Research article

Enhanced antidiabetic efficacy and safety of compound K/β-cyclodextrin inclusion complex in zebrafish

Youn Hee Nam1,*, Hoa Thi Le2,*, Isabel Rodriguez1, Eun Young Kim1, Keonwoo Kim1, Seo Yule Jeong1, Sang Ho Woo1, Yeong Ro Lee1, Rodrigo Castañeda1, Jineui Hong1, Min Gun Ji3, Ung-Jin Kim1, Bin Na Hong3,4, Tae Woo Kim2,**, Tong Ho Kang1,3,*
1Graduate School of Biotechnology, Kyung Hee University, Global Campus, Gyeonggi, Seoul, Korea
2Graduate School of East-West Medical Science, Kyung Hee University, Gyeonggi, Seoul, Korea
3Department of Oriental Medicine Biotechnology, College of Life Sciences, Kyung Hee University, Global Campus, Gyeonggi, Seoul, Korea
4Department of Audiology, Nambu University, Gwangju, Seoul, Korea

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ABSTRACT

Background: 20(S)-Protopanaxadiol 20-O-D-glucopyranoside, also called compound K (CK), exerts antidiabetic effects that are mediated by insulin secretion through adenosine triphosphate (ATP)-sensitive potassium (KATP) channels in pancreatic β-cells. However, the antidiabetic effects of CK may be limited because of its low bioavailability.

Methods: In this study, we aimed to enhance the antidiabetic activity and lower the toxicity of CK by including it with β-cyclodextrin (CD) (CD-CK), and to determine whether the CD-CK compound enhanced pancreatic islet recovery, compared to CK alone, in an alloxan-induced diabetic zebrafish model. Furthermore, we confirmed the toxicity of CD-CK relative to CK alone by morphological changes, mitochondrial damage, and TdT-UTP nick end labeling (TUNEL) assays, and determined the ratio between the toxic and therapeutic dose for both compounds to verify the relative safety of CK and CD-CK.

Results: The CD-CK conjugate (EC50 = 2.158 μM) enhanced the recovery of pancreatic islets, compared to CK alone (EC50 = 7.221 μM), as assessed in alloxan-induced diabetic zebrafish larvae. In addition, CD-CK (LC50 = 20.68 μM) was less toxic than CK alone (LC50 = 14.24 μM). The therapeutic index of CK and CD-CK was 1.98 and 9.58, respectively.

Conclusion: The CD-CK inclusion complex enhanced the recovery of damaged pancreatic islets in diabetic zebrafish. The CD-CK inclusion complex has potential as an effective antidiabetic efficacy with lower toxicity.

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1. Introduction

20(S)-Protopanaxadiol 20-O-D-glucopyranoside, also called compound K (CK, Fig. 1A), is a minor ginsenoside of Panax ginseng [1]. Compund K is the main metabolite obtained through biotransformation by human intestinal bacteria after the oral administration of protopanaxadiol (PPD)-ginsenosides [2–4]; therefore, CK is manufactured by the biodeglycosylation of PPD-ginsenosides [5]. Compound K has anticancer [6–8], anti-inflammatory [9–12], and antidiabetic activity related to insulin secretion through adenosine triphosphate (ATP)-sensitive potassium (KATP) channels [13]. Compound K has low solubility in water; therefore, these bioactivity effects may be limited and many investigators have focused on increasing its solubility. For example, CK including in glycol chitosan increased its solubility and antitumor activity [14]. In addition, CK has been included...
2. Materials and methods

2.1. Reagents and equipment

β-Cyclodextrin was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan) and 20(S)-protonaxadiol 20-D-glucopyranoside (compound K) was obtained from Climax Biotech Co., Ltd. (Chengdu, China). Alloxan monohydrate and sea salts were obtained from Sigma Chemical Co. (St. Louis, MO, USA), and 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG) was purchased from Invitrogen Co., Ltd. (Grand Island, NY, USA). Glimepiride and diazoxide were purchased from Cayman Chemical Co. (Ann Arbor, MI, USA) and Santa Cruz Biotechnology Inc. (Dallas, TX, USA), respectively. Fluorescence microscopy was conducted using an Olympus 1X70 microscope (Olympus Co., Ltd., Tokyo, Japan). Focus Lite (Focus Co, Daejeon, Korea) and Image J (National Institutes of Health, Bethesda, MD, USA) software were used for image analysis.

