Original Research

Buformin increases radiosensitivity in cervical cancer cells via cell-cycle arrest and delayed DNA-damage repair

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Impact statement

Our idea originated in the thought of discovering new effects of old drugs. Although this study is a basic research, it is very close to clinical treatment. Flow cytometry and immunofluorescence were used to verify that buformin increases radiosensitivity. We aimed to address one of the thorniest problems in treatment process. Based on discovering new effects of old drugs, it is feasible to use buformin as an anticancer drug in clinical application. This will provide new ideas for clinical treatment.

Abstract

Buformin is a commonly used hypoglycemic agent, and numerous studies have shown that buformin has potent antitumor effects in several malignancies. In this study, we aimed to assess the cytotoxic effect of buformin combined with ionizing radiation (IR) on two human cervical cancer cell lines (Hela and Siha). Cytotoxicity was detected by colony formation assays; impacts on the cell cycle and apoptosis were detected by flow cytometric analyses. Changes in histone H2AX (γ-H_2AX) phosphorylation and impacts on the AMPK/S6 pathway were also explored. Our data show that the combination of buformin and IR had a much stronger antiproliferative effect and resulted in more apoptosis than did buformin or IR alone. Combination treatment with a low dose of buformin (10 μM) and IR (4 Gy) caused G2/M-phase cell cycle arrest. Consistent with these findings, Western blotting showed that the combination of buformin and IR activated AMPK and suppressed S6. In addition, delayed disappearance of γ-H2AX was detected by immunofluorescence in cervical cancer cells treated with buformin plus IR. Taken together, the data indicate that the combination of a low concentration of buformin and IR increases the radiosensitivity of cervical cancer cells via cell cycle arrest and inhibition of DNA repair. Based on these results, we strongly support the use of buformin as an effective agent for improving IR treatment efficiency in the context of cervical cancer.

Keywords: Cervical cancer, buformin, radiosensitivity, cell cycle arrest, DNA damage

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Introduction

Cervical cancer is the most common malignancy of the female reproductive system. It is estimated that there were as many as 98,900 newly diagnosed cases of cervical cancer and 30,500 cervical cancer-related deaths in China in 2015.[1,2] Surgery alone or combined with radiotherapy may be used to treat early cervical cancer, but advanced disease is mainly treated with radiotherapy with or without chemotherapy.[3,4] Some patients still develop metastasis and recurrence after radical radiotherapy. Therefore, research efforts should focus on predicting the radiosensitivity of cervical cancer and determining how other agents can improve the efficacy of radiotherapy in the treatment of cervical cancer.

The commonly used biguanides include metformin, phenformin, and buformin, which have been widely used to treat type 2 diabetes mellitus.[5,6] Evans *et al*.[7] first proposed biguanides to reduce cancer incidence in patients with type 2 diabetes in 2015; biguanides have since become a research focus in the field of malignant tumor treatment. As research progresses, the pharmacological action and mechanism of metformin are gradually being elucidated. Biguanides can inhibit tumorigenic activity by activating AMPK, inhibiting the mTOR pathway and inducing cell cycle arrest in tumors such as endometrial cancer, ovarian cancer, and breast cancer.[8–11]

To date, most studies have focused mainly on metformin, whereas there is a lack of research on the anticancer

effect of buformin. Nonetheless, a previous report showed that buformin has a better anticancer effect than metformin in endometrial cancer,[10] and another study found that buformin can reduce the development of breast cancer by regulating stem cell function in mice.[11] Our previous research showed that buformin can restrain the proliferation of cervical cancer cells and enhance their chemosensitivity by activating AMPK signaling and inhibiting its downstream targets. Based on the results of these studies, we aimed to investigate the effects and mechanism of biguanide combined with radiotherapy in cervical cancer cells.

Materials and methods

Chemicals and reagents

Buformin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and dissolved in DMSO. DMEM, MEM, RPMI-1640 medium, fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin were obtained from HyClone (Logan, UT, USA). All other reagents were purchased from Sigma-Aldrich (CA, USA) unless otherwise noted.

