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# To probe the effect of Resveratrol on caspase - 3 & caspase -9 activity in Human Papilloma Virus positive Ca Ski cell-line in 48 hours cell cultures

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Conflicts of Interest: Nil

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**Abstract:**

**Objectives:** Cervical cancer is the leading cause of cancer mortality, accounting for a significant percentage of all cancer deaths amongst women. Resveratrol(3,5,4-trihydroxystilbene),a natural pytoalexin ,belongs to a class of polyphenolic compounds called stilbenes. The rich natural sources of resveratrol are grapes,nuts,red wine and roots and stalks of Japanese knotweed(polygonum cuspidatum).It has potent antioxidant,anti-aging,anti-cancer and anti-inflammatory properties **.** As it was demonstrated in a previous study that Resveratrol causes inhibition of cancer cell proliferation in a dose and tiume dependent manner using cell viability assays , our objective was to probe further that Resveratrol treated cells have augmented caspase -3 and caspase - 9 activites than the untreated Ca Ski cells .

**Methods:** Caspase -3 and Caspase-9 activity levels were measured in Resveratrol(90µg/ml) treated and untreated cells that were taken as control using the colorimetric substrates DEVD-Pna and absorbance was read at 405nm to demonstrate augmentation of caspase 3 & 9 activities and GPx activity in Resveratrol treated cells in 48 hours culture.

**Results & conclusion:** Resveratrol treated Ca Ski cell lines that were cultured for 48 hrs showed high levels of caspase-3 and caspase-9 thereby indicating that Resveratrol has an apoptotic and anti-proliferative effect on Ca Ski cells.

**Keywords:** Resveratrol, Cervical cancer, caspase -3 , caspase- 9

## Introduction

The tumor growth and progression happens when inhibition of cell suppression pathways occurs by the carcinogens.This leads to imbalance between apoptosis and cell proliferation resulting in uninhibited growth of cells and ultimately cancer. When a cell receives stimulus ,the activated proteolytic caspases make it undergo organized degradation of cellular organelles. Resveratrol ( trans – 3,4,5- trihydroxystilbene ) , a naturally occurring polyphenol found in red grapes , peanuts and berries lead to cell death in cervical cancer cells through apoptosis and autophagy **(12)**

## MATERIALS AND METHODS:

The present was study done at Department of Biochemistry, Jawaharlal Nehru Medical College and Hospital from June 2015- May 2016. The reagents and procedures that were used are the following:

### Reagents:

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

RPMI-1640 medium were from HiMedia, India. 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, Institutional ethical clearance was also taken.

**Caski Cell Line**

The Caski cell line of cervical cancer was ordered from National Centre for Cell Sciences, Pune, India. Cells were maintained in $CO_2$ Incubator maintained at 10% $CO_2$ using RPMI 1600 Growth medium. Gentamycin and fetal calf seum (10%) were used in culturing. Cells were harvested after fifth day and aliquots 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 used in the present study 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 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 vaccum to minimize the loss of carbon dioxide, and stored at $4^{o}$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 ($5x10^6$ cells/well) (Costar Corp. Cambridge, MA) in complete RPMI-1640 medium. Thereafter, the plates were subsequently incubated at 37°C, 5% $CO_2$ 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% $CO_2$ 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.

**Caspase activity assay in Caski Cell Line Cultures**

Caspase-3 related protease activities in Caski cell line lysates was determined as described elsewhere (**4 ,13)**. **The substrates employed were** Asp-Glu-Val-Asp-*p*-nitroanilide (DEVD-pNA) (Biomol.) for caspases-3. Substrate cleavage was monitored at 405 nm and the activities were calculated according to the instruction **of** the manufacturer. **(4, 7 )**

**Statistical Analysis:** SPSS version 23.0 was used for the analysis of the data. Standard error of difference between the two proportions, Chi-square test and logistic regression analysis were applied wherever applicable. The value of $p<0.05$ was considered as significant for this study.

**Ethical Considerations:** Institutional Ethics Committee, Faculty of Medicine, A.M.U., Aligarh granted the permission.

**RESULTS**

**Caspases 3 and 9 Activity Assays in Caski cell line cultures**

**1. Resveratrol-Induced Activation of Caspase-3 during Apoptosis**

In the present study, an attempt was made to know whether caspases play some role in the apoptosis of Caski cells induced by Resveratrol. Therefore, we determined the caspase activities in Caski cells treated / cultured for 48 hr with Resveratrol.

