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Luteolin suppresses oral carcinoma 3 (OC3) cell growth and migration via modulating polo-like kinase 1 (PLK1) expression and cellular energy metabolism

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Oral squamous cell carcinoma (OSCC) is a prevalent malignant tumor affecting the head and neck region (Leemans et al., 2018). It is often diagnosed at a later stage, leading to a poor prognosis (Muzaffar et al., 2021; Li et al., 2023). Despite advances in OSCC treatment, the overall 5-year survival rate of OSCC patients remains alarmingly low, falling below 50% (Jehn et al., 2019; Johnson et al., 2020). According to statistics, only 50% of patients with oral cancer can be treated with surgery. Once discovered, it is more frequently at an advanced stage. In addition, owing to the aggressively invasive and metastatic characteristics of OSCC, most patients die within one year of diagnosis. Hence, the pursuit of novel therapeutic drugs and treatments to improve the response of oral cancer to medication, along with a deeper understanding of their effects, remains crucial objectives in oral cancer research (Johnson et al., 2020; Bhat et al., 2021; Chen et al., 2023; Ruffin et al., 2023).

Luteolin (3',4',5,7-tetrahydroxy flavone) is a natural flavonoid that is extensively present in fruits and vegetables, such as celery, green pepper, chamomile tea, lonicera, and medicinal herbs (Lin et al., 2008). Of note, plants rich in luteolin have been widely used in Chinese traditional medicine for thousands of years to treat diseases such as allergies, hypertension, bronchitis, other respiratory diseases, and cancer (Sun et al., 2021; Mao et al., 2022; Muruganathan et al., 2022). Numerous studies have reported that luteolin possesses antiapoptotic, antioxidant, anti-inflammatory, and neuroprotective activity, both in vivo and in vitro (Seelinger et al., 2008a, 2008b; Lopez-Lazaro, 2009; Nabavi et al., 2015; Tuorkey, 2016; Imran et al., 2019; Liang et al., 2022). Luteolin's antioxidant activity plays a crucial role in its various protective effects against renal failure, mucosal damage, hepatoxicity, neurodegenerative diseases, and cardiovascular disorders. Its ability to scavenge free radicals and modulate antioxidant enzymes contributes to its therapeutic potential in these conditions. Luteolin has shown promise in some studies as an anti-diabetic agent for diabetic nephropathy, a kidney complication that occurs as a result of long-standing diabetes mellitus (Wang et al., 2011; Al-Ishaq et al., 2019; Davella and Mamidala, 2021; Zhou et al., 2022; Zhang et al., 2023). One of the mechanisms through which luteolin exhibits renoprotective effects is by enhancing the expression of heme oxygenase-1 (HO-1), an enzyme that plays a critical role in protecting cells from oxidative stress and inflammation. Additionally, the above-mentioned antioxidant activity contributes to the prevention of morphological damage to the kidneys associated with diabetes

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mellitus. Moreover, studies conducted in vitro and on animal models have shown promising results regarding the potential benefits of luteolin in Alzheimer's disease and Parkinson's disease, and for cardiac health (Wang et al., 2022; Bhusal et al., 2023; He et al., 2023).

