Cycloastragenol activation of telomerase improves β-Klotho protein level and attenuates age-related malfunctioning in ovarian tissues

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ABSTRACT

Age-related deterioration in the reproductive capacity of women is directly related to the poor developmental potential of ovarian follicles. Although telomerase plays a key role in female fertility, TERT-targeting therapeutic strategies for age-related female infertility have yet to be investigated. This study elucidated the effect of Telomerase activation on mice ovaries and more specifically on Klb (β-Klotho) gene expression, which is linked to ageing, female hormonal regulation, and cyclicity. The homology-based 3D model of hTERT was used to predict its binding mode of Cycloastragenol (CAG) using molecular docking and molecular dynamics simulations. Based on docking score, simulation behavior, and interaction with hTERT residues it was observed that CAG could bind with the hTERT model. CAG treatment to primary cultured mouse granulosa cells and activation of telomerase was examined via telomerase activity assay (Mouse TE (telomerase) ELISA Kit) and telomere length by quantitative fluorescence in situ hybridization. CAG mediated telomerase also significantly improved β-Klotho protein level in the aged granulosa cells. To demonstrate that β-Klotho is telomerase dependent, the TERT was knocked down via siRNA in granulosa cells and protein level of β-Klotho was examined. Furthermore, CAG-mediated telomerase activation significantly enhanced the level of Klb and recovered ovarian follicles in the D-galactose (D-gal)-induced ovarian ageing mouse model. Moreover, Doxorubicin-induced ovarian damage, which changes ovarian hormones, and inhibit follicular growth was successfully neutralized by CAG activated telomerase and its recovery of β-Klotho level. In conclusion, TERT dependent β-Klotho regulation in ovarian tissues is one of the mechanisms, which can overcome female infertility.

1. Introduction

Impaired gamete development, a well-established ageing-related phenomenon, is the major etiological factor for infertility, miscarriage, and other pregnancy-related complications among aged women. The rapid advances in modern assisted reproductive technologies have not contributed to alleviating infertility among geriatric mothers. One of the major mechanisms of reproductive ageing is the degeneration of oocyte-surrounding somatic cells (Tatone and Amicarelli, 2013). Several studies have demonstrated that ovarian tissues and follicular microenvironment undergo marked changes during reproductive ageing (Pertynska--Marczewska and Diamanti-Kandarakis, 2016). Although the genetic basis for ovarian ageing is known, most studies have focused on the free radical theory of ovarian ageing (Wang et al., 2020; Ito et al., 2008). Even though the role of critical proteins and signaling pathways in age-related female infertility is significant and has been investigated by many researchers (Ito et al., 2008, Briley et al., 2016, Liberos et al., 2021), anti-ageing genes in ovarian tissues have gotten very little attention.

Telomere length (TL) is a suitable biomarker for ageing, and TERT is

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a predominant determinant of TL (Vasilopoulos et al., 2019). TERT play critical functions in various tissues, and its dysfunction is associated with the impairment of tissue repair or regeneration in several pathologic conditions (Ito et al., 2008). Previous studies have reported that the highly conserved telomeric DNA sequence (TTAGGG)n is maintained through telomerase activation and telomerase dysfunction leads to morphological, biochemical, and functional changes in the tissues (Vasilopoulos et al., 2019). Transgenic expression of TERT promotes the proliferation of primary cell culture and enhances the reprogramming efficiency of induced pluripotent stem cells (Hidema et al., 2016). In addition to maintaining TL, TERT is reported to be involved in various cellular functions (Martínez and Blasco, 2011; Vasilopoulos et al., 2019; Butts et al., 2009; Treff et al., 2011). Female infertility results from shortened TL in various reproductive tissues (Ito et al., 2008). The downregulation of TERT mRNA and telomerase activity in oocyte-surrounding granulosa cells (GCs) is one of the major mechanisms involved in female gamete deterioration (Tomanek et al., 2008, Lavranos et al., 1999, Kosebent et al., 2018). According to reports, TERT is said to have a role in the corpus luteum, proliferation of cellular processes in addition to TL maintenance (Butts et al., 2009). Several proteins are involved in TERT transcription and activation (Yuan et al., 2019). Conversely, TERT is reported to regulate various proteins (Xu et al., 2021). A previous study identified the correlation between Klotho (KL) and TERT and demonstrated that the downregulation of KL impairs telomerase activity in stem cells (Park et al., 2009). The silencing of KL results in various pre-mature ageing symptoms and decreases the lifespan of mutant mice (Jung et al., 2017). KLB, a subfamily of KL, exerts anti-ageing effects by forming a complex with FGFR1 and facilitating FGFR21 signaling (Suku et al., 2020; Fan and Sun, 2016, Kim et al., 2011, Yan et al., 2021, Yie et al., 2012). Furthermore, the FGFR1/KLB complex promotes gonadotropin-releasing hormone (GnRH) secretion in the pituitary gland and regulates the onset of puberty (Misrahi, 2017). Moreover, KL can individually regulate the onset of female puberty (Misrahi, 2017; Xu et al., 2017). Ovary-specific KLB knockout significantly alters cyclicity, downregulates luteinizing hormone secretion, and consequently decreases female fertility (Xu et al., 2017).

The above studies suggest that telomerase activation may positively affect fertility. Telomerase activation or telomerase-based therapies have been developed to improve health span, and reduce age-related diseases (Retiliet et al., 2020, Rafat et al., 2022, Blasco and Bär, 2016, Jaiyjan et al., 2022). In traditional Chinese medicine, Astragalus membranaceus root extracts has been used as an antiaging compound. Cycloastragenol (CAG) is triterpenoid saponin and an active ingredient of Astragalus membranaceus root (Szabo, 2014; Yu et al., 2018a). CAG was previously used to treat diabetes, reduce inflammation, and treat macular degeneration (Dow and Harley, 2016, Zhu et al., 2021, Zhang et al., 2020). Anti-cancer and neuroprotective properties of CAG have also been discovered by researchers (Hwang et al., 2019, Li et al., 2020; Fan and Sun, 2016, Kim et al., 2011, Yan et al., 2021, Yie et al., 2020). One of the exciting properties of CAG is telomerase activation, enhancing the protein level of TERT, and improving the telomere length (Yilmaz et al., 2022, Hong et al., 2021). However, it is unknown how CAG could affect female fertility, particularly in ageing conditions. Therefore, the present study hypothesized that CAG may postpone female fecundity by upregulating TERT and KLB.

Age-related infertility is one of the leading factors affecting a woman’s ability to conceive and deliver a healthy child. The above literature suggest that telomerase and KLB are crucial for female fertility and may have therapeutic potential. We investigated whether the role of telomerase processes therefore TERT and FGFR1/KLB pathway in human and mouse GCs and in mouse ovarian tissues. We noticed a drastic reduction in the protein levels of TERT and KLB in D-galactose (D-gal)-induced ovarian ageing model and solo activation of TERT recovered the FGFR1/ KLB pathway and ovarian follicles. Furthermore, Doxorubicin (DOX) which dose dependently alter ovarian hormones and inhibit follicular formation. CAG mediated Telomerase activation and recovery of FGFR1/KLB pathway, not only improved the hormonal level, but also recovered the number of developing follicles in ovary.

