Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2025.320

RESEARCH ARTICLE

C6 Ceramide Induces Apoptosis of Canine Oral Melanoma by Attenuating the Wnt/β-catenin Signaling

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ARTICLE HISTORY (25-809)

Received: August 16, 2025
Revised: November 20, 2025
Accepted: November 22, 2025
Published online: December 15, 2025

Key words:
Apoptosis
C6 ceramide
Canine oral melanoma
Mechanism
Wnt/β-catenin signaling

ABSTRACT

Melanoma is prevalent in canine oral tumors. C6 ceramide exerts powerful antitumor activity in multiple types of cancer in various ways. In this study, we investigated the functions of C6 ceramide in canine melanoma cells and xenograft models and elucidated its mechanism. The results showed that C6 ceramide significantly inhibits canine oral melanoma cell growth, migration, and invasion, as well as in xenograft models. Moreover, significant alterations were observed in cell cycle distribution and apoptosis. Subsequently, upregulations of caspase 8 and 3 were found, indicating that C6 ceramide activated the extrinsic apoptotic pathway. Finally, the expressions of p-GSK3βSer9 and β-Catenin were significantly reduced following C6 ceramide treatment, while no changes were observed in GSK3β. In conclusion, C6 ceramide exhibits remarkable antitumor activity by triggering the extrinsic apoptotic pathway and inactivating Wnt/β-catenin signaling in canine oral melanoma.

To Cite This Article: Diao H, Chen S, Tao Q, Zhang Y, Huang X, Hu Y, Chen JL and Yan S, 2025. C6 ceramide induces apoptosis of canine oral melanoma by attenuating the wnt/β-catenin signaling. Pak Vet J. http://dx.doi.org/10.29261/pakvetj/2025.320

INTRODUCTION

Oral melanoma exhibits markedly aggressive biological behavior in veterinary clinics. Due to a high propensity for metastasis to regional lymph nodes, lungs, and other organs (Kawabe *et al*., 2015; Raleigh *et al*., 2022; Smedley *et al*., 2022), oral melanoma poses a serious threat to the life and health of affected dogs. Wide-margin surgical resection remains as primary approach, but it can be used for metastatic patients. In canine oral tumors, malignant melanoma has the shortest median cause-specific survival (206 days) and the highest distant metastasis rate (30%) (Sarowitz *et al*., 2017). Radiation therapy provides an alternative option for controlling local tumors, either as a standalone treatment, adjuvant treatment, or palliative treatment, with median survival times verified from 80 to 758 days. Notably, approximately 30% to 40% of melanoma cases are amelanotic, which complicates diagnosis as they tend to mimic other oral tumors and are associated with a poorer prognosis (Smedley *et al*., 2022; Reynolds and Bell, 2025). Without treatment, the median survival time for canine oral melanoma is approximately 2 months (Kim *et al*., 2021). Additionally, the human and canine genomes share 85% homology (Chibuk *et al*., 2021) and are exposed to similar environments, making naturally occurring canine melanoma a valuable model for studying human melanomas (Hernandez *et al*., 2018). Therefore, exploring treatments for canine oral melanoma holds significance for both veterinary clinics and human medicine.

Ceramides, as a key metabolite of sphingolipids, function as a second messenger regulating cancer cell growth and therapeutic resistance, and are dubbed a "tumor suppressor lipid" due to their potent mediation of various signaling events, such as aopotosis, necroptosis, cell cycle arrest, angiogenesis, and autophagy (Hannun and Obeid, 2017; Ogretmen, 2018; Ung *et al*., 2022). C6 ceramide, a short-chain fatty acid, is widely used because of its high membrane permeability (Fiorani *et al*., 2023). A variety of evidence confirmed that C6 ceramide holds great potential as a tumor suppressor due to its ability to promote cell death through multiple mechanisms. Liu et al. demonstrate that C6 ceramide targets EGR3 to modulate JAK1/STAT3 signaling to inhibit the growth and metastasis of canine mammary tumors (Liu *et al*., 2024). Additionally, nanoliposome-

encapsulated C6 ceramide can reduce immunosuppression and overcome hepatocellular cancer-induced immune tolerance by boosting tumor-infiltrating $CD8^+$ T cells (Li *et al*., 2018; Qi *et al*., 2022), and no dose-limiting toxicities or hepatic metabolic toxicity are observed when treating cancer metastasis patients with ceramide nanoLiposomes (Ciner *et al*., 2023). These findings demonstrate that C6 ceramide holds promise as a novel therapeutic agent with few toxicities in cancer treatment. Nevertheless, its effects on canine oral melanoma remain unclear.

The Wnt/β-catenin signaling is conserved across species, which has been confirmed to participate in cancer progression in various ways, including tumorigenesis, chemotherapy resistance, metastasis, and modulation of the immune microenvironment (Zhu *et al*., 2022; Wu *et al*., 2025; Yang *et al*., 2025). Interestingly, ceramide and its synthase (CERS/LASS) have regulatory effects on the Wnt/β-catenin signaling. For instance, studies have demonstrated that the increase of ceramide reduces nuclear β-catenin levels, thereby inactivating Wnt signaling, resulting in suppressing colon cancer development and enhancing chemosensitivity of cancer cells to cisplatin (Kansal *et al*., 2014; Shi *et al*., 2024). Additionally, overexpression of LASS2 prevents the dephosphorylation of β-catenin by dissociating PP2A from β-catenin, with an increase of cytosolic β-catenin phosphorylation, thus attenuating Wnt/β-catenin signaling (Zhang *et al*., 2021). Conversely, silencing CerS6 contributes to the invasion and glycolysis of melanoma cells by upregulating GLUT1 expression, which in turn downregulates WNT5A expression (Tang *et al*., 2016). Based on the above evidence, we hypothesize that C6 ceramide may modulate canine tumorigenesis via the Wnt/β-catenin signaling.