Cell lines and treatment

Human cervical cancer cells (HeLa and SiHa) were provided and preserved by the Tumor Institute of the Affiliated Hospital of Jiangnan University (Wuxi, Jiangsu, China). The cells were cultured at 37°C and 5% CO_2. The cells were treated with 10 μM buformin and exposed to several doses of radiation (0–6 Gy) using an Elekta SYNERGY accelerator (Sweden). The depth was set at 1.5 cm from the bottom of the 6-well plates.

Colony formation assay

Three hundred cells were seeded in each well of 6-well plates in 2 ml of medium containing 10% FBS and cultured for 12 h; then, the cells were treated with buformin (10 μM) for 1–2 h followed by IR (2, 4, or 6 Gy) and grown for 10 days. Ten days later, cells were stained with 0.1% crystal violet, and colonies (≥50 cells) were counted. This procedure was performed in duplicate and repeated three times.

Flow cytometry

We analyzed the cell cycle using a cell cycle detection kit purchased from CoWin Biosciences (Beijing, China). The cells (1×10^6) were seeded in 6-well plates. After 12 h, the cells were treated with buformin (10 μM) followed by IR (4 Gy). After 48 h, the cells were harvested by trypsinization, fixed with 70% ethanol, and stained with PI. The procedural steps were performed in strict accordance with the instructions. The cell cycle distribution was then analyzed by a FACSCalibur cytometer (BD Bioscience, Mountain View, CA, USA). This procedure was repeated three times.

Western blotting analysis

We used RIPA containing 1% phosphatase inhibitors to extract total protein from cells, which was quantified by the BCA method (Beyotime). The protein samples were separated by 10% SDS-PAGE and then transferred to PVDF membranes, which were blocked with 5% skim milk. Appropriate primary and secondary antibodies were incubated with the membranes, and specific bands were developed using ECL reagents (Beyotime). The primary antibodies against AMPK, p-AMPK, S6, p-S6, cyclin D1, CDK4, and beta-actin (internal control) were from Cell Signaling Technology (Danvers, MA, USA).

Immunofluorescence assay

Cells were plated in 6-well plates with glass cover slips and then treated with buformin and IR. After washing with PBS, the cells were fixed for 15 min with 4% polyformaldehyde. The appropriate primary antibody (γ-H_2AX) and labeled fluorescent secondary antibody were added to the glass cover slips (see below), and nuclei were stained with DAPI. The glass slides were sealed with an antifluorescence quenching sealant and photographed under a laser confocal microscope. ImageTool software was used to count the cells that were positive for γ-H_2AX, and at least 200 cells were counted in each group.

Statistical analyses

Student's t tests, χ^2 tests, and ANOVA were used according to the data category. SPSS version 19.0 (Chicago, IL, USA) was used for data analysis. A $P < 0.05$ value indicated statistical significance.

