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Research Article

Matrine Intensifies the Sensitivity of Cisplatin in NPC Cells via mTOR-Mediated Autophagy

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Abstract

Background and Objective: Matrine plays a crucial role in suppressing tumour growth, while the contribution of matrine combining Cisplatin (DDP) to regulate Nasopharyngeal Carcinoma (NPC) remains unelaborated. Herein, This study demonstrated that matrine could attenuate NPC cells proliferation and accelerate its apoptosis. **Materials and Methods:** CCK-8 assay, colony formation assay, TUNEL assay and western blot were performed to examine the effect of matrine on the proliferation and apoptosis of NPC cells. Wound healing assay and transwell assay were applied to test the role of matrine on the migration of NPC cells. Furthermore, the activation of the mTOR signalling pathway by the combination of matrine and DDP were verified by the western blot and immunofluorescence staining. Student's t-test and Analysis of Variance (ANOVA) were used for the statistical tests. **Results:** To reveal the fundamental mechanism, we noticed that matrine caused the upregulation of apoptosis-related protein C-caspase-3, autophagy indicator LC3-II and the activation of the mTOR signalling pathway and meanwhile induced the down regulation of PCNA, survivin and p62, which could be further intensified by applying DDP. **Conclusion:** To summarize, our findings suggested that matrine could enhance the sensitivity of DDP against NPC through mTOR-mediated autophagy, which provided a novel route for the therapy of NPC.

Key words: Nasopharyngeal carcinoma, autophagy, cisplatin, matrine, mTOR signalling pathway, apoptosis, proliferation

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Nasopharyngeal Carcinoma (NPC) is a malignant tumour with a high incidence in the mucosal epithelium of the nasopharyngeal area¹, generally occurring on the top and sidewalls of the nasopharyngeal, especially the pharyngeal recess². Fundamentally, the occurrence and development of NPC are highly related to Epstein-Barr (EB) virus infection³, genetic⁴ and environmental factors⁵ and is distinctively characterized with regional and ethnic differences⁶. Clinically-pathologically, the lesions of NPC present nodular, ulcerative and submucosal infiltrates⁷, causing symptoms such as tinnitus, nasal congestion, headache and even nerve paralysis^{8,6}. Currently, the WHO recognized pathological classifications of NPC consist of keratinizing squamous cell carcinoma (type I), differentiated non-keratinizing carcinoma (type II) and undifferentiated non-keratinizing carcinoma (type III)⁹. In addition, radiotherapy, chemotherapy, surgical treatment, molecular targeted therapy, immunotherapy and traditional Chinese medicine treatment are extensively adopted to relieve NPC¹⁰. Although the above-mentioned therapeutic strategies have significantly increased the overall survival rate of NPC, the risk of recurrence and metastasis still contribute to the high NPC mortality rate and it is urgently required to find out effective diagnostic and therapeutic methods.

Matrine (molecular formula: C₁₅H₂₄N₂O)¹¹, belonging to alkaloids, is derived from the dried roots, plants and fruits of the legume *Sophora flavescens*¹² and also has been found in other plants such as *Sophora alopecuroides* and *Sophora subprostrata*^{13,14}. Clinically, matrine is functionally regarded as a diuretic¹⁵ and anti-pathogenic¹⁶ active ingredient. According to increasing research, a total of 46 types of traditional Chinese medicines had significantly contributed to inhibiting tumours or acted as chemo-sensitizers in affecting cancer cell metabolism¹⁷. Specifically, Toosendanin (TSN) has been proved to activate WWOX to inhibit tumour growth and metastasis via Wnt/ β -catenin signalling in hepatocellular carcinoma and Galangin could facilitate STAT3-induced ROS to hinder the tumour growth in gastric cancer¹⁸, in addition, Liu M and colleagues revealed the contribution of cucurbitacins to the therapy of lung cancer¹⁹. Recently, matrine derivatives have been reported to participate in NPC cell proliferation²⁰, whereas the contribution of matrine combining cisplatin to the autophagy in NPC remains uncovering.

Autophagy is a process of engulfing one's cytoplasmic proteins or organelles and enveloping them into vesicles,

followed by fusing with lysosomes to form autophagic lysosomes to degrade the contents of the lysosomes, thereby fulfilling the metabolic requirements of the cell itself and the renewal of certain organelles^{21,22}. According to the different pathways of cellular materials transported to the lysosome, autophagy is classified into large autophagy, small autophagy and chaperone-mediated autophagy²³. Notably, DDP has been proved to participate in affecting the onset and progression of autophagy²⁴. However, whether matrine mediates the autophagy process in NPC has not been demonstrated.

