THE APOPTOTIC EFFECT OF RESVERATROL ON HUMAN PAPILLOMA VIRUS POSITIVE CASKI CELL-LINE: A TIME AND DOSE DEPENDENCE STUDY.

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Abstract:

Background & objectives: Resveratrol (trans-3,4,5'-trihydroxystilbene) is an active compound in food, such as red grapes, peanuts, and berries. A body of evidence shows that resveratrol is able to inhibit the growth of many cancers such as leukemia, breast cancer and primary brain tumors. The objective of the present study was to demonstrate the dose and time dependent apoptotic effect of Resveratrol on Human Caski cell line.

Methods: We used cell viability assays like MTT and Trypan blue assays to demonstrate the inhibition of cancer cell proliferation in a dose and time dependent manner.

Results: Our results have shown that Resveratrol inhibited proliferation of Caski cells in a concentration and time dependent manner in 48 hr cultures. No significant effect was observed in 24 hour cultures of cell viability assays.

Interpretation & conclusions: The results indicated that Resveratrol and NAC inhibited cell growth in Caski cells as a function of dose and time.

Keywords: Resveratrol, Cervical cancer, apoptosis

Introduction

Cervical cancer lesions are a major threat to the health of women and represents the second most common cancer worldwide amongst women. Cervical cancer is the third largest cause of cancer mortality in India after cancers of the mouth & oropharynx, and oesophagus, accounting for nearly 10% of all cancer related deaths in the country¹. Among women, it is the leading cause of cancer mortality, accounting for 26% of all cancer deaths²⁻⁴. New approaches to prevention and therapy for cancer including enhancement of natural killer cells, anti-angiogenic process, antioxidant defence systems and direct cytotoxicity against cancer cells without harming the normal cells are urgently needed⁵. Frequent drug resistance and severe toxicities damage patient's life quality and it is therefore of clinical value to explore

more reliable and less toxic therapeutic approach in the adjuvant treatment of cervical cancers⁶.

An established etiological factor in the causation of cervical cancer is the infection with human papilloma virus (HPV). Many naturally occurring polyphenols have been found to be promising agents toward cervical cancer. Resveratrol is a natural polyphenolic phytochemical that has received considerable interest on the basis of its potential as a chemopreventive agent against human cancer⁷. Resveratrol acts on the process of carcinogenesis by affecting tumor initiation, promotion and progression phases and also suppresses the final steps of carcinogenesis (angiogenesis and metastasis). The chemical formula of resveratrol is 3,5,4'-trihydroxystilbene and the natural sources are red wine, grapes, nuts, mulberries etc. Resveratrol is a potent antioxidant⁸. It can inhibit cellular events associated with tumor initiation, promotion, and progression⁹.

Resveratrol provides a number of anti-aging health benefits including improved metabolism, cardio-protection, and cancer prevention¹⁰.

MATERIALS AND METHODS:

Experimental study done at Department of Biochemistry, J.N. Medical College and Hospital from June 2015- May 2016. The following reagents and procedures were used:

Reagents:

N-acetyl-cysteine (NAC) and Resveratrol were from Sigma Chemical Company, U.S.A.

RPMI-1640 medium were from HiMedia, India. MTT cell viability assay kit was from R & D Systems, U.S.A. 12-wells tissue culture plates and tissue culture flasks were obtained from Techno Plastic Products (TPP), Switzerland. Polystyrene microtitre flat bottom ELISA plates having 96 wells (7 mm diameter) were from NUNC, Denmark. All other chemicals were of the highest analytical grade available. The protocol of study was in conformity with the guidelines of the Institutional Ethical Committee, and had Institutional ethical clearance.

Caski Cell Line

Caski cell line of cervical cancer were brought from National Centre for Cell Sciences, Pune, India. Cells were maintained in CO₂ Incubator maintained at 10% CO₂ using RPMI 1600 Growth medium. Gentamycin and fetal calf serum (10%) were used in culturing. Cell was harvested at after fifth day and aliquats the stock were kept in liquid Nitrogen until use. The viability of the stock remained >99% at 1 year. Before use, aliquots were defrosted, then vortexed.

