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Effects of klotho protein or klotho knockdown in porcine oocytes at different stages

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Research Article

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Summary

Klotho is a protein that plays different functions in female fertility. We have previously reported that klotho protein supplementation during *in vitro* maturation improves porcine embryo development, while klotho knockout for somatic cell cloning completely blocks full-term pregnancy *in vivo*. However, the effects of the microinjection of klotho protein or klotho knockdown dual vector in porcine embryos at different time points and the specific molecular mechanisms remain largely unknown. In this study, we injected the preassembled cas9 + sgRNA dual vector, for klotho knockdown, into the cytoplasm of the germinal vesicle stage of oocytes and into porcine embryos after 6-h parthenogenetic activation. Similarly, the klotho protein was inserted into the cytoplasm of germinal vesicle stage oocytes and porcine embryos after 6-h parthenogenetic activation. Compared with the controls, the microinjection of klotho dual vector markedly decreased the blastocyst formation rates in germinal vesicle stage oocytes and activated embryos. However, the efficiency of blastocyst formation when klotho protein was inserted before *in vitro* maturation was significantly higher than that after klotho protein insertion into parthenogenetically activated embryos. These results indicated that klotho knockdown may impair embryo development into blastocyst irrespective of injection timing. In addition, klotho protein injection timing in pig embryos may be an important factor for regulating embryo development.

Introduction

Pigs are widely used as animals for xenotransplantation (Kim *et al*., 2017, 2019) as well as human disease models (Perleberg *et al*., 2018) because of their physiological similarity to humans. Therefore, many studies have produced cloned transgenic animals using pig embryos. Despite the success of this technique, embryo viability, implantation rate, and efficiency of piglet production have remained low. Therefore, practical applications in animal production will require an increase in its efficiency through modifications in oocyte maturation, embryo culture, embryo manipulation methods, and maintenance of the pregnant recipients until full term.

Two main events in the production of pig embryos *in vitro* are *in vitro* maturation (IVM) and *in vitro* culture (IVC). IVM involves cellular changes that transform immature oocytes into mature oocytes that are capable of withstanding fertilization and embryonic development competence with nuclear and cytoplasmic maturation (Romar *et al*., 2019); whereas naturally ovulated oocytes resume the first meiotic divisions within 20 h. IVM is characterized by an artificial initial breakdown of the germinal vesicle (GV), the rearrangement of microtubule networks during the first meiosis (MI), followed by an extrusion of the first polar body and subsequent arrest of the oocytes in metaphase during the second meiosis (MII). In embryonic IVC, during the last stage of *in vitro* production (IVP), the zygotes undergo cleavages and evolve to morula and then to blastocysts. The IVP conditions create a higher oxidative stress burden than that in *in vivo* production conditions. Antioxidants such as melatonin are added to the IVM or IVC medium to reduce oxidative stress, with positive effects (Liang *et al*., 2017). In our previous study, 1 pg/ml of klotho protein was added to the IVM medium and cumulus cell expansion degree and blastocyst formation rate of parthenogenetically activated (PA) embryos was increased through klotho treatment by inhibiting Wnt signalling and regulating the several genes downstream (Kim *et al*., 2020). Therefore, it can be assumed that improving the production efficiency of porcine embryos is possible by the microinjection of klotho proteins directly into porcine embryos. Nonetheless, whether the direct injection of klotho protein or microinjection timing of this protein (in the GV or during MII) would further benefit porcine embryo competence, remains unclear.

In addition, we had tried to produce a klotho knockout pig model for studying the ageing phenotype; however, the pregnancy could not be maintained until full term, and live piglets

could not be obtained (Kim *et al.*, 2021). The placenta from klotho monoallelic knockout fetuses expressed lower levels of IGF1, FOXO1, and downstream antioxidant genes (*MnSOD* and *CAT*) and higher levels of the BAX/BCL2 ratio and CASPASE 3, compared with wild-type placentas. However, studies of the klotho knockout or knockdown embryo production were not performed. Therefore, the purpose of this study was to ascertain the functions of the klotho protein in oocytes and embryos by injecting the dual vectors of cas9 + sgRNA for klotho knockdown that can inhibit the expression of klotho or klotho protein into porcine embryos at the GV stage before starting IVM and into PA embryos, by microinjection; we also assessed the efficiency of blastocyst formation after treatment with the klotho protein and analyzed the molecular mechanism underlying its effects.