Here, we investigated the effects of C6 ceramide on canine oral melanoma and explored the mechanisms through Wnt/β-catenin signaling. This study aims to provide a new potential therapeutic option for the treatment of canine oral melanoma, even supporting theoretical data for comparative medicine.

MATERIALS AND METHODS

Cell lines: Canine oral melanoma cells were provided by Dr. Douglas Thamm and characterized in a previous study (Fowles *et al*., 2016).

Cell viability assay: Cells were seeded in 96-well plates and incubated with various concentrations of C6 ceramide (USA and Cayman Chemical, MI, USA) for 48hours. Each well was added with Cell Counting Kit-8 (10μL/well) (Beyotime, Shanghai, China) and incubated for 1 hour. The optical density was obtained at 450nm. Each experiment was performed in five biological replicates.

Colony formation assay: Cells were seeded in 6-well plates and incubated with 15μM C6 ceramide for 48 hours, then the cells were further cultured in complete DMEM medium for 8 to 10 days. The cells that had successfully attached were stained with crystal violet and photographed to document the results. Each experiment was performed in triplicate biological replicates.

Apoptosis by Hoechst-PI staining: Cells were seeded in 6-well plates and incubated with 15μM C6 ceramide for 48 hours, then cells were stained with an Apoptosis and Necrosis Assay Kit (C0003, Beyotime, Shanghai, China) and observed by fluorescence microscopy (Olympus Corporation, Japan). Each experiment was performed in triplicate biological replicates.

Flow Cytometry: Flow cytometry was applied for detection to further confirm the occurrence of apoptosis. Cells were treated similarly to the Hoechst-PI staining, then an annexin V-FITC/PI kit (40302ES50, Yeasen BioTechnologies Co., Ltd., Shanghai, China) was used following the manufacturer's protocol. For cell cycle investigation, cells were processed similarly to the apoptosis assay. After ice-cold PBS washed, cells were fixed and stained with 50μg/mL PI (C0080, Solarbio, China) for 15 minutes in the dark. Cell apoptosis and cycle distribution were determined using a NovoCyte Fluidics Station S (Agilent Technologies Inc., CA, USA). Each experiment was performed in triplicate biological replicates.

Cell migration assay: Cells were seeded in 6-well plates. A uniform wound was created by a 200μL pipette tip, and then gently washed the wells twice to remove any dislodged cell debris. Cell migration was monitored and quantified by ImageJ software. Each experiment was performed in triplicate biological replicates.

Invasion assay: Cells were seeded in the upper chamber, which was pre-coated with Matrigel (BD Biosciences, CA, USA), in 100μL of DMEM with or without 15μM C6 ceramide, while 500μL of complete DMEM medium was in the lower chamber. After 48-hour incubation, invading cells were fixed, stained, and photographed. Invading cells were subsequently quantified by ImageJ software. Each experiment was performed in triplicate biological replicates.

Western blotting: Cells were seeded in 6-well plates and incubated with 15μM C6 ceramide. After a 48-hour incubation period, cells were lysed with RIPA, and total protein (20μg) was separated by SDS-PAGE. The primary antibodies Phospho-GSK3β (Ser9) (F0299, Selleckchem, Houston, TX, USA, 1:1000), GSK-3β (F0142, Selleckchem, Houston, TX, USA, 1:1000), β-Catenin (F0487, Selleckchem, Houston, TX, USA, 1:1000), Caspase 3 (F1080, Selleckchem, Houston, TX, USA, 1:1000), Caspase 8 (bs-0052R, Beijing Biosynthesis Biotechnology Co. Ltd., China,1:1000), Bcl-2 (F0125, Selleckchem, Houston, TX, USA, 1:1000), BAX (50599-2-Ig, Proteintech, China, 1:1000), β-tubulin (AF2835, Beyotime Institute of Biotechnology, China, 1:2000), and GAPDH (GB15004-100, Servicebio, China,1:3000) were incubated overnight. HRP-conjugated anti-rabbit/mouse (SA00001-2, SA00001-1, Proteintech, China, 1:2000) was incubated for 1 hour. Images were captured, and then the grey of the protein was analyzed with ImageJ software.

Tumorigenicity assay in mice: Tumor models were effectively established in BALB/c nude mice by implanting 5×10^6 Jones cells (N=3). Measure the tumor's length and width, body weights of the mice, every third day until day 15. When the tumor volume reached 50mm³, 30mg/kg C6 ceramide was administered for 15 days, and the same volume of PBS was used as a control. After the study, the

xenograft tumor tissues were collected for histopathology and immunohistochemical analysis of Ki-67 (27309-1-AP, Proteintech, China, 1:1400).

Statistical Analysis: Data were presented as the mean ± standard deviation (SD). A Student's t-test and One-way ANOVA followed by Tukey's post hoc test were employed for statistical comparisons using GraphPad Prism 8. Significance was set at *P<0.05, **P< 0.01, and ***P<0.001.

RESULTS

C6 ceramide inhibits the proliferation of canine melanoma cells by causing cell cycle arrest in the S phase: Treatment with C6 ceramide led to a dose-dependent reduction in the survival of canine melanoma cells (Fig. 1A). The IC50 values for C6 ceramide after 48hours of treatment in the Jones and Parks cell lines were 10.56μM and 12.21μM, respectively. In subsequent experiments in vitro, we treated Jones and Parks cells with 10μM and 12μM. Colony formation assay showed that C6 ceramide exerted long-term effects on cell proliferation (Fig. 1B). Next, flow cytometry was used to verify whether the proliferation inhibition of C6 ceramide was correlated with cell cycle distribution. As depicted in Fig. 1C and 1D, treatment with C6 ceramide increased the proportion of cells in the S phase, which was statistically significant in the Jones cells. These findings indicate that C6 ceramide exerts a proliferation suppression in canine melanoma cells by arresting cells at the S phase.