Preparation of Resveratrol Solution

Resveratrol was purchased from Sigma, USA. The Resveratrol was dissolved in distilled water, and that, the resulting solution was passed through the membrane (0.22 µm) filtered for in vitro uses.

Preparation of RPMI-1640 medium

Dehydrated RPMI-1640 medium of one unit vial (16.3 gm) (HiMedia, India), was suspended in 950 ml of tissue culture-grade water at room temperature with constant, gentle stirring until the medium was completely dissolved. The container was rinsed with tissue culture grade water to remove all traces of powder and added to the above solution. 3.7 gm sodium bicarbonate was added to the medium and stirred until dissolved. The final volume was brought

to 1000 ml with tissue culture grade water. The medium was sterilized immediately by filtering through a sterile membrane filter with a porosity of 0.22 micron using positive pressure rather than vacuum to minimize the loss of carbon dioxide, and stored at 4°C until use.

Treatment with Resveratrol and viability assay

The effect of resveratrol (0–100 µg/ml) on the viability of Caski cells was assessed by using MTT Cell Viability Assay Kit (R & D Systems) according to the manufacturer's instructions provided.

Table 1: Reagents supplied in the kit:

Component	Quantity	Storage conditions
MTT reagent	25 ml	2 – 8°C
Detergent reagent	250 ml	18 – 24°C

Assay procedure:

Adherent cells were gently scraped with RPMI-1640 medium. After this, 5×10^3 cells/well were seeded in 96-well flat-bottomed plates and allowed to attach overnight. Cells were incubated in RPMI-1640 with 2% autologous serum containing Resveratrol (0, 20, 40, 80 and 100 µg/ml) for 48 hours at 37°C, 10% CO₂. After 48 hours, 10 µl of MTT reagent (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) was added to each well and incubation was continued for an additional two hours. When a purple precipitate was clearly visible under the microscope, 100 µl of detergent reagent was added to all wells, including control wells and incubated for two hours in the dark at 20°C. After incubation, the precipitate was solubilized and the absorbance of the resulting solution was measured at 630 nm using a microplate reader. Control cells were treated exactly the same except that no Resveratrol was added to the wells. The percentage of viable cells was calculated by the formula as described by Islam et al., 2000¹¹ and the results were expressed as "Viable cells (% of control cells)".

$$\text{Viable (\% of control cells)} = \frac{\text{Absorbance value of control cells}}{\text{Absorbance value of treated cells}} \times 100$$

Trypan blue exclusion assay for Cell viability

The exponentially growing Caski cells were seeded onto 12-well, flat-bottomed plates at a density of 5×10^3 /mL and allowed to attach overnight. The cells were treated with the Resveratrol of varying concentration (0-100 µg/ml) for 48hr at 37°C in a CO₂ chamber (5%). The cells were collected by

trypsinization and counted by a hemocytometer (Shanghai, China) under light microscope using the Trypan blue dye exclusion method for viability. The cells taking up Trypan blue (dead cells), and cells excluding the dye (viable cells) were counted. Percentage of viable cells was calculated by the following formula

$$\% \text{ Cell viability} = \frac{\text{Total viable cells (unstained)}}{\text{Total cells (stained plus unstained)}} \times 100$$

Preparation of RPMI-1640 medium for Caski cell line cultures

Dehydrated RPMI-1640 medium (HiMedia Laboratories, India) of one unit vial (16.3 gm) was suspended in 950 ml of tissue culture-grade water at room temperature with constant, gentle stirring until the medium was completely dissolved. The container was rinsed with tissue culture grade-water to remove all traces of powder and added to the above solution. 3.7 gm sodium bicarbonate was added to the medium and stirred until dissolved. The final volume was brought to 1000 ml with tissue culture-grade water. The medium was sterilized immediately by filtering through a sterile membrane filter with a porosity of 0.22 micron using positive pressure rather than vacuum to minimize the loss of carbon dioxide, and stored at 4°C till use.