Given that the noticeable significance of matrine in diverse cancers progression, we aim to reveal the functional influence of matrine in NPC cells apoptosis and autophagy and to investigate the combined therapy impact of matrine and DDP on NPC. These insights may provide efficient cancer drugs and serve as a therapeutic target against NPC.

MATERIALS AND METHODS

Study area: The study was carried out at the Department of Otolaryngology, The First Affiliated Hospital of Jinan University from June, 2020-2021.

Cell culture: NPC cell lines HONE1 and SUNE1 were purchased from American Type Culture Collection (ATCC, USA) and were cultured in RPMI-1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS (Gibco, Inc., Grand Island, NY, USA). At logarithmic growth stage, HONE1 and SUNE1 cells were divided into following groups: Control (DMSO), matrine (0.25, 0.50 and 1.00 mg mL⁻¹), DPP (0.2 mg L⁻¹), combined group (DPP+1.00 mg mL⁻¹ matrine, DPP+0.50 mg mL⁻¹ matrine, DPP+0.25 mg mL⁻¹ matrine).

Cell counting kit-8 (CCK-8): To test cells viability, a CCK-8 assay was introduced. Following the testing methods¹¹, transfected HONE1 and SUNE1 cells were cultured into 96-well plates and administrated with different drugs for the indicated time, ranging from 12-72 hrs and the CCK-8 (Sigma-Aldrich, St. Louis, MO, USA) was supplemented at a specific time for another 1 hr incubation. A spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) was implemented for the measurement of OD value at 450 nm.

Colony formation assay: To estimate cells viability, HONE1 and SUNE1 cells were added into a 6-well plate for cultivation for 2 weeks. After then, cloned cells were, respectively carried

out fixation and stain introducing methanol and 0.1% crystal violet. The visualization and counts were fulfilled utilizing ImageJ software.

TUNEL: To evaluate cells apoptosis, a TdT-mediated dUTP nick-end labelling (TUNEL) apoptosis detection kit (Sigma-Aldrich, St. Louis, MO, USA) was implemented. In brief, HONE1 and SUNE1 cells were added in 4% paraformaldehyde, then the TUNEL solution was supplemented and incubated for 1 hr, while the luciferin-labelled dUTP solution was added into the control group, followed by adding DAPI and culturing in dark. The images were captured by using a fluorescence microscope (Mshot, Guangzhou, China).

Transwell: Transwell was introduced for the estimation of cells invasion. In brief, treated HONE1 and SUNE1 cells were suspended in solution without serum. then the prepared suspension was filled into the upper chamber with matrigel (BD Biosciences), while 20% FBS was added into the lower chamber. After 1 day of incubation, the invasive cells located in the bottom of the chamber were carried out fixation applying methanol and stain utilizing crystal violet. Finally, a microscope (Olympus, $\times 100$) was implemented for the visualization and measurement.

Wound healing: To conduct a wound healing assay, HONE1 and SUNE1 cells were plated in a 6-well plate and incubated for 16 hrs following indicated protocols. Then, a 10 μ L pipette tip was applied to create wounds, followed by adding serum-free DMEM and maintained for 1 day. With $\times 20$ magnification, a microscope was implemented to calculate the wound width and achieve the visualization.

Immunofluorescence staining: To examine LC3-II expression levels, pre-treated HONE1 and SUNE1 cells were mixed with primary antibody LC3-II (Abcam, ab192890, 1 μ g mL⁻¹) at 4°C overnight, followed by cultivating with secondary antibodies labelled by fluorescence for 30 min, then 50 μ L of DAPI was supplemented. A fluorescence inverted microscope was introduced for visualization.

Western blotting (WB): For western blotting analysis, total proteins were isolated from HONE1 and SUNE1 cells by RIPA lysis buffer then were separated utilizing SDS-PAGE, followed by transferring to PVDF membranes. About 5% skim milk was adopted to block these membranes and then were mixed with

primary antibodies provided by Abcam (UK) at 4°C overnight, these antibodies including LC3-II (ab192890, 1:2000), P62 (ab109012, 1:10000), p-mTOR (ab109268, 1:5000), mTOR (ab134903, 1:10000), C-caspase-3 (ab32042, 1:500), CASPASE3 (ab32351, 1:5000), PCNA (ab29, 1 μ g mL⁻¹) surviving (ab76424, 1:2000). Then were cultivated with horseradish peroxidase-labelled secondary antibody. A biomolecule ChemiDoc™ imaging system was introduced to detect the protein levels. GAPDH was regarded as the normalization.