2. Material and Methods

All experiments were performed using female ICR mice (bodyweight, 20–25 g; aged 12–14 weeks), which were maintained at the Animal Care Facility of the Gyeongsang National University Institute of Animal Care Committee (GNU-130902-A0059). The animal experiments were performed according to the Code of Practice for Care and Use of Animals for Experimental Purposes. Chemicals and reagents used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Thermo Fisher Scientific (Waltham, MA, USA) unless otherwise stated.

2.1. Experimental design

Primary mouse GCs were collected from mouse ovaries and cultured in the presence or absence of CAG for 24 and 48 h and analyzed the gene expressions and protein levels of Tert and Klb. Next, to develop an ovarian-ageing model and to explore the influence of ageing on GCs, D-gal was given to Human GCs (COV434 cell line). The effective concentration of CAG for mitigating D-gal-induced toxicity in COV434 cells was determined using the MTT assay. Furthermore, Tert was inhibited via Doxorubicin (DOX), and Knockdown via siRNA and KLB protein was analyzed. Moreover, cycloastragenol (CAG catalog no. SMO00372) was treated to DOX-treated and D-gal-treated granulosa cells and KLB and other ovarian follicular growth related proteins (ER-α, SF-1, FOXL2, ERK1/2, PI3K, AKT, and mTOR) were examined using immunofluorescence and western blotting.

ICR strain female mice were used in two investigations. In the first experiment, the female mice aged 8–10 weeks were randomly assigned to one of three groups: saline, D-gal, or D-gal + CAG (Liu et al., 2019). The mice in the D-gal + CAG group were given D-gal intraperitoneally for 15 days, then CAG (20 mg/kg bodyweight/d) intraperitoneally for 28 days in combination to D-gal. Mice in the control group were intraperitoneally administered with an equal volume of saline. In the second experiment the female mice were randomly divided into the following three groups: saline, DOX, and DOX + CAG groups. In both the experiments the ovarian follicles were examined. Furthermore, follicles and granulosa cells specific proteins, and TERT and KLB/FGFR1proteins were examined via western blot and immunofluorescence.

2.2. Treatment

2.2.1. DOX and CAG treatment

ICR strain of female mice aged 8–10 weeks were randomly divided into the following three groups (10 mice per group): saline, DOX, and DOX + CAG groups. DOX was intraperitoneally administrated at a dose of 20 mg/kg of mice for 7 days (20 mg/kg bodyweight; equivalent to a dose of 6.5 mg/m² in patients) (Fig. S1A) (Liu et al., 2019).

2.2.2. D-Gal and CAG treatment

ICR strain of female mice aged 8–10 weeks were randomly divided into the following three groups (10 mice/group): saline, D-gal, and D-gal + CAG groups (Liu et al., 2019). D-gal (200 mg/kg bodyweight/day) was intraperitoneally administrated on alternate days for 42 days. Mice in the control group were intraperitoneally administered with an equal volume of saline. In the D-gal + CAG group, the mice were intraperitoneally injected with D-gal for 15 days, followed by intraperitoneal administration of CAG (20 mg/kg bodyweight/d) for 28 days along with D-gal (Fig. S1B). The protein levels of FGFR1, ESR1, p-MAPK1/3, p-AKT1, and p-MTOR were analyzed via immunofluorescence and western blot.
2. Cell culture

2.1. Primary mouse GC (pGC) culture

Mouse pGCs were obtained from the ovaries of euthanized females. Female mice were injected with 5 IU of pregnant mare serum gonadotropin (Daesung Microbiological Labs, Gyeonggi do, Republic of Korea) and the ovaries were collected in L-15 Leibovitz-Glutamax medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) after 72 h. Cumulus oocyte complexes (COCs) containing immature oocytes in the outer follicles from both the outer (ovarian surface) and inner (deep ovarian tissue) layers of the ovarian cortex were isolated. Mouse pGCs were obtained from COCs and cultured in previously described media and conditions (Baufeld and Vanselow, 2013). Briefly, pre-ovulatory COCs were plated on collagen-coated 24-well plates (1.25 × 10^6 viable cells/well). The GC suspension was cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% FBS and 1% penicillin-streptomycin solution (Gibco) at 37 °C and 5% CO₂ in a humidified atmosphere.

2.2. Human GC line culture and MTT assay

The human GC line COV434 was a kind gift from Professor Jeehyeon Bae (Chung-Ang University, Seoul, Republic of Korea) (Idrees et al., 2022). The cell line was cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin at 37.0 °C and 5% CO₂ (Idrees et al., 2021). The viability of cells was examined using the MTT (3-(4, 5-dimethylthiazol-2-yl)– 2,5-diphenyltetrazolium bromide) assay, following the manufacturer’s instructions (Sigma). Briefly, COV434 cells were cultured in the wells of a 96-well plate (0.8 × 10^4 per well; 60% confluent cells) containing 200 µL DMEM for 24 h. The medium was replaced with fresh medium containing various concentrations of CAG and the cells were incubated for 24 h. The cells were then incubated with MTT solution for 3 h. Next, 100 µL dimethyl sulfoxide was added to the wells and the samples were agitated for 10–20 min on a shaker. The absorbance of the mixture at 550–570 nm (L1; absorbance of live cells) and 620–650 nm (L2; absorbance of cell debris) was examined using a microplate reader.

2.3. Female embryo gonad culture and treatment

Female mouse fetal gonads were cultured as described previously (Morohaku et al., 2016). Briefly, fetal mouse gonads obtained at day 12.5 post-coitum were separated from the mesonephros and cultured in Transwell-COL membranes (Sigma cat. # CLS3491) for 23 days. The basal medium was alpha-minimal essential medium (Gibco, Thermo Fisher Scientific) supplemented with 1.5 mM 2-O-α-D glucopyranosyl-L-ascorbic acid (Tokyo Chemical Industry, Tokyo, Japan), 10 U/mL penicillin, and 10 µg/mL streptomycin (Sigma-Aldrich), FBS (Gibco, Thermo Fisher Scientific), serum protein substitute (SAGE from day 5 to day 11. On day 24 of culture, the gonads were fixed in 4% formaldehyde and stored at 4 °C until analysis.

2.4. RNA extraction, complementary DNA (cDNA) synthesis, and quantitative real-time polymerase chain reaction (qRT-PCR)

The mRNA was extracted using an RNA isolation kit (PicoPure, Arcturus, Thermo Fisher Scientific), following the manufacturer’s instructions. The concentration of mRNA was examined by measuring the absorbance at 260 nm using Nanodrop. The mRNA was reverse-transcribed into first-strand cDNA using iScript reverse transcriptase (Bio-Rad, Hercules, CA, USA). The primers for qRT-PCR analysis were designed using the National Center for Biotechnology Information nucleotide database and Primer3 (v. 0.4.0) software and are listed in Table 1.