As discussed above, the cell growth inhibition / suppressed viability by Resveratrol or NAC was strongly dose and time dependent; and that, negligible effects were recorded at 24 hr cultures, thus we focused our Caspases activity assays on 48 hr cultures only and not on 24 hr cultures.

Also, since in a previous study of MTT cell viability assays, it was established in our system in the present study that 90 ug/ml of Resveratrol as well as 10 mM of NAC was efficient enough to inhibit around 50% of cell viability / cell proliferation, hence in Caspases activity assay studies, a fixed concentration of 90 ug/ml of Resveratrol and 10 mM of NAC were used in cultures. **(10)**

Next, Caski cells were treated with Resveratrol (90 ug/ml) for 48 hr. Cytosolic proteins were extracted

and assayed for caspase activities by incubation with a chromogenic substrate, LEHD-pNA (for caspase-9) or DEVD-pNA (for caspase-3).

Our results showed a significant induction of caspase-3 activities in Caski cells after treatment of 90 ug/ml of Resveratrol (Table 1; $p<0.001$). In untreated cells, as evident from Table 1, the Caspase-3 activity for various experiments carried out in duplicates (n=24) was found to be of very low order / insignificant, as it was recorded to be in the range of 3.12 to 12.87 pMol substrate hydrolyzed / minute.

On the contrary, Caski cells co-cultured with Resveratrol (90 ug/ml) for 48 hr, exhibited a high magnitude of Caspase-3 activity. Thus, as evident from Table 1, the Caspase-3 activity for various experiments carried out in duplicates (n=24) was found to be of very high magnitude, where it was observed to be in the range of 102.45 to 169.25 pMol substrate hydrolyzed / minute.

**2. N-Acetyl Cysteine (NAC) -Induced Activation of Caspase-3 during Apoptosis**

Next, Caski cells were treated with NAC (20 mM) for 48 hr. Cytosolic proteins were extracted and assayed for caspase activities by incubation with a chromogenic substrate, LEHD-pNA (for caspase-9) or DEVD-pNA (for caspase-3).

As evident from our results depicted in Table 2 (experiments (n=24) carried out in duplicates; $p<0.001$), showed that in untreated cells, the Caspase-3 activity was of very low magnitude, as it was recorded to be in the range of 2.33 to 11.98 pMol substrate hydrolyzed / minute. On the contrary, Caski cells co-cultured with NAC (20 mM) for 48 hr, exhibited a high magnitude of Caspase-3 activity. Thus, as evident from Table 2, the Caspase-3 activity for various experiments carried out in duplicates (n=24) was found to be of appreciable magnitude, where it was observed to be in the range of 92.12 to 147.22 pMol substrate hydrolyzed / minute. Upon comparative analysis of data in Table 1 with that of Table 2, it was found that the degree of caspase-3 activity in treated cells were a bit higher in Caski cells co-cultured with Resveratrol (90 ug/ml) than in Caski cells co-cultured with NAC (20mM).

**3. Resveratrol-Induced / NAC-induced Activation of Caspase-9 during Apoptosis**

Next, an attempt was made to evaluate / probe the caspase-9 activity. As evident from our results depicted in Table 3 (experiments (n=24) carried out in duplicates; $p<0.001$), the Caspase-9 activity was of very low magnitude in untreated cells,, as it was recorded to be in the range of 2.33 to 11.98 pMol substrate hydrolyzed / minute. On the contrary, Caski cells co-cultured with Resveratrol (90 ug/ml) and NAC (20 mM) for 48 hr, exhibited a high magnitude of Caspase-9 activities. Thus, as evident from Table 3 and Table 4, the Caspase-9 activities were found to be of appreciable magnitude, where with resveratrol (90 ug/ml), it was observed to be in the range of 100.28 to 166.72 pMol substrate hydrolyzed / minute, and that with 20 mM NAC, it was observed to be in the range of 93.98 to 149.11 pMol substrate hydrolyzed / minute. Again, as was observed above for caspase-3, upon comparative analysis of data in Table 3 with that of Table 4, it was found that the degree of caspase-9 activity in treated cells were a bit higher in Caski cells co-cultured with Resveratrol (90 ug/ml) than in Caski cells co-cultured with NAC (20mM).

