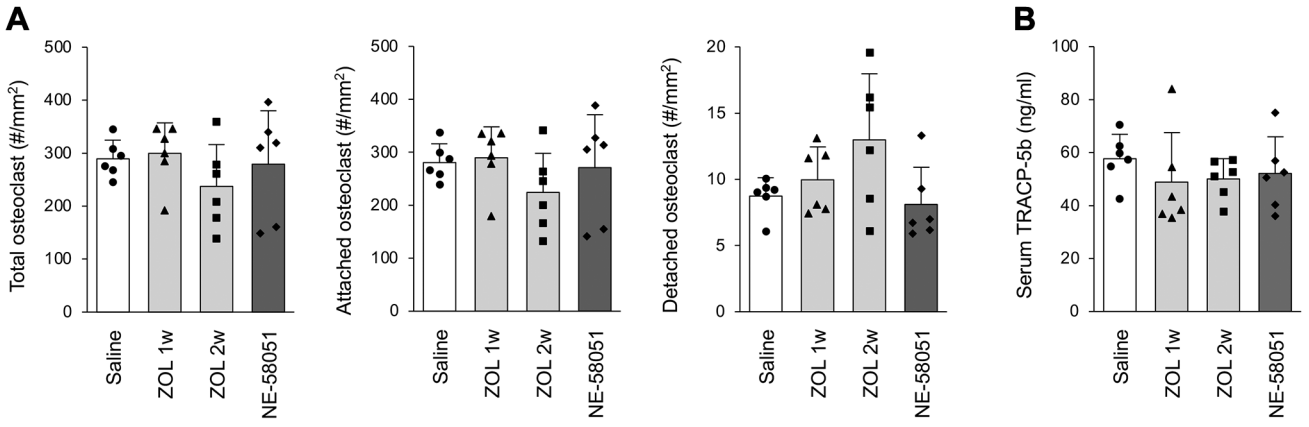

Figure 6. *Quantification of TRAP-positive cells in tooth extraction sockets and TRAcP-5b levels in serum in mice that had been subjected to saline, zoledronate (ZOL) 1 week, ZOL 2 weeks, and NE-58051. (A) Numbers of detached osteoclasts and (B) TRAcP-5b levels in serum.*

pronounced histological changes than those treated for one week, suggesting that duration and cumulative dose of anti-resorptive agents contribute to the severity of MRONJ-like lesions. This observation is consistent with clinical reports in which prolonged exposure to N-BPs correlates with increased risk of MRONJ (1).

The assessment of osteoclast activity revealed no significant differences in total TRAP-positive cell counts among the groups. However, there was a trend toward increased numbers of detached TRAP-positive cells in the ZOL 2w group. This could represent a compensatory response where osteoclast precursors are present but unable to adhere and function effectively due to the inhibitory effects of ZOL on osteoclast maturation and activity (11, 20).

The importance of the effects of N-BPs on bone remodeling has been demonstrated in several pathological conditions, including both osteonecrosis and bone metastasis (21). Previous studies have characterized NE-58051 as a unique BP analog with distinct biological properties (13-16, 22-24). While this compound is a poor inhibitor of FPPS activity compared to risedronate, it retains the ability to inhibit cancer cell proliferation *in vitro* in a dose-dependent manner (14). In contrast to other N-BPs such as ZOL and ibandronate, NE-58051 shows no inhibitory effect on tumor cell invasion (24). However, like other N-BPs, NE-58051 can inhibit matrix metalloproteinase (MMP) activity through its phosphonate groups (24), suggesting that its biological effects are structure-dependent: the R_2 side chain is responsible for cellular effects while the phosphonate groups mediate MMP inhibition. These distinct properties make NE-58051 a valuable tool for dissecting the diverse biological activities of BPs beyond their effects on bone resorption.

Importantly, the mouse model cannot fully replicate the complexity of human MRONJ, as factors such as systemic diseases, concomitant medications, and the influence of oral microorganisms were not taken into consideration. Nevertheless, this study specifically focused on the bone resorption inhibitory ability to approach the pathology of MRONJ, isolating this factor to understand its direct impact. Future research should focus on elucidating the molecular mechanisms by which BPs with varying anti-resorptive activities influence osteoclast function and bone remodeling. Additionally, exploring the role of other cell types, such as osteocytes and immune cells, could enhance our understanding of MRONJ pathogenesis.

Conclusion

Our findings support the notion that potent inhibition of bone resorption is a key driver in the development of MRONJ-like lesions. High bone affinity alone, without

significant anti-resorptive activity, does not appear to induce osteonecrosis in the jaw. These findings have important implications for the design of safer therapeutic agents and highlight the need for careful monitoring of bone turnover in patients undergoing BP therapy.

Conflicts of Interest

All Authors declare no conflicts of interest in relation to this study.

Authors' Contributions

Conceptualization, Y.M.; Data curation, M.K., Y.M. and M.S.; Formal analysis, M.K. and Y.M.; Funding acquisition, Y.M.; Investigation, M.K., Y.M., K.Y. and A.U.; Methodology, M.K. and Y.M.; Project administration, Y.M.; Resources, Y.M. and H.N.; Software, Y.M.; Supervision, M.K., H.N. and T.M.; Validation, M.S. and Y.K.; Visualization, M.K. and Y.M.; Writing - original draft preparation, M.K. and Y.M.; Writing – review and editing, M.K., Y.M., M.S., Y.K., A.U., M.K., H.N. and T.M. All Authors have read and agreed to the published version of the manuscript.

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