C6 ceramide inhibits canine melanoma cells migration and invasion: The wound healing assay revealed that C6 ceramide treatment led to a greater inhibitory effect in the treated groups than the control groups, with open wound area percentages of 23.29% vs 71.43% in Jones cells, 3.77% vs 40.97% in Parks cells, respectively (Fig. 2A, B). In the transwell assay, fewer invaded cells were found in the C6 ceramide-treated groups than in the non-treated groups; notably, this reduction reached statistical significance in Parks cells (Fig. 2C). These findings indicate that C6 ceramide effectively inhibits the migrative and invasive capacity of canine melanoma cells, a process essential for cancer metastasis.

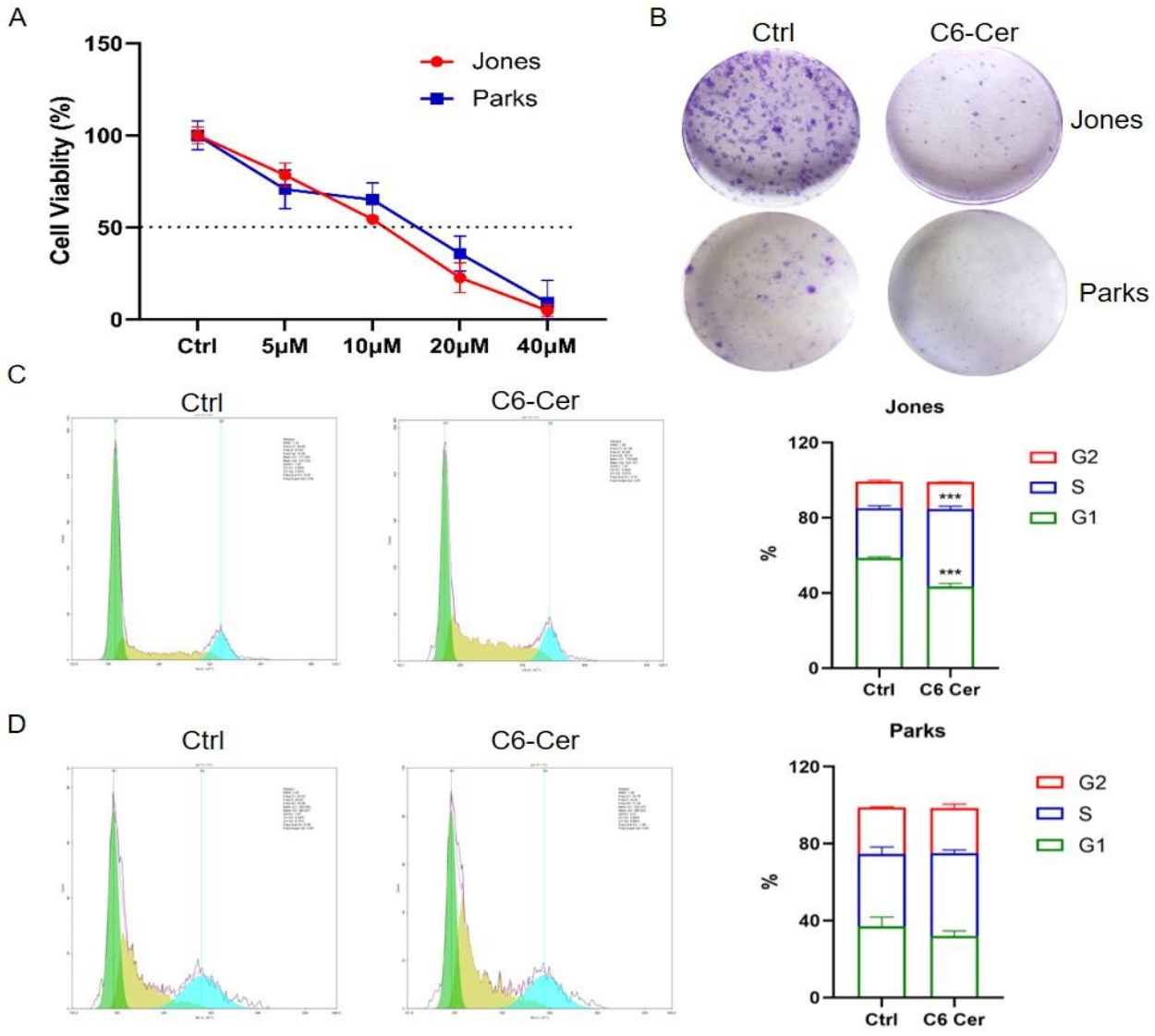

Fig. 1: C6 ceramide inhibited canine melanoma cell growth by regulating the cell cycle. (A) Cell viability was assessed using the CCK-8 assay after exposure to C6 ceramide. (B) Colony formation assays were performed on Jones and Parks cells treated with C6 ceramide. (C) and (D) Cell cycle distribution was assessed in Jones and Parks cells treated with C6 ceramide using flow cytometry. The data were analyzed from triplicate biological replicates and presented as the mean±standard deviation (SD). *P<0.05, **P<0.01, ***P<0.001.