2.5. Modeling of hTERT and molecular docking

The homology modeling based in silico study aimed to predict the binding mode of Cycloastragenol (CAG) with 3D model of human telomerase. According to the literature review, only the structure of Tribolium castaneum telomerase is currently available in Protein Data Bank (PDB) (Baginski and Serbakowska, 2020). The first human telomerase modeled 3D structure reveals that it’s a multidomain enzyme and its catalytic domain found in telomerase reverse transcriptase (TERT) (Steczkiewicz et al., 2011). We therefore, modeled human TERT (hTERT) domain using Tribolium castaneum inhibitor bound structure PDB id 6E53 as a template (Hernandez-Sanchez et al., 2019). The sequence of hTERT was obtained from UniProt (https://www.uniprot.org/) and aligned to the Tribolium castaneum TERT structure in Discovery Studio (DS) v18 (www.accelrys.com Accelrys Inc. San Diego, USA). Following that, the Build Homology Model protocol available in DS was utilized to build the 3D hTERT model. In addition, two well-known modeling servers (Swiss-Model and I-TASSER) were utilized to model TERT for comparative analysis and model selection. The Swiss-Model performs template-based modeling whereas I-TASSER uses threading or multiple templates based approach (Roy et al., 2010). To select the final model the best model from each approach was validated using PROCHECK using PDBsum and ProSA webserver (Laskowski et al., 1997). The final model was energy minimized and prepared using Clean Protein protocol of DS for molecular docking with CAG. Due to the lack of binding site information between hTERT and CAG we utilized CB-Dock2 web server for the identification of potential cavities on hTERT (Y. T. Liu et al., 2022; Y. Liu et al., 2022). Both hTERT and CAG were submitted to the server and the potential cavities were detected. Subsequently the blind docking was also performed by server using AutoDock vina (Trott and Olson, 2010). The CB-Dock2 predicted docking sites and affinities were further validated using another docking run in Genetic Optimization of Ligand Docking (GOLD v5.2.2) program (Verdonk et al., 2003). For comparison we additionally docked CAG on known TERT active site. The binding site was defined using the template superimposition within the 9 Å radius in DS. A total of fifty conformers were generated for each binding cavity and ranked according to GOLD scoring function (Sapundzhi et al., 2019).

2.6. Molecular dynamics (MD) simulations

MD simulations were performed to further validate the hTERT-CAG complex under physiological conditions using Groningen Machine for Chemical Simulations (v5.1.5) (Van Der Spoel et al., 2005). The simulation parameters for the hTERT model and CAG were generated using the CHARMM27 force field and Swiss Param, respectively (Lindahl et al., 2010). The simulation box was hydrated with the TIP3P water model, and the simulation system was neutralized using 52 Cl ions. Subsequently, energy minimization and equilibration were performed. Finally, the simulation was performed under periodic boundary conditions. The detailed procedure used is summarized elsewhere. Finally, the MD simulation trajectory was used to calculate the binding free energy.

| Table 1 |
| --- |
| **Forward and reverse primers.** |
| hTERT | 5'-GGAGAGGTCTGGAGCAAA-3' |
| hTR | 5'-GGATGGAGGAGGTCGAA-3' |
| GAPDH | 5'-TGGGCTGACAGTGCGAGG-3' |

(146 bp)
(ΔG) of the hTERT-CAG complex using the molecular mechanics Poisson-Boltzmann surface area, a computationally rigorous method (Kumari et al., 2014). In total, 40 frames were generated from the simulation trajectories. The final binding free energy was calculated as follows:

$$\Delta G_{\text{bind}} = G_{\text{complex}} - (G_{\text{protein}} + G_{\text{ligand}})$$

The final ΔGbind value for the hTERT-CAG complex was the average value from 40 to 50 ns of the MD simulation trajectories.

2.7. Immunoblotting

The ovaries were freshly collected from mice after treatment, frozen in liquid nitrogen, and stored at −80 °C. The tissues were then homogenized in a protein extraction solution pro-prep™ (INRION Biotechnology, Burlington, NJ, USA, cat. # 17081), following the manufacturer’s instructions (INRION Biotech, Inc.) using a homogenizer (Idrees et al., 2021). The tissue lysate (protein homogenate) was centrifuged at 13200 rpm at a controlled temperature (4 °C) for 25 min. The proteins (supernatants) were collected and stored at −80 °C for further analysis. The concentrations of the protein were determined using the Bradford assay (Bio-Rad Laboratories Hercules, CA, USA, cat. # 5000002), following the manufacturer’s instructions. Equal amounts of proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% or 12% gel. The resolved proteins were then transferred to polyvinylidene difluoride (Sigma-Aldrich, cat. # GE 10600023) membrane. The membrane was blocked with blocking solution (5% skim milk) for 1 h. Next, the membrane was incubated with the primary antibodies at 4 °C overnight, followed by incubation with the horseradish peroxidase (HRP)-conjugated secondary antibodies at room temperature for 90 min. After washing thrice with PBST for 10 min, immuno-reactive signals were detected using the iBright™ Super Signal™ West Pico Chemiluminescent Substrate (Thermo Fisher cat. # 34095) and analyzed using the iBright™ FL1500 Imaging System (Thermo Fisher cat. # A44115). A protein ladder (Abcam, USA, cat. # ab116029) was used to detect proteins based on their molecular weights. ImageJ software (National Institutes of Health, Bethesda, MD, USA; https://imagej.nih.gov/ij) program was used to detect the optical densities of bands.

2.8. Ovarian histology and follicle counting

Mice were sacrificed after the drug treatment to perform morphological analysis and follicle counting. The ovaries were fixed in 4% ice-cold paraformaldehyde in phosphate-buffered saline (PBS) for 12 h and fixed with 4% paraformaldehyde for 48 h and 20% sucrose solution for 72 h. The ovaries were then embedded in optimal cutting temperature compound and stored at −80 °C. The ovary Section (12 μm) were prepared on plus-charged slides using a Leica cryostat (CM 3050 C, Germany). The follicles in the ovaries were counted according to a previously described method (Bernal et al., 2010). Briefly, the number of primordial follicles was counted in all serial sections of the ovary. The follicles were classified based on the GC layers. The primordial follicle has one layer of flattened granulosa surrounding the oocyte. The primary follicle has two layers of cuboidal GCs, while the secondary follicle has more than two layers of cuboidal GCs surrounding the follicles. The antral follicle was easily classified because it contained one or more antral spaces and multiple layers of cuboidal GC layers (Borgeest et al., 2002).