2.2. β-Cyclodextrin-compound K (CD-CK) inclusion complex

Compound K (CK) (1.3 mg) was added to 2 mL of 10 mM CD in water solution and stirred for 20 h. After equilibrium, the sample was filtered through a 0.45-μm membrane filter. Compound K at 1mM in dimethyl sulfoxide (DMSO) was prepared as the reference sample.

2.3. Turbidity study

Turbidity changes, based on the molar ratio of CD to CK (i.e., 0:1, 1:1, 3:1, 5:1, 10:1), were determined by photography after 20 h of stirring at room temperature.

2.4. Zebrafish maintenance and egg collection

Adult zebrafish were maintained in a zebrafish S-type housing system [1500 mm (width) × 400 mm (depth) × 2050 mm (height); Genomic Design Bioengineering Co., Daejeon, Korea]. Two pairs of zebrafish were placed in a spawning box overnight. The next day, the zebrafish initiated spawning after a 30-min light period. Zebrafish eggs were collected 3 h postfertilization (hpf) and incubated in Petri dishes in a 0.03% sea salt solution. Embryos were maintained in a 14-h light:10-h dark photoperiod in an incubator at 28.5°C. Zebrafish were cared for in accordance with standard zebrafish protocols approved by the Animal Care and Use Committee of Kyung Hee University (Seoul, Korea).

2.5. Compound K and CD-CK efficacy on alloxan-induced pancreatic islet damage zebrafish larvae

We evaluated CK and CD-CK using zebrafish larvae. Wild-type zebrafish larvae 5 days postfertilization (dpf) were placed into a 96-well plate. The larvae were exposed to 25μM 2-NBDG for 12 h and rinsed three times using 0.03% sea salt solution. To induce pancreatic islet damage, we used alloxan at a concentration of 100μM. After 6 h of alloxan treatment, the larvae were stained for 1 h with 25μM 2-NBDG. The larvae were mounted in 96-well plates, and observed using fluorescence microscopy. To determine the efficacy of CK, CD-CK, and GLM as a positive control, zebrafish were treated with 5μM CK, CD-CK, or GLM for 1 h, after which the larvae were restained for 1 h with 25μM 2-NBDG and observed under fluorescence microscopy. All captured images were analyzed for pancreatic islet size and histogram using Focus Lite and Image J Software. Before and after treatment, we analyzed pancreatic islet size via images. All values are expressed as the size change value, based on the following equation: size change value = post-treatment pancreatic islet size − pretreatment pancreatic islet size.

2.6. The 50% effective concentration

Zebrafish were treated with nine different concentrations (i.e., 0.05μM, 0.1μM, 0.5μM, 1.0μM, 2.5μM, 5.0μM, 7.5μM, 10.0μM, and 15.0μM) of CK alone or CD-CK. The 50% effective concentration (EC50) values were calculated by nonlinear regression using

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Fig. 1. The molecular structure of the compounds used in the study. (A) 20(S)-protopanaxadiol 20-D-glucopyranoside (i.e., compound K). (B) β-cyclodextrin.
2.7. Action of diazoxide on alloxan-induced pancreatic islet-damaged zebrafish larvae

Wild-type zebrafish larvae 5 dpf were divided into the following seven groups: normal, 100μM alloxan, 25μM diazoxide as a negative control, 5μM CK, 5μM CK + 25μM diazoxide, 5μM CD-CK, and 5μM CD-CK + 25μM diazoxide. Zebrafish larvae were treated with 25μM 2-NBDG for 12 h. To damage the pancreatic islets, zebrafish larvae were treated with 100μM alloxan and stained with 25μM 2-NBDG for 1 h. Fluorescence microscopy images were then obtained. After first capture, zebrafish larvae were treated with CK and CD-CK with diazoxide. Second images were captured by fluorescence microscopy. All images were analyzed using Focus Lite and Image J software.