**4. Effect of Hydrogen Peroxide (2 mM) on Activities of Caspase-3 and Caspase-9**

Next, as already stated above that since reactive oxygen species (ROS) is known to be a major player for contribution to progression of a variety of cancers, thus, in the present study, attempts were also made to probe the effect of Hydrogen Peroxide (2 mM) in Caski cell line cultures by evaluating Caspases-3 and 9 activities in 48 hr Caski cell cultures.

As evident from our results depicted in Table 5 and 6 (experiments (n=24) carried out in duplicates; $p<0.001$), showed that in untreated cells, the Caspase-3 and 9 activities were of very low magnitude, as it was recorded to be in the range of 1.55 to 11.23 as well as 2.12 and 14.54 pMol substrate hydrolyzed / minute for caspase-3 and caspase-9 respectively.

Interestingly, Caski cells co-cultured with Hydrogen Peroxide (2 mM) for 48 hr, exhibited a bit low magnitude of Caspase-3 ( Range=1.03 to 5.98 pMol substrate hydrolyzed / minute) and Caspase-9( Range=1.01 to 6.32 pMol substrate hydrolyzed / minute) activities in comparison to untreated cells as shown above. Thus, the data obtained indicates that hydrogen peroxide was further augmenting the proliferation of cancer cells.

**5. Modulation Study on Activities of Caspase-3**

Next, an attempt was made to probe the modulation of Caspase-3 activity by co-culturing the Caski cells

for 48 hr with hydrogen peroxide (2 mM) along with Resveratrol (90 ug/ml). As evident from our results depicted in Table 7 (experiments (n=24) carried out in duplicates; $p<0.001$), showed that in untreated cells, the Caspase-3 activity was of very low / insignificant, as it was recorded to be in the range of 1.77 to 12.56 pMol substrate hydrolyzed / minute for caspase-3. Interestingly, as evident from Table 7, co-culturing the Caski cells for 48 hr with hydrogen peroxide (2 mM) along with Resveratrol (90 ug/ml) ameliorated the Caspase-3 activity (Range= 68.34 to 84.89 pMol substrate hydrolyzed / minute).

**Table 1: Caspase 3 activity assay in untreated and Resveratrol treated Caski Cell Lines**

| **Untreated Caski cell lines (Controls)** | **Caspase-3 activity** (pMol Substrate Hydrolyzed/Minute) | **Resveratrol (90 ug/ml) + Caski Cell Lines** | **Caspase-3 activity** (pMol Substrate Hydrolyzed / Minute) |
|---|---|---|---|
| Control Sample 1 | 4.11 | Test sample 1 | 135.22 |
| Control Sample 2 | 5.18 | Test sample 2 | 156.98 |
| Control Sample 3 | 9.22 | Test sample 3 | 123.22 |
| Control Sample 4 | 11.25 | Test sample 4 | 149.44 |
| Control Sample 5 | 4.56 | Test sample 5 | 112.55 |
| Control Sample 6 | 10.09 | Test sample 6 | 134.04 |
| Control Sample 7 | 6.21 | Test sample 7 | 160.21 |
| Control Sample 8 | 10.03 | Test sample 8 | 122.57 |
| Control Sample 9 | 7.92 | Test sample 9 | 166.11 |
| Control Sample 10 | 8.25 | Test sample 10 | 123.67 |
| Control Sample 11 | 12.21 | Test sample 11 | 159.43 |
| Control Sample 12 | 5.82 | Test sample 12 | 162.98 |
| Control Sample 13 | 10.13 | Test sample 13 | 129.67 |
| Control Sample 14 | 8.13 | Test sample 14 | 135.05 |
| Control Sample 15 | 3.12 | Test sample 15 | 149.32 |
| Control Sample 16 | 5.11 | Test sample 16 | 114.34 |
| Control Sample 17 | 12.02 | Test sample 17 | 107.04 |
| Control Sample 18 | 4.32 | Test sample 18 | 144.23 |
| Control Sample 19 | 11.04 | Test sample 19 | 119.21 |
| Control Sample 20 | 4.33 | Test sample 20 | 112.12 |
| Control Sample 21 | 9.12 | Test sample 21 | 169.25 |
| Control Sample 22 | 13.08 | Test sample 22 | 144.77 |
| Control Sample 23 | 7.98 | Test sample 23 | 111.21 |
| Control Sample 24 | 12.87 | Test sample 24 | 102.45 |

**Table 1: Caspase-3 activity assay with Resveratrol:**

Resveratrol (90 ug/ml) treated Caski cell lines (n=24) that were cultured for 48 hrs showed high activity levels of caspase-3. Untreated cells (n=24) showed insignificant levels of caspase-3. Cervical Caski cell lines were left untreated or treated with 90 ug/ml of Resveratrol as described in methods. Nuclei free cell lysates were prepared from sample and used in the caspase-3 activity assay using the colorimetric substrates DEVD-pNA. Absorbance was read at 405 nm. One unit of caspase

activity is the amount of enzyme activity liberating 1 pmol of substrates/min. Values shown is means of 24 independent experiments carried out in duplicates for caspase-3 ($p<0.001$ for all).

