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Apigenin modulates aging-associated stromal-epithelial cross-talk in prostate cells

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Abstract

Background: The therapeutic potential of phytonutrients has gained significant attention due to their bioactive properties and effectiveness in disease management, including cancers. Apigenin, a flavone abundant in fruits and vegetables is known for its anti-inflammatory, antioxidant and anti-cancer effects. **Methods**: In this study, we investigated the ameliorative potential of apigenin on aberrant proliferation of normal prostate epithelial cells, driven by the paracrine factors of stromal origin, when conditioned with estradiol (20 pM) and dihydrotestosterone (10 nM). **Results**: Apigenin (5 μM) significantly inhibited the aberrant epithelial cell proliferation, both in isolation as well as in stromal co-culture conditions. Notably, the treatment reduced spheroid size and branching patterns three-dimensional (3D) overlay culture models. Live cell staining with Calcein acetoxymethyl ester (Calcein AM) revealed a marked decrease in viable cell numbers following treatment. Additionally, apigenin increased the expression of estrogen receptor beta (ERβ), a recognized tumor suppressor and induced apoptosis, while also inhibiting stromal cell proliferation. **Conclusions**: These results provide valuable insights into the preventive potential of apigenin against prostate pathologies driven by stromal-epithelial interactions, offering a foundation for further research into dietary strategies for cancer prevention.

Keywords

Apigenin; Prostate; Stromal-epithelial interaction; Sex-steroids (Estrogen, DHT); Endocrine cancers

La apigenina modula la comunicación cruzada estromal-epitelial asociada al envejecimiento en las células de la próstata

Resumen

Antecedentes: El potencial terapéutico de los fitonutrientes ha ganado una atención significativa debido a sus propiedades bioactivas y su eficacia en el manejo de enfermedades, incluidos los cánceres. La apigenina, una flavona abundante en frutas y verduras, es conocida por sus efectos antiinflamatorios, antioxidantes y anticancerígenos. **Métodos**: En este estudio, investigamos el potencial mejorador de la apigenina en la proliferación aberrante de células epiteliales de próstata normales, impulsada por los factores paracrinos de origen estromal, cuando se acondicionó con estradiol (20 pM) y dihidrotestosterona (10 nM). **Resultados**: La apigenina (5 μM) inhibió significativamente la proliferación aberrante de células epiteliales, tanto en condiciones de aislamiento como en condiciones de co-cultivo estromal. En particular, el tratamiento redujo el tamaño de los esferoides y los patrones de ramificación en los modelos de cultivo de superposición tridimensional (3D). La tinción de células vivas con Calcein acetoxymethyl ester (Calcein AM) reveló una marcada disminución en el número de células viables después del tratamiento. Además, la apigenina aumentó la expresión del receptor de estrógeno beta (ERβ), un supresor tumoral reconocido, e indujo la apoptosis, al tiempo que inhibía la proliferación de células del estroma. **Conclusiones**: Estos resultados brindan información valiosa sobre el potencial preventivo de la apigenina contra las patologías de próstata impulsadas por interacciones estromales-epiteliales, lo que ofrece una base para futuras investigaciones sobre estrategias dietéticas para la prevención del cáncer.

Palabras Clave

Apigenina; Próstata; Interacción estromal-epitelial; Esteroides sexuales (estrógeno, DHT); Cánceres endocrinos

1. Introduction

The prostate gland, a key male accessory reproductive organ, is highly susceptible to age-related conditions that can significantly impact quality of life. Three major conditions affecting the aging prostate are prostatitis, benign prostatic hyperplasia (BPH) and prostate cancer (PCa) [1]. BPH and PCa, in particular, are the most common prostate-related disorders in men over 50 years of age. Globally, BPH affects approximately 50% of men between the ages of 51 and 60, with prevalence increasing to 90% in men older than 80 years. PCa is the second most common cancer and the fifth leading cause of cancer death in men worldwide [2, 3]. The socio-economic burden of these diseases is substantial, with BPH leading to lower urinary tract symptoms (LUTS) that impact quality of life by disrupting daily activities, and PCa posing significant mortality risks when not diagnosed early. Pharmacological treatments for BPH, including α-blockers and 5α-reductase inhibitors, alleviate symptoms by targeting smooth muscle tone and reducing androgen-driven prostate growth, but are often associated with side effects like dizziness, fatigue and sexual dysfunction, limiting their long-term use. Surgical interventions, such as transurethral resection of the prostate (TURP), are effective but carry risks of complications including bleeding, infection and urinary incontinence [4]. For PCa, androgen deprivation therapy (ADT) is commonly used in advanced cases to reduce tumor growth, yet resistance to ADT frequently develops, leading to castration-resistant prostate cancer (CRPC), a more aggressive and treatment-refractory form [5]. These limitations underscore the need for alternative therapeutic approaches, particularly those that can target early disease processes without adverse side effects.

Structurally, the prostate is a heterocellular organ composed of basal and luminal (secretory) epithelial cells, neuroendocrine cells and a supportive stromal matrix. The stroma primarily consists of smooth muscle cells and fibroblasts, alongside smaller populations of endothelial cells, nerve cells and infiltrating inflammatory cells [6]. Prostate function and homeostasis are tightly regulated by sex steroids, notably androgens such as dihydrotestosterone (DHT) and estrogens (like estradiol, E2). These hormones exert their effects through androgen receptors (AR) and estrogen receptors (ERα and ERβ), which coordinate cellular processes such as proliferation, differentiation and apoptosis, ensuring the maintenance of prostate health [7].

