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Original Article

Influence of di(2-ethylhexyl) phthalate on dysregulation of testosterone production via alteration of aromatase expression

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Abstract

Di(2-ethylhexyl) phthalate (DEHP), an endocrine-disrupting chemicals (EDCs), is commonly used as a plasticizer to improve the flexibility and durability of plastics. On a daily basis, we are exposed to varying amounts of this compound. Several studies have demonstrated that exposure to DEHP and its metabolite, mono(2-ethylhexyl) phthalate (MEHP), leads to testosterone deficiency (TD) in both humans and animals. However, the precise mechanism that causes DEHP-induced TD is still not completely understood. This study aims to determine the effects of DEHP on testosterone levels and elucidate the underlying mechanisms. C57BL/6 mice and Leydig cells were exposed to various doses of DEHP (0, 0.5, and 5 mg/kg/day) for 9 weeks and MEHP (0, 0.05, 0.5, and 5 μM) for 24 hours, respectively. Both in vivo and in vitro results indicated significant reductions in testosterone levels due to DEHP and MEHP. Additionally, DEHP and MEHP increased the expression of aromatase, a gene that converts testosterone to estradiol and induced an increase in the expression of inflammatory cytokines such as IL-6, IL-1β, and TNF-α. Moreover, DEHP activated NF-κB, a key transcription factor regulating numerous genes associated with inflammation. These results suggest that sustained exposure to DEHP increases inflammatory factors, which may elevate aromatase activity and result in decreased testosterone levels in the body. Furthermore, this study provides a basis for discussing the potential correlation between persistent DEHP exposure and TD characterized by low testosterone levels in the body.

Keywords: Di(2-ethylhexyl) phthalate, Leydig cell, Aromatase, Inflammatory cytokine, Testosterone deficiency

1. Introduction

Testosterone, the principal male sex hormone, plays a crucial role in various biological processes, including sexual performance, bodily functions, and development [1]. Serum testosterone levels in men decline by 1-2 % annually after age 40 [2], with approximately 30 % of men aged 50-70 experiencing testosterone deficiency (TD) [3]. TD can significantly impact the quality of life, leading to symptoms such as fatigue, negative mood, decreased libido, and erectile dysfunction [4-6]. Therefore, identifying the molecular mechanisms by which endocrine-disrupting chemicals (EDCs) reduce testosterone levels is crucial.

Testosterone synthesis occurs primarily in Leydig cells within the testes, where cholesterol is converted to testosterone through enzymes including steroidogenic acute regulatory protein (StAR), CYP11α1, and HSD17β [7, 8]. Synthesized testosterone can be converted to 6β-hydroxytestosterone by CYP1β1, to dihydrotestosterone by 5α-reductase, or to estradiol by CYP19α1 (aromatase), which reduces testosterone levels [9]. Overexpression of CYP19α1 in animal models, such as AROM+ mice, leads to Leydig cell hyperplasia, reduced steroidogenesis, and decreased serum testosterone levels [10].

Testosterone levels are influenced by various external and internal factors. Inflammatory cytokines have been shown to reduce testosterone production in men, and patients with inflammatory diseases tend to exhibit lower testosterone levels [11, 12]. This suggests a bidirectional relationship between inflammation and testosterone levels. Additionally, testosterone supplementation therapy has been observed to decrease the expression of inflammatory cytokines in patients with inflammatory diseases [13].

EDCs interfere with hormone effects, biosynthesis, transport, and metabolism, altering endocrine system physiology [14]. These chemicals are prevalent in pesticides, plastic products, cosmetics, electronics, and clothing [15]. Di(2-ethylhexyl) phthalate (DEHP) is a significant phthalate derivative due to its widespread utilization and environmental prevalence [16]. In the body, DEHP is metabolized into mono(2-ethylhexyl) phthalate (MEHP), which is preferentially absorbed [17]. DEHP and MEHP induce oxidative stress, increase inflammatory cytokine expres-

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sion, and can lead to various illnesses, including cardiovascular disease, obesity, and reproductive toxicity [18-20].