2.9. Immunofluorescence

Tissue sections or cultured cells (4 chamber culture slides (cat. No. 15491TPK) fixed in 4% formaldehyde) were subjected to immunofluorescence staining as previously described (Idrees et al., 2021). Briefly, the slides containing samples were rinsed thrice with 0.3% polyvinyl alcohol (PVA) in 1 × PBS and permeabilized with proteinase K solution (0.1%) for 5 min. The sections were blocked with serum at room temperature for 1 h and incubated with the primary antibodies overnight at 4 °C. Next, the samples were washed thrice with PBS-PVA and incubated with the fluorescein isothiocyanate (FITC)-conjugated secondary antibodies at room temperature for 1 h overnight. After washing thrice with PBS-PVA, the samples were incubated with the second primary antibodies overnight. On day three, the samples were washed thrice with PBS-PVA and incubated with the second tetra methyl rhodamine (TRITC)-conjugated secondary antibodies at room temperature for 1 h. The samples were then washed and stained with 4',6-diamidino-2-phenylindole (DAPI) for 5 min. The images were captured using a confocal laser-scanning microscope (Fluoview FV 1000, Olympus, Japan) after washing the samples thrice. The relative integrated density of the signals was measured using the ImageJ analysis program (National Institutes of Health, Bethesda, MD, USA; https://imagej.nih.gov/ij) software.

2.10. Quantitative fluorescence in-situ hybridization (FISH) using a Telomere-specific DNA Probe

The human telomeric probe containing the telomeric DNA sequence TTAGGG was simultaneously amplified and labeled with digoxigenin (dig) coupled to ddUTP using PCR with human genomic DNA as the template, 5-(CCCTAA)-3’ primer, and a dig-labeling kit (Roche, Mannheim, Germany). Quantitative FISH analysis was performed following the previous protocol with minor modifications (Sohn et al., 2012). Briefly, the cultured cells were incubated in RNase A (Sigma) and dehydrated using an increasing ethanol gradient. Dig-labeled probes containing hybridization solution (Roche) were dropped onto the slides and the samples were denatured at 78 °C for 10 min and hybridized at 37 °C overnight. Next, the samples were incubated with the FITC-conjugated anti-dig antibodies at 37 °C for 30 min, washed with PBT buffer, counterstained with propidium iodide (PI), and examined under a fluorescence microscope (Model AX-70, Olympus, Tokyo, Japan) at green (FITC) and red (PI) dual excitation wavelengths. The images were captured using a digital camera (DP-70, Olympus) and analyzed using MetaMorph® (Universal Imaging Co., PA, USA), an image analysis program. Telomere-specific signals in at least 100 interphase nuclei were examined for each specimen.

2.11. Telomerase Activity

Primary cultured mice granulosa cells (isolated form mouse ovaries follicles) were treated with CAG for 24 h. Quantitative determination of mouse telomerase concentrations was performed in plasma preparations using a colorimetric sandwich-ELISA assay, Mouse TE(telomerase) ELISA Kit (catalog no.E-EL-M1125 Elabsciences®, Houston, TX, USA). The optical density (OD) was measured at 450 ± 2 nm.

2.12. Antibodies

The following antibodies were used in this study: TERT (cat. # sc-393013), β-Klotho (cat. # ARP53325_P050), FGFR1 (Thermo Scientific 13–3100), p-AKT (Cell Signaling, cat. # 9271), AKT (Cell Signaling technology cat. # 9272), p-mTOR (cat. # ab84400), RAGE (sc-80652), ER-α (Abcam, Cambridge, Cambs, UK cat. # ab3575), SF-1 (Abcam cat. # ab168380), FOXL2 (LifeSpan Bioscience, Seattle, WA, USA cat. # LS-B142365), p-ERK1/2 (Cell Signaling, cat. CST 9101 S), ERK1/2 (Cell Signaling, cat. CST 9102 S), p38 (cat. # sc-374534), BCL-2 (cat. # sc-783 Santa Cruz Biotechnology, Dallas, TX, USA), CASP3 (cat. # sc-1225 Santa Cruz Biotechnology, Dallas, TX, USA), NRF-1 (sc-365651), KEAP-1 (cat. # sc-515432), and β-actin (cat. # sc-47778 Santa Cruz Biotechnology, Dallas, TX, USA). Secondary antibodies used in this study were mouse-FITC (cat. # sc-516140), rabbit-TRITC (Thermo Fisher Scientific, Waltham, MA USA cat. # A16101), mouse-HRP (cat. #
NA931 Amersham, Buckinghamshire, UK), and rabbit HRP (catalogue no. NA934 Amersham, Buckinghamshire, UK).

2.13. Statistical analysis

All data were statistically analyzed using the GraphPad Prism software (version 6.0 for Windows; GraphPad Software, San Diego, California, USA; www.graphpad.com). The comparison of the mean values between multiple groups was performed by the analysis of variance (ANOVA) followed by Sidák’s multiple comparison test and Tukey’s multiple comparison test. For all comparisons, p values < 0.05 was considered statistically significant and numerical data were expressed as the mean ± SD.

4. Results

4.1. Ageing dysregulates the Klb and Tert protein levels in cultured pGCs

To initiate our experiment, we qualitatively examined the protein levels of KLB and FGFR1 in a human granulosa cell line (COV434) and Klb and Fgfr1 proteins in mouse ovaries (Fig. 1A). We hypothesized that KLB gene is Telomerase dependent in granulosa cells and to activate telomerase we used small molecule cycloastragenol (CAG) (Ip et al., 2014). In order to determine the effective concentration of CAG, the COV434 cell line was treated with D-gal and various concentrations of CAG (1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 7.5, and 10.0 µM), and D-gal-induced toxicity was assessed via MTT assay (Fig. S2A). D-gal-induced cytotoxicity against GCs was considerably reduced by 2.5 µM of CAG treatment. Since previous research has shown a connection between prolonged pGCs culture and the induction of ageing-like phenotypes (Kulus et al., 2020), we next assessed the effects of CAG on pGCs cultured for 24 and 96 h. Intriguingly, CAG mitigated the down-regulation of the anti-ageing proteins Tert and Klb in prolonged cultured pGCs (Fig. 1B).

FGF21 is the ligand of FGFR1/KLB co-receptors and its treatment to granulosa cells enhances estradiol production via AKT/mTOR signaling. We supplied fgf21 to the primary cultured mouse granulosa cells and examined AKT and mTOR via western blot. Interestingly, the levels of p-Akt1 and p-mTOR proteins did not differ significantly between the control and FGF21-treated samples, but were markedly increased (p < 0.05) after CAG treatment (Fig. 1C). After obtaining the aforementioned data, we argued that if CAG increases the FGFR1/KLB downstream markers, it may be because Tert is activated. Furthermore, if TERT is involved, than its protein level should be directly proportional to KLB. Using immunofluorescence, we measured the amounts of TERT and KLB proteins in control and CAG-treated pGCs grown at 24 h and 96 h (Fig. 1D). The protein levels of Tert and Klb in CAG-treated pGCs were significantly (p < 0.05) higher than that in control pGCs. Next, we analyzed the telomerase activity in primary culture granulosa cells using CAG (Fig. 1E). The results showed significant (p < 0.05) enhancement in the Telomerase activity in the presence of CAG. Even more, the telomere length was examined in 24 h and 96 h cultured control and CAG treated pGCs using FISH with a telomere-specific DNA probe (Fig. 1F).