As aging progresses, significant alterations in the hormonal milieu occur. Circulating DHT levels decline due to reduced 5α-reductase activity, while estradiol (E2) levels increase as a result of sustained peripheral aromatase activity [8, 9]. This shift in the androgen-to-estrogen ratio creates a distinct endocrine signature of male aging, which has been increasingly linked to the development of BPH and PCa [10, 11]. Understanding how these hormonal imbalances influence stromal-epithelial interactions in the prostate is crucial, as these interactions play a central role in disease progression. The stroma, primarily composed of smooth muscle cells and fibroblasts, secretes paracrine factors that modulate the behavior of adjacent epithelial cells. Disruption of this cross-talk due to hormonal changes is believed to contribute to aberrant epithelial proliferation, a hallmark of BPH and PCa.

Epidemiological evidence suggests that dietary factors, particularly plant-based diets can play an important role in reducing the risk of prostate cancer [12, 13]. For instance, Asian men, who consume diets rich in plant-derived compounds, have markedly lower incidences of prostate cancer and possibly BPH compared to their western counterparts [14]. Among the bioactive compounds found in such diets, apigenin, a naturally occurring flavonoid (4′,5′,7′-trihydroxyflavone) present in foods such as parsley, onions, oranges, chamomile and tea, has emerged as a promising agent due to its broad range of biological activities [15, 16]. Numerous *in vitro* studies have demonstrated its anti-proliferative, pro-apoptotic, anti-angiogenic, anti-inflammatory and anti-mutagenic effects across various cancer cell lines [17–19]. Despite these promising properties, the precise mechanisms by which apigenin influences prostate stromal-epithelial interactions, especially in the context of age-related hormonal changes, remain poorly understood. To address this knowledge gap, the present study aims to investigate the therapeutic potential of apigenin in modulating stromal-epithelial interactions in the aging prostate. Altered stromal-epithelial interactions, corresponding to the aging prostate *in vivo*, were mimicked *in vitro* by culturing normal prostate stromal (WPMY-1) cells under an optimized ratio of E2 (20 pM) to DHT (10 nM). The conditioned medium (CM) containing stromal cell-secreted growth factors was subsequently applied to normal prostate epithelial cells (RWPE-1) to evaluate its potential to induce aberrant proliferation. Apigenin, known for its anti-proliferative effects in various cancer cell lines, was utilized to assess its role in modulating this stroma-mediated epithelial proliferation, both in isolation and co-culture. This approach allows us to explore the mechanistic underpinnings of stromal-epithelial dysregulation in the aging prostate and the therapeutic potential of apigenin in regulation of these interactions.

2. Materials and methods

2.1 Reagents

Dulbecco's modified Eagle medium (DMEM, high glucose, pyruvate) (catalog number 11995065); Keratinocyte serum-free medium (K-SFM 1×) (catalog number 17005042); supplements for K-SFM (containing epidermal growth factor human recombinant and bovine pituitary extract) (catalog number 37000015, lot number 2401388); heat inactivated fetal bovine serum (HI FBS catalog number 10082147, lot number 2719155P) were procured from Gibco (Life Technologies Corporation, Grand Island, NY, USA). β-Estradiol (E2) (catalog number E2758); 5α-androstan17β-ol-3-one (dihydrotestosterone, DHT) (catalog number D-073); thiazolyl blue tetrazolium bromide (catalog number M5655); antibiotic antimycotic solution (100×) (catalog number A5955); molecular grade dimethyl sulfoxide (DMSO) (catalog number D8418); culture grade dimethyl sulfoxide (DMSO) (catalog number D2650, lot number RNBH5033); apigenin (catalog number 10798, lot number WE445301/1); and finasteride (catalog number F1293) were procured from

Sigma Aldrich (St. Louis, MO, USA). All the cell-culture plastic wares (6-well, 24-well, 96-well plates), co-culture inserts, phenol red-free matrigel (catalog number E6909) were obtained from Corning Inc. (One Riverfront Plaza, Corning, NY, USA).

2.2 Cell culture

Non-neoplastic human prostate stromal (WPMY-1) and epithelial (RWPE-1) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). RWPE-1 cells were cultured in keratinocyte serum-free medium (K-SFM) supplemented with 5 ng/mL epidermal growth factor (EGF) and 0.05 mg/mL bovine pituitary extract (BPE), maintained at 37 °C with 5% CO_2 in a humidified incubator. WPMY-1 cells were grown in high-glucose, pyruvate Dulbecco's modified Eagle medium (DMEM) supplemented with 5% fetal bovine serum (FBS) under the same conditions. Cell morphology and growth were regularly monitored using an inverted phase-contrast microscope (Olympus IX53, Olympus Corporation, Tokyo, Japan), and the culture media were replaced every 2 days.

2.3 Conditioned medium (CM) preparation

Conditioned medium was prepared from WPMY-1 cells, cultured in 75 cm^2 tissue culture flasks with 15 mL of complete DMEM. Once the cells reached approximately 80% confluence, the culture medium was replaced with 15 mL of DMEM containing 5% charcoal-stripped FBS, and 20 pM E2 & 10 nM DHT were added. This particular ratio of hormones has been previously optimized in our laboratory (unpublished data). The cells were further allowed to grow under this treatment for 72 hours at 37 °C in a 5% CO_2 atmosphere. After incubation, the medium was collected and centrifuged at 2000g for 10 minutes at 4 °C to remove cellular debris. The supernatant, now referred as CM was aliquoted and stored at −80 °C for future use.

For apigenin-treated conditioned media, apigenin was added to the medium along with the hormonal treatments before the incubation period, and the resulting medium was processed and stored following the same protocol.