Materials and methods

Chemicals

All chemicals used in this study were purchased from the Sigma-Aldrich Chemical Company unless otherwise specified.

Oocyte collection and in vitro maturation

From a local slaughterhouse, porcine ovaries from pre-pubertal gilts were collected and transferred to the laboratory in a saline solution at 32–37°C. The ovarian follicles of 4–8 mm in diameter were aspirated with an 18G needle connected to a 10-ml syringe. The cumulus–oocyte complexes (COCs) were extracted and rinsed three times by using medium comprising 9.5 g/l tissue culture medium-199 (Thermo Fisher Scientific, Waltham, MA, USA), 10 mM *N*-piperazine-*N*′-[2-ethanesufonic acid] (HEPES), 5 mM sodium hydroxide, 0.3% polyvinyl alcohol (PVA), 2 mM sodium bicarbonate, and 1% penicillin–streptomycin (Invitrogen). After, at least three rinses, the COCs with more than three layers of cumulus cells and homogeneous round cytoplasm were collected and incubated in a 4-well culture dish for 44 h at 39°C under conditions of 5% CO_2 in 95% humidified air. The first IVM medium consisted of TCM-199 liquid form (cat. no. 11150.059), 0.57 mM cysteine, 10 ng/ml epidermal growth factor, human chorionic gonadotropin (hCG), and equine chorionic gonadotropin (eCG) at 10 IU/ml each, 10% porcine follicular fluid, 0.91 mM sodium pyruvate, and 10 µl/ml insulin–transferrin–selenium mixture solution. In addition, after 22 h of maturation, the IVM medium was changed to a hormone-free IVM medium until 44 h.

Parthenogenetic activation and embryo development evaluation

Immediately after IVM of the porcine oocytes, the COCs were denuded using 1% hyaluronidase in Tyrode's albumin lactate pyruvate (TALP) medium with gentle pipetting. Subsequently, mature oocytes whose first polar bodies were visible and cumulus cells were removed, were transferred to the activation medium containing mannitol 0.28 M, HEPES 0.5 mM, $MgSO_4$ 0.1 mM, and $CaCl_2$ 0.1 mM. The oocytes were, then, placed in a 3.2-mm double electrode BTX chamber for electrical stimulation in an activation medium, and the chamber was connected to BTX-2001 (BTX Inc., San Diego, CA, USA). Next, the oocytes were cultured in a 40-µl drop of porcine zygote medium-5 (Wako Chemicals, Osaka, Japan; CSR-CK024) covered with mineral oil at 39°C under conditions of 5% CO_2 and 5% O_2 in 90% humidified air. The assessment of embryo development was performed on Day 2 (48 h) for a cleavage rate check and on Day 7 (168 h) for blastocyst formation rate. Finally, the blastocysts were washed in PBS three times and stored at −80°C for further gene analysis.

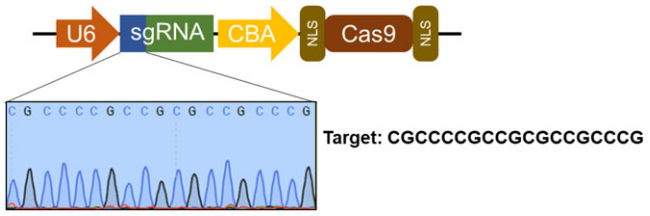

Figure 1. Schematic for the Cas9–sgRNA dual expression vector for targeting exon 1 of porcine klotho.

Klotho knockdown vector preparation

For the construction of gRNA and Cas9 dual expression plasmids, an oligonucleotide targeting porcine klotho exon 1 was cloned using the restriction enzyme *Bbs*I (New England Biolabs, NEB) at GenKOre. pSpCas9 (BB)-2A-EGFP (PX458) v.2.0 (Addgene) was used as a backbone plasmid as Figure 1. The ligated plasmids were transformed into DH5α *E. coli* cells. Cloning was confirmed using Sanger sequencing analysis. The confirmed plasmid vectors were purified using a NucleoBond® Xtra Midi EF kit (MN). The final concentration of this vector was 2290 ng/µl and was diluted at a concentration of 20 pg/µl. As a control, DEPC was used.

