Different effects of H_2O_2 treatment on cervical squamous carcinoma cells and adenocarcinoma cells

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Abstract

Introduction: This study aims to compare the antioxidant abilities of cervical squamous carcinoma cells and cervical adenocarcinoma cells and to study the related mechanisms.
Material and methods: Cervical squamous carcinoma and adenocarcinoma cells were treated with H_2O_2. Cell proliferation was determined with the MTT assay. The reactive oxygen species (ROS) level was detected by the 2',7'-dichlorofluorescein-diacetate (DCFH-DA) method. The 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) method was performed to measure intracellular concentrations of reduced glutathione (GSH) and oxidized glutathione (GSSG). The nitrite formation method, the molybdate colorimetric method, and the DTNB colorimetric method were used to determine activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), respectively.
Results: Compared with untreated control cells, cell proliferation of cervical squamous carcinoma cells and cervical adenocarcinoma cells was significantly inhibited by H_2O_2 treatment ($p < 0.05$). Reactive oxygen species levels and GSSG levels were significantly increased ($p < 0.01$), whereas GSH levels were significantly decreased ($p < 0.05$ or 0.01) in both cells after H_2O_2 treatment. Thus the ratio of GSH/GSSG was significantly decreased by H_2O_2 treatment in both cells ($p < 0.01$). In addition, H_2O_2 treatment significantly increased activities of SOD, CAT, and GPx in both cells ($p < 0.05$ or 0.01). Furthermore, the above-mentioned changes induced by H_2O_2 treatment were more dramatic in cervical squamous carcinoma cells.
Conclusions: The antioxidant ability of cervical squamous carcinoma cells is lower than that of cervical adenocarcinoma cells, which may be related to the increased ROS levels in cervical squamous carcinoma cells induced by H_2O_2 treatments.

Key words: cervical neoplasm, reactive oxygen species, hydrogen dioxide, glutathione, antioxidative enzyme.

Introduction

Cervical cancer is one of the most common gynecologic malignancies. The pathologic types of cervical cancers are mainly composed of squamous cell carcinoma (80–85%), adenocarcinoma (15–20%), and adenosquamous

carcinoma (3–5%) [1]. There have been many studies on the genesis and development of cervical cancers, but the mechanism remains unclear [2, 3].

The intracellular free oxygen radicals have dual effects, since they may be toxic but also have important physiological effects in some conditions. At low concentrations, superoxide can be a growth signal that is involved in cell growth and proliferation control. At high concentrations, it damages cells and even leads to cell apoptosis or cancer formation [4, 5]. The cells have oxidation reduction control mechanisms, such as the non-enzyme system, including reduced glutathione (GSH), pyridine nucleotides, ascorbic acid, retinoic acid, thioredoxin and vitamin E. The other system is the enzyme system, including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). Superoxide dismutase converts the superoxide anion (O^{2-}) to hydrogen peroxide (H_2O_2), while CAT and GPx convert H_2O_2 into water [4, 6].

Chronic cervical inflammation is associated with cervical cancer to a certain degree, and reactive oxygen species (ROS) are an important factor for the occurrence and development of chronic inflammation. Chiou and Hu [7] found that the *in vivo* lipid peroxide levels in cervicitis patients were high but the antioxidant system was lacking. Chen *et al*. [8] showed that the level of inducible nitric oxide synthase (iNOS) in cervical cancer patients was increased, leading to tumor metastasis. These findings suggest that intracellular redox levels and antioxidant systems in cervical carcinomas are closely correlated with formation and development of cervical carcinomas.

Since the oxygen free radical levels and cellular antioxidant enzyme activity changes contribute to tumor formation, imbalanced redox states may be related to tumorigenesis and tumor development [9–13]. However, the mechanisms underlying the relationship between the redox status, oxidative capacity and cervical cancers are not clear. In this study, the ROS levels, redox status, and antioxidant capacity of cervical squamous cell carcinoma cells and adenocarcinoma cells were compared in order to investigate the mechanisms of their resistance to oxidative stress.