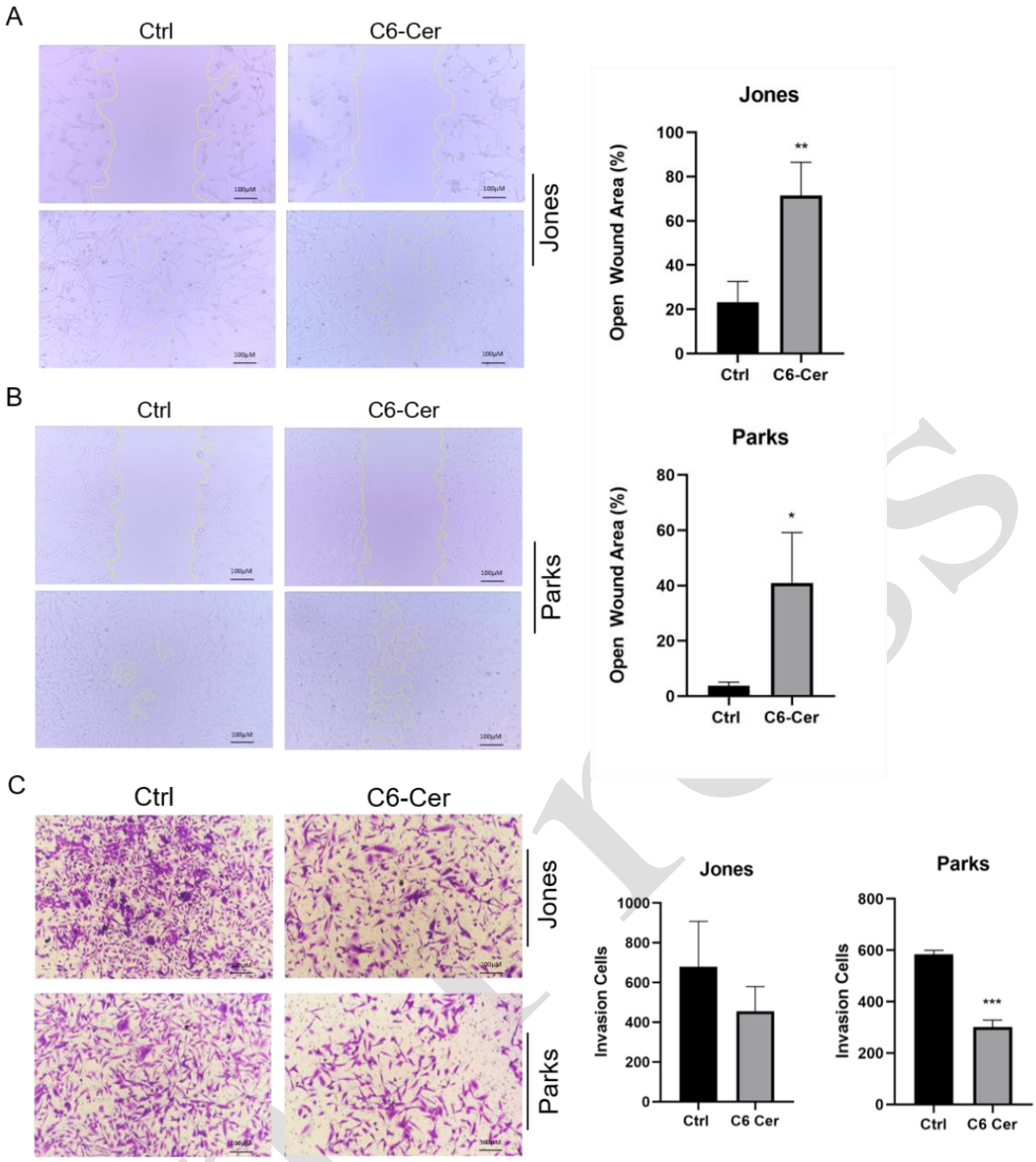

Fig. 2: C6 ceramide inhibited the migration and invasion of canine melanoma cells. (A) and (B) The migratory ability of Jones and Parks cells was evaluated at 48 hours post-treatment with C6 ceramide. Magnification×10, scale bar=100μm. The migration rate was quantified using Image J software. (C) Invading cells were stained with 0.1% (w/v) crystal violet at 48 hours post-treatment with C6 ceramide. Magnification×10, scale bar=100μm. The number of invading cells was quantified using Image J software. The data were analyzed from triplicate biological replicates and presented as the mean±SD. *P<0.05, **P<0.01, ***P<0.001.

C6 ceramide induces apoptosis in canine melanoma cells: To further verify the antitumor mechanisms of C6 ceramide was associated with apoptosis, we conducted Hoechst/PI and annexin V-FITC/PI staining. As depicted in Fig. 3A and B, the cell nuclei appeared with dense or fragmented staining following C6 ceramide treatment (yellow arrow). Subsequently, the flow cytometry also confirmed that the apoptotic percentage of cells was much higher in both C6 ceramide treatment groups (Fig. 3C, D). To identify the pro-apoptotic mechanism, we directly investigated apoptosis-related proteins. As depicted in Fig. 4, C6 ceramide treatment significantly altered the expression of caspase 3 and 8 in two cell lines. Nevertheless, in neither cell line were significant differences detected in the expression of BAX and Bcl-2 when comparing the C6 ceramide-treated and control groups (Fig. 4E, F, I, and K). Collectively, these findings suggest induction of apoptosis primarily via the extrinsic pathway.

***In Vivo* suppression of Jones xenograft tumor growth by C6 Ceramide**: To investigate the in vivo impact of C6 ceramide on Jones cell proliferation, a xenograft tumor model was established by implanting Jones cells into BALB/c nude mice. Following 15 days of treatment, xenograft tumors were excised (Fig. 5A, B), and significant reductions in tumor volume and weight were observed in the