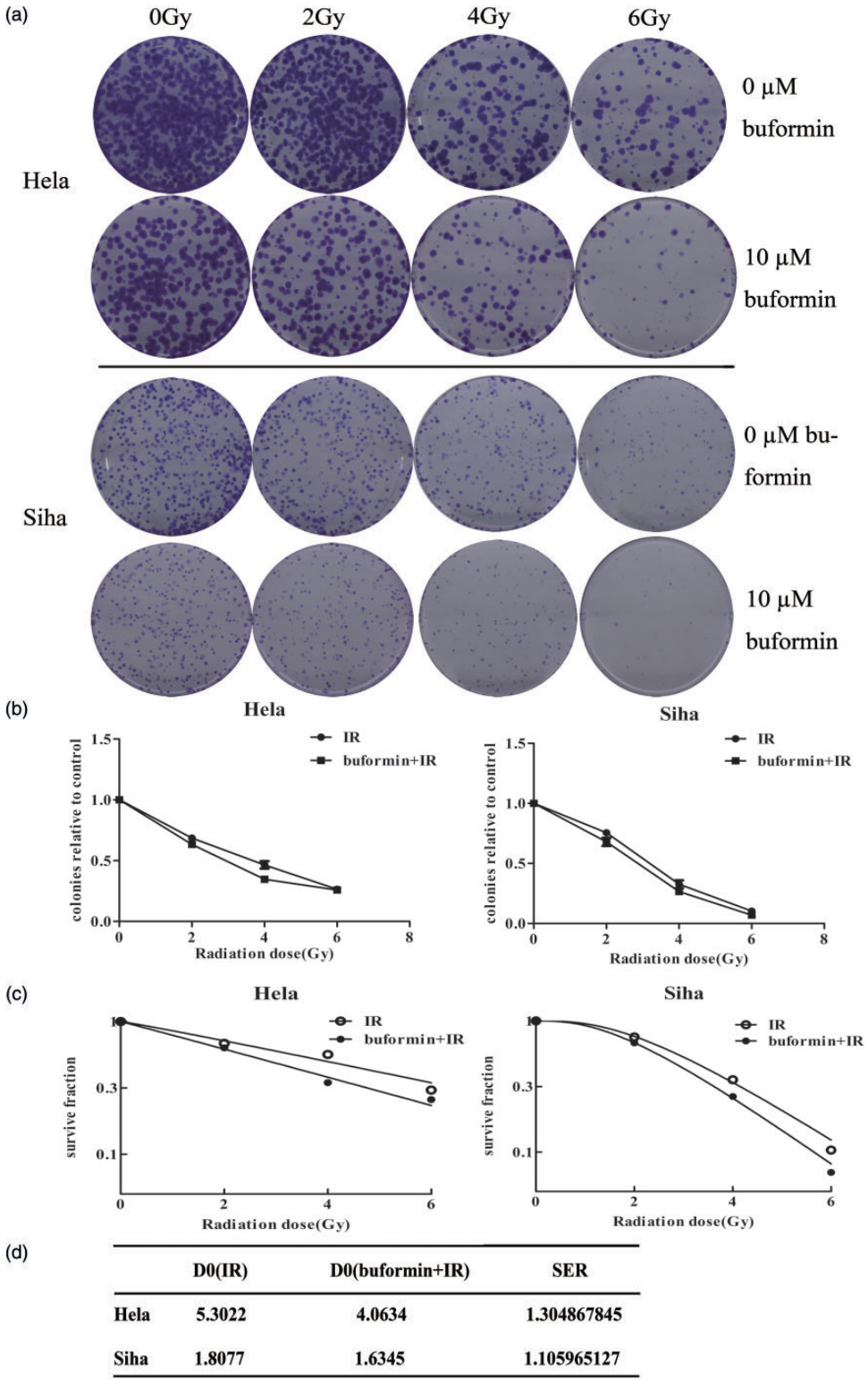
Results

Effect of buformin plus ionizing radiation on cervical cancer cell proliferation

We assessed the ability of the two cell lines (HeLa and SiHa) to form colonies in 6-well plates. After treatment with 10 μmol/L buformin and radiation (0, 2, 4, and 6 Gy) for 10 days, there were fewer colonies than those after treatment with radiotherapy or buformin alone (Figure 1(a) and (b)). The cell survival curves and sensitivity enhancement ratios (SERs) were obtained from the clonogenic assay data after analysis with GraphPad software (Figure 1(c)). SERs were 1.3 and 1.1 for the combination treatment in HeLa and SiHa cells, respectively (Figure 1(d)). The above results indicate that a low dose of buformin sensitizes cervical cancer cells to radiotherapy.

The combination of buformin and IR results in cell cycle arrest and increased apoptosis

Although rare, we observed increased apoptosis in the combination group after 48 h of different treatments (Figure 2). Flow cytometric analysis also showed that the combination treatment caused increased arrest at G2/M phase. Cells at this stage are more sensitive to radiation than cells in other states (Figure 3).