Material and methods

Reagents

Fetal bovine serum was purchased from Tianjin TBD Company. DMEM, L-15 culture medium and trypsin were purchased from Gibco. DCFH-DA and thiazolyl blue (MTT) were purchased from Sigma. Dimethyl sulfoxide (DMSO) was purchased from Beijing Solarbio Technology Co. Ltd. The GSH and GSSG detection kits were purchased from Jiangsu Haimen Beyotime Institute of Biotechnology. The SOD, CAT, and GPx assay kits were purchased from Nanjing Jiancheng Bioengineering Institute. Coomassie brilliant blue G250 was purchased from Shanghai Chemical Reagent Company. Bovine serum albumin (BSA) was purchased from TAKARA Biotechnology (Dalian) Co. Ltd. H_2O_2 was purchased from Shandong Laiyang Chemical Reagent Factory (30%, w/w, AR Level).

Cell lines and culture

Siha (cervical squamous cell carcinoma cell) and HeLa (cervical adenocarcinoma cell) were purchased from Cell Library of Chinese Academy of Sciences. SW756 (cervical squamous cell carcinoma cell) and GH329 (cervical adenocarcinoma cell) were purchased from American Type Culture Collection (ATCC). HeLa, Siha and GH329 cells were cultured with DMEM, and SW756 cells were cultured with L-15 medium. Both culture media were supplemented with 10% fetal bovine serum, penicillin (60 µg/ml) and streptomycin (100 mg/ml). Cells were cultured in an incubator with 5% CO_2 at 37°C and under saturated humidity conditions. Cells were digested with 0.25% trypsin for cell subculture. Cells in the logarithmic growth phase were selected.

MTT

The cell suspension was incubated in a 96-well plate with density of 1×10^5 /ml after trypsin digestion. Hundred µl of medium and cell suspension was added to each well and cultured for 24 h. Medium was replaced with serum free medium. Cells treated with H_2O_2 formed the treatment group, and cells without treatment constituted the control group. The final concentration of H_2O_2 was 250 µM, 500 µM, 1000 µM, and 1500 µM, respectively. Three wells were set up in each group. Then 10 µl of MTT solution was added to each well after 48 h of culture. Four hours later, culture was terminated and culture medium was abandoned. To each well we added 100 µl of DMSO, and a micro-plate reader (BIO-RAD Model 680) was applied for measurement of the A value at 490 nm wavelength. Cell survival rate = A value of treatment group/a value of control group.

2',7'-dichlorofluorescein-diacetate (DCFH-DA) method

After the medium was changed, Siha, SW756, HeLa and GH329 cells were treated with 250 µM H_2O_2 for 24 h, and cells were collected by trypsin digestion. After rinsing with PBS buffer once, cells were cultured in serum-free cell culture medium to adjust the density to 1×10^6 /ml. The final concentration was 5 µM after the DCFH-DA fluorescence probe was added. The cell suspension was incubat-

ed in a cell culture box at 37°C in the dark for 30 min, and then rinsed with PBS buffer twice. Flow cytometry (BD FACSCalibur) recorded fluorescence changes of dichlorofluorescein (DCF) (excitation wavelength of 488 nm, and emission wavelength of 525 nm). CellQuest software was applied to analyze the mean fluorescence intensity of DCF (MFI), which indirectly shows levels of cell ROS [14].

5,5'-dithiobis-2-nitrobenzoic acid (DTNB) method

Cells were treated with H_2O_2 as the method described above. The DTNB method was applied for detection of intracellular GSH and GSSG content, and the operation was in accordance with instructions of the reagent kit. The microplate reader was BIO-RAD Model 680.

Nitrite formation method, molybdate colorimetric method, and DTNB colorimetric method

Cells were treated with H_2O_2 as described above. The nitrite formation method, the molybdate colorimetric method, and the DTNB colorimetric method were applied to determine SOD, CAT, and GPx activities, respectively. The specific operations were in accordance with instructions of reagent kits. Enzyme activity was detected with a 722S spectrophotometer.