Statistical analysis: In this study, all statistical analyses were finished implementing graphpad prism (version 5.01) and were exhibited as Mean $\pm$ Standard Deviation (SD). The differences were estimated by adopting the Student's t-test and analysis of variance (ANOVA). The $p < 0.05$ was regarded as statistical significance.

RESULTS

Matrine represses NPC cells proliferation and drives apoptosis: To determine the functional impact of matrine on NPC cells growth, we pre-treated NPC cell lines (HONE1 and SUNE1) with different concentrations of matrine (0, 0.25, 0.50 and 1.00 mg mL⁻¹). Compared with a control group, we noticed that matrine efficiently weakened the viability of the HONE1 and SUNE1 cells, following a concentration-dependent manner (Fig. 1a, b). Similarly, matrine in the concentration ranging from 0.25-1.00 mg mL⁻¹ induced a significant reduction in the counts of cloned cells (Fig. 1c, d). In addition, the TUNEL assay exhibited that matrine accelerated HONE1 and SUNE1 cells apoptosis rate in a dose-dependent manner (Fig. 1e-h). Taken together, these findings concluded that matrine could exert an anti-proliferation influence upon NPC cells and meanwhile facilitate its apoptosis.

Matrine amplifies the suppressive impact of DPP against NPC cells growth: Given that the inhibitory effect that matrine exerted on NPC cells proliferation, we further aim to investigate the combined influence of matrine and DPP. After co-treating HONE1 and SUNE1 cells with matrine and DPP, we found that matrine efficiently enhanced the inhibited effect of DPP against cells viability in a concentration-dependent manner (Fig. 2a, b). Consistent with CCK-8 results, colony formation assay clarified that the combination of matrine and DPP caused a less number of colonies, in comparison with that in single drug groups (Fig. 2c, d). Furthermore, DPP exerted