**Table 2: Caspase 3 activity assay in untreated and NAC (10 mM) treated Caski Cell Lines**

| **Untreated Caski cell lines (Controls)** | **Caspase-3 activity** (pMol Substrate Hydrolyzed/Minute) | **NAC (20 mM) + Caski Cell Lines** | **Caspase-3 activity** (pMol Substrate Hydrolyzed/Minute) |
|---|---|---|---|
| Control Sample 1 | 4.11 | Test sample 1 | 128.23 |
| Control Sample 2 | 4.98 | Test sample 2 | 147.22 |
| Control Sample 3 | 7.31 | Test sample 3 | 118.35 |
| Control Sample 4 | 11.68 | Test sample 4 | 129.33 |
| Control Sample 5 | 3.04 | Test sample 5 | 103.23 |
| Control Sample 6 | 9.87 | Test sample 6 | 121.87 |
| Control Sample 7 | 6.03 | Test sample 7 | 134.32 |
| Control Sample 8 | 11.22 | Test sample 8 | 111.99 |
| Control Sample 9 | 5.79 | Test sample 9 | 149.21 |
| Control Sample 10 | 9.45 | Test sample 10 | 114.76 |
| Control Sample 11 | 13.91 | Test sample 11 | 134.98 |
| Control Sample 12 | 5.03 | Test sample 12 | 138.34 |
| Control Sample 13 | 10.21 | Test sample 13 | 129.32 |
| Control Sample 14 | 7.98 | Test sample 14 | 130.32 |
| Control Sample 15 | 2.33 | Test sample 15 | 137.32 |
| Control Sample 16 | 5.97 | Test sample 16 | 110.22 |
| Control Sample 17 | 11.98 | Test sample 17 | 98.34 |
| Control Sample 18 | 3.51 | Test sample 18 | 127.32 |
| Control Sample 19 | 9.89 | Test sample 19 | 100.34 |
| Control Sample 20 | 5.01 | Test sample 20 | 99.43 |
| Control Sample 21 | 10.06 | Test sample 21 | 138.34 |
| Control Sample 22 | 11.12 | Test sample 22 | 125.56 |
| Control Sample 23 | 7.98 | Test sample 23 | 98.23 |
| Control Sample 24 | 11.29 | Test sample 24 | 92.12 |

**Table 2: Caspase-3 activity assay with NAC:** N-Acetyl Cysteine (NAC) (20 mM) treated Caski cell lines (n=24) that were cultured for 48 hrs showed high activity levels of caspase-3. Untreated cells (n=24) showed insignificant levels of caspase-3. Cervical Caski cell lines were left untreated or treated with 20 mM of NAC as described in methods. Nuclei free cell lysates were prepared from sample and used in the caspase-3 activity assay using the colorimetric substrates DEVD-pNA. Absorbance was read at 405 nm. One unit of caspase activity is the amount of enzyme activity liberating 1 pmol of substrates/min. Values shown is means of 24 independent experiments carried out in duplicates for caspase-3 ($p<0.001$ for all).

**Table 3: Caspase 9 activity assay in untreated and Resveratrol (90 ug/ml) treated Caski Cell Lines**

| **Untreated Caski cell lines (Controls)** | **Caspase-9 activity** (pMol Substrate Hydrolyzed/Minute) | **Resveratrol (90 ug/ml) + Caski Cell Lines** | **Caspase-9 activity** (pMol Substrate Hydrolyzed/Minute) |
|---|---|---|---|
| Control Sample 1 | 3.67 | Test sample 1 | 138.23 |
| Control Sample 2 | 5.78 | Test sample 2 | 155.12 |