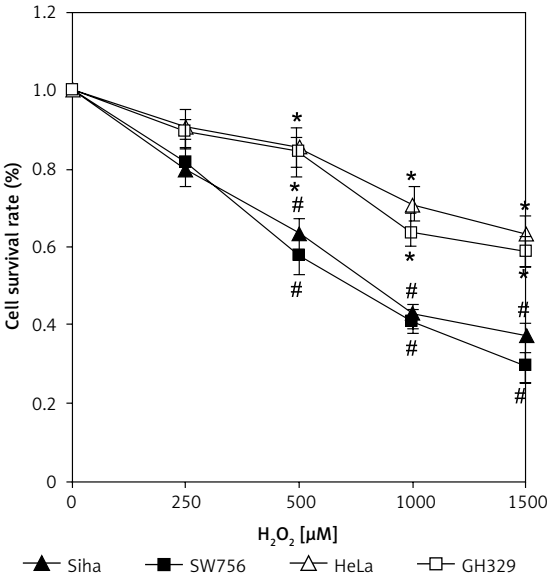

Figure 1. Effects of H_2O_2 on proliferation of the Siha, SW756, HeLa, and GH329 cells. Two cervical squamous cell lines (Siha and SW756) and two cervical adenocarcinoma cell lines (HeLa and GH329) were treated with H_2O_2 (250 μM, 500 μM, 1000 μM, or 1500 μM) for 24 h in the treatment groups. The cells treated with medium only served as the control groups. The MTT assay was performed. The survival rate of cells was calculated. Compared with HeLa and GH329 cells treated with medium only, $^*p < 0.05$. Compared with Siha and SW756 cells treated with medium only, $^\#p < 0.05$

Protein determination

Protein content was detected with Coomassie brilliant blue G250, and the standard protein was bovine serum albumin (BSA).

Statistical analysis

Experiment data were processed by SPSS13.0 statistical software, and all experiments were repeated 3 times. Analysis of variance was used for the significance test. Value of $p < 0.05$ was considered statistically significant.

Results

H_2O_2 inhibits cell proliferation of Siha and SW756 cells more significantly than inhibition of HeLa and GH329 cells.

To investigate whether H_2O_2 affects the proliferation of cells, an MTT assay was performed. Two cervical squamous cell lines (Siha and SW756) and two cervical adenocarcinoma cell lines (HeLa and GH329) were treated with H_2O_2 (250 μM, 500 μM, 1000 μM, or 1500 μM) for 24 h in the treatment groups. The cells treated with medium only served as the control groups. As shown in Figure 1, when compared with the untreated controls, H_2O_2 significantly inhibited cell growth of Siha, SW756, HeLa and GH329 cells ($p < 0.05$). However, the growth inhibition by H_2O_2 in Siha and SW756 cells was more effective than that in HeLa and GH329 cells. For example, after treatment with 500 μM H_2O_2, the survival rates of Siha and SW756 cells were 63.4 ±4.3% and 58.6 ±5.4%. Meanwhile, the survival rates of HeLa and GH329 cells were 87.5 ±8.3% and 86.1 ±5.7%. These results suggest that the four different histological types of cervical carcinoma cells have different responses to oxidative stimuli. H_2O_2 inhibited cell proliferation of Siha and SW756 cells more significantly than inhibition of HeLa and GH329 cells.

H_2O_2 increases the ROS levels more effectively in the Siha and SW756 cells than in the HeLa and GH329 cells

To investigate whether H_2O_2 affects the ROS levels of cells, the cells were treated with or without H_2O_2 (250 μM) for 24 h. The ROS levels were measured with the DCFH-DA method. As shown in Figure 2 A, ROS levels of all of these 4 types of cells were significantly increased by H_2O_2 treatments when compared with the untreated cells ($p < 0.05$ or 0.01). However, the ROS levels in Siha and SW756 cells were increased much more significantly, up to 1.9 and 2.3 times the levels in

their control groups, respectively ($p < 0.01$). These results indicated that H_2O_2 treatments increase the ROS levels more effectively in the Siha and SW756 cells than in the HeLa and GH329 cells.

Levels of GSH and GSSG, and GSH/GSSG ratios were altered in cells by the H_2O_2 treatments

To investigate whether H_2O_2 affects the GSH levels of cells, the cells were treated with or without H_2O_2 (250 μM) for 24 h. The GSH levels were measured with the DTNB method. As shown in Figure 2 B, H_2O_2 treatments decreased GSH levels in all of these 4 types of cells ($p < 0.01$). The decrease in GSH levels was more dramatic in Siha and SW756 cells.