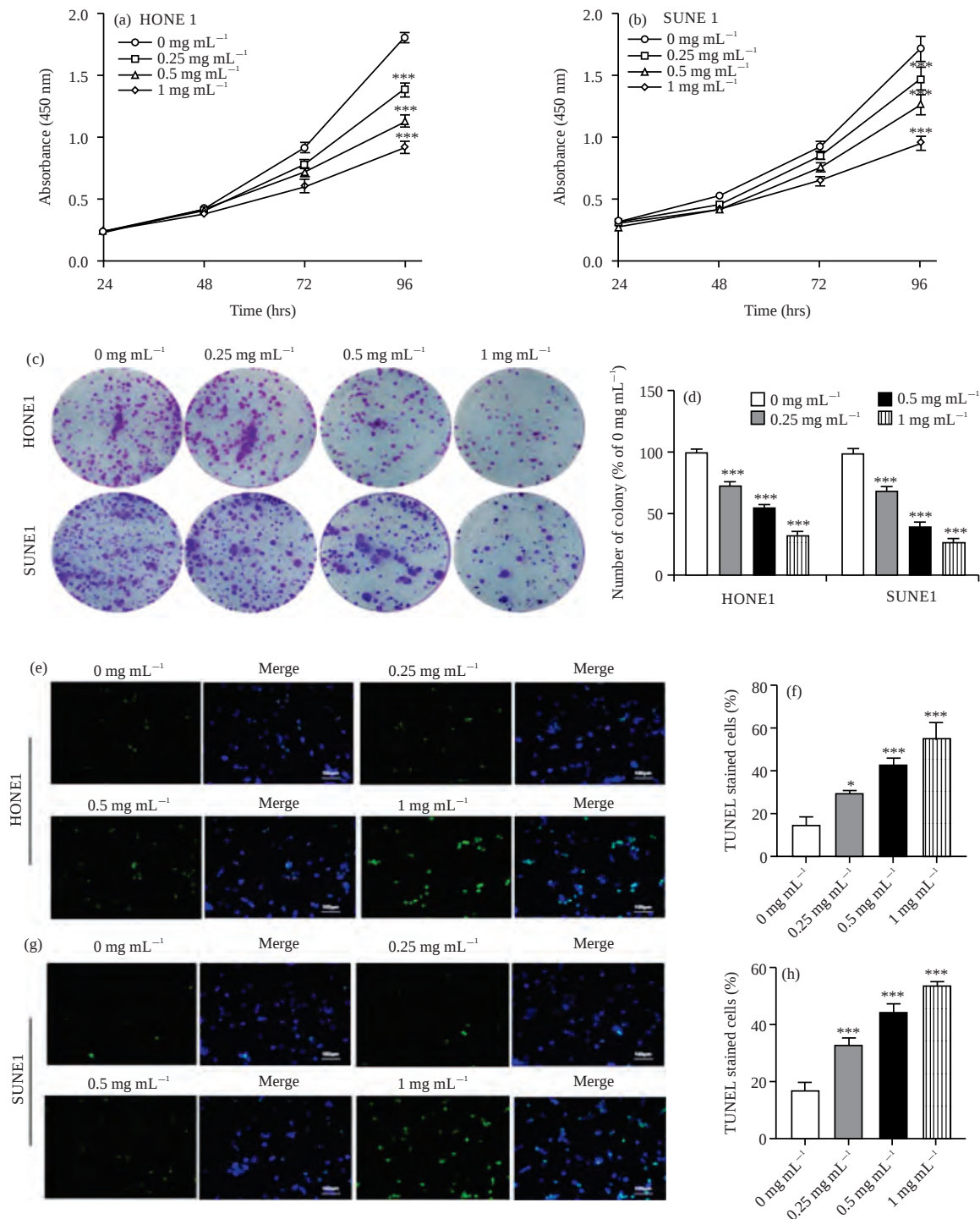


Fig. 1(a-h): Matrine hinders NPC cells proliferation and promotes apoptosis, NPC cells were treated with (a, b) Matrine, CCK-8, (c and d) Colony formation and (e-h) TUNEL assays were utilized to detect cells proliferation and apoptosis rate. Data are reported as Mean $\pm$ SD of 3 independent experiments, * $p < 0.05$ and *** $p < 0.001$.

promoted impact on HONE1 and SUNE1 cells apoptosis rate, which could be further strengthened by matrine (Fig. 2e-h). Mechanistically, the accelerated apoptosis rate was mainly

derived from the higher levels of pro-apoptosis protein C-caspase-3 as well as the lower levels of anti-apoptosis protein PCNA and growth factor survivin in matrine+DPP