4.2. CAG-activated Telomerase upregulates KLB Protein level in the human GC line

Previous research has shown that DOX dose dependently alters ovarian hormone and inhibits follicular growth (Aziz et al., 2020). To clarify the importance of KLB for ovarian functioning and its link with telomerase, COV434 cells were treated with DOX. Confocal microscopy analysis revealed that DOX significantly (p < 0.05) downregulated the protein level of KLB but not that of FGFR1 (Fig. 2A). Next, the DOX-treated GCs were transfected with the hTERT plasmid, which resulted in the upregulation of KLB (Fig. 2B). However, neither the control-treated nor the DOX-treated GCs’ TERT protein levels were significantly changed by KLB plasmid transfection (Fig. 2C). In contrast, CAG significantly increased the TERT protein level.

We then investigated the effect of D-gal on granulosa cells by examining the TERT and KLB protein levels. We treated the COV434 granulosa cell line with D-gal and added CAG (2.5 µM for 24 h). TERT plasmid was also transfected to the D-gal-treated granulosa cells. Both the CAG treatment and TERT plasmid transfection significantly (p < 0.05) reduced the RAGE (main marker of ageing) level in granulosa cells (Fig. 2D).

Next, the mRNA level of Telomerase RNA Component (hTR), and TERT were examined in the D-gal, and CAG co-treated samples (Fig. S2B). Which also showed that CAG significantly (p < 0.05) retained the TERT gene expression in the CAG co-treated groups as compared to control, and D-gal groups. Next, the effect of D-gal on TERT and KLB was examined in D-gal-treated COV434 cells (Fig. 2E). We observed that treatment with CAG markedly mitigated the D-gal-induced downregulation of TERT and KLB. Moreover, the enhanced TERT protein level was also detected in the CAG treated group.

To gain a more in-depth picture regarding the GCs functioning, we examined estrogen receptor 1 (ER-α), and Nuclear Receptor Subfamily 5 Group A Member 1 (NR5A1) (Fig. S2C). The results demonstrate that CAG dramatically increased both female hormone-regulating proteins, even when co-treated with D-gal. To clarify whether CAG activated Telomerase is responsible for KLB enhanced expression, TERT was knocked down via siRNA and CAG was treated to the GCs (Fig. 2F). The gene expression of KLB was downregulated even in the presence of CAG. The results were further confirmed by a western blot analysis of KLB protein (Fig. 2G). Furthermore, the telomere-specific DNA Probe was used to examine the telomere length in the D-gal treated, DOX-treated, and CAG co-treated GCs using Fluorescence In situ hybridization (Fig. S2D). The results showed that CAG significantly mitigated the DOX-induced and D-gal-induced downregulation of telomerase protein level and telomere length.

4.3. Homology modeling of hTERT and molecular docking

The TERT is catalytic subunit of human telomerase protein, which comprise of TEN (telomerase essential N-terminal domain), TRBD (telomerase RNA binding domain), RT (reverse transcriptase domain) and CTE domains (C-terminal extension) (Fig. 3A). The BLASTp template search revealed that sequence alignment of human TERT and Tribolium castaneum PDB 6E53 showed 26.82% identity (Fig. 3B) and E value 3e-13. As a result, the available X-ray structures of T. castaneum structures were analyzed and inhibitor bound structure of T. castaneum (PDB is 6E53) was selected as a template for modeling of hTERT (Fig. 3C) (Hernandez-Sanchez et al., 2019). In this work, hTERT catalytic subunit (excluding TEN domain) was modeled. The aligned sequence file of hTERT and template was submitted to Build Homology Model protocol in DS (Fig. 3D) (Chen et al., 2017). The modeler produced 10 models and ranked them according to probability density function (PDF) total energy and discrete optimized potential energy (DOPE) score (Table S1). The model Human.M0001 displaying lowest PDF total energy and DOPE score was selected initially (Fig. 3D). The quality of the model was assessed using superimposition of the on the template which showed acceptable root mean square deviation (Fig. 3F). Additionally, for the selection of better model, further modeling was carried out with Swiss-Model and I-TASSER server (Fig. S3). The best model from each approach was selected and superimposed on each other, revealed all the models adopt similar confirmations (Fig. S3A-D). Additional the models were validated using PDBsum webserver for Ramachandran Plot analysis which analyses the stereochemical properties of the three-dimensional structure (Laskowski et al., 1997; Wiederstein and Sippl, 2007). Ramachandran plot analysis revealed that model from DS displayed 96.1% residues are in allowed region this is followed by Swiss-Model model and I-TASSER model (Fig. 3F and Table S2). The detailed validation of models demonstrated that model
Fig. 1. Ovarian ageing dysregulate the protein level of KLB and the functions of granulosa cells. (A) Representative western blot images of KLB and FGFR1 proteins in the human granulosa cell line (COV434 cells) and mouse ovaries. KLB and FGFR1 both the proteins are expressed in granulosa cells and ovary, with β-actin used as a loading control. (B) The image represents the western blot results of Tert, Klb, and Fgfr1 in primary granulosa cells after 24 and 96 h of culture in the absence or presence of CAG. All the proteins showed significant (P < 0.05) downregulation with the increase in the culture time. (C) Representative images of western blot of pAkt1, total akt, p-mTOR, and total mTOR. The protein levels of pAkt1, p-mTOR were not significantly affected by fgf21 supplement (1 µg/mL) to primary cultured mouse granulosa cells, whereas they were significantly increased by CAG treatment. The data are expressed as the mean ± SEM for the indicated proteins in vitro (n = 5/group). (D) Representative confocal microscopy images of Klb (green) and Tert (red) in mouse granulosa cells cultured for 24 and 96 h. The cells were counterstained with DAPI (blue) to visualize DNA. Klb and Tert both showed significant (P < 0.05) enhancement in the protein levels with CAG treatment. Scale bar = 20 µm. The data are indicated as the mean ± SEM for the indicated proteins (n = 3/group). (E) Histogram presents the telomerase activity (TA) in cultured primary mouse granulosa cells. Quantitative determination of TA was performed on cell samples obtained from both control and CAG groups (n = 3/group). (F) Image of Fluorescent In situ hybridization of telomere length (TL) in mouse primary cultured granulosa cells. The telomeric DNA is indicated by bright (green-yellow) spots against the genomic DNA (red background). At least 100 interphase nuclei were examined for each specimen. TL showed significant enhancement in the presence of CAG after 96 h of culture. Scale bar = 20 µm. The data are indicated as the mean ± SEM and the n = 3 per each group. Data are represented as mean ± standard error of mean. *P < 0.05; **P < 0.01; ***P < 0.001.
Fig. 2. Cycloastragenol (CAG)-activated TERT upregulated the protein levels of KLB and FGFR1. (A) Human granulosa cell line (COV434) were cultured in the DOX, DOX + CAG, and control conditions and were immune-labeled with fluorescein isothiocyanate-conjugated anti-KLB (green) and tetra methyl rhodamine-conjugated anti-FGFR1 (red) antibodies. The cells were counterstained with DAPI (blue) and analyzed using confocal microscopy. Scale bar = 20 µm. The data are indicated as the mean ± SEM (n = 4/group). (B) COV434 cells were treated with Dox and with TERT plasmid and the protein level of Klb was examined via immunoblot with β-actin used as a loading control. Klb significantly decreased its protein level when DOX was added, but increased when transfected with TERT plasmid. The bands were quantified using ImageJ software, and the differences are represented by histograms. (C) Western blot image of TERT protein in COV434 cells treated with CAG, DOX, and transfected with KLB plasmid. TERT protein was significantly (P < 0.05) high in CAG treated group, while DOX and KLB plasmid have no significant effects on TERT. The data are expressed as the means ± SEM for the indicated proteins in vitro (n = 4/group). (D) Representative image of western blot of RAGE protein in COV434 cells. RAGE protein level was considerably downregulated by TERT plasmid and CAG, whereas it was significantly increased by D-gal treatment. (E) Immunofluorescent co-localization of KLB and TERT in the D-gal-treated and CAG-treated COV434 cells. (F) TERT was silenced via siRNA and the mRNA level of TERT and β-Klotho was examined via quantitative RT-PCR (n = 6 biological replicates). (G) Protein level of β-Klotho was also examined in the TERT knockdown GCs via Western blot. The results confirmed that KLB gene expression and protein level is TERT dependent. The data are indicated as the mean ± SEM. *P < 0.05; * *P < 0.01; * * *P < 0.001; NS indicate non-significant.
obtained from DS could be considered for further studies.