In addition, the GSSG levels were also measured in this experiment. As shown in Figure 2 C, the GSSG levels were increased in all of these 4 types of cells compared with the untreated cells ($p < 0.05$ or 0.01). The GSSG levels in HeLa and GH329 cells were lower than those in Siha and SW756 cells.

To determine whether the balance between GSH and GSSG was altered, GSH/GSSG ratios in the cells treated or untreated with H_2O_2 (250 μM) for 24 h were calculated. The GSH/GSSG ratios are shown in Figure 2 D. With H_2O_2 treatment, GSH/GSSG ratios in all of the 4 types of cells were decreased when compared with the untreated controls ($p < 0.01$). Especially, in the Siha cells, the treatment resulted in up to 63% decreases in GSH/GSSG ratios when compared with the levels in the untreated control group ($p < 0.01$). Altogether, the above results suggest that levels of GSH and GSSG, and the GSH/GSSG ratios were altered in cells by the H_2O_2 treatments.

SOD, CAT, and GPx activities were altered by the H_2O_2 treatments

To investigate whether H_2O_2 affects the SOD activity in the Siha, SW756, HeLa, and GH329

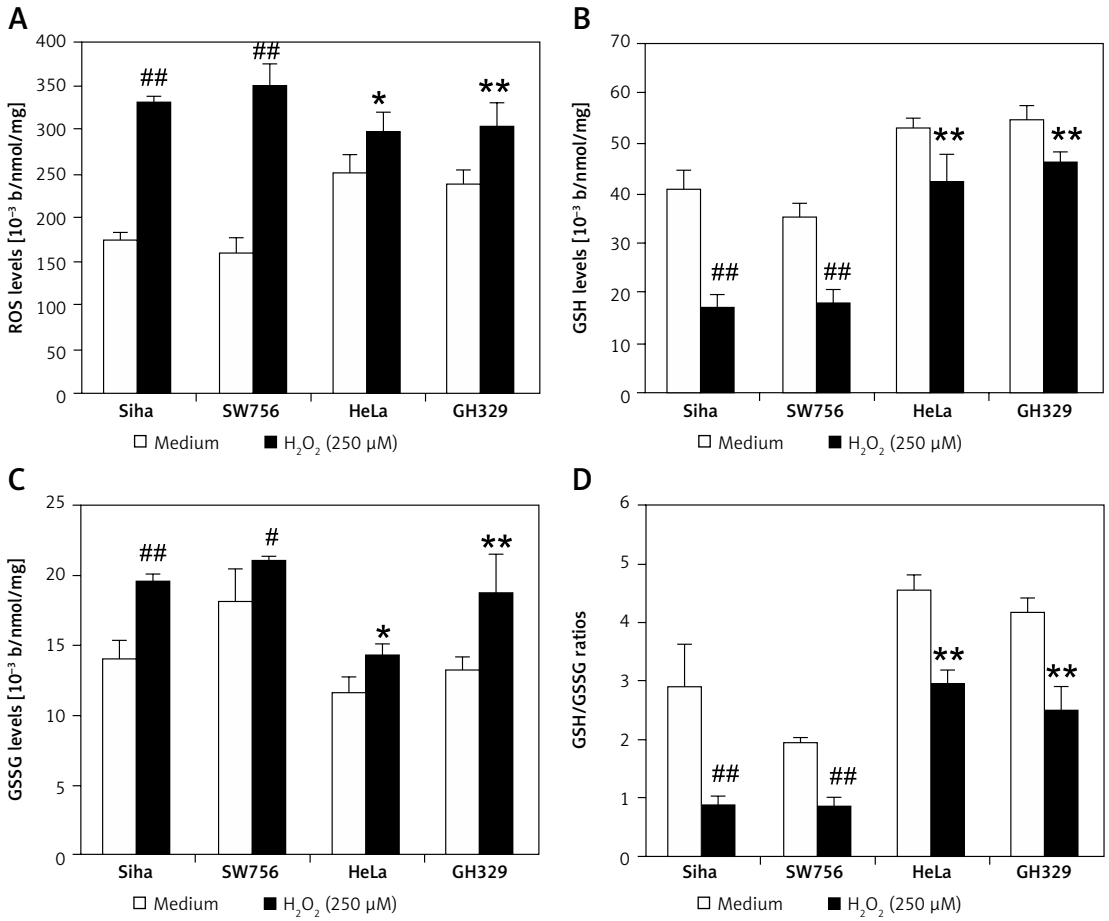

Figure 2. Determination of the ROS, GSH, GSSG levels, as well as the GSH/GSSG ratios, in cells treated with or without H_2O_2. The cells were treated with or without H_2O_2 (250 μM) for 24 h. The ROS levels of those cells were measured with the DCFH-DA method. The DTNB method was applied for detection of intracellular GSH and GSSG content. **A** – ROS levels in cells. **B** – GSH levels in cells. **C** – GSSG levels in cells. **D** – GSH/GSSG ratio in cells. Compared with HeLa and GH329 cells treated with medium only, $^*p < 0.05$, $^{**}p < 0.01$. Compared with Siha and SW756 cells treated with medium only, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$

cells, the cells were treated with or without H_2O_2 (250 μM) for 24 h. As shown in Figure 3 A, SOD activities were increased by H_2O_2 ($p < 0.05$ or 0.01).

As shown in Figure 3 B, the treatments with H_2O_2 significantly increased CAT activities in all of these 4 types of cells ($p < 0.01$). In Siha and SW756 cells, H_2O_2 significantly increased CAT activities by 2.8- and 2.2-fold, respectively, compared with the untreated controls.