2.4 Treatment of cells

RWPE-1 cells were seeded at a density of 5000 cells per well and treated with varying concentrations of apigenin (stock prepared in culture grade DMSO) to determine a non-cytotoxic range using a cell viability assay. To assess apigenin's potential in reducing stromal conditioned medium (CM)-induced epithelial proliferation, epithelial cells were treated with CM alone and with non-toxic concentrations of apigenin for 72 hours. Finasteride, an anti-BPH drug, was used as a positive control. Additionally, to examine the effects of apigenin on the stromal secretome, conditioned medium from apigenin-treated stromal cells was applied to RWPE-1 cells for 72 hours, followed by evaluation of cell proliferation.

For the stromal cells (WPMY-1), 5000 cells were plated per well and treated with hormones and apigenin to evaluate the anti-proliferative effects of apigenin on these stromal cells.

2.5 Co-culture of cells

RWPE-1 epithelial cells were plated at a density of 0.5×10^5 cells per well in a 6-well plate and allowed to adhere for 48 hours. Simultaneously, WPMY-1 stromal cells were seeded at a density of 1.0×10^5 cells per insert and cultured on hanging inserts for 24 hours. The co-culture was then established by placing the hanging inserts with stromal cells into the wells containing epithelial cells and maintained for 72 hours in a 1:1 mixture of stromal medium (DMEM supplemented with 5% charcoal-stripped fetal bovine serum) and epithelial medium (K-SFM). Treatments included 20 pM estradiol, 10 nM dihydrotestosterone, and a non-toxic concentration of apigenin. After the 72-hour treatment period, the inserts were removed, and cell viability was assessed using the Calcein AM viability assay.

2.6 Cell viability assays

2.6.1 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Cell viability was assessed using the MTT assay. Briefly, 96-well plates were seeded with 5.0×10^3 cells per well and incubated at 37 °C with 5% CO_2 for the specified time points. Following the treatment, cells were exposed to 0.5 mg/mL of thiazolyl blue tetrazolium bromide (catalog number M5655, Sigma Aldrich, St. Louis, MO, USA) for 4 hours. The resulting formazan crystals were then dissolved in molecular grade dimethyl sulfoxide (DMSO) for 2 hours. The absorbance of the colored product was measured at 570 nm using a microplate reader (Infinite M200 pro, Tecan Group Ltd., Männedorf, Switzerland).

2.6.2 Calcein acetoxymethyl ester (Calcein AM) staining

Cell viability was assessed using Calcein AM staining, which relies on the activity of intracellular esterases in live cells. After treatments, the medium was removed, and cells were washed with phosphate buffered saline (PBS). Then cells were incubated with 4 μM Calcein AM (reference number C3100MP, lot number 2610316, Invitrogen, Life Technologies Corporation, Willow Creek Road, Eugene, OR, USA) in PBS for 30 minutes at 37 °C. Live cells, containing active esterases, convert Calcein AM to the green fluorescent Calcein. Fluorescence intensity, indicative of viable cell numbers, was measured with a microplate reader (Infinite M200 pro, Tecan Group Ltd., Männedorf, Switzerland) at excitation and emission wavelengths of 494 nm and 517 nm, respectively.

2.7 Three-dimensional (3D) culture

For 3D top-up culture experiments, 96-well tissue culture plates were used following a protocol adapted for acinar morphogenesis in Michigan Cancer Foundation 10A (MCF10A) and RWPE-1 cells [20]. Matrigel, thawed overnight at 4 °C, was applied (50 μL) per well and allowed to solidify at 37 °C for 1 hour. RWPE-1 cells were seeded at 5.0×10^3 cells/cm^2 in medium containing K-SFM, 2% FBS, 5 ng/mL EGF, and 2% (v/v) Matrigel. After 3 days, cells were treated

with stromal-CM and apigenin for an additional 3 days. Post-treatment, at least 50 acinar structures per well were quantified. Bright-field imaging was conducted with a ZEISS LSM 980 and Axio Observer 7 confocal microscope (Carl Zeiss AG, Oberkochen, BW, Germany), and images were analyzed using ImageJ software. Live cell staining with 4 μM Calcein AM for 30 minutes allowed visualization of viable cells, with fluorescence imaged to assess cell viability within the acinar structures.

2.8 RNA isolation and real-time polymerase chain reaction

Total RNA was extracted from cells using Trizol® reagent (reference number 15596018, lot number 510710, Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA) according to the manufacturer's protocol. The isolated RNA was then reverse transcribed into complementary DNA (cDNA) using the High-Capacity cDNA Reverse Transcription Kit (reference number 4368814, lot number 00974750, Applied Biosystems, Thermo Fisher Scientific Baltics UAB, V. A. Graiciuno 8, Vilnius, Lithuania). Quantitative polymerase chain reaction (qPCR) was performed to quantify the expression levels of target genes, including estrogen receptor α (*ESR1*), estrogen receptor β (*ESR2*), androgen receptor (*AR*) and actin beta (*ACTB*), with the KAPA SYBR® FAST universal qPCR Master Mix (2×) kit (reference number KK4602, lot number 0000134685, Kapa Biosystems Pty Ltd, Salt River, Cape Town, South Africa). Primer sequences are provided in **Supplementary Table 1**. C_t values were used to determine the relative expression of target genes, normalized against β-Actin. Fold changes in gene expression were calculated using the $\Delta\Delta C_t$ method, and statistical significance was assessed with an unpaired, one-tailed Student's *t*-test.