(d)

	D0(IR)	D0(buformin+IR)	SER
Hela	5.3022	4.0634	1.304867845
Siha	1.8077	1.6345	1.105965127

Figure 1. Buformin combined with IR has an inhibitory effect on cervical cancer cell proliferation. (a, b) IR inhibited colony formation in a dose-dependent manner, while IR combined with buformin showed increased inhibition of colony formation by HeLa and SiHa cells. (c) The cell survival curve was fitted to a one target, one hit model, and the sensitization enhancement ratio (SER) was then calculated. The SERs were 1.3 and 1.1 in HeLa and SiHa cells, respectively. (d) The curve was fitted to a one target, one hit model, and D0 and SER = D0 (IR)/D0 (buformin+IR) were calculated. SER > 1 indicates sensitization to radiotherapy. $^{*}P < 0.05$; $^{**}P < 0.01$.

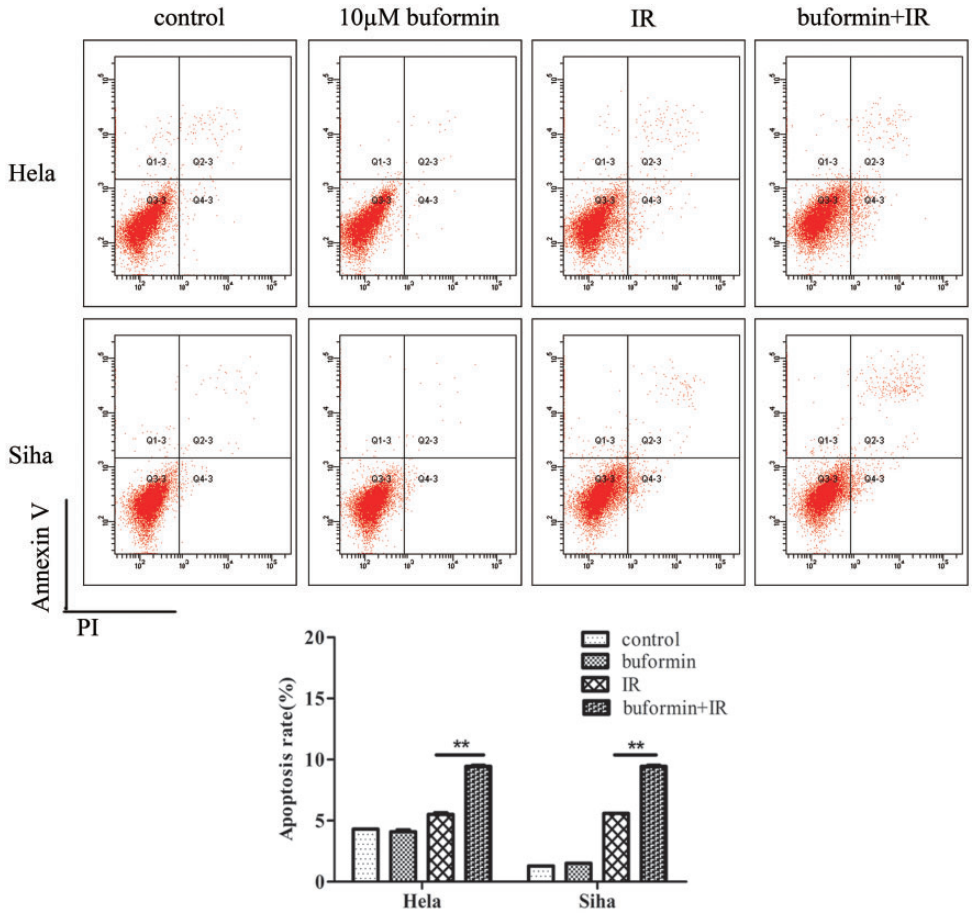


Figure 2. The combination of buformin and IR results in increased apoptosis. Treatment with 10 μM buformin and exposure to several doses of radiation (4 Gy) induced more apoptosis than treatment with radiation only in both cell lines. $^{*}P < 0.05$; $^{**}P < 0.01$.

The combination of buformin and IR suppresses proliferation by activating AMPK signaling and inhibiting the mTOR pathway in cervical cancer cells

The mTOR pathway is highly activated in malignant tumors. As shown by Western blotting analysis, buformin increased AMPK phosphorylation and significantly suppressed S6 phosphorylation. The combination of buformin and IR (4 Gy) resulted in a greater decline in p-S6 levels than did either buformin or IR alone (Figure 4). Activation of the mTOR signaling pathway was inhibited to a greater extent by the combination treatment than by either treatment alone.