To investigate the mechanisms behind testosterone reduction due to exposure to DEHP and MEHP, we conducted in vivo studies in male C57BL/6 mice and in vitro studies using TM3 Leydig cells. Our findings indicate that DEHP and MEHP reduce testosterone levels by inducing inflammatory cytokines and increasing CYP19α1 expression. This study aims to provide a better understanding of the potential correlation between continuous exposure to DEHP and TD, characterized by low levels of testosterone in the body.

2. Materials and methods

2.1. Animals and Treatment

Male C57BL/6 mice (7 weeks old; Central Lab Animal Inc., Korea) were individually housed in cages under controlled conditions: a room temperature of 22 (±1)°C and a humidity of 50 %. Mice were fed on chow food and water ad libitum and maintained on a 12-hour light-dark cycle. The mice adapted for one week before treatment. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Kyungsung University (Approval No. 21-020B). Eight-week-old mice were divided into three groups with four mice in each group: a control group, 0.5 and 5 mg/kg/day DEHP groups. DEHP (Sigma-Aldrich, St. Louis, MO, USA) dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich) was diluted in water and administered to the mice for nine weeks. At the end of the treatment period, the mice were sacrificed, and the testes were immediately collected, frozen with liquid nitrogen, and stored at -80 °C.

2.2. Cell Culture

Leydig cells from mice were obtained from the Korea Cell Line Bank (Seoul, Korea). Cells were maintained in a 1:1 (v:v) mixture of Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Grand Island, NY, USA) and Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12, Gibco) supplemented with 2.5 % fetal bovine serum (FBS, Gibco), 5 % horse serum (Gibco), 100 units penicillin, and 100 µg/mL streptomycin (Sigma-Aldrich) at 37 °C in a humidified atmosphere with 5 % CO_2. Subculture was performed after reaching 70-80 % confluence, and cells were detached by incubation in 0.25 % trypsin-EDTA (Gibco).

2.3. Cell Viability Assay

Cell viability was determined using an EZ-Cytox Cell Viability Assay kit (DoGenBio, Seoul, Korea) according to the manufacturer's protocol. Leydig cells were plated in a 96-well plate at a density of 5.0×10^4 cells/well and incubated to adhere for 24 h. After 24 h, cells were treated with MEHP (0, 6.25, 12.5, 25, 50, and 100 µM) for 24 h. Absorbance was measured at 450 nm using a Varioskan™ LUX spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA).

2.4. Measurement of Testosterone Level

Testes tissues and Leydig cells were prepared as follows: the frozen testes from each group were cut into small pieces weighing 10 mg. ProEX™ CETi Lysis Buffer (Translab, Daejeon, Korea) was added to homogenize them using a Teflon pestle. The homogenized testes were centrifuged at 16,100 × *g* for 5 to 10 min. Leydig cells were plated in a 48-well plate at a density of 1.7×10^5 cells/well and exposed for 24 h with or without 5 µM MEHP. Testosterone levels were analyzed using a commercially available testosterone ELISA kit (#ADI-900-065, Enzo Life Sciences, Farmingdale, NY, USA) according to the manufacturer's instructions. Samples and varying doses of standard diluents were added along with the antibody to each well of a goat anti-mouse IgG microtiter plate and incubated for 1 h at room temperature with shaking. Wells were washed three times, and substrate solution was added to each well and incubated for 1 h at 37 ℃ without shaking. A stop solution was added to each well, and absorbance was measured at 405 nm using a microplate reader (Epoch Microplate Spectrophotometer, Biotek, Winooski, VT, USA). Testosterone concentrations were calculated using the standard curve generated during the experiment.

2.5. Quantitative Real-Time PCR Analysis

Quantitative real-time PCR procedures were performed as follows: total RNA was extracted using a Total RNA Extraction kit (SJ BioScience, Daejeon, Korea) according to the manufacturer's instructions. Extracted total RNA was reverse-transcribed into complementary DNA (cDNA) using a Compact cDNA Synthesis Kit (SJ BioScience). Real-time RT-PCR was performed in a 20 µL volume using a SYBR Green PCR Master Mix (SJ BioScience) on a QuantStudio™ 1 Real-Time PCR Instrument (Thermo Fisher Scientific Inc.). The relative expression of each gene was calculated by the $2^{-(\Delta\Delta CT)}$ method normalized to 18S. The primer sequences used in this study are listed in Table 1.