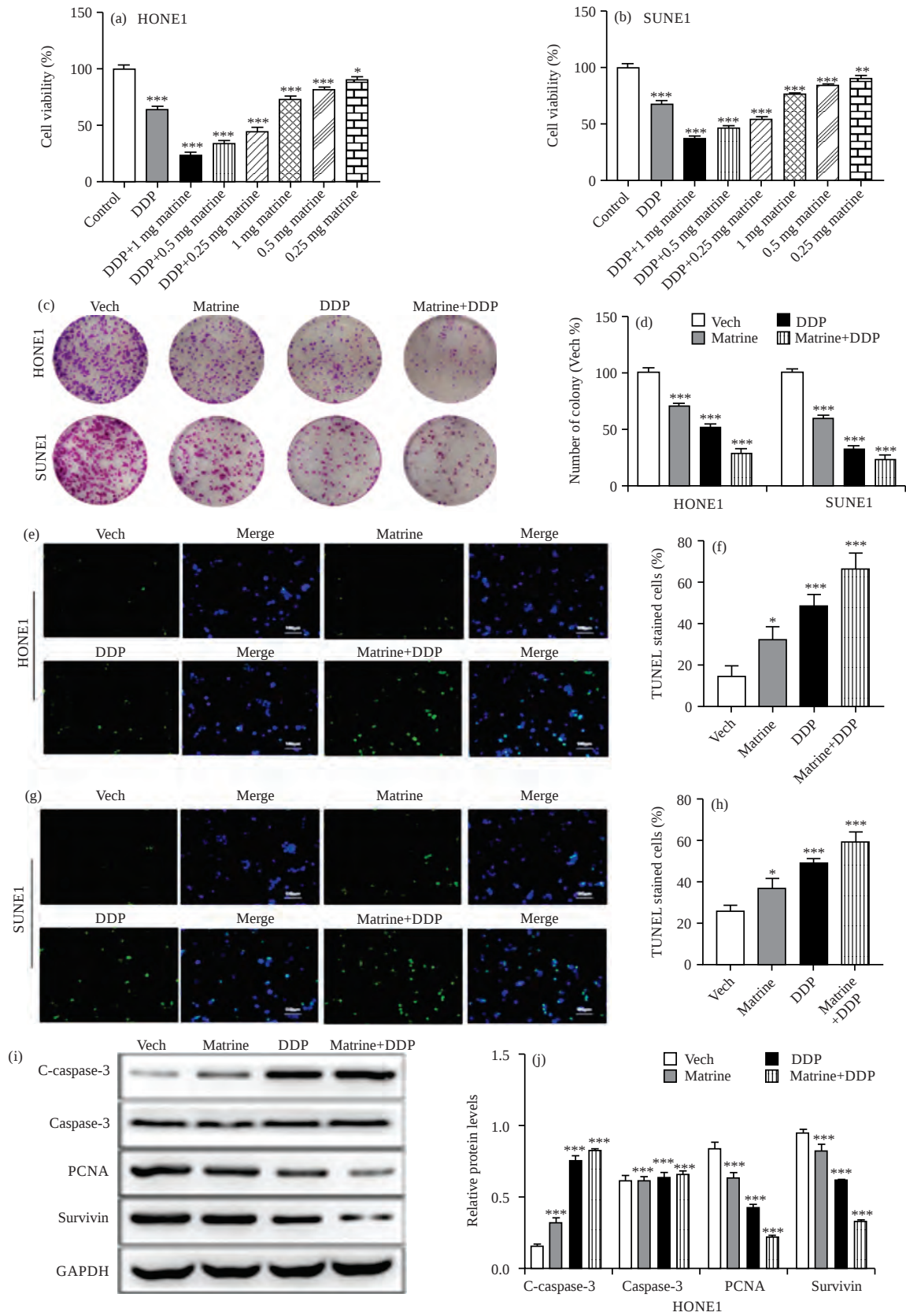


Fig. 2a-l: Continue

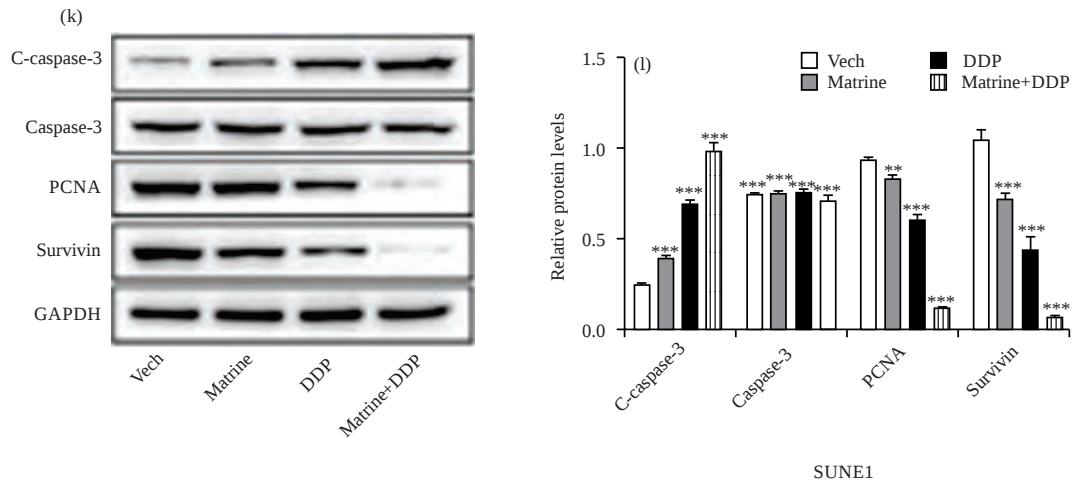


Fig. 2(a-l): Matrine enhances the repressive effect of DPP against NPC cells growth, NPC cells were co-treated with (a-b) Matrine (0.5 mg mL⁻¹) and DPP (0.2 mg L⁻¹), CCK-8 and (c-d) Colony formation and (e-h) TUNEL assays were adopted to examine cells proliferation and apoptosis rate. Western blotting was employed to evaluate proliferation and apoptosis-related factors levels (l-l)