4.4. Molecular docking and MD simulations

Molecular docking approach was used to predict the binding mode of CAG with hTERT. Initial docking sites were predicted using CB-Dock2 (Y. T. Liu et al., 2022; Y. Liu et al., 2022). The hTERT and CAG both were provided as an input for the prediction of cavities on hTERT (Fig. S4A–B). The three top predicted sites were subsequently docked using CB-Dock2 and the docking score, molecular interactions were observed (Table S3). It is noteworthy to mention that Cavity1 predicted by CD-Dock2 lies in the close proximity to the inhibitor binding site (Fig. S4B). The CB-Dock2 results were further validated using second docking run in GOLD program (Verdonk et al., 2003). A total of 50 conformers of CAG were generated for each predicted site and inhibitor binding site as well. The best conformer was selected from largest cluster

Fig. 3. (A) The domain representation of human TERT (hTERT). (B) Sequence alignment of Tribolium castaneum TERT (PDB: 6E53) and hTERT. Dark color indicates increased similarity between the two proteins. (C–D) The three-dimensional structures of T. castaneum TERT and model of hTERT. (E) The structural superimposition of hTERT (peach color) and T. castaneum TERT (purple color). (F) The Ramachandran plot analysis displaying the distribution of backbone dihedral angles $\varphi$ on X-axis and $\psi$ on Y-axis.

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which showed acceptable docking Goldscore and desirable molecular interaction with residues of hTERT (Sapundzhi et al., 2019). Both the docking runs indicates that CAG displayed highest affinity towards Cavity1 with vina score − 8.1 kcal/mol and Goldscore of 55.17, this is followed by Cavity3 and Cavity2 (Table S3). Interestingly the inhibitor binding site displayed a lower Goldscore of 44.64 when compared with Goldscore of Cavity1, 55.17. This indicates that Cavity1 may the binding site of CAG on hTERT (Fig. S4C–E).

The selected binding conformation of CAG-hTERT from Cavity1 was further simulated under physiological conditions. The main objective of the molecular simulations was to calculate the binding affinity of the CAG towards hTERT using computationally exhaustive methodology.

Fig. 4. Molecular dynamics simulation analysis. (A–B) The root means square deviation and root mean square fluctuation of human TERT (hTERT) backbone atoms. (C–D) The binding free energy plot generated using molecular mechanics Poisson-Boltzmann surface area and per-residue contribution in molecular interactions. (E) The binding mode of cycloastragenol (CAG) with hTERT. (F–G) The enlarged three-dimensional and two-dimensional representations of the hTERT binding site showing the molecular interactions with key residues. The protein backbone and residues are represented in peach color. CAG is represented as light purple color sticks. The dark green, light green, and pink colors indicate hydrogen bond, van der Waals, and π-alkyl interactions, respectively.
The stability of the 50 ns simulation trajectories were analyzed for root mean square deviation and fluctuations (RMSD and RMSF) (Kumar et al., 2021). The analysis revealed that the CAG-hTERT complex displayed stable RMSD and acceptable RMSF fluctuations (Fig. 4A–B). Further, the binding free energy analysis of last 10 ns stable simulation trajectory revealed that CAG could bind with good reasonable binding affinity (−101.67 kJ/mol) with the hTERT protein (Fig. 4C). The per residue contribution of analysis of CAG-hTERT complex revealed that residues Val596, Lys626, Asn899, and Arg901 can contribute significantly via hydrophobic interactions (Fig. 4D). The binding mode of the hTERT-CAG complex was studied using the average structure from last 5 ns simulation trajectory (Fig. 4E). It can be seen from Fig. 4F that CAG forms three hydrogen bonds with Leu630, Val897 and Leu900 of hTERT. Additionally, CAG also forms van der Waals interactions with Gly588, Gly629, Arg631, Asp628, Val898, Asn899, Arg901 and α-alkyl interactions with Val596, Lys626 and Cys896 (Fig. 4G).

4.5. CAG-activated Telomerase protects ovarian follicles against D-gal-induced ovarian ageing

Previous studies have reported that D-gal adversely affects female reproduction and promotes premature follicular loss (Wang et al., 2019). To understand the effect of D-gal on TERT and FGFRI/KLB signaling, fertile female mice were treated with D-gal and D-gal + CAG and their ovarian morphology was examined using hematoxylin and eosin (H&E) staining (Fig. 5A). H&E staining revealed that CAG significantly (p < 0.05) mitigated D-gal-induced follicular loss. Next, the protein levels of several key GC function-related proteins, such as Esr1, Nr5a1, and Foxl2, were examined using western blotting (Fig. 5B). Treatment with CAG mitigated the D-gal-induced downregulation of Esr1, Nr5a1, and Foxl2 protein levels. Similarly, treatment with CAG upregulated the Klb and its downstream signaling proteins, such as ERK1/3 and P13K (Fig. 5C). Next, the effects of D-gal and CAG on female mouse embryonic gonads was analyzed. The gonads obtained from 12.5-day female mouse embryos were cultured for 23 days and treated with D-gal and D-gal + CAG (Fig. 5D). Treatment with D-gal significantly (p < 0.05) inhibited follicle formation and decreased gonad size, while CAG alleviated D-gal-induced ovarian toxicity. Furthermore, Klb protein was also significantly (p < 0.05) downregulated in cultured gonads, whereas that of Fgfr1 was not significantly affected upon treatment with D-gal (Fig. 5E). Treatment with CAG mitigated the D-gal-induced downregulation of Klb, impaired follicle development, and decreased gonad size.