2.6. Western Blot Analysis

Nuclear and cytoplasmic fractions were isolated using the EpiQuik™ Nuclear Extraction Kit (EpigenTek, Farmingdale, NY, USA). Proteins were quantified by the Bradford protein assay (Bio-Rad, Hercules, CA, USA), separated by 10 % SDS-PAGE, and transferred to transfer paper for 1.5 h. Membranes were blocked in a membrane-blocking solution (Translab) for 0.5 h and incubated overnight at 4 ℃ with primary antibodies. Membranes were washed with TBS-T buffer three times and incubated with a 1:10000 dilution of goat anti-rabbit IRDye® 680RD fluorescent secondary antibodies (LI-COR Inc., Lincoln, NE, USA) in a blocking solution for 1.5 h at room temperature. Target protein analysis was conducted using the Odyssey Infrared Imaging System (LI-COR Inc.).

2.7. Statistical Analysis

All data are presented as means ± standard error of the mean (SEM). Analysis of variance (ANOVA) with the Dunnett test was performed using GraphPad Prism (version 5.0, GraphPad Software Inc., USA). Statistical significance was determined at $p < 0.05$.

3. Results

3.1. Effects of DEHP on Testosterone Levels in the Testes

To evaluate the impact of DEHP on testosterone levels, we measured testosterone levels in the testes of mice treated with varying doses of DEHP (0, 0.5, and 5 mg/kg/day) for nine weeks. The results demonstrated that testosterone

Table 1. Primer sequences used for RT-PCR analysis.

Gene	Forward primer (5'→3')	Reverse primer (5'→3')
StAR	TGCCCATCATTTCATTCATCCTT	AAAAGCGGTTTCTCACTCTCC
HSD3β	TGGACAAAGTATTCCGACCAGA	GGCACACTTGCTTGAACACAG
HSD17β	ACTTGGCTGTTCGCCTAGC	GAGGGCATCCTTGAGTCCTG
CYP11α1	AGGTCCTTCAATGAGATCCCTT	TCCCTGTAAATGGGGCCATAC
CYP17α1	GCCCAAGTCAAAGACACCTAAT	GTACCCAGGCGAAGAGAATAGA
CYP19α1	ATGTTCTTGGAAATGCTGAACCC	AGGACCTGGTATTGAAGACGAG
CYP1β1	CACCAGCCTTAGTGCAGACAG	GAGGACCACGGTTTCCGTTG
Srd5α1	GAGTTGGATGAGTTGCGCCTA	GGACCACTGCGAGGAGTAG
Srd5α2	GATCCTGTGCTTTGGGAAACC	GCATCCCTACCGACACCAC
Srd5α3	CTACGTCATCTCAGTTGTGTGG	GAGCAGAGCACTAAGCCAGT
18S	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG

levels were significantly reduced in the DEHP-exposed groups compared to the control group (Figure 1). Specifically, mice treated with 0.5 mg/kg/day DEHP exhibited a moderate decrease in testosterone levels, whereas those treated with 5 mg/kg/day DEHP showed a substantial reduction. The control group maintained normal testosterone levels throughout the experiment.

3.2. Effect of DEHP on Testosterone Production-Related Gene Expression in Testes

To determine the molecular mechanism underlying the decline in testosterone levels in the testes, we evaluated the impacts of DEHP on the expression of genes associated with testosterone synthesis, including HSD3β, StAR, CYP17α1, and HSD17β, as well as enzymes involved in the attenuation of testosterone levels, such as Srd5α1, CYP1β1, and CYP19α1. Exposure to DEHP (0.5 and 5 mg/kg/day) did not affect mRNA expression levels of genes involved in testosterone synthesis (data not shown). On the other hand, the expression of testosterone-attenuating genes indicated that only the CYP19α1 gene was upregulated in the DEHP 5 mg/kg/day group, while Srd5α1 and CYP1β1 did not (Figure 2 A-C). Increased protein expression of CYP19α1 was further confirmed by RT-PCR, showing a concentration-dependent increase in the DEHP-treated group (Figure 2 D). Based on these results, we concluded that DEHP regulates the expression of CYP19α1, an enzyme that converts testosterone to estradiol, thereby attenuating testosterone levels.