2.9 Apoptosis assay

Apoptosis was assessed using CellEvent® Caspase-3/7 Green reagent (reference number R37111, lot number 2145013, Invitrogen, Life Technologies Corporation, Eugene, OR, USA), which detects caspase-3/7 activity via a peptide-dye conjugate. In non-apoptotic cells, the Asp-Glu-Val-Asp (DEVD) peptide blocks dye binding to DNA, resulting in no fluorescence. Conversely, in apoptotic cells, caspase-3/7 cleaves the peptide, enabling the dye to bind DNA and emit green fluorescence (530 nm), which is measured using a fluorescein isothiocyanate (FITC) filter set in the Countess II FL Automated Cell Counter (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA).

RWPE-1 cells (1.5×10^5 cells per well) were seeded into 6-well plates and incubated for 72 hours. After incubation, cells were treated with stromal CM and Apigenin for 72-hours at 37 °C with 5% CO_2. Following treatment, cells were trypsinized, centrifuged at 120g for 10 minutes and resuspended in 1 mL of complete medium. A drop of CellEvent® Caspase-3/7 Green reagent (as per the manufacturer's protocol) was added to the cell suspension and incubated for 30 minutes. After incubation, 10 μL of the cell suspension was loaded into the counting chamber of the automated cell counter to quantify fluorescence-positive cells in the suspension.

2.10 Statistical analysis

The data were analyzed using Microsoft Excel 2019 (Microsoft Corporation, Redmond, WA, USA) and GraphPad Prism 8.01 (GraphPad Software, Inc., San Diego, CA, USA) with one way analysis of variance (ANOVA) or Student *t*-test (unpaired, one tailed). The level of statistical significance was set at $p < 0.05$.

3. Results

3.1 Effect of apigenin on aberrant proliferation of prostate epithelium

Mimicking the aged prostate environment *in vitro*, CM derived from stromal cells treated with different combinations of E2 and DHT induced aberrant proliferation in prostate epithelial cells (Fig. 1a). Based on these results the optimum parameters (20 pM E2, 10 nM DHT and 72-hour time point) were used for further experiments to prepare the stromal CM. The potential therapeutic effect of apigenin was investigated on the epithelial cells, for which first a non-toxic concentration range was established by exposing epithelial cells with variable doses of apigenin (**Supplementary Fig. 1**). Apigenin at concentrations of 5 and 10 μM, was quite effective in significantly reducing the aberrant proliferation induced by the stromal-CM (Fig. 1b). Furthermore, in the co-culture model with both epithelial and stromal cells subjected to hormonal conditions, apigenin treatment notably decreased epithelial cell proliferation (Fig. 1c).

In a 3D culture system, stromal CM treatment led to substantial morphological changes in the epithelial spheroids, including increased size, enhanced branching, and complex network formation. These changes reversed after apigenin supplementation, as evident by the reduced spheroid size and branching, also the morphology got restored similar to control conditions (Fig. 2a,b). Additionally, Calcein AM live-cell staining revealed a significant decrease in the proportion of viable cells in the spheroids following apigenin treatment as compared to CM alone, implying towards an anti-proliferative activity (Fig. 2c).

In our previous study, stromal CM was shown to drive aberrant proliferation through the upregulation of androgen receptor (AR) and estrogen receptor alpha (ERα) [21]. This study demonstrated that although apigenin supplementation upregulated the mRNA expression levels of androgen receptor (AR) and estrogen receptor alpha (*ESR*1), it also significantly upregulated the relative mRNA expression of estrogen receptor beta (*ESR*2/ERβ)), a recognized tumor suppressor (Fig. 3a). Furthermore, apigenin treatment induced a marked increase in cells positive for caspase-3/7 activity, indicating a promotion of apoptotic pathways (Fig. 3b,c). Overall, these findings underscore the efficacy of apigenin in mitigating CM-induced aberrant epithelial proliferation. Apigenin effectively reduces spheroid size and branching, diminishes cell viability and promotes apoptosis, highlighting its potential as a therapeutic agent for controlling abnormal epithelial cell growth.

3.2 Effect of apigenin on prostate stromal cells

The prostate stroma interacts with the epithelium and modulates the release of various andromedins promoting the growth