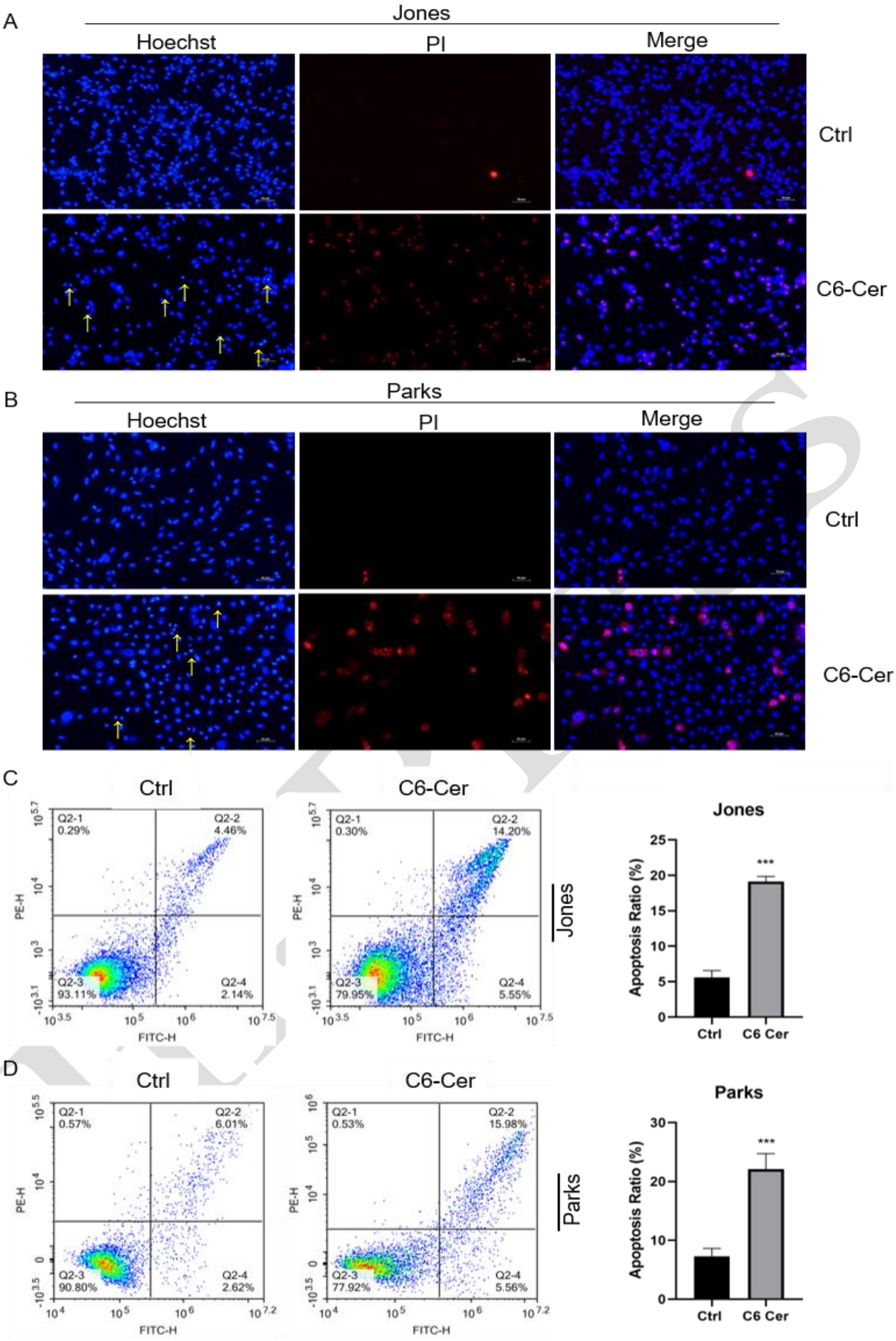

Fig. 3: C6 ceramide induced apoptosis in canine melanoma cells. (A) and (B) Hoechst/PI staining was performed in Jones and Parks cells 48 hours post-treatment with C6 ceramide. Magnification×20, scale bar=50μm. (C) and (D) Annexin V-FITC/PI staining was performed in Jones and Parks cells 48 hours post-treatment with C6 ceramide, and apoptosis was analyzed using flow cytometry. The data were analyzed from triplicate biological replicates and presented as the mean±SD. *P<0.05, **P<0.01, ***P<0.001.