Data are reported as Mean ± SD of 3 independent experiments, *p<0.05, **p<0.01 and ***p<0.001

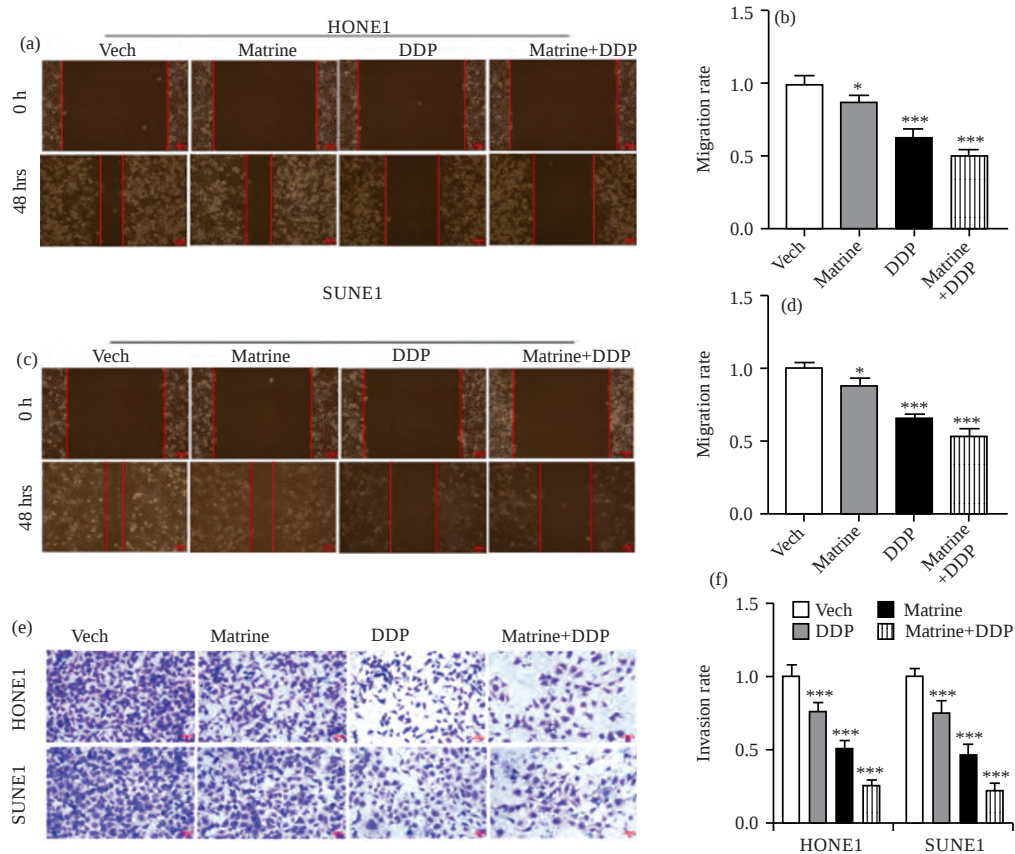


Fig. 3(a-f): Matrine strengthens the inhibitory impact of DPP against NPC cells migration and invasion, NPC cells were co-treated with (a-d) Matrine (0.5 mg mL⁻¹) and DPP (0.2 mg L⁻¹), wound healing and (e, f) Transwell assays were utilized to assess cells migratory and invasive capacities

Data are reported as Mean ± SD of 3 independent experiments, *p<0.05 and ***p<0.001