Microinjection at the GV stage

Microinjection of porcine oocytes with more than three layers cumulus cells was performed immediately after selection for IVM. The 30 oocytes were moved to a 4-µl drop of rinsing medium of COCs for the microinjection process. Briefly, porcine COCs were injected using a microscope (Eclipse TE2000-S, Nikon, Tokyo, Japan) connected to the microinjection machine Femtojet (Eppendorf, Hamburg, Germany). Cas9 mRNA and sgRNA dual vector (20 pg/µl) or klotho protein (1 pg/µl, standard, MBS9359499, MyBioSource, San Diego, CA, USA) was microinjected into the cytoplasm of the immature COCs with three cumulus cell layers, and the COCs were cultured in TCM-based IVM medium at 39°C under conditions of 5% CO_2 in 95% humidified air. As a control for klotho protein, DEPC was microinjected with six independent replications. The control group in the GV stage injections was marked as control 1.

Microinjection 6 h after electrical activation

The proper timing of 6 h after the electrical activation of the oocytes has been explained previously (Ridlo *et al.*, 2021, 2022). At 6 h after activation, the zygotes were held with a holding pipette in a 4-µl drop of PZM-5. The klotho protein (1 pg/µl) or knockdown vector (20 pg/µl) was injected into the perivitelline space of embryos using a microinjection machine Femtojet (Eppendorf, Hamburg, Germany) in six independent replications. The developmental stage of the oocytes at 6 h after activation corresponded to the MII stage (Ikeda *et al.*, 2001). Thus, this group was marked as the MII stage, and the control group for this experimental group was indicated as control 2.

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Quantitative real-time RT-PCR of mRNA

The mRNA transcripts of blastocysts were measured using a standard SYBR Green real-time PCR assay. RNA was isolated using the easy-spin™ Total RNA Extraction Kit (Intron Biotechnology, Seoul, Republic of Korea), and 500 ng of the total RNA was reverse transcribed using the cDNA synthesis kit (Invitrogen). Real-time PCR was performed on a StepOnePlus Real-Time PCR System (Applied Biosystems, Waltham, MA, USA, #4376600) with gene-specific primers using the 2× SYBR Green PCR Master Mix (Applied Biosystems, Waltham, MA, USA, #4309155) similar to our previous study (Kim *et al.*, 2020). GAPDH was used as a housekeeping gene. Amplicons were analyzed using the $2 - (\Delta Ct\ sample - \Delta Ct\ control)$ method, and the data were represented as the mean of six replications ± standard error of the mean (SEM).

Statistical analysis

Statistical analysis of the embryo development rate and gene expression was performed using GraphPad Prism 5. The embryo developments were evaluated with univariate variance (ANOVA) with post Dunn's test of all comparisons and gene expression levels using an unpaired *t*-test with Welch's correction. In addition, any values at $P < 0.05$ were considered statistically significant differences.

Results

Embryo development after klotho protein microinjection at different stages

We observed the effects of the microinjection of klotho protein on *in vitro* embryo development in the first experiment. The cleavage rate and blastocyst formation rate are presented in Figure 2. Although the differences in the cleavage rate of embryos were not significant, the blastocyst formation rate of klotho protein injection at the GV stage, which means microinjection before IVM, had the highest rate compared with control 1 group ($P < 0.05$). The cleavage rate and blastocyst formation rate of klotho protein microinjection at the MII stage, which means microinjection after 6 h of electrical activation, did not show any differences compared with the control 2 group.

Embryo development after klotho knockdown microinjection at different stages

Next, we examined the development of porcine embryos after microinjecting them with a klotho knockdown vector with DEPC as a control (Figure 3). The results demonstrated that the klotho knockdown vector at the GV and MII stages yielded a significantly lower percentage in blastocyst formation rate compared with the control 1 and control 2 groups for each value ($P < 0.05$).

Transcripts of porcine blastocyst derived from microinjection of klotho protein at the GV stage

We evaluated the expression levels of mRNA transcripts associated with apoptosis (*BAX*, *BCL2*), Wnt signalling pathway (*GSK3A*, *GSK3B*, and *AXIN2*), and klotho transcript in the porcine blastocysts of control 1 and klotho protein microinjection groups at the GV stage (Figure 4). The klotho protein microinjection in the GV stage caused a significant increase in the expression level of *BCL2* and *GSK3B*; however, no differences were observed in *KLOTHO*, *GSK3A*, and *AXIN2* between the two groups. In contrast, *BAX* expression was downregulated in the klotho protein microinjection group.