Figure 3 C shows the different changes in the GPx activities of these 4 types of cells. The treatments with H_2O_2 increased GPx activities in these 4 types of cells ($p< 0.05$ or 0.01), especially in HeLa and GH329 cells. Altogether, the above results suggest that the SOD, CAT, and GPx activities were altered by the H_2O_2 treatments.

Discussion

In recent years, it has been reported [15, 16] that proteins bcl-2 and bax are involved in the occurrence and development of cervical squamous cell carcinomas, and protein BAG-1 is highly expressed in cervical adenocarcinoma cell lines and tissues. Additionally, there are distinct biological behavior differences between cervical squamous cell carcinomas and cervical adenocarcinomas.

Obviously, the development processes of cervical squamous cell carcinoma and adenocarcinoma are different. In this study, it was found that ROS levels, redox status, and antioxidant enzyme activities were different between cervical squamous cell carcinoma and adenocarcinoma. After exogenous oxidation stimulation, cell responses to oxidative stress between cervical squamous cell carcinoma and adenocarcinoma were different. The antioxidant capacity of cervical squamous cell carcinoma cells was weak, possibly due to the increased ROS levels in squamous cells after H_2O_2 treatment, while the adenocarcinoma cells had strong antioxidant abilities, possibly because of the less changed ROS levels in adenocarcinoma cells.

In this study, two main components of the antioxidant system were also examined under oxidative stress conditions. Our results showed that GSH levels and the GSH/GSSG ratio were significantly decreased ($p < 0.01$) and SOD, CAT and GPx activities were significantly increased ($p < 0.05$ or 0.01) under oxidative stress in the cervical squamous cell carcinoma cells. The CAT activity was increased significantly, suggesting that even with a better response of cell antioxidant enzyme systems, increased ROS and their damage to GSH antioxidant system cannot be prevented. In contrast,