Buformin can delay DNA damage repair in cervical cancer cells after radiotherapy

After exposure to 4 Gy of radiation, cells were observed by confocal microscopy for γ-H2AX levels in the nucleus, and DAPI staining showed γ-H2AX localization in the nucleus. After treatment with 10 μmol/L buformin and 4 Gy radiotherapy, the numbers of γ-H2AX foci and cells with γ-H2AX foci were increased. At 24 h after treatment, the γ-H2AX foci in the radiotherapy alone group largely disappeared, whereas the combined treatment group continued to show focal aggregation of γ-H2AX (Figure 5). The above results suggest that buformin can delay the repair of DNA damage in cervical cancer cells after radiotherapy.

Discussion

Based on recent clinical and epidemiological data, biguanides are currently believed to have potential antitumor effects in a wide variety of cancers through the induction of apoptosis and cell cycle arrest, as well as metabolic mechanisms.[9,12,14,15] Due to a higher risk of lactic acidosis compared to metformin, buformin is not currently used clinically; however, studies have reported that this side effect mostly occurs in patients with renal insufficiency.[14,17,19] Based on the better anticancer effect of buformin compared to metformin in endometrial cancer,[9] we explored the anticancer effect of buformin in cervical cancer.

Radiotherapy is a very important treatment for cervical cancer, and many patients who are not candidates for surgery achieve satisfactory results with radiotherapy. However, individual tolerance to its toxic side effects, such as radiation proctitis, vulva edema, and diarrhea, limits the increase in radiotherapy dose and restricts the curative effect.[14] Our study found a synergistic effect of buformin combined with radiotherapy, and this finding may provide a new strategy for clinical diagnosis and treatment. To the best of our knowledge, buformin combined

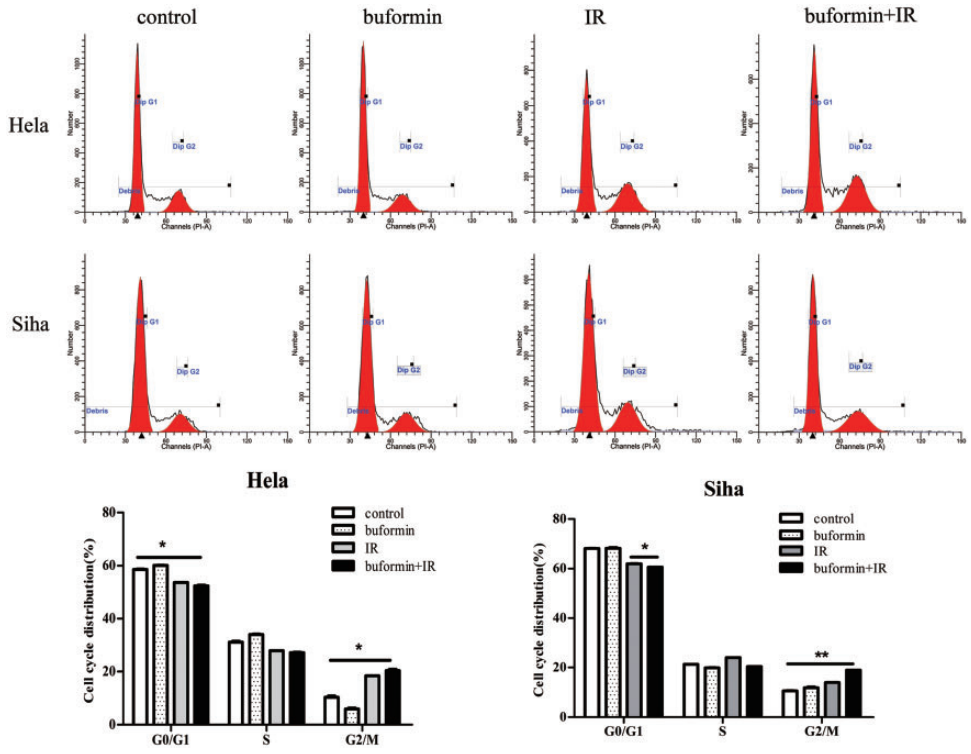


Figure 3. The combination of buformin and IR results in cell cycle arrest. The flow cytometry results revealed significant G2 phase arrest in the combination treatment group. $^{*}P < 0.05$; $^{**}P < 0.01$.