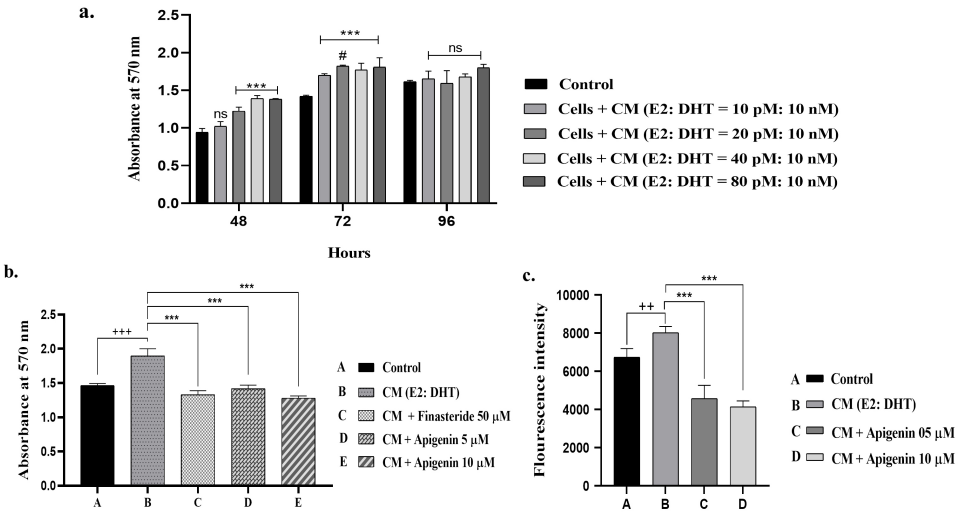

FIGURE 1. Effect of apigenin on prostate epithelial cell proliferation. (a) Growth of RWPE-1 in response to CM from WPMY-1 treated with different concentration ratios of E2 and DHT. *** indicates $p < 0.001$, ns indicates $p > 0.05$; # indicates $p < 0.05$ as compared to the 10 nM DHT 10 pM E2 CM treated group at the 72-hour time point (n = 3). (b) Effect of apigenin on the growth of RWPE-1 cells cultured with stromal derived medium. (c) Effect of apigenin on epithelial proliferation in Co-culture. Data presented as mean ± SD (standard deviation). $^{+++}$ indicates a significant ($p < 0.001$), $^{++}$ ($p < 0.01$) change in the growth of cells in group B as compared to control (group A). *** indicates a significant ($p < 0.001$) change in the growth of cells in groups compared to CM alone treatment (group B) (n = 3). CM: conditioned medium; DHT: dihydrotestosterone.

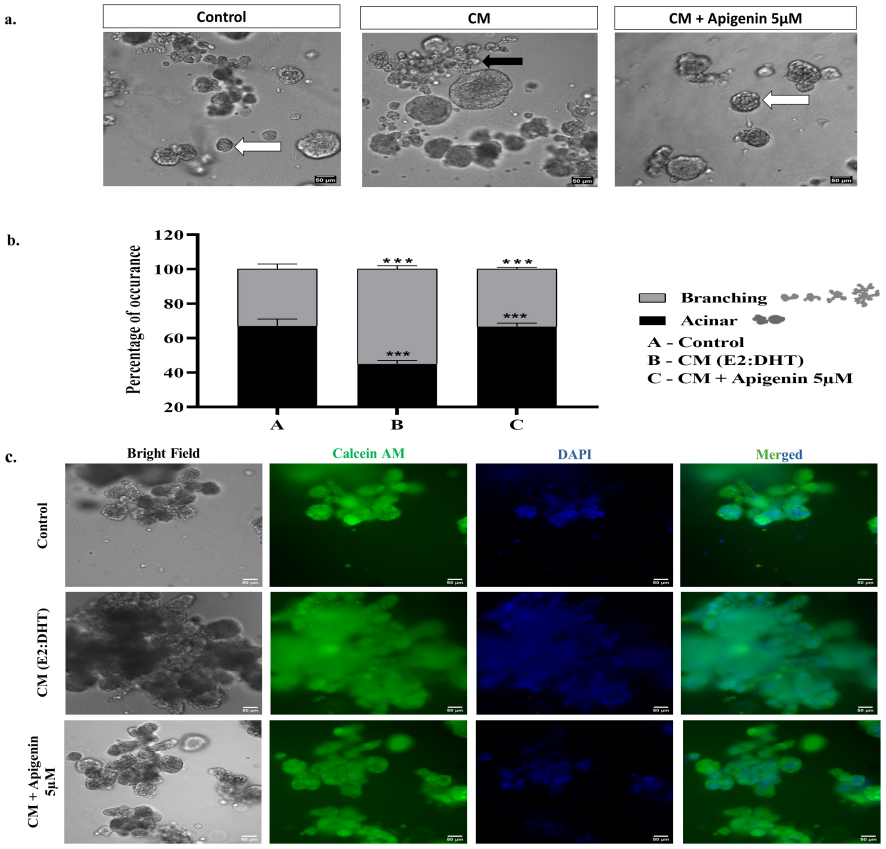

FIGURE 2. Effect of apigenin on prostate epithelial cellular morphology. (a) Bright-field microscope images of epithelial spheroids. Spheroid structures are referred to as acinar (white arrow), and branching (cord-like) (black arrow) as indicated. (b) Frequency distribution of acinar and branching structures; *** Significant differences in acinar and branching structures were observed in Group B compared to Group A, as well as between Group C and Group B (n = 3). (c) Images of spheroid live staining (Calcein AM), (magnification 20×). CM: conditioned medium; DHT: dihydrotestosterone; AM: acetoxymethyl ester; DAPI: 4′,6-diamidino-2-phenylindole.