Cell culture of Caski Cell Lines and treatment with supplements

Human cervical cancer cell line Caski was from the National Centre for Cell Science, Pune, India. Around 80-90 % confluent cultures Caski cell lines were harvested and plated onto 12-well tissue culture plates (5×10^6 cells/well) (Costar Corp. Cambridge, MA) in complete RPMI-1640 medium. Thereafter, the plates were subsequently incubated at 37°C, 5% CO₂ for overnight in RPMI-1640 supplemented with 2% autologous serum. Then, as per experimental design, the Caski cell line cultures were co-cultured for 48 hr at 37°C, 5% CO₂ with varying doses of NAC (0-25 mM), or Resveratrol (0-100 ug/ml) or with fixed doses of Hydrogen peroxide (2 mM) or a combination of 2 mM Hydrogen peroxide along with 90 ug/ml of Resveratrol. Thereafter, the cell cultures were harvested and subjected to investigations of different designed parameters as described by us and others previously¹²⁻¹⁷.

Statistical Analysis: Data was analysed using the SPSS version 23.0. Standard error of difference between

the two proportions, Chi-square test and logistic regression analysis was applied wherever applicable. The value of $p < 0.05$ was considered as significant for this study.

Ethical Considerations: Permission from the Institutional Ethics Committee, Faculty of Medicine, A.M.U., Aligarh was taken.

RESULTS:

Cell Viability Assays

Resveratrol and NAC Inhibited Proliferation of Cervical Cancer Caski Cells

The proliferation rate of exponentially growing Caski cells in the presence of resveratrol and NAC was determined at a density of 5×10^3 cells/well. Cells were incubated with increasing concentrations of resveratrol (0 – 100 ug/ml) and NAC (0 – 25 mM) respectively for growing time periods up to 48 h and their viability was determined by the MTT and Trypan blue dye exclusion assay.

In comparison to untreated cells, insignificant / negligible amount of inhibition of cell proliferation was observed in the presence of a maximum dose of 100 ug/ml of Resveratrol respectively after 24 h of treatment (Fig. 1). Thus, the present study was carried out in 48 hr cultures and not 24 hr cultures.

FIGURES:

Figure 1: MTT cell viability assay (In 24 hr cultures): Dose response effect of Resveratrol (0- 100 µg/ml) after 24hr on Caski cell. Data represents mean $\pm$ SEM of 3 experiments ($P < 0.001$).

Our results have shown that Resveratrol inhibited proliferation of Caski cells in a concentration and time dependent manner in 48 hr cultures (Figs.2 - 4).

Figure 2: MTT cell viability assay (Experiment No's 1-3): Dose response effect of Resveratrol (0-100 µg/ml) after 48 hr on Caski cell. Data represents mean $\pm$ SEM of 3 experiments ($P < 0.001$).

Figure 3: MTT cell viability assay (Experiment No's 4-6): Dose response effect of Resveratrol (0- 100 µg/ml) after 48 hr on Caski cell. Data represents mean $\pm$ SEM of 3 experiments ($P < 0.001$).

Figure 4: MTT cell viability assay (Experiment No's 7-9): Dose response effect of Resveratrol (0- 100 µg/ml) after 48 hr on Caski cell. Data represents mean $\pm$ SEM of 3 experiments ($P < 0.001$).

When Caski cell lines were co-cultured with varying doses of Resveratrol for 48 hrs, then the percent cell viability observed was of the order of 99, 98, 97, 98, 98, 97, 74, 67, 60, 58, 53, 49 and 46 percent with 0, 2,

5, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 µg/ml of Resveratrol respectively (Fig. 2 for experiments no's 1-3; $p < 0.001$). Similar observations were made in MTT Experiment No's 4-6 (Fig. 3; $p < 0.001$) and Experiment No's 7 – 9 (Fig. 4; $p < 0.001$). Therefore, analysis of the MTT data depicted in Figs. 2 to 4, it was observed that Resveratrol concentration till 30 µg/ml had no effect on the viability Caski cells. Thereafter, viability was affected by Resveratrol in a dose-dependent manner i. e from 40 µg/ml to 100 µg/ml. As evident from Figs. 2 to 4, around 50% proliferation of Caski cells in 48 hr cultures were inhibited (i. e. loss in viability) by 90 µg/ml of Resveratrol. Furthermore, similar pattern observations were recorded with NAC (Figs. 5 – 7). Doses of NAC to the order of 20 and 25 mM exhibited around 50% inhibition in proliferation / viability of Caski cells. Therefore, all further studies were carried out by employing 90 µg/ml of Resveratrol and 20 mM of NAC.