In recent years, there have been considerable interests among researchers regarding the potential application of luteolin in cancer treatment, as well as the underlying mechanisms involved. The in-silico analysis of the luteolin molecule from *Tridax procumbens* suggesting high active probability and reduced cardiotoxicity makes it a promising drug candidate for targeting the mini chromosome maintenance protein 7 (MCM7) (Lakhera et al., 2022). Dysregulation of the MCM7 protein has been associated with various types of cancers due to its involvement in DNA replication and cell cycle control. Thus, by targeting MCM7, luteolin may potentially help restore proper DNA regulation and inhibit tumor growth. It has been found to effectively inhibit cancer cell proliferation both at a dosage range of 3 to 50 μmol/L in vitro and at a dosage range of 5 to 10 mg/kg body weight in vivo, including esophageal carcinoma cells, gastric cancer cells, breast cancer cells, lung cancer cells, colon cancer cells, and prostate cancer cells (Fang et al., 2007; Chen et al., 2017; Kang et al., 2017; Cook, 2018; Pu et al., 2018; Reyes-Farias and Carrasco-Pozo, 2019). Moreover, one advantage of luteolin is its ability to penetrate the skin, making it suitable for treating skin cancer. This compound has also exhibited an inhibitory effect on human leukemic cell lines. The signaling pathways targeted by luteolin include phosphoinositide-3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR), Wnt/β-catenin, mitogen-activated protein kinase (MAPK)/forkhead box O3a (FOXO3a), and Notch pathways. In the context of breast and colon cancers, luteolin has been shown to inhibit the expression of transcriptionally active forms of β-catenin to suppress cancer cell epithelial–mesenchymal transition (Pandurangan et al., 2013; Lin et al., 2017). Additionally, luteolin could target Notch1 and Notch4 to regulate metastatic breast cancer. A critical molecular link between the Notch and Wnt/β-catenin pathways is glycogen synthase kinase 3β (GSK3β), and luteolin has been shown to attenuate GSK3β inactivation, potentially through AKT inhibition (Fang et al., 2000; Pandurangan et al., 2013). In bladder cancer, luteolin and its metabolites hinder cell proliferation by upregulating p21 and inhibiting mTOR signaling (Iida et al., 2020). Furthermore, luteolin treatment induces autophagy in SW620 cells, as evidenced by increased phosphorylation of extracellular signal-related kinase 1/2 (ERK1/2), c-Jun N-terminal kinase 1/2 (JNK1/2), and p38, and expression of FOXO3a (Potočnjak et al., 2020). However, there is limited research on the role of luteolin in oral cancer. Thus, this study investigated the effects of luteolin on oral cancer cells and unraveled the underlying mechanisms. Specifically, we evaluated the impacts of luteolin on the cell cycle progression, proliferation, and migration of oral carcinoma 3 (OC3) cells, and analyzed its potential involvement in tumor suppressor p53 signaling and tumor cell energy metabolism.

The OC3 cell line, a human oral carcinoma cell line, was initially established by Lin et al. (2004). The cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, MO, USA) supplemented with 10% (volume fraction) heat-inactivated fetal bovine serum (FBS; Gibco, CA, USA), streptomycin, and penicillin (100 U/mL; Gibco) at 37 °C with humidified 95% air and 5% CO_2. Luteolin (Sigma-Aldrich) was diluted to 10 μmol/L in DMEM, and this concentration was used in all assays. To investigate the effects of luteolin on OC3 cell biology, various aspects of tumor cell behavior, including proliferation, cell cycle progression, and migration, were evaluated, as mentioned in "Materials and methods". The proliferation rate of luteolin-treated OC3 cells was determined by the cell counting kit-8 (CCK-8; Yeasen Biotechnology, Shanghai, China) assay. As shown in Fig. 1a, the relative absorbance value of luteolin-treated OC3 cells was about 40% at 24 h, which represented a slight but not significant decrease compared to the values of the control (about 50%) and dimethyl sulfoxide (DMSO) groups (about 50%). Comparatively, the proliferation of OC3 cells following luteolin treatment was significantly suppressed, with relative absorbance values being about 50% and 60% at 48 and 72 h, respectively. To further evaluate whether luteolin exposure affects the cell cycle progression of OC3 cells, a flow cytometry-based assay was performed and the results demonstrated that, although the percentages of cells at the G0/G1 stage remained comparable among all groups, the percentage of cells at the S stage (about 10%) was significantly reduced following luteolin treatment as compared to about 25% and 20% in the control and DMSO groups, respectively. The percentage of cells