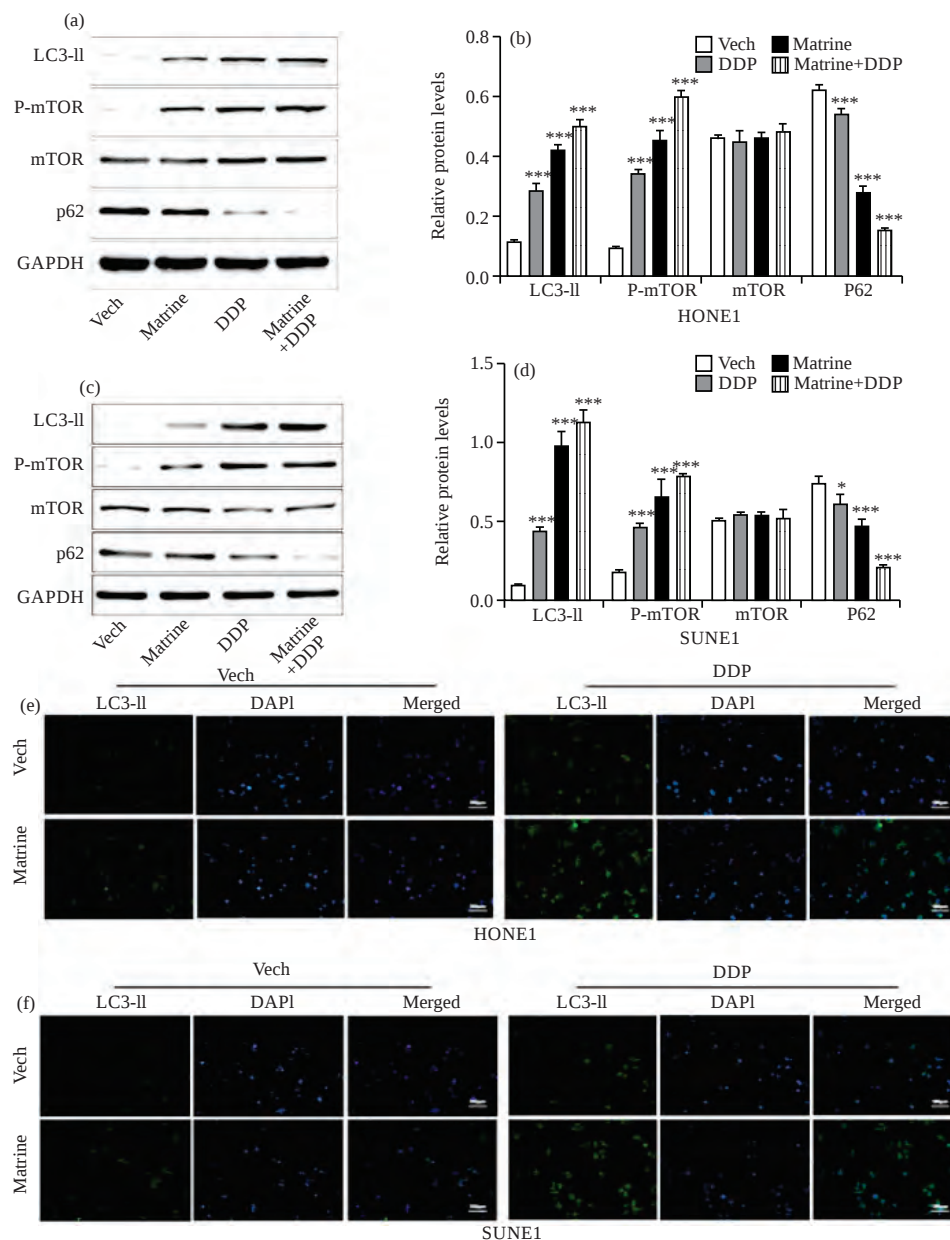


Fig.4(a-f): Combined matrine and DPP induce mTOR-modulated autophagy in NPC cells, NPC cells were co-treated with (a-d) Matrine (0.5 mg L⁻¹) and DPP (0.2 mg L⁻¹), Western blotting and (e and f) Immunofluorescence staining were, respectively used to detect the expression of autophagy-related signalling pathways and factors
Data are reported as Mean ± SD of 3 independent experiments, *p<0.05 and ***p<0.001

group, compared with that in cells treated with a single drug (Fig. 2i-l). Overall, our results highlighted that matrine could enhance the repressive effect of DPP against NPC cells proliferation.

Matrine strengthens the inhibitory impact of DPP against NPC cells migration and invasion: For an in-depth exploration of NPC cells on the migration and invasion after co-treating with matrine and DPP, the wound healing assay

was performed and demonstrated that matrine further shortened the wound width inhibited by DPP of HONE1 (Fig. 3a, b) and SUNE1 (Fig. 3c, d) cells in a dose-dependent manner. In addition, DPP caused a strikingly drop in the counts of invasive cells and which could be further amplified by matrine (Fig. 3e, f). In summary, these data manifested that matrine showed potential in enhancing the suppressive influence of DPP on NPC cells migratory and invasive capacities.