Figure 5: MTT cell viability assay (Experiment No's 1-3): Dose response effect of N-Acetyl-Cysteine (NAC) (0- 25 mM) after 48 hr on Caski cell. Data represents mean $\pm$ SEM of 3 experiments ($P < 0.001$).

Figure 6: MTT cell viability assay (Experiment No's 4-6): Dose response effect of N-Acetyl-Cysteine (NAC)

(0- 25 mM) after 48 hr on Caski cell. Data represents mean $\pm$ SEM of 3 experiments ($P < 0.001$).

Figure 7: MTT cell viability assay (Experiment No's 7-9): Dose response effect of N-Acetyl-Cysteine (NAC) (0- 25 mM) after 48 hr on Caski cell. Data represents mean $\pm$ SEM of 3 experiments ($P < 0.001$).

Similarly, we confirmed our above results with Trypan blue dye exclusion assay (Fig. 8). Since, as stated above in Fig. 1, an insignificant amount of inhibition of cell proliferation, was observed after 24 h of treatment in the presence of varying doses of Resveratrol, and the same with NAC was not carried out. Therefore, as evident from Fig. 8 ($p < 0.001$), trypan blue exclusion assay for 48 hr cultures with varying doses of Resveratrol exhibited a dose-dependent reduced / suppressed viability.

Figure 8: Trypan blue cell viability assay: Viability of Caski cell lines following Resveratrol treatment. Caski cells were incubated with Resveratrol for 48 hr (0-100 µg/ml) and the number of viable cells is counted by trypan blue exclusion assay. Cell viability is represented as percent viable cells, where the vehicle treated cells are regarded as 100%. Data represents

the analysis of three independent experiments in duplicate, expressed as mean viable cells ($\pm$ SEM) percentage of controls.

These results indicated that Resveratrol inhibited cell growth in Caski cells as a function of dose and time. Inhibition of cell proliferation / viability may be the consequence of the induction of cell cycle arrest or apoptosis. Therefore, we hypothesized that Resveratrol induced inhibition of cell proliferation was due to programmed cell death i.e. apoptosis, which in turn, has been substantiated by our data of caspase activity assays as shown elsewhere in this study.

DISCUSSION;

Globally, cervical cancer is the second most common malignancy in women¹⁸. Human papillomaviruses (HPVs) are associated with cervical cancer, particularly a subgroup of HPVs designated as the 'high-risk' subgroup, which includes HPV16 and 18¹⁹. As part of their carcinogenic mechanism, these high-risk human papillomaviruses (HR-HPVs) encode E6 and E7 viral oncoproteins that interfere with the function of the tumor suppressor proteins p53 and retinoblastoma, respectively^{7, 20}. Resveratrol is a phytoalexin present in more than 70 plant species, including a wide variety of fruits and vegetables such as grapes, berries, peanuts, and various herbs²¹⁻²². In the first report of resveratrol as a possible cancer chemopreventive agent, Jang et al. (1997) observed that this compound exerts antitumor properties at all three stages of skin carcinogenesis²³. Resveratrol has been reported to inhibit growth and induce apoptosis in cancer cell lines including promyelocytic leukemia, breast, prostate, lung, rhabdomyosarcoma, and colon cancer cells²²⁻²⁹.

In the present study, keeping in mind the previous reports on Resveratrol, we have probed the dose response effect of Resveratrol on the viability of Caski cell lines. No significant effect was observed in 24 hr cultures of cell viability assays. On the contrary, 48 hr cultures showed high magnitude effect of resveratrol. Our data is in agreement with earlier studies showing resveratrol-induced apoptosis⁷.

CONCLUSIONS:

In 24 hr cultures, Resveratrol failed to show any significant effect on the viability of cervical cancer Caski cells. Resveratrol and N-Acetyl Cysteine (NAC) significantly inhibited / suppressed the viability of Caski cells in 48 hr cultures in a dose-dependent

manner at concentrations higher than 30 ug/ml (Resveratrol) and 20 mM (NAC). The results indicated that Resveratrol and NAC inhibited cell growth in Caski cells as a function of dose and time.

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