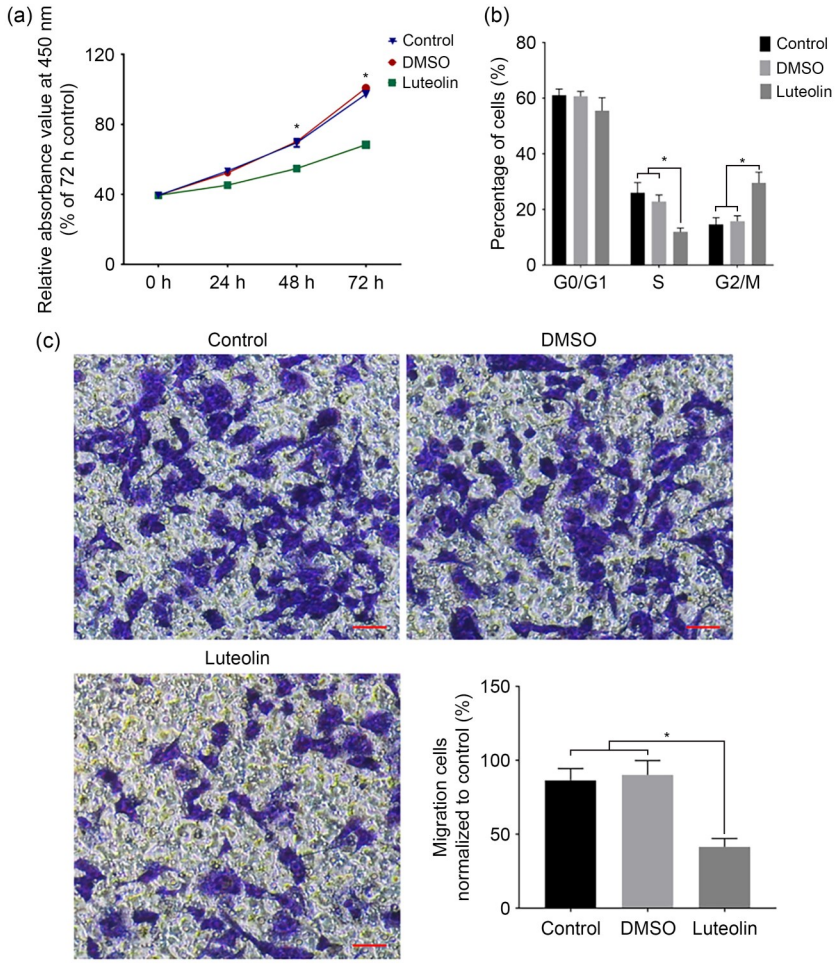

Fig. 1 Effects of luteolin exposure on proliferation, cell cycle progression, and migration of oral carcinoma 3 (OC3) cells in vitro. (a) Proliferation rates of OC3 cells determined by cell counting kit-8 (CCK-8) assay at indicated time points (represented as relative absorbance values at 450 nm, and all the numbers are normalized to the absorbance value of the control group at 72 h). (b) Percentages of OC3 cells at G0/G1, S, and G2/M stages determined by flow cytometry analysis. (c) Migration of OC3 cells (migrated cells on the other side of the filter membrane were stained using crystal violet and manually counted). Scale bar=50 μm. All values are presented as mean±standard deviation (SD), n=3. * P<0.05 vs. control. DMSO: dimethyl sulfoxide.

at the G2/M stage in the luteolin-treated group (about 30%) was almost doubled compared to the control and DMSO groups (Fig. 1b). Lastly, the migration capacity of OC3 cells was investigated and the number of migrated cells following luteolin treatment was significantly reduced to about 40% as opposed to about 80% cells observed in the control and DMSO groups (Fig. 1c).