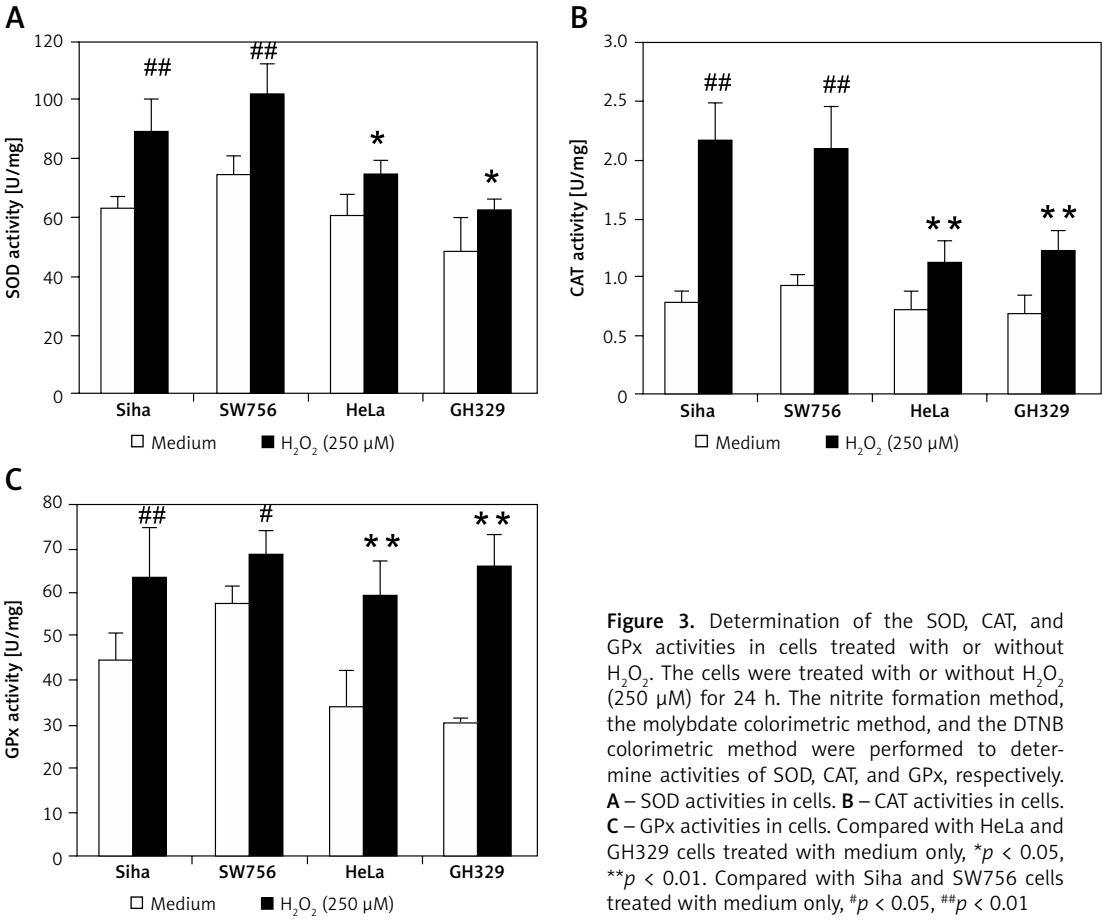

Figure 3. Determination of the SOD, CAT, and GPx activities in cells treated with or without H_2O_2. The cells were treated with or without H_2O_2 (250 μM) for 24 h. The nitrite formation method, the molybdate colorimetric method, and the DTNB colorimetric method were performed to determine activities of SOD, CAT, and GPx, respectively. **A** – SOD activities in cells. **B** – CAT activities in cells. **C** – GPx activities in cells. Compared with HeLa and GH329 cells treated with medium only, $^*p < 0.05$, $^{**}p < 0.01$. Compared with Siha and SW756 cells treated with medium only, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$

under oxidative stress, though GSH levels declined in cervical adenocarcinoma cells, the decline was smaller than that in cervical squamous cell carcinoma cells. Compared with untreated controls, the GSH level in HeLa and GH329 cells was decreased by 19% and 17%, whereas in Siha and SW756 cells it was decreased by 59% and 47%. The intracellular SOD, CAT, and GPx activities were significantly increased ($p < 0.05$ or 0.01) in cervical adenocarcinoma cells treated with H_2O_2. The increase in GPx activities was especially high. The GPx activities in HeLa cells and GH329 cells treated with H_2O_2 were 1.8-fold and 2.3 times those in untreated cells, respectively. These data indicate that the antioxidant enzymes actively responded to oxidative stress, which may result in protection against excessive oxidation.

In conclusion, there were different redox states and responses to exogenous oxidative stress between cervical squamous cell carcinoma and adenocarcinomas.

Acknowledgments

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Conflict of interest

The authors declare no conflict of interest.

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