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# ROLE OF *PTEN* IN MODULATING PREVENTIVE EFFECT OF 3,4-DHPEA AGAINST OXIDATIVE STRESS

# OKSİDATİF STRESE KARŞI 3,4-DHPEA'NIN KORUYUCU ETKİSİNİN MODÜLASYONUNDA PTEN'İN ROLÜ

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**ABSTRACT**

Prostate cancer (PCa) with a Phosphate tensin homolog (*PTEN)* gene mutation can become aggressive. In this study, it was hypothesized that the *PTEN* mutational status in PCa cell lines might modify the chemopreventive effect of 3,4-dihydroxyphenyl ethanol (3,4-DHPEA), thus, determining the cells' ability to manage oxidative stress created by N,N,N′ ,N′ -Tetrakis(2-pyridylmethyl)ethylenediamine (TPEN). The human PCa cell lines with varying *PTEN* status, DU-145 (*PTEN* +/−), 22Rv1 (*PTEN* +/+), and PC3 (*PTEN* −/−), were treated with up to 100 µM of 3,4-DHPEA and/or up to 6.5 µM of TPEN for 24 hours. The viability of cells after treatment was measured with Cell Titer-Glo Luminescent Assay and analyzed with the analysis of variance test. 3,4-DHPEA treatment as high as 50 µM had the greatest cytotoxic effect on 22Rv1 followed by DU-145 and PC3. Similar overall trend was also observed with TPEN treatment. When the cells were treated with TPEN at IC50 doses, 3,4-DHPEA co-treatment still showed cytotoxicity in the same order as 3,4-DHPEA treatment alone. No chemoprotective effect due to 3,4-DHPEA was observed. The data is still consistent with the hypothesis that oxidative stress inducing agents are dependent on the *PTEN* status. This is consistent with 22Rv1 with wild type *PTEN* showing the greatest susceptibility to 3,4-DHPEA.

**Keywords:** *PTEN* status; TPEN; 3,4-DHPEA; oxidative stress; prostate cancer

**ÖZ**

Phosphate tensin homolog (*PTEN)* gen mutasyonuna sahip prostat kanseri (PCa) agresif hale gelebilir. Bu çalışmada, PCa hücre hatlarındaki *PTEN* mutasyonel durumunun, 3,4-dihydroxyphenyl ethanolün (3,4-DHPEA) kemopreventif etkisini değiştirebileceği ve böylece hücrelerin N,N,N′ ,N′ -Tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) tarafından oluşturulan oksidatif stresi yönetme yeteneğini belirlediği hipotezi ileri sürülmüştür. Farklı *PTEN* statüsüne sahip DU-145 (*PTEN* +/−), 22Rv1 (*PTEN* +/+) ve PC3 (*PTEN* −/−) insan PCa hücre hatları 24 saat boyunca 100 µM'a kadar 3,4-DHPEA ve/veya 6,5 µM'a kadar TPEN ile muamele edildi. Muameleden sonra hücre canlılıkları Cell Titer-Glo Luminescent Assay ile ölçüldü ve varyans analizi testi ile analiz edildi. 50 µM kadar yüksek 3,4-DHPEA uygulaması 22Rv1 üzerinde en fazla sitotoksik etki gösterdi ve bunu DU-145 ve PC3 izledi. Benzer bir genel eğilim *TPEN* muamelesi ile de gözlemlendi. *TPEN* uygulamasında IC50 değerleri 22Rv1 için 4.718 µM, DU-145 için 4.963 µM ve PC3 için 5.245 µM idi. Hücrelerin IC50 dozunda *TPEN* ile birlikte 3,4-DHPEA ile muamelesi 3,4-DHPEA'nın yalnız uygulaması ile aynı şekilde sitotoksisite göstermiştir. 3,4-DHPEA'ya bağlı herhangi bir kemopreventif koruma etkisi gözlemlenmemiştir. Sonuçlar oksidatif stres oluşturan ajanların *PTEN* statüsüne bağlı oldukları hipotezi ile örtüşmektedir. Bu, wild tip *PTEN* içeren 22Rv1'in 3,4-DHPEA'ya karşı en büyük duyarlılığı göstermesi ile tutarlıdır.