| | | | |
|---|---|---|---|
| Control Sample 3 | 8.91 | Test sample 3 | 129.12 |
| Control Sample 4 | 13.45 | Test sample 4 | 144.67 |
| Control Sample 5 | 2.98 | Test sample 5 | 111.23 |
| Control Sample 6 | 11.26 | Test sample 6 | 132.32 |
| Control Sample 7 | 5.78 | Test sample 7 | 158.91 |
| Control Sample 8 | 9.23 | Test sample 8 | 126.87 |
| Control Sample 9 | 6.87 | Test sample 9 | 166.11 |
| Control Sample 10 | 9.45 | Test sample 10 | 127.87 |
| Control Sample 11 | 14.21 | Test sample 11 | 155.23 |
| Control Sample 12 | 4.87 | Test sample 12 | 162.98 |
| Control Sample 13 | 9.43 | Test sample 13 | 124.26 |
| Control Sample 14 | 7.23 | Test sample 14 | 133.56 |
| Control Sample 15 | 2.78 | Test sample 15 | 148.98 |
| Control Sample 16 | 6.87 | Test sample 16 | 113.77 |
| Control Sample 17 | 11.23 | Test sample 17 | 105.43 |
| Control Sample 18 | 3.67 | Test sample 18 | 142.41 |
| Control Sample 19 | 10.29 | Test sample 19 | 117.82 |
| Control Sample 20 | 4.81 | Test sample 20 | 110.26 |
| Control Sample 21 | 9.66 | Test sample 21 | 166.72 |
| Control Sample 22 | 12.82 | Test sample 22 | 142.11 |
| Control Sample 23 | 8.29 | Test sample 23 | 109.37 |
| Control Sample 24 | 13.12 | Test sample 24 | 100.28 |

**Table 3: Caspase-9 activity assay with Resveratrol:** Resveratrol (90 ug/ml) treated Caski cell lines (n=24) that were cultured for 48 hrs showed high activity levels of caspase-9. Untreated cells (n=24) showed insignificant levels of caspase-9. Cervical Caski cell lines were left untreated or treated with 90 ug/ml of Resveratrol as described in methods. Nuclei free cell lysates were prepared from sample and used in the caspase-9 activity assay using the colorimetric substrates Z-LEHD-FMK**.** Absorbance was read at 405 nm. One unit of caspase activity is the amount of enzyme activity liberating 1 pmol of substrates/min. Values shown is means of 24 independent experiments carried out in duplicates for caspase-9 ($p<0.001$ for all).

**Table 4: Caspase 9 activity assay in untreated and NAC (10 mM) treated Caski Cell Lines**

| **Untreated Caski cell lines (Controls)** | **Caspase-9 activity** (pMol Substrate Hydrolyzed/Minute) | **NAC (20 mM) + Caski Cell Lines** | **Caspase-9 activity** (pMol Substrate Hydrolyzed/Minute) |
|---|---|---|---|
| Control Sample 1 | 3.67 | Test sample 1 | 124.11 |
| Control Sample 2 | 5.78 | Test sample 2 | 142.12 |
| Control Sample 3 | 8.91 | Test sample 3 | 119.87 |
| Control Sample 4 | 13.45 | Test sample 4 | 134.17 |
| Control Sample 5 | 2.98 | Test sample 5 | 101.25 |
| Control Sample 6 | 11.26 | Test sample 6 | 122.12 |
| Control Sample 7 | 5.78 | Test sample 7 | 148.21 |
| Control Sample 8 | 9.23 | Test sample 8 | 123.29 |
| Control Sample 9 | 6.87 | Test sample 9 | 149.11 |
| Control Sample 10 | 9.45 | Test sample 10 | 110.57 |
| Control Sample 11 | 14.21 | Test sample 11 | 145.33 |
| Control Sample 12 | 4.87 | Test sample 12 | 149.98 |
| Control Sample 13 | 9.43 | Test sample 13 | 120.12 |

| | | | |
|---|---|---|---|
| Control Sample 14 | 7.23 | Test sample 14 | 123.26 |
| Control Sample 15 | 2.78 | Test sample 15 | 138.38 |
| Control Sample 16 | 6.87 | Test sample 16 | 105.07 |
| Control Sample 17 | 11.23 | Test sample 17 | 90.43 |
| Control Sample 18 | 3.67 | Test sample 18 | 122.41 |
| Control Sample 19 | 10.29 | Test sample 19 | 107.82 |
| Control Sample 20 | 4.81 | Test sample 20 | 92.26 |
| Control Sample 21 | 9.66 | Test sample 21 | 143.72 |
| Control Sample 22 | 12.82 | Test sample 22 | 126.11 |
| Control Sample 23 | 8.29 | Test sample 23 | 99.27 |
| Control Sample 24 | 13.12 | Test sample 24 | 93.98 |