4.6. CAG ameliorates DOX induced suppression of TERT and KLB and diminishes ovarian follicles in mouse ovaries

DOX alter ovarian hormone secretion and regulation by inhibiting Follicular development in the ovaries of fertile female mice treated with the DOX and CAG was examined (Fig. 6A). DOX also alter female hormonal regulation which is may be due to reduction in Klb expression. We found that DOX markedly impaired follicular development and significantly (p < 0.05) downregulated the Klb protein in mouse ovaries (Fig. 6B). However, treatment with CAG mitigated DOX-induced impaired follicular development and downregulation of Klb. Furthermore, it has been previously reported that ageing ovarian tissues, especially GCs, are characterized by mitochondrial dysfunction (Ernst and Lykke-Hartmann, 2015). DOX disrupts mitochondrial functioning and compel them to produce more ROS (Y. Liu et al., 2022; T. Liu et al., 2022). Since TERT is involved in mitochondrial functioning and mitochondrial DNA maintenance, we looked at the mitochondrial markers Cytochrome C and Bcl2 (Fig. 6C). Treatment with DOX markedly decreased Bcl2 and significantly increased Cyto C protein levels. In contrast, treatment with CAG mitigated DOX-induced Bcl2 downregulation and Cyto C upregulation. While Bcl2 and Cyto C protein levels were not significantly (p < 0.05) different between the CAG-treated and control groups. High protein level of Cyto C initiate apoptosis and thus we checked the protein level of Casp-3 via western blotting (Fig. 6D). Compared with those in the DOX-treated group, the ovarian protein level of Casp3 was significantly downregulated in the CAG-treated group. Nr2-antioxidant response element pathway-related proteins block mitochondria initiated apoptosis. We found that the protein levels of Nr2 and HO-1 were significantly (p < 0.05) upregulated, whereas those of Keap1 were significantly (p < 0.05) downregulated (Fig. 6E). These results suggest that CAG-activated TERT mitigates DOX-induced ovarian toxicity.

5. Discussion

Despite rapid advancements in assisted reproductive technology, there has not been significant success in the discovery of effective treatments for age-related female infertility. This study examined the role of the anti-ageing proteins TERT and KLB in mitigating ovarian ageing. Age-related ovarian dysfunction is associated with the downregulation of TERT, which has significant effects on the FGFRI/KLB signaling pathway. We investigated the TERT activation effects on the FGFRI/KLB co-receptor pathway and its downstream signaling in granulosa cells and in whole mouse ovaries. We found that D-gal promoted ovarian ageing by dysregulating TERT and increasing FGF21 resistance, which can be attributed to the downregulation of KLB. CAG-activated TERT significantly upregulated the protein level of the FGFRI/KLB in D-gal induced ovarian ageing model. These findings are consistent with the role of telomerase and KLB in female fertility (Xu et al., 2020, Liu and Li, 2010). However, further studies are needed to establish the correlation between TERT and the FGFRI/KLB signaling pathway and the alleviation of age-related follicle depletion in aged females.

Several telomerase-based therapies have been developed to improve the health span, enhance longevity, and reduce mortality (Rehman et al., 2020, Rafat et al., 2022, Blasco and Bar, 2016, Jaijyan et al., 2022). Additionally, various therapeutic strategies, such as activation of telomerase, transfection of telomerase sequences, and reactivation of silenced telomerase, have been employed (Jaijyan et al., 2022; Bernardes de Jesus et al., 2012). Compared with other methods, treatment with pharmacological telomerase activators is a better therapeutic strategy owing to the easy regulation of treatment duration and dosage effects (Harley et al., 2011; Bernardes de Jesus et al., 2012). The activity of CAG, a potent telomerase activator, has been demonstrated in immune cells, neonatal keratinocytes, and fibroblasts in culture (Harley et al., 2011). Several in vivo studies have demonstrated that CAG increases the TL in leukocytes (Harley et al., 2011, Salvador et al., 2016). Similarly, dietary supplementation of CAG in mice promoted the elongation of critically short telomeres and improved organ fitness (Bernardes de Jesus et al., 2012). Furthermore, several studies also reported the role and importance of TERT for female reproductive tissues (Vasi et al., 2019, Butts et al., 2009, Treff et al., 2011). However, the effects of telomerase activation on ovarian tissues and female infertility have not been previously investigated. This study examined the effects of telomerase activation on ovarian tissues and its correlation with the anti-ageing Fgfr1/Fgfr1/Klb signaling pathway. In addition to telomerase, the FGF21 pathway plays a critical role in ageing-related metabolic disorders, such as insulin resistance, dyslipidemia, and obesity in roents (Coskun et al., 2008, Kharitonenko et al., 2005). A previous study reported that KL deficiency impaired telomerase activity in stem cells (Ullah et al., 2019). Treatment with FGF21 did not affect the downstream markers of FGFRI/Klb, such as Akt1 and mTOR in GCs cultured for a prolonged duration, which indicated that Klb downregulation suppresses FGF21 signal transduction (Villarroya et al., 2018, Fisher et al.,
Fig. 5. Effect of cycloastragenol (CAG) and D-gal on cultured female embryo gonads and on adult mouse ovaries. (A) Representative images of hematoxylin and eosin-stained mouse ovaries obtained from mice belonging to the control, D-gal (200 mg/kg), and D-gal + CAG (20 mg/kg) groups. (B) Immunoblot analysis revealed that the protein levels of ER-α, Nr5a1, and Foxl2 in the D-gal-treated group were significantly downregulated when compared with those in the control and CAG-treated groups. β-actin was used as a loading control for western blotting analysis. Band intensities were quantified using ImageJ software and represented as histograms. The data are expressed as the mean ± SEM for the indicated proteins (n = 4 mice per group). (C) Western blotting analysis revealed that the protein levels of Klotho, Fgfr1, PI3K, and ERK1/2 in the D-gal-treated group were significantly (P < 0.05) downregulated when compared with those in the control and CAG-treated groups. (D) The gonads were obtained from 12.5-day embryos and cultured for 23 days. The data are expressed as the mean ± SEM for the indicated proteins in vitro (n = 6 gonads per group). (E) Immunofluorescence images of Klb (green fluorescent protein-labeled), and Fgfr1 (red fluorescent protein-labeled) in female embryonic gonads cultured for 22 days. The cells were counterstained with DAPI (blue) to visualize DNA. CAG treatment significantly improved Klb protein level, but fgfr1 remained non-significant. The data are indicated as the mean ± SEM for n = 4 mice per group. N.S, not significant; *P < 0.05; **P < 0.01; ***P < 0.001.
Fig. 6. Cycloastragenol attenuate Doxorubicin (DOX)-treated malfunctioning in mouse ovaries. (A) Histogram present the number of ovarian follicles in the control, DOX-treated (2 mg/kg), and cycloastragenol (CAG)-treated mice. At least four mice from each treatment group were used for measurements. (B) Immunofluorescence analysis of Klb (green) and Fgfr1 (red), and nucleus with DAPI (blue) revealed that treatment with CAG upregulated Klb. The data are indicated as the mean ± SEM for n = 4 mice/group. (C) The levels of Cycs, Bcl2, and Casp3 in the control, DOX-treated, and CAG-treated groups were analyzed via western blot. β-actin was used as a loading control for western blotting analysis. The data are indicated as the mean ± SEM for n = 4 mice/group. (D) Representative images of western blot of analysis revealed that the levels of Nrf2 antioxidant response element pathway-related proteins (Nrf2, HO-1, and Keap1) were significantly (P < 0.05) downregulated. The data are indicated as the mean ± SEM for n = 4 mice per group. Experiments were repeated at least three times. N.S, not significant; *P < 0.05; **P < 0.01; ***P < 0.001.
Telomerase activation through CAG is an effective strategy to mitigate D-gal-induced ageing (Liu et al., 2019). We found that CAG can neutralize the D-gal induced ovarian toxicity by activating KLB/FGFR1 pathway via Telomerase activation. CAG mitigated the D-gal-induced downregulation of the KLB/FGFR1 complex and upregulated the protein levels of several critical proteins, such as Er1 and Nr5a1 (Banerjee et al., 2012)(Li et al., 2020). Furthermore, the GCs were treated with DOX to analyze the TERT inhibitory effects on the KLB/FGFR1 pathway (Eskiocak et al., 2008; Al-Kawlani et al., 2020a). DOX exerts adverse effects on GCs by inducing aromatase deficiency via downregulating estrogen levels (Al-Kawlani et al., 2020). However, we found that treatment with CAG significantly mitigated the DOX-induced downregulation of estrogen level.