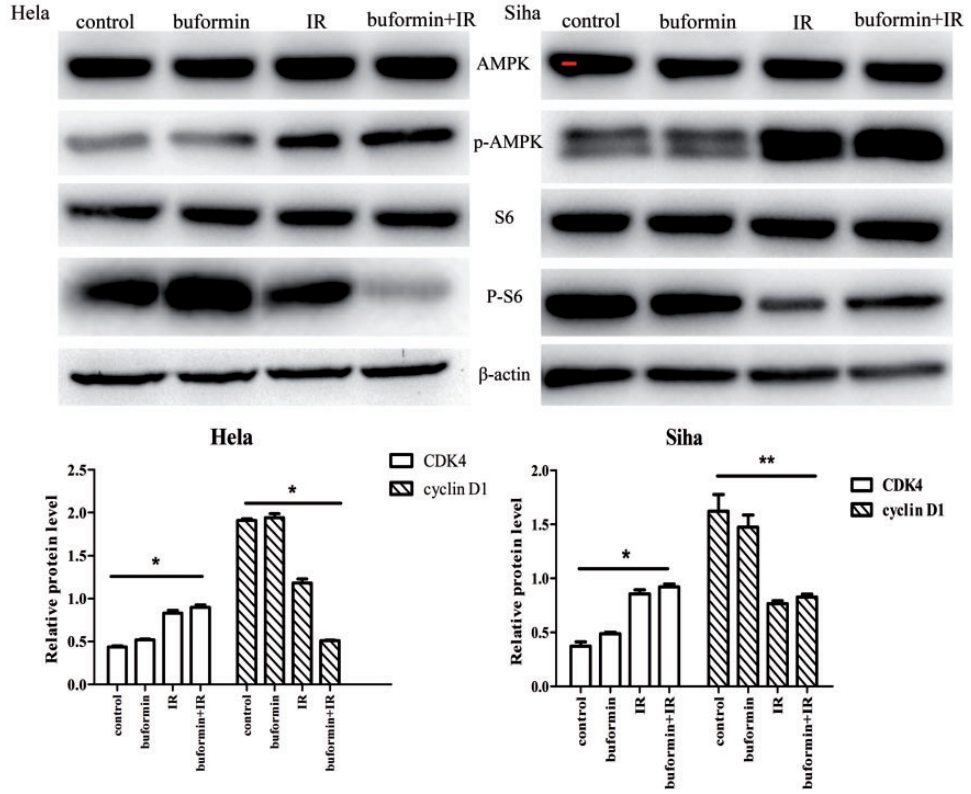


Figure 4. The combination of buformin and IR suppresses cervical cancer cell proliferation by activating AMPK signaling and inhibiting the mTOR pathway. Buformin induced AMPK phosphorylation, while there were higher p-AMPK levels in the combination group than the other groups. Consequently, the phosphorylation of S6 (a key factor downstream of the AMPK signaling pathway) was significantly suppressed. $^{*}P < 0.05$; $^{**}P < 0.01$.