2.8. Identification of the development of toxicity related to CK and CD-CK

Twenty zebrafish embryos were chosen to undergo treatment for toxic testing. Embryos were placed in six-well plates, which were incubated under temperature control at 28.5°C and a 14-h light:10-h dark photoperiod. Sixteen treatments were used: control, CD, CD-CK, and GLM at 5.0 μM, 25.0 μM, and 100 μM concentrations, respectively. The embryos were observed under microscopy at 2 d post-treatment (dpt) and dead embryos were recorded. We evaluated the survival rate, heartbeat, and body length.

2.9. Efficacy of CK and CD-CK in expressing enhanced green fluorescent protein fused to the mitochondrial localization sequence of zebrafish COXVIII

Enhanced green fluorescent protein (EGFP) fused to the mitochondrial localization sequence (MLS) of zebrafish COXVIII (MLS-EGFP) zebrafish larvae were obtained from Genomic Design Bioengineering Co. (Daejeon, Korea). The MLS-EGFP zebrafish larvae at 3 dpf were treated with CK and CD-CK at a 20μM concentration. After 48 h treatment, MLS-EGFP zebrafish larvae were observed under fluorescence microscopy and all images were analyzed using Focus Lite and Image J software.

2.10. TdT-UTP nick end labeling assay

Wild-type zebrafish larvae at 3 dpf were treated with 20μM CK or CD-CK. After 48 h treatment, zebrafish larvae were fixed in fresh 4% paraformaldehyde in phosphate-buffered saline (PBS) with 0.05% Tween 20 (PBST) overnight at 4 °C, dehydrated with methanol, stored overnight at −20°C, and rehydrated using decreasing concentrations of methanol in PBST. A TdT-UTP nick end labeling (TUNEL) assay was performed using the DeadEnd Fluorometric TUNEL System (Promega Ltd., Madison, WI, USA). Fluorescent TUNEL-positive cells were detected and counted under a fluorescence microscope.

2.11. The 50% lethal concentration values of CD and CD-CK

Zebrafish were treated with six different concentrations (i.e., 5μM, 10μM, 15μM, 20μM, 25μM, and 30μM) of CD or CD-CK. The 50% lethal concentration (LC50) values were calculated by nonlinear regression using GraphPad Prism version 5.01 software.

2.12. Therapeutic index

The therapeutic index (TI) (also called the therapeutic window or safety margin) is the ratio between the toxic dose and the therapeutic dose of a drug. It is used to measure the relative safety of the drug for a particular treatment. We calculated the TI by the following equation: TI = LC50/EC50.

2.13. Statistical analysis

Statistical analysis was performed using GraphPad Prism software (version 5.01). The data are expressed as the mean ± standard error of mean and significance was determined using repeated one-way analysis of variance (ANOVA) followed by Tukey’s test. The probability level for statistical significance was p < 0.05.

3. Results

3.1. Turbidity study

To evaluate the turbidity of CK by CD, we confirmed that the vials (1–5) had a molar ratio of CD to CK at 0:1, 1:1, 3:1, 5:1, 10:1, respectively. Bubbles were observed in vials 1–4 and the vials contained free saponin in solution. Vial 5 contained a clear solution without any bubbles and had a complete CD-CK inclusion complex (Fig. 2).