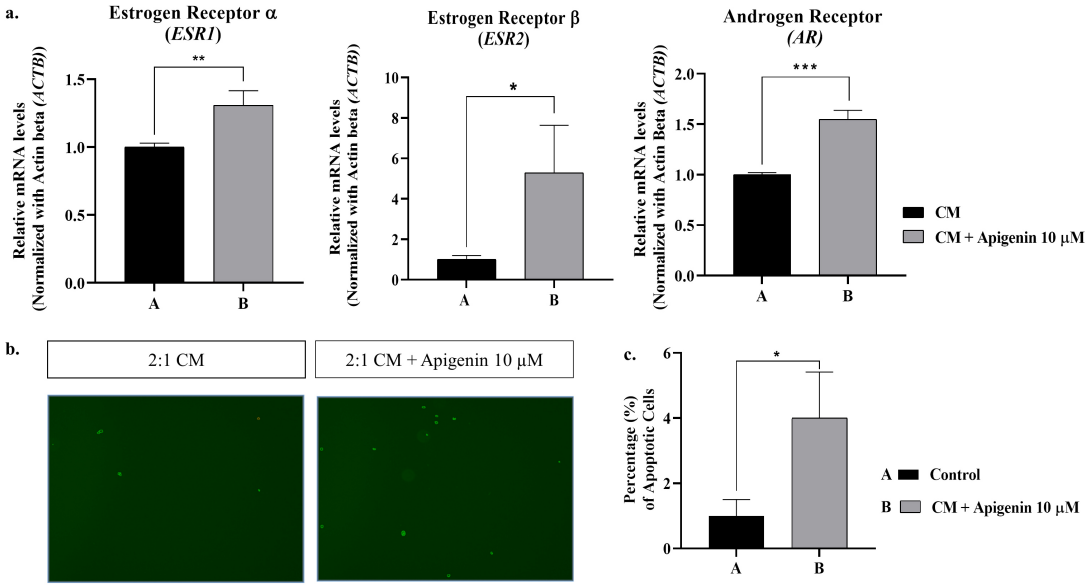

FIGURE 3. Effect of apigenin at the molecular level in prostate epithelial cells. (a) Relative gene expression of *ESR1*(ERα), *ESR2* (Erβ) and *AR*. data are shown as mean ± SEM (standard error of the mean). ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ compared to control group (n = 3); (b) Representative images showing caspase-3/7 positive cells detected by cell counter; (c) Quantification of caspase-3/7 positive cells. Data are shown as mean ± SD. *$p < 0.05$ compared to the 2:1 CM group (n = 3). mRNA: Messenger RNA; CM: conditioned medium.

of complex ductal epithelium and maintenance of prostate micro-environment. During aging and disease progression, there is a disruption of this micro-environment affecting the stromal tissues also. We investigated the impact of apigenin on the stromal cell physiology under the aging hormonal milieu. Initially, a non-toxic concentration range of apigenin was established for stromal cells by assessing their viability across variable apigenin doses (**Supplementary Fig. 2**). The presence of apigenin in concentrations of 5 and 10 μM significantly reduced the proliferation as compared to hormonal treatment alone (Fig. 4a).

We further wanted to investigate if apigenin could suppress the release of growth factors from stromal cells *i.e.*, its impact on the stromal secretome. In order to study this, stromal CM was prepared by adding apigenin to the culture medium along with the hormones (E2 & DHT), and this conditioned medium was analysed for its effects on epithelial cell proliferation. The results indicated that CM from apigenin-treated stromal cells did not mitigate epithelial aberrant proliferation (Fig. 4b). These findings suggest that apigenin exerts its primary effect by directly targeting epithelial cells rather than altering the stromal CM's capacity to modulate epithelial proliferation. Additionally, apigenin's ability to inhibit stromal cell proliferation under hormonal treatment underscores its dual action in modulating both stromal and epithelial cell dynamics.

4. Discussion

The prostate gland is a hormone-dependent organ, with testosterone and estrogen playing critical roles in its development, functional maintenance and homeostasis. The intricate interplay between stromal and epithelial compartments is fundamental to the prostate's structural and functional integrity [22]. Experimental evidence highlights the necessity of both hormones and stromal-epithelial cross-talk for proper prenatal prostate development, as their absence results in impaired growth [23, 24]. In the adult prostate, dihydrotestosterone (DHT), the bioactive metabolite of testosterone, predominantly regulates cellular homeostasis, with estradiol (E2) providing secondary but synergistic effects [25, 26]. DHT and testosterone activate androgen receptors (AR) in both stromal and epithelial cells, inducing the secretion of andromedins, which promote cellular proliferation, differentiation, and tissue maintenance. Estrogen exerts its effects via ERα, which promotes proliferation, and ERβ, which induces apoptosis, thus maintaining epithelial-stromal equilibrium.

As men age, this delicate hormonal balance shifts due to a decline in DHT levels (caused by reduced 5α-reductase activity) and an increase in estradiol levels (due to enhanced aromatase activity). This altered androgen-to-estrogen ratio has been strongly associated with the onset of prostate diseases, particularly benign prostatic hyperplasia (BPH) and prostate cancer (PCa), where disrupted stromal-epithelial interactions contribute to unchecked cellular proliferation and disease progression [27, 28]. The clinical burden of these prostate pathologies is substantial, particularly in Western countries, which exhibit higher disease prevalence compared to Asian populations [29]. This difference has been attributed, in part, to dietary factors, as Asian diets are often rich in plant-derived bioactive compounds that may offer protective benefits against prostate diseases [14].

Apigenin, a naturally occurring flavonoid found in parsley, onions, oranges, chamomile and tea, has been shown to possess significant anti-proliferative, pro-apoptotic, anti-angiogenic and anti-inflammatory effects [30]. In this study, we sought to explore the therapeutic potential of apigenin