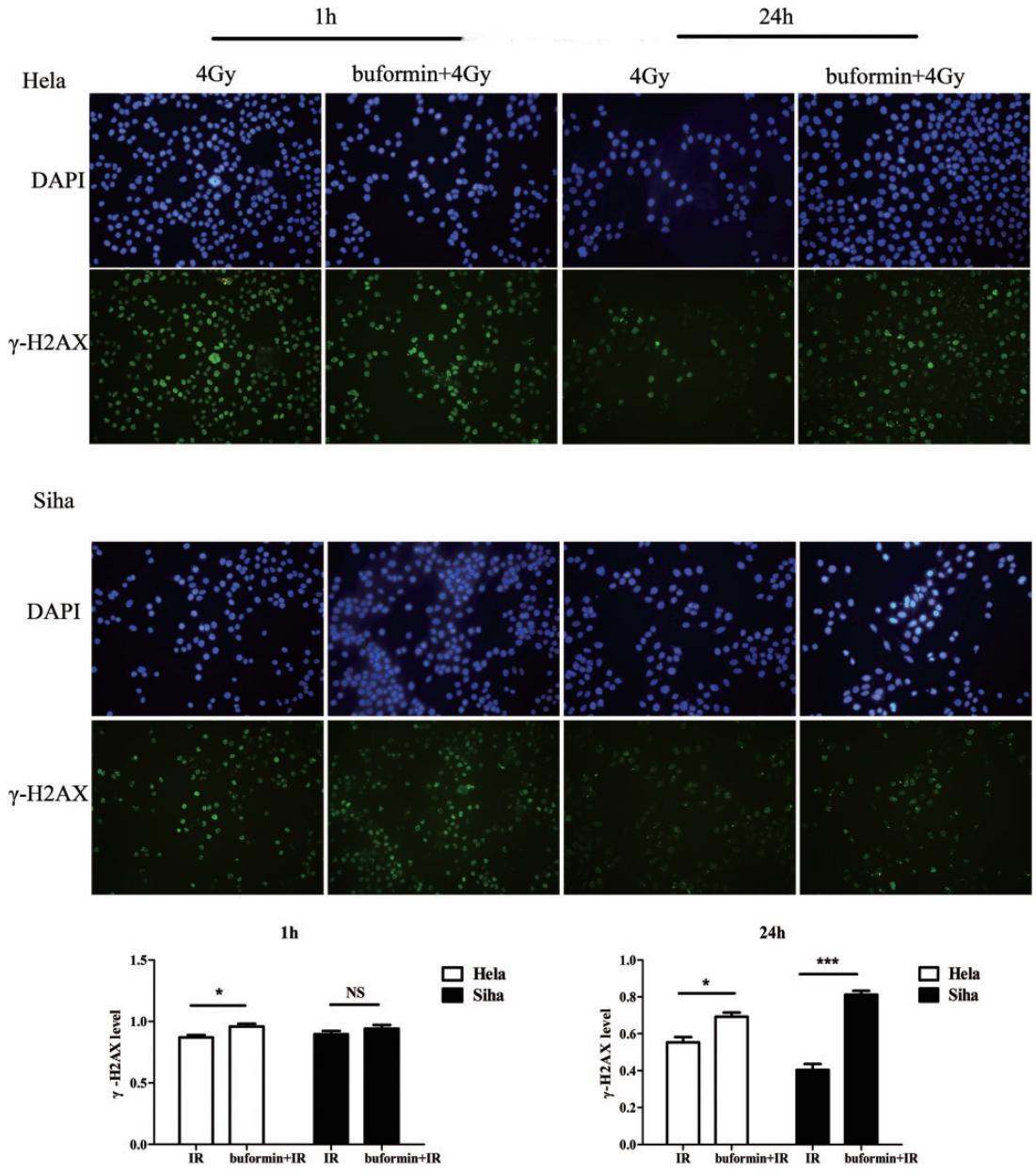


Figure 5. Buformin delays the repair of radiation-induced DNA damage in HeLa and SiHa cells. Representative photomicrographs showing the accumulation of γ-H2AX, a marker of DNA damage, under the indicated treatment conditions. The numbers of γ-H2AX foci and cells with γ-H2AX foci were increased after treatment with 10 μmol/L buformin and 4 Gy radiotherapy. After continuous culture for 24 h, the γ-H2AX foci in the radiotherapy alone group largely disappeared, while focal aggregation of γ-H2AX was still observed in the combination treatment group. $^{*}P < 0.05$; $^{**}P < 0.01$.

with X-ray radiation has not been reported in cervical cancer.