3.2. Optimal CD-CK inclusion complex

To evaluate the bioavailability of CD-CK, damaged zebrafish pancreatic islets were treated with different molar ratios of CD to CK (0:1, 1:1, 3:1, 5:1, and 10:1) and CK in DMSO as the control. Pancreatic islet size in the alloxan-treated group was significantly decreased by 8.81-fold (p = 0.0112), compared to the normal group. The CK-treated group had a 1.72-fold increase in the islet size (p = 0.0045), compared to the alloxan-treated group. Pancreatic islet size in the CD:CK [0:1]- and [1:1]-treated groups was increased by 0.95-fold (p = 0.2700) and 1.16-fold (p = 0.0735), respectively, compared to the alloxan-treated group. In addition, the CD:CK [3:1]-, [5:1]-, and [10:1]-treated groups had a significant increase in the pancreatic size of 1.19-fold (p = 0.0303), 2.09-fold (p = 0.0002), and 2.92-fold (p = 0.0112), respectively (Figs. 3A and 3C).

To investigate the histogram of pancreatic islets, we used Image J software. The histogram is a count of pixel values that indicate fluorescence intensity (green color) ranging from level 0 to level 255. The green level in the alloxan-treated group (the mean count
of pixels ($N = 72, p = 0.0162$) was significantly decreased, compared with that of the normal group ($N = 132$). The CK-treated (the mean count of pixels $= 117, p = 0.0213$) and CD:CK $[10:1]$-treated groups (the mean count of pixels $= 128, p = 0.0183$) were significantly increased, compared to the alloxan-treated group (Figs. 3B and 3C).

### 3.3. The effect CK and CD-CK on alloxan-induced pancreatic islet-damaged zebrafish larvae

To evaluate the efficacy of CK and CD-CK, we investigated the pancreatic islets after alloxan treatment with GLM, CD, CK, and CD-CK at 5μM. As the positive control, GLM promoted insulin secretion by closing $K^+_ATP$ channels [24]. The pancreatic islet size in the alloxan-treated group was significantly decreased by 8.54-fold ($p < 0.0001$), compared to the normal group. The GLM-treated group had a 2.19-fold increase in the islet size ($p = 0.0037$), compared to the alloxan-treated group. The CD-treated group had a 0.87-fold increase in the pancreatic size ($p = 0.0121$), compared to the alloxan-treated group. In addition, the CK-treated and CD-CK-treated groups had a 1.89-fold ($p < 0.0001$) and 2.36-fold ($p < 0.0001$) increase, respectively, in the pancreatic size, which was significant (Fig. 4A).

Moreover, we measured fluorescence intensity under these same treatments. The green level in the alloxan-treated group (the mean count of pixels $= 72, p = 0.0014$) was significantly decreased, compared to the normal group (the mean count of pixels $= 132$). The GLM-treated group was significantly increased (the mean count of pixels $= 117, p = 0.0037$), compared to the alloxan-treated group. The CK-treated group was increased (the mean count of pixels $= 100, p = 0.0818$), compared to the alloxan-treated group. The CD-CK-treated group was significantly increased (the mean count of pixels $= 132, p = 0.0292$), compared to the alloxan-treated group (Fig. 4B). The CD-CK-treated group had a similar recovery pattern as that of the GLM-treated group.

### 3.4. The EC50 values of CK and CD-CK

To evaluate the EC50, we generated a dose-effect curve using zebrafish treated with CK and CD-CK at nine different CK and CD-CK concentrations. The EC50 values of CK and CD-CK were calculated at 7.221μM and 2.158μM, respectively (Fig. 5).
3.5. Action of diazoxide on alloxan-induced pancreatic islet-damaged zebrafish larvae

To evaluate the involvement of CK and CD-CK in pancreatic β-cell KATP channels, we investigated pancreatic islet size after diazoxide treatment. Diazoxide inhibits insulin secretion as a KATP channel opener in the prediabetic and diabetic state [25]. During diazoxide treatment, the pancreatic islet size of the CK with the diazoxide-treated group was decreased by 0.76-fold (p < 0.2437) compared to the CK-treated group. Furthermore, the CD-CK with diazoxide-treated group was significantly decreased by 1.25-fold (p = 0.0099), compared to the CD-CK-treated group (Fig. 6A). However, the fluorescence intensity of the pancreatic islets did not show different changes during diazoxide treatment (Fig. 6B).