**Table 4: Caspase-9 activity assay with NAC:** NAC (20 mM) treated Caski cell lines (n=24) that were cultured for 48 hrs showed high activity levels of caspase-9. Untreated cells (n=24) showed insignificant levels of caspase-9. Cervical Caski cell lines were left untreated or treated with 20 mM of NAC as described in methods. Nuclei free cell lysates were prepared from sample and used in the caspase-9 activity assay using the colorimetric substrates Z-LEHD-FMK**.** Absorbance was read at 405 nm. One unit of caspase-9 activity is the amount of enzyme activity liberating 1 pmol of substrates/min. Values shown is means of 24 independent experiments carried out in duplicates for caspase-9 ($p<0.001$ for all).

**Table 5: Caspase 3 activity assay in untreated and Hydrogen peroxide (2 mM) treated Caski Cell Lines**

| **Untreated Caski cell lines (Controls)** | **Caspase-3 activity** (pMol Substrate Hydrolyzed/Minute) | **Hydrogen peroxide (2 mM) + Caski Cell Lines** | **Caspase-3 activity** (pMol Substrate Hydrolyzed/Minute) |
|---|---|---|---|
| Control Sample 1 | 2.33 | Test sample 1 | 1.33 |
| Control Sample 2 | 2.46 | Test sample 2 | 3.12 |
| Control Sample 3 | 7.23 | Test sample 3 | 5.34 |
| Control Sample 4 | 11.23 | Test sample 4 | 6.32 |
| Control Sample 5 | 1.77 | Test sample 5 | 1.33 |
| Control Sample 6 | 9.34 | Test sample 6 | 3.45 |
| Control Sample 7 | 4.56 | Test sample 7 | 2.12 |
| Control Sample 8 | 8.12 | Test sample 8 | 3.65 |
| Control Sample 9 | 5.77 | Test sample 9 | 2.76 |
| Control Sample 10 | 8.04 | Test sample 10 | 4.12 |
| Control Sample 11 | 12.56 | Test sample 11 | 5.98 |
| Control Sample 12 | 3.87 | Test sample 12 | 2.11 |
| Control Sample 13 | 8.12 | Test sample 13 | 3.67 |
| Control Sample 14 | 5.95 | Test sample 14 | 2.78 |
| Control Sample 15 | 1.55 | Test sample 15 | 1.03 |
| Control Sample 16 | 5.57 | Test sample 16 | 3.87 |
| Control Sample 17 | 9.43 | Test sample 17 | 2.91 |
| Control Sample 18 | 2.65 | Test sample 18 | 1.34 |
| Control Sample 19 | 7.99 | Test sample 19 | 4.02 |
| Control Sample 20 | 3.66 | Test sample 20 | 1.65 |
| Control Sample 21 | 8.54 | Test sample 21 | 2.09 |
| Control Sample 22 | 11.02 | Test sample 22 | 3.87 |
| Control Sample 23 | 6.75 | Test sample 23 | 3.87 |
| Control Sample 24 | 10.92 | Test sample 24 | 5.78 |

**Table 5: Caspase-3 activity assay with Hydrogen peroxide (H2O2):** H2O2 (2 mM) treated Caski cell lines (n=24) that were cultured for 48 hrs showed high activity levels of caspase-3. Untreated cells (n=24) showed insignificant levels of caspase-3. Cervical Caski cell lines were left untreated or treated with 2 mM of hydrogen peroxide as described in methods. Nuclei free cell lysates were prepared from sample and used in the caspase-3 activity assay using the colorimetric substrates DEVD-pNA. Absorbance was read at 405 nm. One unit of caspase activity is the amount of enzyme activity liberating 1 pmol of substrates/min. Values shown is means of 24 independent experiments carried out in duplicates for caspase-3 ($p<0.001$ for all).