We tried to analyze the potency of CAG against hTERT, but we did not find hTERT 3D structure in PDB. Therefore, we modeled our protein using template based and threading based approach with DS studio, Swiss-Model and I-TASSER webservers. The validated model was used for the study of molecular interactions study between CAG and hTERT. For this, molecular docking and molecular dynamics simulations were used. The molecular simulations revealed that CAG could bind with hTERT with strong binding affinity with – 101.67 kJ/mol. The per residue contribution revealed that Arg901 contribute significantly in hydrophobic interactions with hTERT. A recent modeling study revealed that the Arg631 and Tyr717 is important for the inhibitor binding with hTERT protein (Kalathiya et al., 2019). Interestingly, CAG was observed to interact with Arg631 through non-polar interaction. We therefore can conclude that interaction of CAG with hTERT in molecular docking and simulations, supporting the experimental results.

CAG is reported to exhibit various pharmacological activities, including TERT-activation, telomere-elongation, antioxidant, and anti-inflammatory activities (Yu et al., 2018). Pharmacokinetic studies have revealed that CAG is absorbed by the intestinal epithelium through passive diffusion and undergoes first-pass hepatic metabolism (Zhu et al., 2010). Likewise, various experimental and clinical studies have indicated the safety of CAG, which has a wide range of applications (Yu et al., 2018; Ip et al., 2014). CAG is previously been used against age-associated diseases, Neurotrophic dysfunction, depression, and Osteoclastogenesis (Yu et al., 2018, Salvador et al., 2016, Yu et al., 2020). We investigated the effects of CAG on ovarian ageing and more specifically on GCs induced dysfunction due to ageing. It is well established by several studies that D-gal accelerate ovarian ageing by increasing oxidative stress in the ovary and enhances follicular atresia (Wang et al., 2019; Li et al., 2020). We found that CAG significantly mitigated the D-gal induced damage to the ovarian follicles. Telomerase activation via CAG not only reduced the damage, but also enhances the key protein related to the GCs functioning (Nr5a1 and Foxl2). The malfunction of GCs, which results in age-associated reduced oocyte quality, is primarily characterized by altered telomerase activity (Iwata, 2017). We found that ageing not only reduced telomerase activity in the ovary but also alters the KLB signaling pathway.

Doxorubicin (DOX) is an effective antineoplastic drug, commonly used in childhood cancer. DOX is found to reduce the ovarian follicular size by inhibiting the oocyte surrounding granulosa cells (Aziz et al., 2020). Furthermore, DOX also altered the hormonal secretion and we found that DOX treated mice showed significant reduction in FGFR1/KLB pathway which is involved in female hormonal regulation. Interestingly co-treatment of CAG significantly mitigated the DOX induced ovarian follicular loss. DOX also induces mitochondrial apoptosis by disrupting the electron transport chain and compelling mitochondria to produce more ROS (Y. Liu et al., 2022; T. Liu et al., 2022). It has previously been discovered that telomerase can link to mitochondrial DNA and protect it from damage (Haendeler et al., 2009; Singhapol et al., 2013). In order to understand CAG mediated telomerase activation effect on mitochondria, we found that CAG reduced the mitochondrial damage and also enhanced the NRF2 signaling which plays a noteworthy role in mitochondrial membrane potential (Holmstrom et al., 2016). Furthermore our results are in consistent with previous report which stated that telomerase-targeted therapy mitigates DOX-induced cytotoxicity (Chatterjee et al., 2021). We primarily attribute the healing of ovarian follicles to telomerase activation, which results in mitochondrial health, and subsequently to ovarian KLB protein level restoration.

6. Conclusions

This study elucidated novel functions of Telomerase in ovarian ageing. The findings of this study indicate that downregulation of TERT and Klb occurs in mouse ovarian ageing. The binding of CAG to the three-dimensional structure of hTERT revealed that CAG is an effective telomerase activator. CAG-activated Telomerase not only upregulate Klb protein level but also improved mitochondrial functioning (non-nuclear function of Telomerase) to reduce the ovarian damage caused by D-gal or DOX. Furthermore, ovarian follicles are irreparably harmed by Doxorubicin therapy, utilized in the treatment of ovarian cancer. However, CAG prevented the harmful effects of Doxorubicin on the ovarian follicular pool.

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Author Contributions

For Designed research: M.I.; Performed research: M.I., V.K., and M. K.; Analysed data: M.I.; V.K.; Provided reagents and helped in experiments: M.-D.J., S.-H.S., and I.-K.K.; Wrote the paper: M.I.; V.K., and I.-K.K., Review and Supervision: I.-K.K.

Data Availability

Data will be made available on request.

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Conflict of Interest

The authors declared no conflict of interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.mad.2022.111756.

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