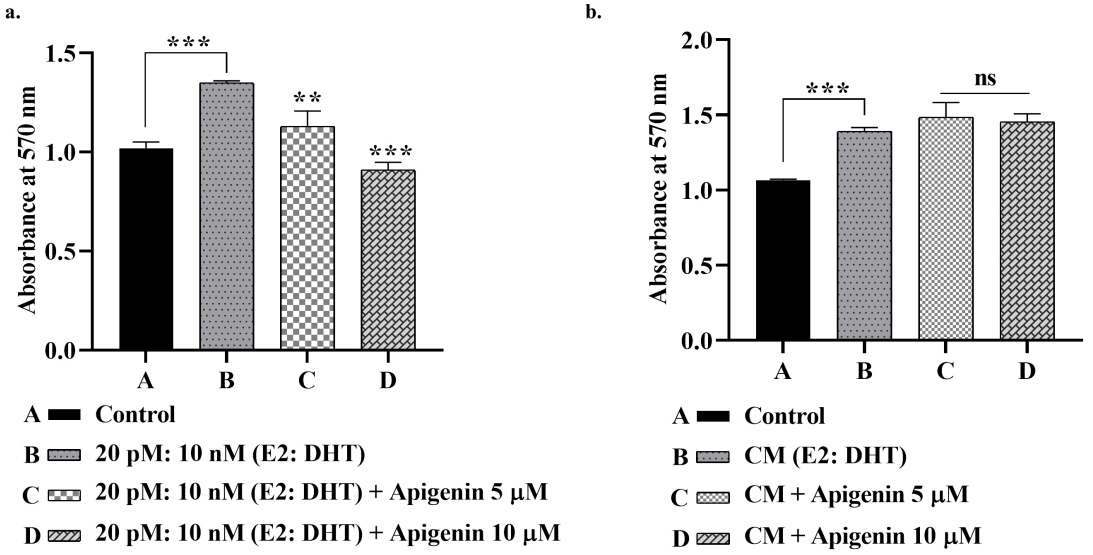

FIGURE 4. Effect of apigenin on prostate stromal cells. (a) Effect of apigenin on the growth of WPMY-1 cells under hormonal treatment; (b) Effect of apigenin treated stromal CM on the epithelial cell proliferation. Data presented as mean ± SD. *** indicates a significant ($p < 0.001$) change in the growth of cells in group B as compared to control (group A). ** indicates a significant ($p < 0.01$), *** ($p < 0.001$), ns ($p > 0.05$) change in the growth of cells in group C, D as compared to group B; (n = 3). CM: conditioned medium; DHT: dihydrotestosterone.

in modulating abnormal stromal-epithelial interactions in the aging prostate, specifically under conditions mimicking the altered hormonal environment associated with aging. We utilized stromal cells (WPMY-1) conditioned with an optimized dose of E2 and DHT to simulate the aging prostate microenvironment, and assessed the impact of apigenin on aberrant epithelial proliferation.

Our findings reveal that apigenin (5 μM) significantly inhibited abnormal epithelial proliferation under both stromal-conditioned medium (CM) and stromal co-culture conditions. In 3D culture models, apigenin treatment notably reduced spheroid size and branching morphogenesis, both of which are indicative of early neoplastic changes, effectively restoring conditions to resemble those of control cultures. Furthermore, live cell staining using Calcein AM demonstrated a substantial reduction in viable epithelial cell numbers following apigenin treatment, reinforcing its inhibitory effect on cell proliferation.

Further investigation revealed that apigenin effectively restricted hormone-induced stromal proliferation, suggesting its capacity to modulate stromal growth under hormonal milieu. Previous studies have demonstrated that apigenin inhibits the proliferation of prostatic stromal cells through the mitogen-activated protein kinas (MAPK) signaling pathway and induces cell-cycle arrest at the G1/S phase [31]. Analysis of the stromal secretome treatment showed that CM from apigenin-treated stromal cells did not alleviate epithelial aberrant proliferation, indicating that apigenin acts primarily through direct effects on epithelial cells. These findings highlight apigenin's dual role in modulating both stromal and epithelial cellular dynamics by inhibiting stromal proliferation and preventing stromal-mediated epithelial dysregulation.

At the molecular level, we observed that apigenin significantly enhanced ERβ expression, a tumor suppressor known for its pro-apoptotic effects. This upregulation of ERβ is consistent with apigenin's known pro-apoptotic actions and suggests a potential mechanism by which it may exert its anti-tumorigenic effects. Additionally, apigenin treatment promoted apoptosis (as evident by increased caspases activity) in epithelial cells, further supporting its role in modulating cell turnover and preventing hyperplastic or neoplastic transformation. Previous studies have shown that apigenin can inactivate protein kinase B (Akt), a key regulator of cell survival, thereby triggering apoptosis in both *in vitro* and *in vivo* models of human prostate cancer [32]. Additionally, research has indicated that apigenin inhibits IκB kinase α (IKKα) activation, further enhancing its capacity to suppress prostate cancer progression [33]. Further, studies on prostate cancer xenograft models and *in vitro* studies further highlights apigenin's influence on key molecular targets, including cell cycle regulatory proteins, ultimately promoting apoptosis [34–36]. These findings are consistent with previous studies showing that apigenin preferentially activates ERβ in DU-145 (human prostate cancer cell line) and MDA-MB-231 (human breast cancer cell line) cancer cells, resulting in stronger antiproliferative effects compared to genistein. By selectively modulating ERβ and activating caspase-3, apigenin exerts more potent pro-apoptotic effects in these cancer models [37]. Our findings reinforce apigenin's potential as a selective ERβ modulator with significant implications for the prevention and treatment of epithelial cell malignancies. The key findings of the study are illustrated in Fig. 5, highlighting apigenin's potential as promising candidate for the management of prostate pathologies.