Discussion

Our study further demonstrated that klotho protein microinjection at the GV stage could improve *in vitro* embryo development compared with microinjection at the MII stage. We also found that klotho depletion at any stage of embryos impairs embryo development. Microinjection is a well established approach for introducing molecules such as CRISPR/Cas9 or protein into oocytes/embryos. Especially, for generating transgenic animals, the microinjection could be used to introduce a foreign gene, the transgene, or gene editing molecule such as CRISPR/Cas9. The major problem of microinjection-mediated transgenesis is mosaicism and a potential alternative to decrease mosaicism is to introduce an editing molecule before DNA replication in the zygote, or even before fertilization (Ma *et al.*, 2017). Considering significant improvement of blastocyst formation when klotho protein was injected in the GV stage compared with control and MII stage, the optimization of microinjection time of porcine embryos may be the important factors for determining the embryo development. As Tian *et al.* (2006) reported that the oocyte injection per se may produce an increase in oocyte activation, the oocyte injection per se by protein in the GV stage before starting IVM may be critical reasons for embryo development. These results also suggest that klotho protein microinjection timing had a critical role in porcine embryo development.

In addition, the molecular mechanism by which klotho inhibits apoptosis was investigated by gene expression analysis. The results showed that klotho protein microinjection in the GV stage increased BCL2 expression and inhibited Bax expression, which meant an increased BCL2/BAX ratio compared with those in the control. These results suggested that introducing the klotho protein into the cytoplasm of porcine oocytes before IVM inhibited apoptosis and supported embryo development. These results were consistent with the findings that klotho inhibited apoptosis in acute kidney injury models (Sugiura *et al.*, 2010) and myocardial cells (Hu *et al.*, 2021). The results of the present study showed that the klotho protein microinjection at the same concentration 6 h after parthenogenetic activation had no effect on the blastocyst formation rate. Here, as the klotho protein was administered at a concentration of 1 pg/μl, the effect of the protein injection at different concentrations could not be predicted. In general, IVM was performed in 500 μl of IVM medium. In our previous study, adding 5 pg/ml of klotho protein to 500 μl of IVM medium favoured COCs for embryo development (Kim *et al.*, 2020). In this study, the microinjection of 1 pg/μl of klotho protein per COC before starting IVM was beneficial enough for improving blastocyst formation. Considering that the duration of the IVM was 44 h and that of IVC was 7 days, it is not reasonable to expect that a treatment of 1 pg/μl in IVM and 1 pg/μl in IVC would have the same effect. In addition, as the composition of the IVM and IVC medium were different, it was easy to assume that both would have different effects. Moreover, the treatment of IVM with 1 pg/μl of klotho protein caused a significant improvement in the blastocyst development, similar to the direct injection of the protein at the GV stage at a concentration of 20 pg/μl, suggesting that the klotho protein may be involved in the development of porcine embryos. Therefore, the direct injection of klotho protein

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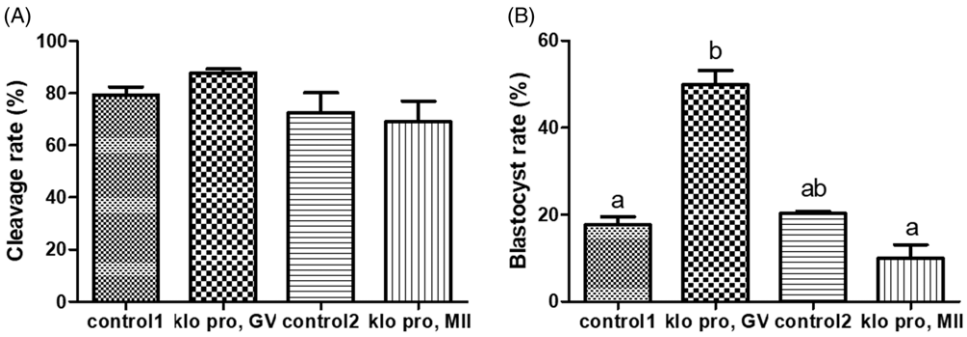

Figure 2. Evaluation of the development of embryos microinjected with klotho protein. (A) Cleavage rate, (B) blastocyst formation rate. The column represents the mean of each replication with standard error of the mean (SEM). Different lowercase letters in each column indicate significant differences ($P < 0.05$). GV, germinal vesicle; klo pro, klotho protein; MII, metaphase II.