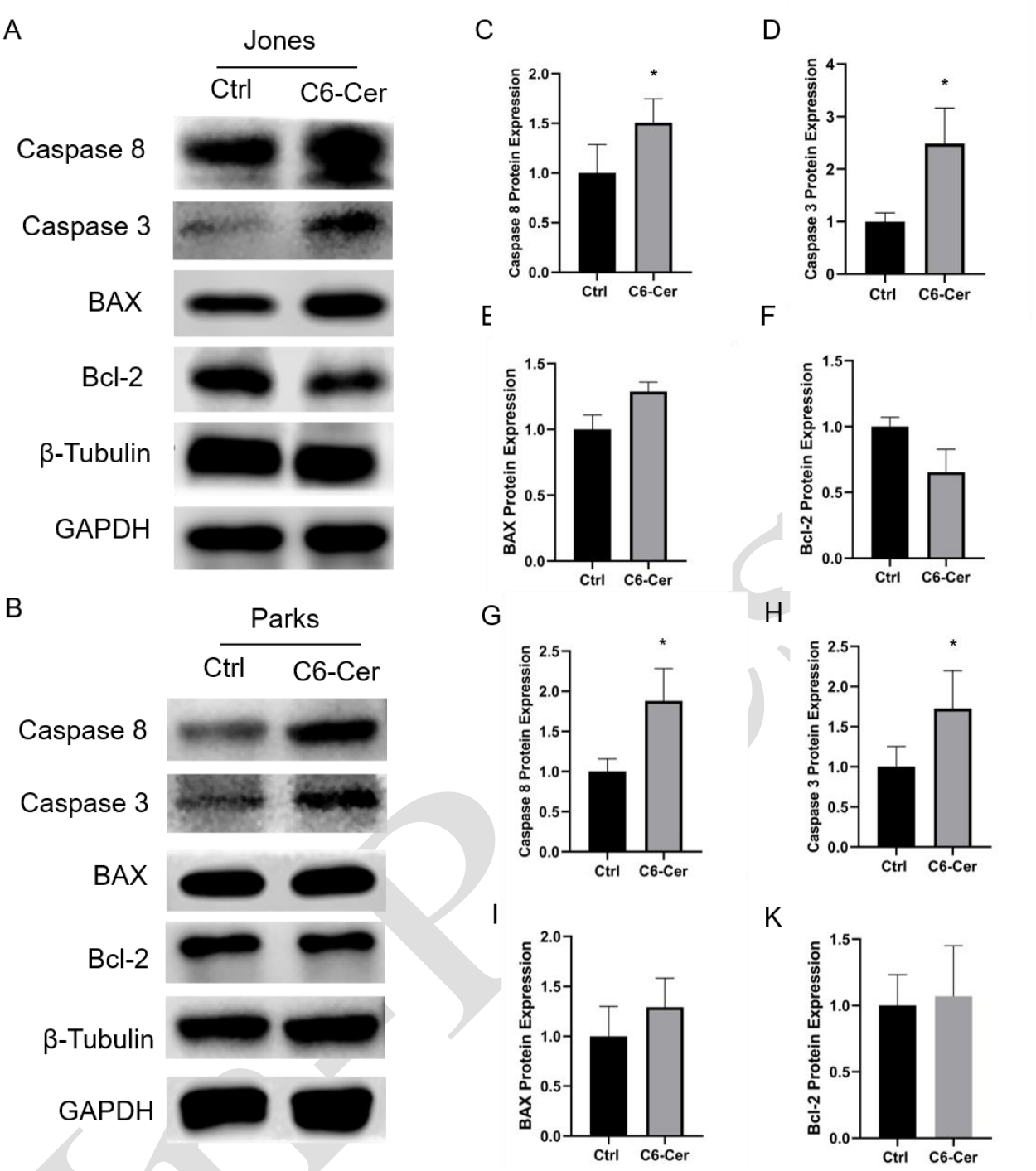

Fig. 4: The effect of C6 ceramide on the expression of caspase 8, caspase 3, BAX, and Bcl-2 in canine melanoma cells. Western blotting was performed to examine the expression of caspase 8, caspase 3, BAX, and Bcl-2 in Jones cells (A) and Park cells (B) after exposure to C6 ceramide. Densitometric analysis of caspase 8, caspase 3, BAX, and Bcl-2 levels in Jones cells (C-F) and Parks cells (G-K). The data were analyzed from triplicate biological replicates and presented as the mean±SD. *P<0.05, **P<0.01, ***P<0.001.

C6 ceramide (30 mg/kg) treatment group (Fig. 5C and D). Except on the 12th day, the ody weight of mice remained similar in the two groups (Fig. 5E). Pathological histological analysis of Jones cell-derived tumor masses revealed abundant poorly differentiated cells with marked atypia (Fig. 5F). Additionally, mitotic figures were observed (indicated by the yellow arrow). Immunohistochemical analysis of the proliferation marker Ki67 (cells that were stained in brown are positive, while those in blue are negative) in tumor tissues revealed a significant intergroup difference (Fig. 5G). These findings reinforce the effects of C6 ceramide in suppressing Jones xenograft tumor growth.

C6 ceramide attenuated the Wnt/β-Catenin Signaling: The activation of Wnt/β-catenin signaling is positively correlated with malignant progression and cancer-related mortality. Thus, western blotting was used to investigate the alterations of primary components of this signaling. As depicted in Fig. 6, the expressions of p-GSK3β^{Ser9} and β-Catenin were notably induced following C6 ceramide exposure, while the expression of GSK3β remained unchanged. These findings suggest that the anti-tumor effects of C6 ceramide on canine oral melanoma are associated with the attenuation of Wnt/β-catenin signaling.