Combined matrine and DPP drive mTOR-mediated autophagy in NPC cells: Considering the significance of DPP in autophagy, we further wonder whether the combination of matrine and DPP regulates the autophagy progress in NPC cells. The western blotting assay exhibited the higher expression levels of LC3-II and P-mTOR and the lower levels of p62 in HONE1 and SUNE1 cells co-treated with matrine and DPP, compared with that in cells treated with a single drug (Fig. 4a-d), suggesting that combined matrine and DPP promotes the autophagy in NPC via activating the mTOR signalling pathway. In addition, immunofluorescence staining further validates the higher expression of autophagy-related marker LC3-II in the matrine+DPP group (Fig. 4e, f). These results indicated that DPP played a crucial role in facilitating autophagy progress in NPC cells, which could be enhanced by matrine.

DISCUSSION

In this study, the proliferation, migration and invasion inhibition of NPC cells elicited by DDP is sharpened by matrine. To verify the underlying mechanism, we examined the expression alteration of P62, LC3-II, m-TOR in HONE1 and SUNE1 cells and it indicated that DPP promotes the autophagy in NPC via activating the mTOR signalling pathway, which was intensified by the application of matrine.

According to increasing references, traditional Chinese medicine has been extensively applied in extenuating multiple cancers, like Kaempferol in prostate cancer²⁵, Sinomenine in hepatic carcinoma²⁶ and astragaloside in gastric cancer²⁷. In our study, we revealed the inhibitory effect of matrine upon NPC cells proliferation, migration and invasion and the promoted impact on apoptosis rate and autophagy progress. Moreover, DPP exerted a similar influence on NPC progression as same as matrine and which could be enhanced by combining with matrine.

Matrine has been proved to efficiently hinder diverse cancers development. For example, Dai *et al.*²⁸ validated that alkaloid matrine restrained cells migration, invasion and EMT under hypoxia in hepatocellular carcinoma through activating miR-199a-5p. Zhu *et al.*²⁹ uncovered that matrine could exert a repressive effect on non-small cell lung cancer cells proliferation, migration and promoted impact on cells apoptosis via circFUT8/miR-944/YES1 axis. In addition, Liang and Ju³⁰ suggested that matrine significantly restrained cells proliferation and facilitated apoptosis rate through mediating the p38MAPK/ERK/JNK signalling pathway. Consistent with previous research²⁸⁻³⁰, we confirmed matrine could restrain

cells proliferation, migration and invasion and accelerate apoptosis and autophagy rate in a dose-dependent manner, which could be mechanistically embodied in the upregulation of C-caspase-3, autophagy indicators LC3-II and the mTOR signalling pathway, whereas the downregulation of PCNA, survivin and p62 levels. Among them, C-caspase-3, PCNA and survivin were acknowledged as the apoptosis and proliferation-related indicators, which implied the cells proliferation and apoptosis rate. In addition, mTOR has been verified as a signalling pathway mediating autophagy³¹. In our study, the application of matrine activated the expression levels of mTOR signalling and meantime upregulated autophagy indicators LC3-II, meaning that the promoting effect of matrine on NPC cells autophagy.

The therapeutic contribution of cisplatin to cancers progression has been proved based on accumulating investigations. For instance, Montanino *et al.*³², revealed the combined effect of etoposide and cisplatin in inhibiting small cell lung cancer progression and Dai *et al.*³³ suggested that mortaline/glucose-mediated protein facilitated the sensitivity of cisplatin against gastric cancer through modulating metabolic reprogramming. Herein, we first confirmed the inhibitory influence of matrine on NPC cells progression and autophagy and then determined that matrine could intensify the impairment on NPC cells proliferation, migration and invasion caused by DPP, which further provided rational evidence to consider the combination of DPP and matrine as a potential chemotherapy method into NPC treatment.

CONCLUSION

To summarize, we highlighted that matrine could efficiently inhibit NPC cells proliferation, migration and invasion in a dose-dependent manner and meanwhile facilitate its apoptosis and autophagy rate. Notably, matrine amplified the chemotherapeutic efficiency of DPP against NPC, which could be regarded as promising and efficient candidates against NPC progression.

SIGNIFICANCE STATEMENT

This study discovered the combined effect of matrine and DPP against NPC cells malignant behaviours that can be beneficial for the therapy of NPC. This study will help the researchers to uncover the critical areas of introducing the traditional Chinese medicines to enhance the therapeutic effect of DPP against NPC that many researchers were not able to explore. Thus a new theory on ameliorating NPC may be arrived at.

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