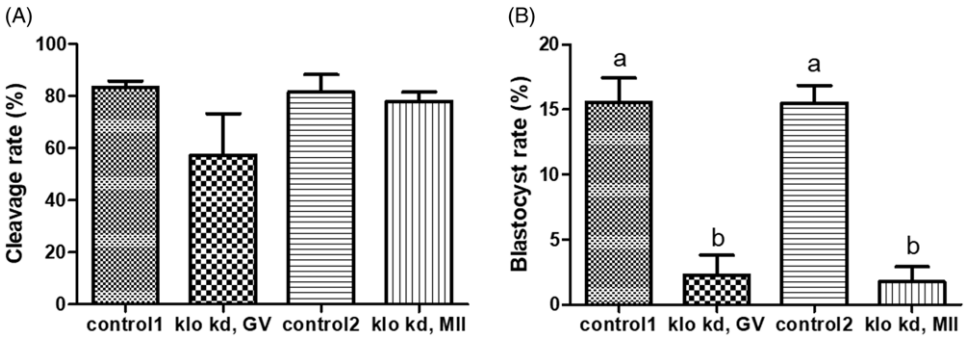

Figure 3. Evaluation of the development of embryos microinjected with klotho knockdown vector. (A) Cleavage rate, (B) blastocyst formation rate. The column represents the mean of each replication with standard error of the mean (SEM). Different lowercase letters in each column indicate significant differences ($P < 0.05$). GV, germinal vesicle; klo kd, klotho knockdown; MII, metaphase II.