3.3. Effect of DEHP on Inflammatory Cytokine Expression in Testes

Several studies suggest a close link between inflammation and testosterone levels [12, 21]. To determine the effect of DEHP on the expression of inflammatory cytokines, the levels of IL-1β, IL-6, and TNF-α were evaluated in the testes of mice. Experimental results showed that DEHP exposure increased the expression levels of IL-1β, IL-6, and TNF-α, with significant increases observed in the 5 mg/kg/day DEHP treatment group (Figure 3 A-C). To understand the underlying mechanism, we examined NF-κB activation, which is essential for the transcriptional regulation of these inflammatory cytokines. Nuclear translocation of NF-κB was increased in a concentration-dependent manner in the DEHP-treated group compared to the control group (Figure 3 D).

3.4. Effect of MEHP on Cell Viability

To assess the impact of MEHP on cell viability, mouse Leydig cells were incubated with various doses of MEHP. The viability of Leydig cells was measured after exposure to different concentrations of MEHP (0, 6.25, 12.5, 25, 50, and 100 μM) for 24 h using the MTT assay (Figure 4 A). The results indicated that MEHP at these concentrations did not significantly affect cell viability, as no substantial differences were observed compared to the control group

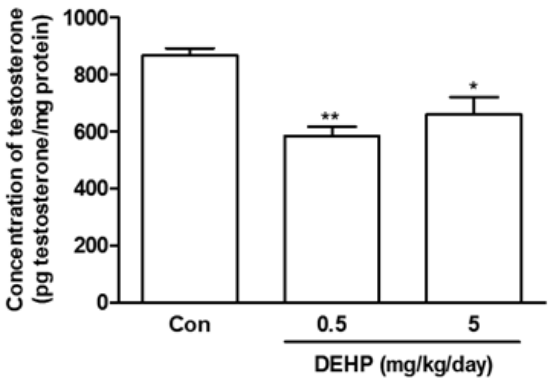

Fig. 1. Effects of DEHP on testosterone concentration in the mouse testes. Testosterone concentration was reduced by the DEHP exposure group. One-way ANOVA was used to determine the significance of differences: $*p < 0.05$ and $**p < 0.01$ when compared to the control group.

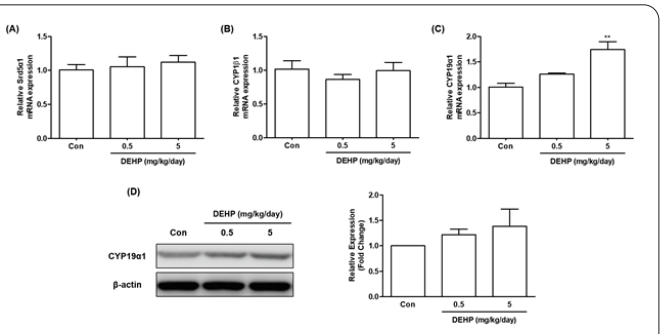

Fig. 2. Effects of DEHP on mRNA expression and protein expression of testosterone attenuation-related genes in the mouse testes. The results of mRNA expression were as follows: Srd5α1 (A), CYP1β1 (B), and CYP19α1 (C). The result of the protein expression of CYP19α1 (D). One-way ANOVA was used to determine the significance of differences: $**p < 0.01$ when compared to the control group.

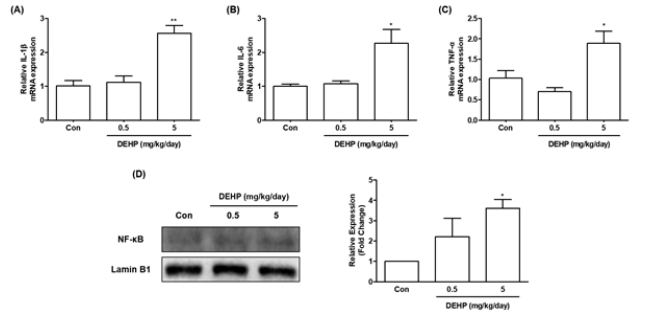

Fig. 3. Effects of DEHP on mRNA expression and protein expression of inflammatory cytokines in the mouse testes. The results of mRNA expression were as follows: IL-1β (A), IL-6 (B), and TNF-α (C). The result of the protein expression of NF-κB (D). One-way ANOVA was used to determine the significance of differences: $*p < 0.05$ and $**p < 0.01$ when compared to the control group.