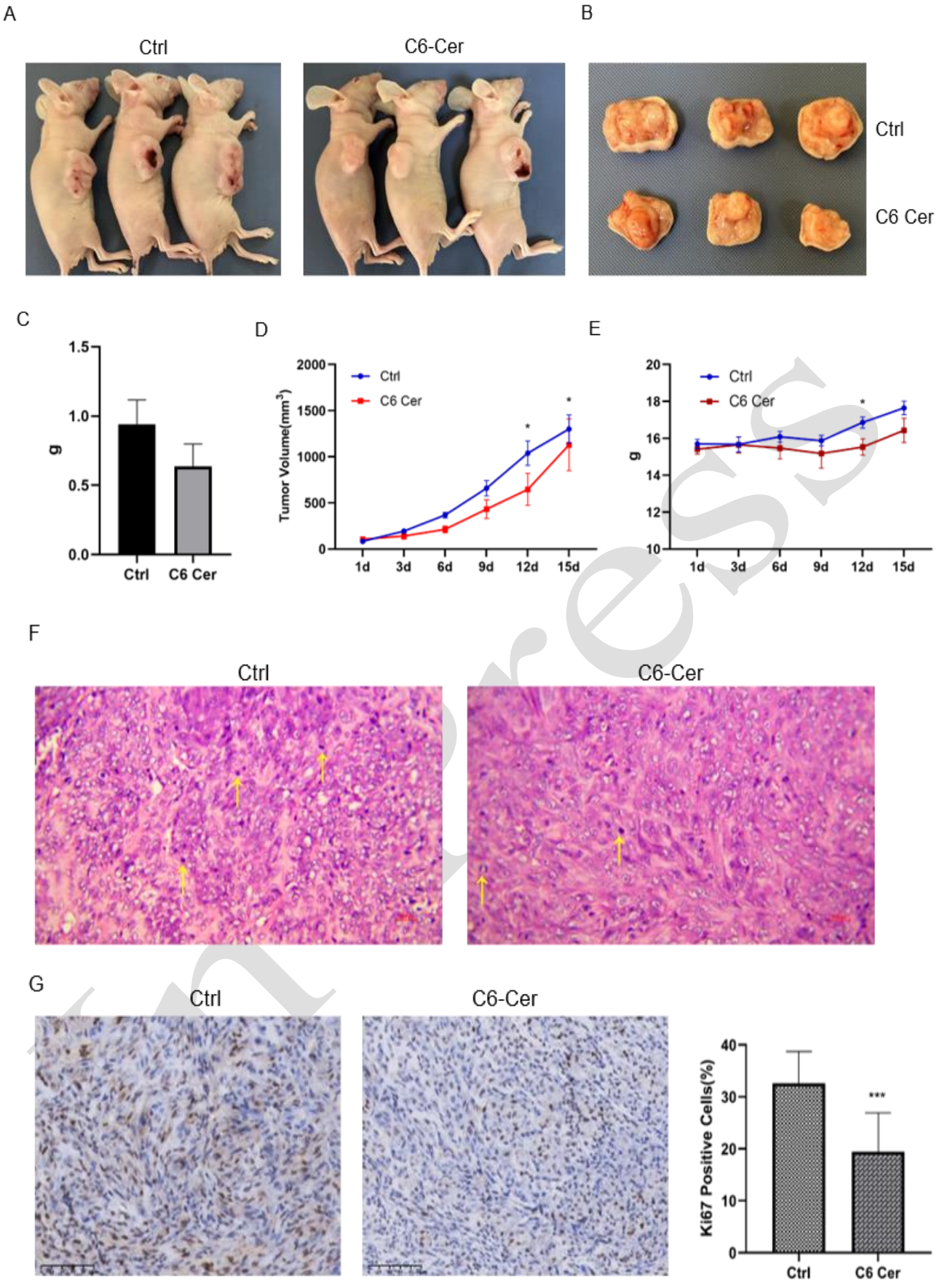

Fig. 5: C6 ceramide inhibited xenograft tumor growth. (A) Mice bearing subcutaneously transplanted tumors; (B) Photographs of tumors after the experiment; (C) Weight measurements of the subcutaneously transplanted tumors; (D) Tumor volumes; (E) Mice body weights. (F) Hematoxylin and eosin staining of the tumor masses. Magnification×10, scale bar=100μm; (G) Representative images of immunohistochemistry (IHC) staining for Ki67 expression, with a quantitative analysis of Ki67 staining. Magnification×40, scale bar=50μm. The data were analyzed from triplicate biological replicates and presented as the mean±SD. *P<0.05, **P<0.01, ***P<0.001.

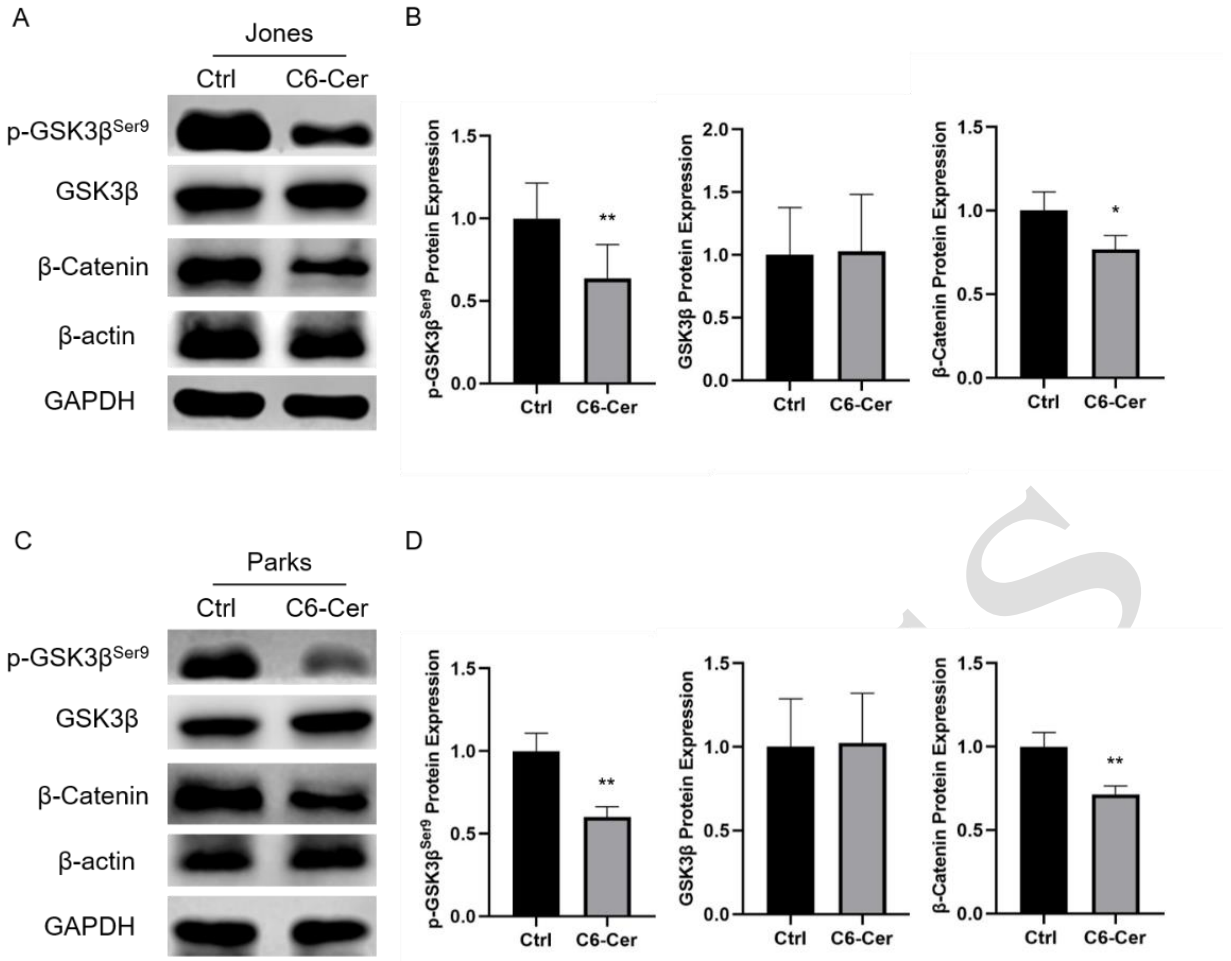

Fig. 6: The effect of C6 ceramide on the Wnt/β-catenin signaling. Western blotting examined the expression of p-GSK3β^{Ser9}, GSK3β, and β-Catenin in Jones cells (A) and Park cells (B) after exposure to C6 ceramide. Densitometric analysis of p-GSK3β^{Ser9}, GSK3β, and β-Catenin levels in Jones cells (C) and Parks cells (D). The data were analyzed from triplicate biological replicates and presented as the mean±SD. *P<0.05, **P<0.01, ***P<0.001.