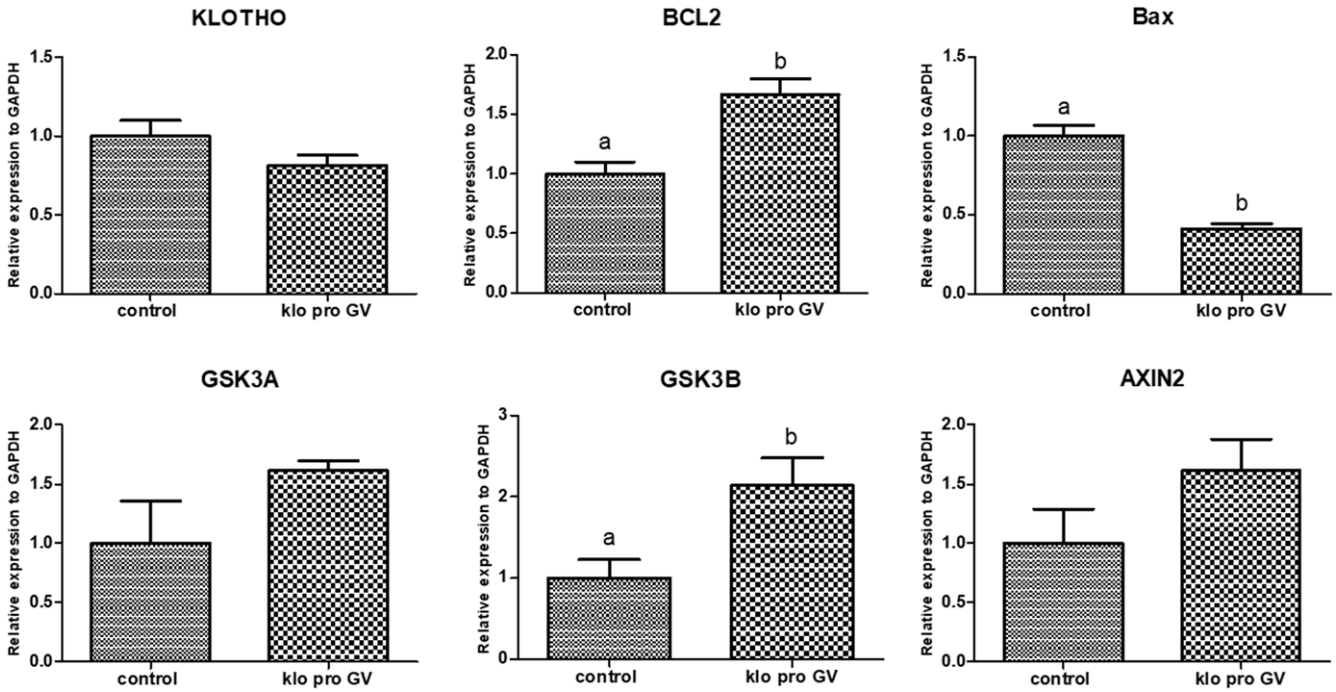

Figure 4. mRNA expression levels of genes associated with klotho, apoptosis (*BCL2*, *BAX*), and Wnt signalling (*GSK3A*, *GSK3B*, and *AXIN2*). The column represents the mean of each replication with standard error of the mean (SEM). The experiment was performed using at least six technical replicates during qRT-PCR analysis on mRNA obtained from at least 30 blastocysts per sample in six replications. Groups labelled with different lowercase letters indicate statistically significant differences ($P < 0.05$).

plays a critical role in regulating genes related to apoptosis of blastocyst formation.

Gene editing systems such as ZFN, TALEN, and CRISPR/Cas9 have been widely used to generate knockout piglets by somatic cell nuclear transfer (SCNT). However, these methods are unable to generate either piglets with embryonic or postnatal lethal mutations. While previous results have demonstrated that piglets could not be produced by SCNT using the CRISPR/Cas9 system to knockout klotho (Kim *et al.*, 2021), in the present study, porcine embryos produced by microinjection using the CRISPR/Cas9 system to knockout klotho also failed to develop into blastocyst compared with control *in vitro*. To generate knockout or

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knockdown mice with lethal gene mutations and prolong the survival of knockout mice, one such study has used one-step two-cell embryo microinjection (Wu *et al*., 2019). Therefore, we aimed to apply this method to produce piglets with klotho knockout or knockdown mutations for our future studies.

Klotho is reported to inhibit Wnt signalling in porcine cumulus–oocyte complex in our previous study. The classical Wnt pathway describes a series of the dishevelled receptor family proteins and the changes of β-catenin levels. Dishevelled is a key component in the membrane associated Wnt receptor complex, wherefore inhibits downstream protein complex including Axin and glycogen synthase kinase-3 (GSK-3) proteins. The GSK-3 family of serine/threonine kinase was first identified as a rate limiting enzyme in glycogen synthesis (Woodgett *et al*., 1982). This family contains two isoforms, α and β, which is typically active only in unstimulated cells. In contrast with most protein kinase (Dorn and Force, 2005). Therefore, the inhibition of GSK-3 is functionally activation of its downstream substrate. After activation of the Wnt signal, the activity of GSK3β decreased which weakened its ability to degrade β-catenin. Likewise, the gene expression of *GSK3β* of klotho protein microinjection at the GV stage was improved compared with the control, which can be an effect of suppression of Wnt signalling, and it can be assumed that the efficiency of embryo was improved due to Wnt downregulation by klotho activation.

To the best of our knowledge, this is the first study that identified the direct effect of klotho protein, thereby confirming its significance in porcine embryo development through the regulation of apoptosis. In addition, the direct microinjection of klotho knockdown Cas9-sgRNA dual vector in porcine oocytes impaired embryo development regardless of when it was micro-injected. Collectively, we concluded that klotho may be a direct regulator of porcine embryo development.

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Author contributions. Conceptualization, GA Kim; methodology, EP Kim, DY Kim and GA Kim; formal analysis, EP Kim, C Park, SM Yoo, MS Lee and GA Kim; investigation, and GA Kim; data curation, EP Kim and GA Kim; writing—original draft preparation, EP Kim and GA Kim; writing—review and editing, EP Kim, DY Kim C Park, SM Yoo, MS Lee and GA Kim; visualization, EP Kim, DY Kim and GA Kim; supervision, GA Kim.; project administration, MS Lee and GA Kim; funding acquisition, C Park, SM Yoo, MS Lee and GA Kim. All authors have read and agreed to the published version of the manuscript.

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Competing interests. The authors declare no conflict of interest.

Ethical statement. The authors assert that no live animals were used in this study. Therefore, ethical approval was not required.

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