To elucidate the expression profiles of key molecules involved in regulating cell cycle progression, OC3 cells were treated with luteolin for 24 and 48 h. Western blot analysis showed a reduction in the protein levels of polo-like kinase 1 (PLK1), p53, and the phosphorylation of p53 at Serine 15 (p53-Ser15(p)) in luteolin-treated OC3 cells compared to the control and DMSO groups (Fig. 2). PLK1 is a crucial protein involved in the regulation of cell division and the maintenance of genome stability during mitosis. It plays a role in several important processes such as spindle assembly, centrosome maturation, chromosome segregation, and DNA damage response (Iliaki et al., 2021). The reduction in PLK1 protein levels observed in luteolin-treated OC3 cells suggested that luteolin treatment might inhibit or downregulate PLK1 expression, potentially affecting these processes. p53 is a well-known tumor suppressor protein that plays a critical role in preventing cancer development by regulating cell cycle arrest, DNA repair, senescence, and apoptosis.

p53-Ser15(p) is an important post-translational modification that usually occurs in response to DNA damage and activates p53's functions. The decrease in both total p53 protein levels and p53-Ser15 phosphorylation in luteolin-treated OC3 cells indicates that luteolin treatment may affect p53-mediated signaling pathways and their ability to respond to DNA damage. Overall, the findings suggested that luteolin might exert its anticancer effects by modulating key regulatory proteins involved in cell division, genome stability, and DNA damage response. Further studies are needed to elucidate the exact molecular mechanisms underlying these effects and their implications for cancer therapy. Several studies have investigated the impact of luteolin on cellular bioenergetics, which refers to the processes involved in energy production and utilization within cells (Naia et al., 2021; Rehfeldt et al., 2022). Luteolin has been shown to affect mitochondrial function, which is crucial for cellular bioenergetics (Han et al., 2007). It has been reported to modulate glycolytic pathways and activate adenosine monophosphate (AMP)-activated protein kinase (AMPK), which contributes to cellular bioenergetics by providing substrates for adenosine triphosphate (ATP) generation (Lim et al., 2016). To evaluate the impact of luteolin treatment on cellular bioenergetics of OC3 cells, the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of OC3 cells were measured using a Seahorse instrument. After sequential addition of oligomycin, carbonyl cyanide 4-trifluoromethoxy-phenylhydrazone (FCCP), and rotenone, the OCR was measured, and the OCR curves were very similar between the control and DMSO groups throughout the experiment, with no significant differences at all tested time points (Fig. 3a).

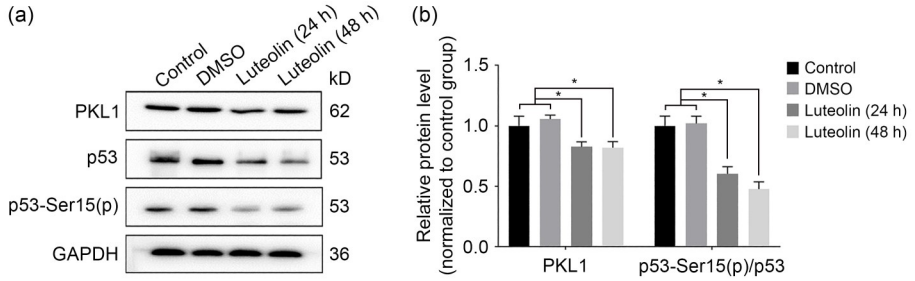

Fig. 2 Effects of luteolin exposure on the expression of PLK1, p53, and p53-Ser15(p) at protein levels in OC3 cells. (a) Expression of PLK1, p53, and p53-Ser15(p) at protein levels examined by western blotting. (b) Quantification of protein levels performed by ImageJ software. All values are presented as mean±standard deviation (SD), *n*=3. * *P*<0.05. DMSO: dimethyl sulfoxide; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; PLK1: polo-like kinase 1; p53-Ser15 (p): phosphorylation of p53 at serine 15; OC3: oral carcinoma 3.