3.6. Toxicity test

We investigated morphological changes, heartbeat, and body length of the zebrafish embryos to evaluate the toxicity of CK and CD-CK. We did not observe morphological changes during CD-CK treatment. However, 20.0 μM CK-treated zebrafish had a mortality rate of 100% (Fig. 7A). The GLM-treated zebrafish had bent spines and yolk sac edema at a 20.0 μM concentration. Furthermore, zebrafish treated with 25.0 μM GLM did not develop eyes or heads, and also developed bent spines. At 2 dt, there was no significant difference between CK- and CD-CK-treated zebrafish in the heartbeat and body length, compared to the normal group (Figs. 7B–7E).

3.7. Toxicity of CK and CD-CK in MLS-EGFP

To evaluate mitochondrial changes due to CK and CD-CK, MLS-EGFP zebrafish larvae were treated with 20μM CK and 20μM CD-CK. The digestive system size of the CK-treated group was significantly decreased by 36.43% (p = 0.0029), compared to the normal group. The CD-CK-treated group was significantly decreased by 23.57% (p = 0.0076), compared to the normal group. There was also a significant size difference in the digestive system between the CK and CD-CK-treated groups, whereby the CK-treated group size was decreased by 16.82% (p = 0.0335) relative to the CD-CK-treated group (Figs. 8A and 8B).
The digestive system of the CK-treated group (mean number of pixels = 131; p = 0.0018 and p = 0.0002, respectively) had significantly decreased fluorescence intensity, compared to the normal group (the mean count of pixels = 184) and the CD-CK-treated group (the mean count of pixels = 174) (Figs. 8A and 8C). These results indicate that CK decreased the expression of mitochondria in the digestive system of treated zebrafish. In addition, CD-CK suppressed the mitochondrial damage incurred by CK alone.

3.8. The TUNEL assays of CK- and CD-CK-treated digestive systems in zebrafish larvae

We examined apoptosis of the digestive system using the TUNEL assay. The TUNEL staining of the digestive system showed apoptotic cells in the CK- and CD-CK-treated zebrafish larvae. There were differences in the TUNEL-positive apoptotic cells. The CK-treated zebrafish larvae had an increased number of TUNEL-positive cells, compared to the CD-CK-treated zebrafish larvae (Figs. 8D and 8E).

3.9. The LC50 values of CK and CD-CK

To evaluate LC50, we investigated mortality related to CK and CD-CK in zebrafish. Zebrafish were treated with CK and CD-CK at six different concentrations. The LC50 values of CK and CD-CK were calculated at 14.24 μM and 20.68 μM, respectively (Fig. 9).

3.10. Therapeutic index

The TI of CK and CD-CK were calculated by LC50/EC50. The TI is an index of drug safety: safer drugs have a higher TI. The TI of CK and CD-CK were calculated at 1.98 and 9.58, respectively (Fig. 10).