However, several limitations must be acknowledged. First, the reliance on *in vitro* models may not adequately reflect the intricate stromal-epithelial interactions present *in vivo*. While three-dimensional cultures and co-culture systems offer a more accurate representation of *in vivo* conditions than traditional monolayer cultures, they still do not encompass the systemic

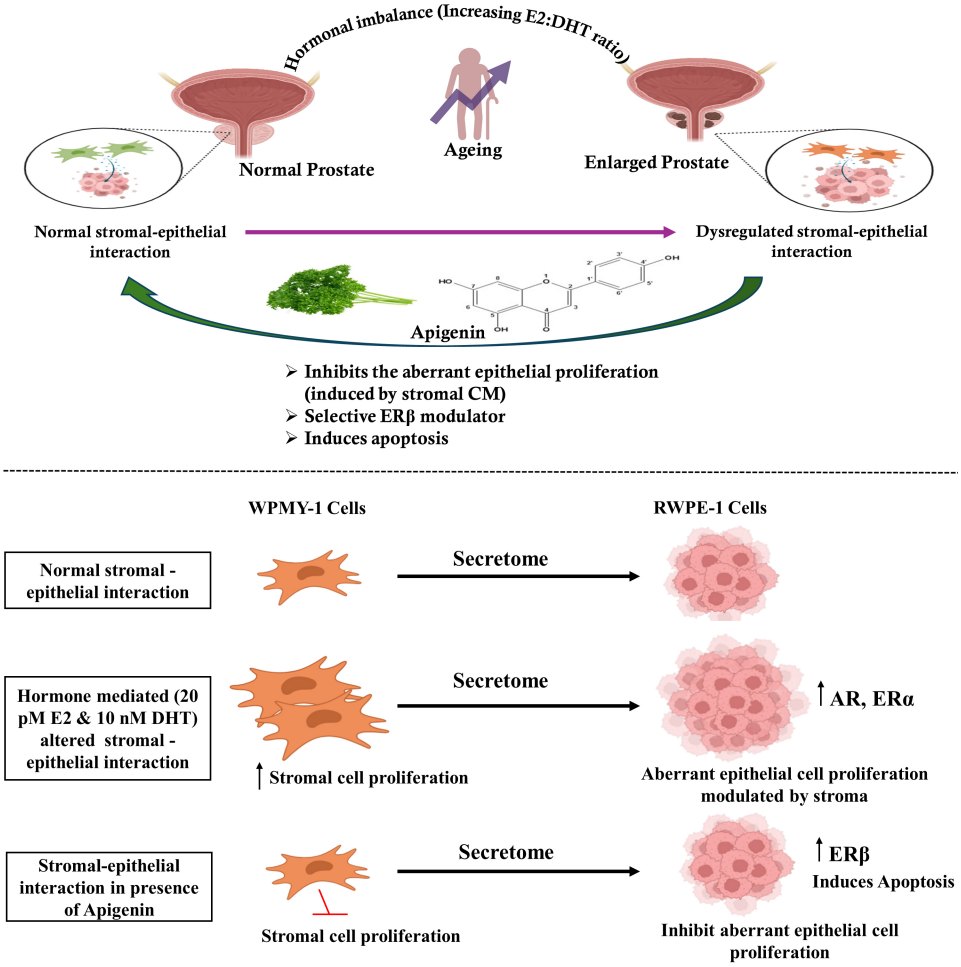

FIGURE 5. Illustration of role of apigenin in modulating stromal epithelial interaction in prostate. ERα: estrogen receptor alpha; ERβ: estrogen receptor beta; DHT: dihydrotestosterone; E2: estradiol; CM: Conditioned medium; WPMY-1: normal prostate stromal; RWPE-1: normal prostate epithelial cells; AR: androgen receptors.

factors and tumor microenvironment characteristic of biological systems. Therefore, further investigations utilizing *in vivo* models are crucial to validate our findings and examine the efficacy and systemic effects of apigenin in a clinical context. Additionally, our study focused on normal prostate epithelial and stromal cells, highlighting the necessity to assess whether apigenin produces similar effects in malignant prostate tissues or under conditions that simulate more advanced stages of the disease.

Future research should focus on translating these findings into *in vivo* studies, ultimately leading to clinical trials that evaluate the therapeutic potential of apigenin in prostate diseases. Apigenin's selective enhancement of estrogen receptor beta (ERβ)—known for its tumor-suppressive and pro-apoptotic properties—suggests it may serve as a promising therapeutic strategy for benign prostatic hyperplasia (BPH) and early-stage prostate cancer. Clinical trials assessing apigenin's efficacy in preventing disease progression, reducing prostate size, and improving lower urinary tract symptoms (LUTS) in BPH patients could yield valuable insights into its therapeutic viability. Furthermore, apigenin's role in modulating stromal-epithelial interactions may help prevent the progression of pre-cancerous lesions to invasive cancer, particularly in aging populations with hormonal imbalances.

5. Conclusions

In conclusion, this study elucidates the therapeutic potential of apigenin in modulating stromal-epithelial interactions within the aging prostate. Apigenin exhibited significant anti-proliferative and pro-apoptotic effects on epithelial cells, primarily through the upregulation of estrogen receptor beta (ERβ). It's ability to inhibit stromal proliferation while concurrently preventing aberrant epithelial growth underscores its promise as a novel therapeutic strategy for benign prostatic hyperplasia (BPH) and early-stage prostate cancer. Further investigation in *in vivo* models is imperative to validate these findings and guide therapeutic strategies, ultimately aiming to establish innovative treatment options for age-related prostate diseases.

AVAILABILITY OF DATA AND MATERIALS

The data are contained within this article (and **Supplementary material**).

AUTHOR CONTRIBUTIONS

DP and AMN—conceived and designed the research study, analyzed and interpreted the data. AMN—performed the research experiments and wrote the manuscript. DP—contributed reagents, materials, analysis tools or data, acquisition of funding and revising the manuscript. ND—contributed in writing and revising the manuscript. PKC, NK and SG—provided help and advice on analysis and interpretation of data. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

SUPPLEMENTARY MATERIAL

Supplementary material associated with this article can be found, in the online version, at https://files.intandro.com/files/article/1932309226092150784/attachment/Supplementary%20material.docx.

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