In a previous study, we found that buformin inhibits the proliferation of cervical cancer cells in a concentration-dependent manner.[15] Functional analysis showed that buformin inhibited cell cycle progression and cell invasion. Our results strongly support the potential of buformin to treat cervical cancer. All of these phenomena were accompanied by AMPK activation and inhibition of the mTOR signaling pathway.[17,21] Notably, we also found that buformin increases the sensitivity of cervical cancer cells to chemotherapeutic drugs (paclitaxel, cisplatin, and 5-fluorouracil). In this study, we found that buformin enhances the efficacy of radiotherapy and inhibits colony formation by cervical cancer cells (HeLa/SiHa). During the pre-experiment, we tried different buformin administration methods, such as giving buformin for three days or more, stopping buformin for radiotherapy, using buformin after first irradiation, or performing radiotherapy and dosing simultaneously. After doing a lot of screening, we adopted a more effective method, which was buformin was used 1–2 h before radiotherapy, and then cell culture was continued using culture medium with buformin after radiotherapy. After determining the administration mode, we took buformin with different concentrations from 1 μM to 20 μM(successively increasing by 2.5 μM) for combined radiotherapy, and selected the best concentration of 10 μM buformin with better inhibitory effect for subsequent experimental treatment. Although

few apoptotic cells were observed in each experimental group, the combined group showed more apoptosis did than the other groups. We hypothesized that this finding might be related to the low doses of radiation. The combination of buformin and radiotherapy causes HeLa and SiHa cell arrest in G2/M phase, which shows the highest instability and sensitivity to radiotherapy. Many studies have confirmed that IR can cause DNA damage. γ-H2AX is the most important DNA damage-sensing molecule; it binds to sites of double-strand DNA damage and coordinates multiple signals and/or repair proteins to form foci. Thus, there is a one-to-one correspondence between the number of foci and DNA damage repair induced by γ-H2AX. As shown in our experiments, radiation can cause DNA damage in HeLa and SiHa cells, but after 24 h, the foci in the control group essentially disappeared, and microcellular DNA injury was repaired. However, the numbers of γ-H2AX foci and cells with γ-H2AX foci were significantly higher in the buformin treatment group than in the radiotherapy alone group. Therefore, we believe that buformin can delay the repair of DNA damage after radiation treatment in HeLa and SiHa cells, thus inducing these cells to undergo other forms of death.

The four main factors influencing the biological effects of radiation are repair, regeneration, redistribution, and reoxidation. After radiation, the cell first suffers DNA damage, which is then repaired. Second, reproliferation is the proliferation of cells during radiation therapy. Third, there is a significant difference in the radiosensitivity of cells that are redistributed during the cell cycle. Radiation biology studies have shown that cells at M and G2 stages are the most sensitive, with cells at S and G0 stages being the most resistant to radiation.[16,18,23,25] There is a redistribution of cells from the relatively radiation-resistant phases to the radiosensitive phases, which helps to improve the effect of radiation on tumor killing. These are closely related to AMPK activation.[16] Fourth, reoxidation under aerobic conditions can produce oxygen and free radicals formed by organic peroxides (RO2), ameliorating the effects of IR on the target substance produced by free radical damage. Our experimental results showed that buformin can delay the repair of DNA damage in cervical cancer cells after radiotherapy, induce cell cycle arrest, and inhibit tumor cell proliferation; accordingly, this treatment achieves three of the above changes. In combination with previous research results, the data presented herein indicate that buformin itself has an anticancer effect, and low-dose buformin can sensitize cancer cells to chemotherapy and radiotherapy. Therefore, buformin may be a promising therapeutic for cervical cancer, providing new options for the treatment of cervical cancer. On the basis of this cytological study, we will conduct further animal model tests. All of these will provide reliable evidence for clinical patients to take buformin. There is still a gap in the application of buformin in clinical practice, and our team will continue to further study.

INSTITUTIONAL REVIEW BOARD APPROVAL

This study has been approved by the ethics committee of the Affiliated Hospital of Jiangnan University.

Authors' contributions: All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript. LC, MT and Q-LL conducted most of the experiments and wrote the manuscript, J-JY, H-FQ and YW supplied critical reagents and contributed to the design and review of the study, TZ and Y-EY participated in the statistics of experimental data and the preparation of reagents.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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