4. Discussion

In our study, CD included in CK (CD-CK) enhanced bioavailability through recovery of pancreatic islet damage in an alloxan-induced diabetes zebrafish model. Alloxan causes pancreatic β-cell necrosis, which decreases β-cell mass, blocks insulin secretion, and thereby induces diabetes [26–28]. We previously reported a decreased size in the pancreatic islets and β-cells in alloxan-induced diabetic zebrafish, which verified zebrafish as a diabetes model [29]. We therefore used alloxan-induced diabetes zebrafish and treated them with GLM, CD, CD-CK, and CK. Glimepiride was the positive control because it promotes insulin secretion by closing K⁺ATP channels and permits calcium (Ca²⁺) inflow [24]. These processes involve glucose utilization at the cellular level and regulate blood glucose level in various tissues [30]. Compound K shows antidiabetic activity by insulin secretion in β-cells, which acts on the K⁺ATP channels and improves insulin sensitivity [13]. The CK-treated group showed recovery of damaged pancreatic islets and the CD-CK-treated group exhibited a significantly higher recovery, compared to the CK-treated group, and improves the antidiabetic effect of CK. This finding is consistent with previous CK treatment results in type 2 diabetic mice and MIN6 β-cells [31].
Fig. 7. The toxicity of compound K (CK), β-cyclodextrin included in compound K (CD-CK), and glimepiride (GLM), based on zebrafish embryo testing. (A) Comparison of the toxicity results of CK, CD-CK, and GLM on zebrafish embryonic development. The data are 2 d post-treatment (dpt) statistics. (B) The heartbeat/min in zebrafish treated with CK at 2 dpt. (C) The heartbeat/min in zebrafish treated with CD-CK at 2 dpt. (D) The body length of zebrafish treated with CK at 2 dpt. (E) The body length of zebrafish treated with CD-CK at 2 dpt. ND, not determined.
Based on these results, we investigated the pathway of insulin secretion by modulating K<sub>ATP</sub> channels. Closure of the K<sub>ATP</sub> channels leads to membrane depolarization, the opening of voltage-gated Ca<sup>2+</sup> channels, and Ca<sup>2+</sup> influx and increase, which result in insulin secretion [32,33]. Many diabetic models use diazoxide to open K<sub>ATP</sub> channels, which inhibits insulin secretion in alloxan-induced diabetic mice and zebrafish [29,34]. Compound K increases insulin secretion by the K<sub>ATP</sub> channel pathway in HIT-T15 cells, which have decreased insulin secretion with cotreatment with diazoxide [13]. Therefore, we investigated how CD-CK enhanced insulin secretion in diabetic zebrafish exhibiting significantly decreased pancreatic islet size, compared to CD-CK-treated zebrafish. These results suggest that CD-CK may stimulate insulin secretion by closing K<sub>ATP</sub> channels in pancreatic β-cells.

To verify the improved bioavailability of CD-CK, we investigated the EC<sub>50</sub> in the recovery effect on alloxan-related pancreatic islet damage because CD-included compounds remove toxicity and increase the rate of compound absorption [19,21]. The EC<sub>50</sub> value of CD-CK was 2.158 μM, which showed that CD-CK has a recovery effect on damaged pancreatic islets at lower concentrations, compared to CK alone (EC<sub>50</sub> value 7.221 μM). In addition, the heartbeat and body length of CD-CK- and CK-treated zebrafish indicated no toxicity. We also calculated the LC<sub>50</sub> by the zebrafish survival rate. Our results demonstrated that CD-CK had better antidiabetic effects and lower toxicity, compared to CK, with LC<sub>50</sub> values 20.68 μM and 14.24 μM, respectively.

Systematic toxicity of CK has not been reported; however, in a subchronic toxicity study [22], CK showed possible hepatotoxicity. In addition, CK cytotoxicity against tumor cells has been reported [23]. The relationship between the EC<sub>50</sub> and LC<sub>50</sub> can be calculated as the TI, which was used in quantitative comparisons of drugs as the ratio of LC<sub>50</sub> to EC<sub>50</sub> [35]. The larger the TI, the safer the drug. However, if the TI is small, the drug must be dosed carefully and the patient receiving the drug should be monitored closely for any signs of drug toxicity [35]. In this study, we demonstrated that CD-CK is a safer drug, relative to CK, through the TI.
The mechanism for CK toxicity is by mitochondrial damage. Mitochondria are very sensitive to endogenous and exogenous environmental stresses such as toxicants, iatrogenic medications, immune activation, and metabolic disorders [36]. For CD-CK, the expression of mitochondria was not changed, compared to the control, but the CK-treated zebrafish showed decreased mitochondrial expression. Therefore, CK toxicity may be related to mitochondrial damage. In addition, the TUNEL assays showed increased apoptosis in the digestive system of CK-treated zebrafish. Such aberrant apoptosis may contribute to the consequent morphologic anomalies and damage of mitochondria in CK-treated animals.

In conclusion, CD-CK inclusion complexes exhibit improved solubility. We determined enhanced antidiabetic efficacy and lowered toxicity of CD-CK relative to CK treatment alone.

Conflicts of interest
The authors have no conflicts of interest to declare.

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