DISCUSSION

Canine oral melanoma remains a challenge for veterinary clinicians due to its strong metastatic capacity and high mortality rate, urging the exploration of new treatment strategies. This study demonstrates that C6 ceramide effectively inhibits canine oral melanoma cell growth, which is associated with the regulation of the cell cycle distribution and the induction of apoptosis. Enhancing apoptosis has been a key objective in the development of improved antitumor agents (Dalseno *et al*., 2025; Wang *et al*., 2025), including chemotherapy, immunotherapy, and targeted therapies. Apoptosis is primarily categorized into the intrinsic pathway and the extrinsic pathway. In this study, induction of apoptosis by C6 ceramide in canine oral melanoma is probably through extrinsic pathways, as the upregulated of caspase-8 and -3 and no significant expression alterations of BAX and Bcl-2 (Fig. 4). Studies have confirmed that the binding of death ligands to their receptors activates initiator caspase-8, which subsequently cleaves and activates caspases-3 and -7, trigger cell death (Tummers and Green, 2017; Dalseno *et al*., 2025). Previous studies have shown that C6 ceramide triggers apoptosis by regulating caspases-3 and/or PARP in various cancers, such as pancreatic cancer, cutaneous T cell lymphoma, and hepatocellular cancer (Zhao *et al*., 2016; Wilhelm *et al*., 2021; Qi *et al*., 2022). Additionally, ceramide also triggers Bax-dependent apoptosis and commits cells to death (Jain *et al*., 2020). Therefore, ceramide has been regarded as a novel mechanism in apoptosis induction (Wang *et al*., 2025), which is valuable for systematic and further investigation.

Cancer cells negotiate multiple strategies to form metastases, including metastasis-promoting genes, driver mutations, cancer stem cells, phenotypic plasticity, and epithelial-mesenchymal transition, which eventually lead to death (Ganesh and Massague, 2021). The ability of cell migration and invasion is essential for cancer metastasis. Kazunori *et al*. demonstrate that C6-ceramide effectively inhibits the formation of lamellipodia, thereby decreasing the motility of anaplastic thyroid carcinoma cells (Fujiwara *et al*., 2020). Similarly, C6-ceramide within exosomes mediated miR-29b to further participate in endothelial cells' migration and angiogenesis by modulating the Akt signaling (Liu *et al*., 2020). In our study, a significant inhibition in the metastasis of canine melanoma cells was observed following C6 ceramide (Fig. 2). Further study is required to elucidate the mechanism of C6 ceramide in metastasis suppression.

The canonical Wnt/β-catenin signaling is a driver of a variety of cancers, as it is frequently maintained at an activated status because of the migration of β-catenin to the nucleus (Stamos and Weis, 2012; Valenta *et al*., 2012). The nuclear localization and phosphorylation of β-catenin are

closely regulated by glycogen synthase kinase 3β (GSK3β). Simply, the higher GSK3β phosphorylation, the lower β-catenin phosphorylation. The upregulation of phosphorylated GSK3β impairs the degradation of β-catenin, which causes excessive accumulation of β-catenin and transfer to the nucleus, and activates the Wnt signaling (Luan *et al*., 2020; Huang *et al*., 2023). Conversely, downregulated phosphorylation of GSK3β suppresses nuclear localization of β-catenin, inactivating Wnt signaling (Hu *et al*., 2024). In our study, treatment with C6-ceramide significantly downregulated the expression of $GSK3\beta^{Ser9}$ in canine oral melanoma cells, while GSK3β expression remained unchanged (Fig. 6). And the notable decreased expression of β-catenin further supports this finding. Collectively, these results indicate that C6-ceramide attenuates Wnt/β-catenin signaling activity by downregulating the expressions of $GSK3\beta^{Ser9}$ and β-catenin.

Further research should be conducted to explore the exact molecular mechanisms of how C6-ceramide-mediated activation of caspase 8 and 3 occurs. Additionally, the destruction complex that regulates β-catenin phosphorylation contains four components; it remains unclear whether the altered expression of β-catenin is associated with the other three components, such as APC, CK1α, and Axin. Finally, phosphorylation of the N-terminal Serine 9 residue and Tyrosine 216 residue of GSK3β has opposite effects on the regulation of β-catenin: $GSK3\beta^{Ser9}$ activates β-catenin, while $GSK3\beta^{Tyr216}$ inactivates β-catenin (Park *et al*., 2013; Xu *et al*., 2017). Here, we solely examined the expression of $GSK3\beta^{Ser9}$, adding the examination the $GSK3\beta^{Tyr216}$ will make this research more complete.

Conclusions: The present study firstly demonstrates that C6 ceramide effectively inhibits the proliferation, migration, and invasion of canine oral melanoma cells, as well as tumor growth in vivo, by inducing the extrinsic apoptotic pathway and inactivating the Wnt/β-catenin signaling. These findings suggest that C6 ceramide serves as a potential therapeutic candidate for canine oral melanoma.

Author Contributions: Hongxiu Diao conceived and designed the study. Shichao Chen and Qianting Tao executed the experiment. Yanqin Zhang, Xinzi Huang, and Yuqing Hu contributed to the data collection. Ji-Long Chen provided technical support and reviewed this manuscript. Shihong Yan supervised the entire project and provided critical revisions to the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by the National Natural Science Foundation of China (grant number 32302946), the Fujian Provincial Natural Science Foundation of China (grant number 2024J01384), and the Innovation Training Program for College Students of Fujian Province (grant number 202410389194).

Institutional Review Board Statement: All procedures involving animals were conducted with the approval of the Institutional Animal Care and Use Committee at Fujian Agriculture and Forestry University (Permit Number PZCASFAFU23006).

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in the study are included in the article; further inquiries can be directed to the corresponding authors.

Acknowledgments: We are grateful to Dr. Douglas Thamm at the Flint Animal Cancer Center (FACC) of Colorado State University for their gift of the canine oral melanoma cell lines.

Conflicts of Interest: The authors declare no conflicts of interest.

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