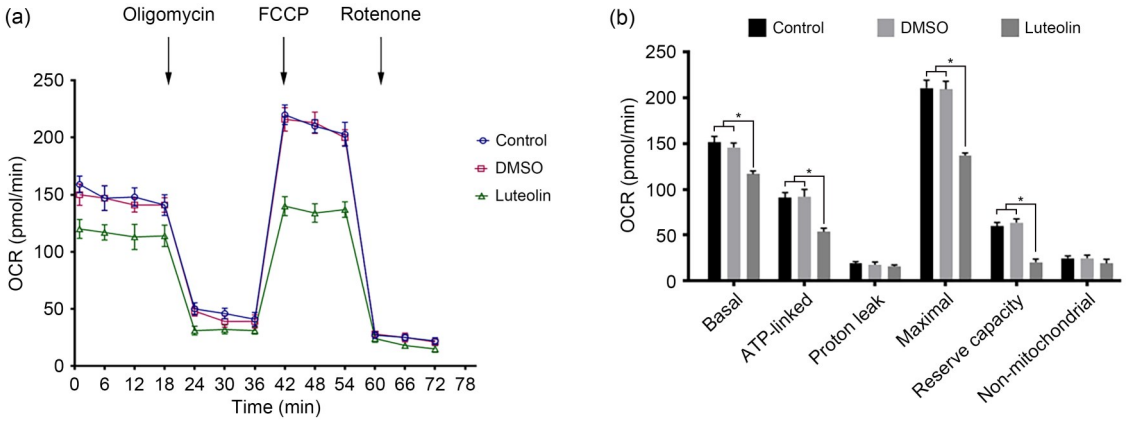

Fig. 3 Effect of luteolin exposure on the mitochondrial dysfunction of oral carcinoma 3 (OC3) cells. (a) Representative graph of the oxygen consumption rate (OCR). Oligomycin (1.5 μmol/L), carbonyl cyanide 4-trifluoromethoxy-phenylhydrazone (FCCP) (0.5 μmol/L), and rotenone (1 μmol/L) were added at indicated time points to determine different parameters of mitochondrial functions. (b) The average basal OCR, adenosine triphosphate (ATP)-linked production, proton leakage, maximal OCR, and reserve respiratory capacity, as well as non-mitochondrial production were determined. All values are presented as mean±standard deviation (SD), *n*=3. * *P*<0.05. DMSO: dimethyl sulfoxide.

In contrast, luteolin exposure pronouncedly altered mitochondrial functions in OC3 cells so that basal OCR, ATP-linked OCR, maximal OCR, and the reserve capacity in luteolin-treated cells were significantly reduced compared to the control and DMSO groups. The only exceptions observed were proton leak OCR and non-mitochondrial OCR, which remained almost unchanged among all groups (Fig. 3b). To further examine the effects of luteolin exposure on glycolytic flux (glycolysis and glycolytic capacity) represented as ECARs, it was assessed following the sequential addition of glucose, oligomycin, and 2-deoxy-D-glucose (2-DG). Similar to the OCR plots, ECAR plots of the control and DMSO groups exhibited almost identical trends at all tested time points (Fig. 4a), while following luteolin exposure, the glycolysis and glycolytic capacity of luteolin-treated cells were reduced to about 15 and 18 mpH/min, respectively. This represented a significant decrease in ECAR values compared to the control group (Fig. 4b). To further confirm the perturbation of mitochondrial function following luteolin treatment in OC3 cells, the products (ATP and reactive oxygen species (ROS)) of mitochondrial respiration were quantified and the results demonstrated a significant decrease in ATP production in luteolin-treated cells (Fig. 5a). Although a slight increase in ROS production in luteolin-treated cells was observed, no significant difference among all groups was noted (Fig. 5b)

Altogether, the findings of this study suggested that luteolin administration represents a promising energy suppression strategy for cancer treatment. Luteolin appears to be a promising candidate for ancillary therapy of oral cancer. The next step will be to discover and test the effects of combined luteolin and chemotherapeutic drugs in vitro and in vivo, which could be invaluable for oral cancer therapy.

Materials and methods

The detailed methods are provided in the electronic supplementary materials of this paper.