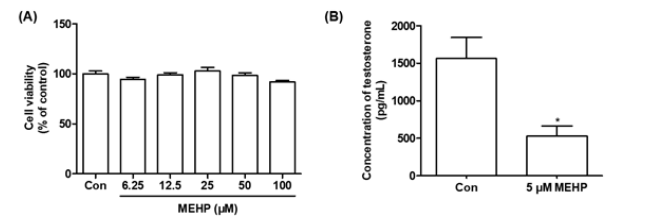

Fig. 4. Effects of MEHP on cell viability in Leydig cells (A). There was no significant impact on cell viability. Effects of MEHP on testosterone concentration in Leydig cells (B). Testosterone concentration was reduced by the 5 μM MEHP group. One-way ANOVA was used to determine the significance of difference: $*p < 0.05$ when compared to the control group.

(0 μM). The results suggest that MEHP does not exert cytotoxic effects on Leydig cells within the tested concentration range, confirming their viability remained stable under these conditions.

3.5. Effects of MEHP on Testosterone Levels in Leydig Cells

Similar to the experiments conducted with DEHP, we performed a study using mouse Leydig cells to determine whether MEHP reduced testosterone levels. Leydig cells were treated with various concentrations of MEHP, and testosterone levels were measured. The results demonstrated a significant reduction in testosterone levels in the MEHP 5 μM treatment group compared to the control group (0 μM) after 24 h of incubation (Figure 4 B). Specifically, the data indicated that exposure to 5 μM MEHP led to a marked decrease in testosterone production, highlighting the potential endocrine-disrupting effects of MEHP on Leydig cells. This finding demonstrates the similarity in the impact of MEHP and DEHP on testosterone synthesis, emphasizing the need for further investigation into the mechanisms underlying this effect (Figure 4 B).

3.6. Effect of MEHP on Gene Expression in Leydig Cells

To investigate the molecular mechanism underlying the decrease in testosterone levels in Leydig cells, we examined how MEHP affects the expression of factors related to testosterone synthesis such as HSD3β, StAR, CYP17α1, and HSD17β, as well as enzymes involved in testosterone attenuation, including CYP19α1 and Srd5α1, 2, and 3. Exposure to MEHP (0.05, 0.5, and 5 μM) did not significantly affect mRNA expression levels of genes implicated in testosterone synthesis (data not shown). On the other hand, the expression of CYP19α1, an enzyme that converts testosterone to estradiol, was increased in both the MEHP 0.5 μM and 5 μM groups (Figure 5). As with DEHP, we measured the expression of inflammatory cytokines to explain the increased expression of CYP19α1 following exposure to MEHP. We found that the expression levels of inflammatory cytokines such as IL-6, IL-1β, and TNF-α were elevated in the MEHP-exposed group, with significant increases observed in the 5 μM MEHP group for all three enzymes (Figure 6).

4. Discussion

Although numerous synthetic substances have been developed to simplify our lives, their complete biological and hazardous impacts remain uncertain. DEHP, the most commonly used plasticizer, is classified as an EDCs. It can enter the human body through drinking, eating, direct skin contact, and breathing. This can result in negative impacts on reproductive development, kidney function, respiratory system, immune system, and nervous system [22, 23]. Several studies are underway to investigate the association between inflammation and TD caused by DEHP and its metabolite MEHP [20, 24]. However, the direct association with the aromatase gene, involved in testosterone production in the body, remains unknown.

For many years, studies have consistently shown that exposure to DEHP and MEHP leads to male reproductive disorders, including reduced testosterone concentration in the male body and impaired sperm motility [25-27]. To

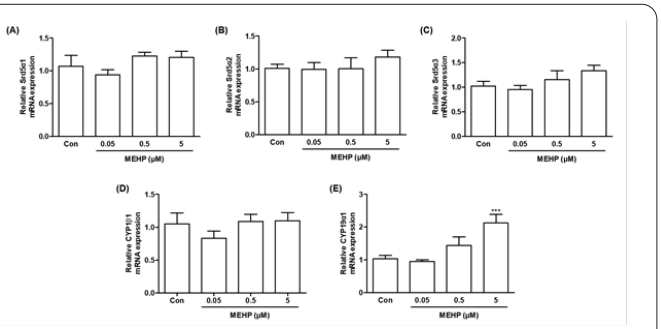

Fig. 5. Effects of MEHP on mRNA expression of testosterone attenuation-related genes in Leydig cells. The results of mRNA expression were as follows: Srd5α1 (A), Srd5α2 (B), Srd5α3 (C), CYP1β1 (D), and CYP19α1 (E). One-way ANOVA was used to determine the significance of differences: $***p < 0.001$ when compared to the control group. One-way ANOVA was used to determine the significance of differences.