**Table 6: Caspase 9 activity assay in untreated and Hydrogen peroxide (H2O2) treated Caski Cell Lines**

| **Untreated Caski cell lines (Controls)** | **Caspase-9 activity** (pMol Substrate Hydrolyzed/Minute) | **Hydrogen peroxide (2mM) + Caski Cell Lines** | **Caspase-9 activity** (pMol Substrate Hydrolyzed/Minute) |
|---|---|---|---|
| Control Sample 1 | 6.23 | Test sample 1 | 2.12 |
| Control Sample 2 | 2.87 | Test sample 2 | 1.34 |
| Control Sample 3 | 6.23 | Test sample 3 | 4.12 |
| Control Sample 4 | 10.22 | Test sample 4 | 5.33 |
| Control Sample 5 | 2.13 | Test sample 5 | 1.01 |
| Control Sample 6 | 8.22 | Test sample 6 | 6.32 |
| Control Sample 7 | 5.12 | Test sample 7 | 3.09 |
| Control Sample 8 | 7.98 | Test sample 8 | 4.76 |
| Control Sample 9 | 6.28 | Test sample 9 | 3.05 |
| Control Sample 10 | 9.54 | Test sample 10 | 6.11 |
| Control Sample 11 | 10.23 | Test sample 11 | 4.47 |
| Control Sample 12 | 4.27 | Test sample 12 | 2.54 |
| Control Sample 13 | 7.43 | Test sample 13 | 4.65 |
| Control Sample 14 | 4.98 | Test sample 14 | 3.12 |
| Control Sample 15 | 3.54 | Test sample 15 | 1.23 |
| Control Sample 16 | 7.12 | Test sample 16 | 2.87 |
| Control Sample 17 | 4.91 | Test sample 17 | 2.98 |
| Control Sample 18 | 7.98 | Test sample 18 | 2.12 |
| Control Sample 19 | 11.34 | Test sample 19 | 3.77 |
| Control Sample 20 | 14.54 | Test sample 20 | 4.87 |
| Control Sample 21 | 5.87 | Test sample 21 | 3.19 |
| Control Sample 22 | 10.29 | Test sample 22 | 5.11 |
| Control Sample 23 | 9.43 | Test sample 23 | 2.91 |
| Control Sample 24 | 4.87 | Test sample 24 | 2.11 |

**Table 6: Caspase-9 activity assay with Hydrogen peroxide (H2O2):** H2O2 (2 mM) treated Caski cell lines (n=24) that were cultured for 48 hrs showed high activity levels of caspase-9. Untreated cells (n=24) showed insignificant levels of caspase-9. Cervical Caski cell lines were left untreated or treated with 2 mM of hydrogen peroxide as described in methods. Nuclei free cell lysates were prepared from sample and used in the caspase-9 activity assay using the colorimetric substrates Z-LEHD-FMK**.** Absorbance was read at 405 nm. One unit of caspase-9 activity is the amount of enzyme activity liberating 1 pmol of substrates/min. Values shown is means of 24 independent experiments carried out in duplicates for caspase-9 ($p<0.001$ for all).

**Table 7: Modulation Study: Caspase 3 activity assay in untreated and Hydrogen peroxide (2 mM) along with Resveratrol (90 ug/ml) treated Caski Cell Lines**

| **Untreated Caski cell lines (Controls)** | **Caspase-3 activity** (pMol Substrate Hydrolyzed/Minute) | **Hydrogen peroxide (2 mM) + Resveratrol (90 ug/ml) + Caski Cell Lines** | **Caspase-3 activity** (pMol Substrate Hydrolyzed/Minute) |
|---|---|---|---|
| Control Sample 1 | 2.33 | Test sample 1 | 72.12 |
| Control Sample 2 | 2.46 | Test sample 2 | 77.34 |
| Control Sample 3 | 7.23 | Test sample 3 | 78.12 |
| Control Sample 4 | 11.23 | Test sample 4 | 82.11 |
| Control Sample 5 | 1.77 | Test sample 5 | 70.23 |
| Control Sample 6 | 9.34 | Test sample 6 | 79.56 |
| Control Sample 7 | 4.56 | Test sample 7 | 75.47 |
| Control Sample 8 | 8.12 | Test sample 8 | 78.33 |
| Control Sample 9 | 5.77 | Test sample 9 | 76.63 |
| Control Sample 10 | 8.04 | Test sample 10 | 79.89 |
| Control Sample 11 | 12.56 | Test sample 11 | 83.12 |
| Control Sample 12 | 3.87 | Test sample 12 | 74.62 |
| Control Sample 13 | 8.12 | Test sample 13 | 77.78 |
| Control Sample 14 | 5.95 | Test sample 14 | 77.12 |
| Control Sample 15 | 1.55 | Test sample 15 | 68.34 |
| Control Sample 16 | 5.57 | Test sample 16 | 77.47 |
| Control Sample 17 | 9.43 | Test sample 17 | 70.43 |
| Control Sample 18 | 2.65 | Test sample 18 | 78.33 |
| Control Sample 19 | 7.99 | Test sample 19 | 81.69 |
| Control Sample 20 | 3.66 | Test sample 20 | 84.89 |
| Control Sample 21 | 8.54 | Test sample 21 | 76.22 |
| Control Sample 22 | 11.02 | Test sample 22 | 80.64 |
| Control Sample 23 | 6.75 | Test sample 23 | 77.34 |
| Control Sample 24 | 10.92 | Test sample 24 | 73.11 |