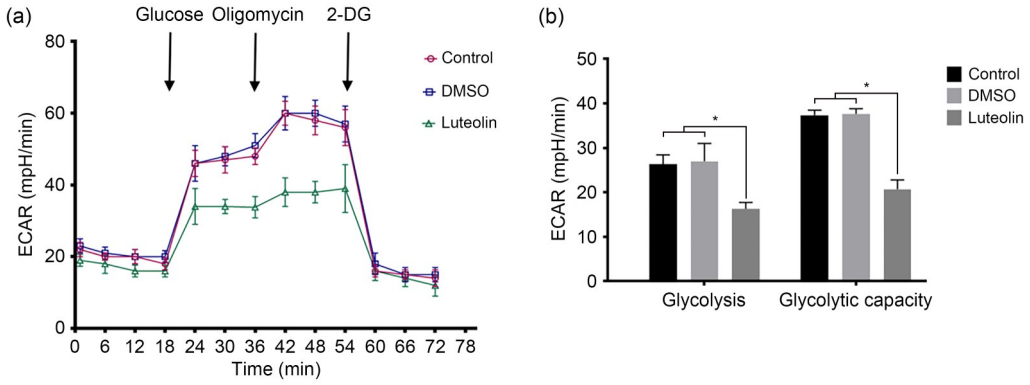

Fig. 4 Effect of luteolin exposure on glycolysis of oral cancer 3 (OC3) cells. (a) Extracellular acidification rate (ECAR) was assayed using a Seahorse analyzer in OC3 cells. (b) Glycolysis and glycolytic capacity were calculated and summarized. All values are presented as mean±standard deviation (SD), n=3. * P<0.05. 2-DG: 2-deoxy-D-glucose; DMSO: dimethyl sulfoxide.

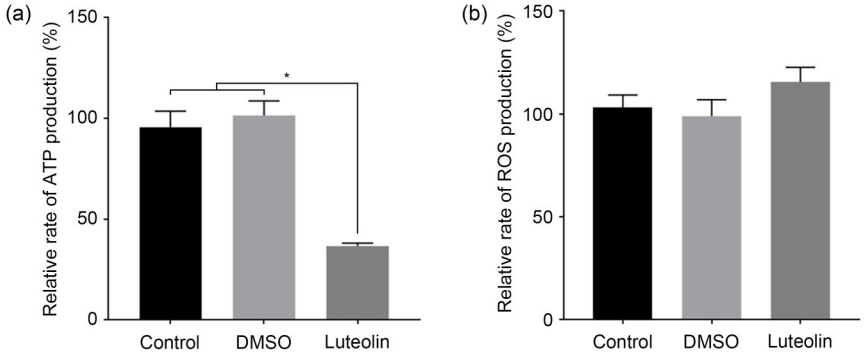

Fig. 5 Effects of luteolin exposure on the changes of adenosine triphosphate (ATP) and reactive oxygen species (ROS) production in oral carcinoma 3 (OC3) cells. (a) Measurements of ATP production. (b) Relative levels of ROS production. All values are presented as mean±standard deviation (SD), n=3. * P<0.05. DMSO: dimethyl sulfoxide.

Data availability statement

All data generated or analyzed during this study are included in this paper and its supplementary information files.

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Author contributions

All authors contributed to the study's conception and design. Pengfei GAO and Wentao ZHANG designed the experiment and drafted the manuscript. Yujie LIN, Ruijie LU, and Zijian LOU performed the study. Gang LU analyzed data. Ruolang PAN and Yunfang CHEN designed the experiment, revised the manuscript, and monitored the project progression. All authors have read and approved the final manuscript, and therefore, have full access to all the data in the study and took responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Pengfei GAO, Wentao ZHANG, Yujie LIN, Ruijie LU, Zijian LOU, Gang LU, Ruolang PAN, and Yunfang CHEN declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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Supplementary information

Materials and methods