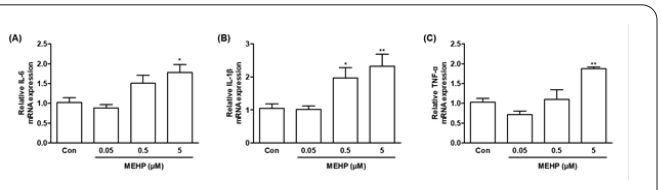

Fig. 6. Effects of MEHP on mRNA expression of inflammatory cytokines in the Leydig cells. The results of mRNA expression were as follows: IL-6 (A), IL-1β (B), and TNF-α (C). One-way ANOVA was used to determine the significance of differences: $*p < 0.05$ and $**p < 0.01$ when compared to the control group.

confirm the mechanism behind decreased testosterone levels caused by DEHP and MEHP, we analyzed total testosterone levels in mouse testes exposed to DEHP and Leydig cells exposed to MEHP. The experimental results confirmed that DEHP and MEHP exposure reduces total testosterone levels compared to the control group. This is consistent with numerous studies showing that DEHP and MEHP directly cause testosterone reduction.

Recent literature suggests that when cells, including macrophages, are exposed to EDCs, they initiate an inflammatory response by inducing the secretion of various inflammatory mediators and pro-inflammatory cytokines [28, 29]. We observed a significant increase in inflammatory cytokine expression levels in both testicular and Leydig cells exposed to DEHP and MEHP compared to the control group. The activation of inflammation was confirmed through the measurement of NF-κB, showing a significantly higher inflammatory response activation in the DEHP exposure group compared to the control group. Based on these results, we conclude that DEHP and MEHP exposure can cause inflammation.

Testosterone is converted to estradiol through a reaction with the aromatase enzyme [30]. Studies have shown that aromatase deficiency in men results in gonadotropism, fertility problems, and increased serum testosterone levels [31, 32]. Another study used AROM(+) male mice, an animal model containing an aromatase fusion gene, and found that increased aromatase activity decreased testosterone levels while increasing estrogen levels [33]. This suggests that changes in aromatase expression closely relate to levels of testosterone. We examined changes in aromatase expression upon exposure to DEHP and MEHP and found an increase. Many studies have shown that high expression of inflammatory cytokines increases aromatase expression through the formation of the cAMP/PKA/CREB signaling pathway [34]. According to related studies, inflammatory adipose tissue involvement leads to an increase in aromatase gene expression, resulting in decreased testosterone levels due to conversion to estradiol [35, 36]. Based on these studies, we suggest that increased inflammatory cytokines expression due to DEHP exposure enhances aromatase expression.

5. Conclusion

This study confirms that DEHP reduces testosterone levels by inducing inflammatory cytokines and increasing aromatase gene expression. Furthermore, this study can serve as a foundation for discussing the potential correlation between continuous DEHP exposure and TD, characterized by low testosterone levels in the body.

Authors' contributions

Conceptualization: HKK, MHP; Methodology: HKK, MHP; Formal analysis: MJP, SJL; Investigation: MJP, SJL; Writing - original draft: MJP, SJL, YJP, EY, HKK, MHP; Writing-review & editing: YJP, EY, HKK, MHP; Funding Acquisition: YJP; Supervision: HKK, MHP. All authors read and approved the final manuscript.

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Conflict of Interests

The authors have no competing interests to declare that are relevant to the content of this article.

Ethics approval

All the animal experimental procedures were approved by the Institutional Animal Care and Use Committee of Kyungsung University (Approval No. 21-020B).

Data availability statement

The datasets generated during and/or analysed during the current study are available from the corresponding author upon reasonable request.

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