**Table 7: Caspase-3 activity assay with Competition between Hydrogen peroxide (H2O2) and Resveratrol (90 ug/ml):** H2O2 (2 mM) along with Resveratrol (90 ug/ml) treated Caski cell lines (n=24) that were co-cultured for 48 hrs showed augmented / ameliorated activity levels of caspase-3. Untreated cells (n=24) showed insignificant levels of caspase-3. Cervical Caski cell lines were left untreated or treated with 2 mM of hydrogen peroxide + 90 ug/ml Resveratrol as described in methods. Nuclei free cell lysates were prepared from sample and used in the caspase-3 activity assay using the colorimetric substrates DEVD-pNA. Absorbance was read at 405 nm. One unit of caspase activity is the amount of enzyme activity liberating 1 pmol of substrates/min. Values shown is means of 24 independent experiments carried out in duplicates for caspase-3 ($p<0.001$ for all).

**DISCUSSION**:

Globally, cervical cancer is the second most common malignancy in women **(9)**. Resveratrol is a phytoalexin present in a wide variety of fruits and vegetables such as grapes, berries, peanuts, and various herbs **(1,5)**.

During apoptosis, the downstream signals are transmitted via caspases. Caspases are synthesized as inactive proenzymes and their activation during apoptosis results in cleavage at specific aspartate cleavage sites. While initiator caspases-8 and -9 undergo autocatalytic activation, executioner pro-caspase-3 is processed by initiator caspases. Caspase-3 is one of the key executioners of apoptosis, being responsible either partially or totally for the proteolytic cleavage of many key proteins, such as the nuclear enzyme PARP, followed by DNA fragmentation.

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 as well as on Caspases-3 and 9 activities. Moreover, Resveratrol treated Caski cells also exhibited an augmented caspase-9 and -3 activities which were found to be dose dependent and time dependent. 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 apoptosis **(3)**.

Another interesting observation in the present study was with respect to Caski cells culture along with hydrogen peroxide as well as separate cultures receiving a combination of both hydrogen peroxide and resveratrol. Our data showing hydrogen peroxide further augmenting proliferation / or unresponsive towards caspase-3 activity reflects upon as well as in agreement with earlier reports that reactive oxygen species (ROS) contributes to progression of a variety of cancers including cervical cancer **(8).**

The most interesting observation in the present study was that the Resveratrol induced amelioration of caspase-3 activity in hydrogen peroxide treated Caski cells. Recent report shows that several polyphenols/natural antioxidants possess synergistic characteristics with cancer chemotherapeutic agents. Hence, an appropriate combination of polyphenols with existing chemotherapeutics will lead to a reduction in side effects without decreasing the chemotherapeutic effects **(6)**. Therefore, the preliminary data obtained in the present study may probably be helpful in carrying out in-depth studies for using an appropriate combination of Resveratrol with existing chemotherapeutics of cervical cancer, which may in turn, lead to a reduction in side effects without decreasing the chemothe- rapeutic effects.

Therefore, in summary, it is hoped that the present study may throw some light in understanding and moving a step further about elucidating the complete molecular mechanism, and in turn, exploring of Resveratrol to be used as an adjunct in the management of cervical cancer.

**CONCLUSION:**

Untreated Caski cells exhibited insignificant or negligible Caspases -3 and 9 activities.Resveratrol (90 ug/ml) and NAC (20 mM) exhibited high magnitude Caspases 3 and 9 activities, thereby indicating the induction of apoptosis in 48 hr Caski cell cultures.The Resveratrol induced a slightly higher apoptotic effect than NAC.Resveratrol appreciably abrogated the augmented cell proliferation effect of Hydrogen peroxide in 48 hr Caski cell cultures.Therefore, Resveratrol could probably be employed as safe and economical alternative / adjunct in the management of cervical cancer.

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