**Anahtar kelimeler:** *PTEN* statüsü; TPEN; 3,4-DHPEA; oksidatif stres; prostat kanseri

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## INTRODUCTION

*PTEN* is a tumor suppressor gene on chromosome 10q23.3. Loss of *PTEN* is frequently encountered in PCa, thus a definite prognosis cannot be made (1-3). The *PTEN* gene expresses a lipid/protein phosphatase that negatively impacts the phosphatidylinositol-3′-OH-kinase (PI3K)/AKT pathway. Loss of expression by either single or double alleles of *PTEN* gene has been reported in PCa progression leading to critical clinical outcomes (2,3). Prostate cancers which have a *PTEN* gene mutation can become very aggressive. This mutation is a characteristic of PC3 and C4-2 cell lines of PCa. In these cell lines, both alleles have either parts of, or the whole gene, deleted to have the mutation. Cell line 22Rv1 does not have a mutation in the *PTEN*; causing this particular tumor suppressor to function normally. And the *PTEN* alleles in DU-145 are heterozygous for the mutation; the presence of one wild type copy could make the cells intermediate in response to oxidative stress (3).

Olive oil is composed of a vast array of compounds that includes phenolic substances such as 3,4-DHPEA and their secoiridoid derivatives (4). The olive oil phenolics have the potential cancer preventive activity that may show effect during either cancer initiation, promotion, or progression phases. One potential cancer preventive mechanism may be the antioxidant effect by these phenolic compounds. In the presence of reactive oxygen species (ROS), DNA may react with ROS and the resulting mutations may lead to cancer initiation (5). Several studies conducted on human chondrocytes, breast and prostate cancer cell lines, blood monocytes, neuroblastoma cells lines, and Jurkat cells have reported that 3,4-DHPEA has the ability to lower the oxidative DNA damage inflicted by the hydrogen peroxide exposure (6-10).

A membrane permeable zinc chelator, TPEN, was reported to cause apoptosis in several cancer studies (11-15) through several modes of action including the p53 transcription factor (14,15), and the X-linked inhibitor of apoptosis protein (XIAP) (13). However, the exact molecular cascade of events triggered by TPEN that results in apoptosis is not fully discovered. Hashemi et al. (2007) reported that toxic effect of TPEN might be through increased oxidative stress as a result of zinc deficiency as the cytotoxic effect of TPEN was inhibited by the antioxidant N-acetyl-L-cysteine (NAC) (12).

In this study, it was hypothesized that the *PTEN* mutational status in PCa cell lines might modify the chemopreventive effect of 3,4-DHPEA, thus, determining the cells' ability to manage oxidative stress created by TPEN.

## MATERIALS-METHOD

### *Cells Lines and Culture*

The human prostate adenocarcinoma cell lines DU-145, 22Rv1, and PC3 were obtained from Dr. William Gmeiner from Wake Forest University Medical School (Winston Salem, NC, USA) and the study was performed in Dr. Gmeiner's Laboratory. PCa cell lines in the study had different *TPEN* status as follows; DU-145 (*PTEN* +/−), 22Rv1 (*PTEN* +/+), and PC3 (*PTEN* −/−).

Human PCa cell lines were grown in RPMI-1640 (Lonza, Allendale, NJ, USA) media supplemented with 10% fetal bovine serum (FBS) (Lonza, Allendale, NJ, USA) at 37°C in the presence of 5% $CO_2$ in a humidified incubator.

### *Chemicals and Treatments*

The PCa cells were seeded in 96-well plates at $6x10^3$ cells/well concentration. After an overnight incubation, TPEN (Sigma, #P4413, St. Louis, MO, USA) was used as an exogenous oxidant to introduce oxidative stress to the PCa cells through zinc chelation. TPEN concentrations applied ranged from 0-6.5 μM. Cells were also treated with 3,4-DHPEA (Cayman Chemical, #10597-60-1, Ann Arbor, MI, USA) (0-100 μM) for 24 hours.

### *Study Design*

TPEN and 3,4-DHPEA were prepared in dimethyl sulfoxide (DMSO) and ethanol, respectively, and further diluted in RPMI-1640 medium with 10% FBS to the desired concentration prior to each experiment. To investigate the effect of 3,4-DHPEA treatment on the PCa cells in response to oxidative stress experiments were set as follows: (1) 24 h treatment of 3,4-DHPEA on all three cell lines; (2) 24 h treatment of TPEN on all three cell lines; and (3) 24 h co-treatment of 3,4-DHPEA in the presence of TPEN at IC50 concentration on all three cell lines.

### *Luminescent Cell Viability Assay*

The remaining live cell count after treatment was measured with the CellTiter-Glo® luminescent cell viability assay by Promega (Madison, WI, USA) following the manufacturer's instructions. Luminescence was measured using a microplate reader and the IC50 values were calculated.

### *Statistical Analysis*

The cytotoxic effects of treatments were determined by consolidating the results of three independent experiments. Results were compared using 2-way ANOVA (Factor 1: Treatment concentration, Factor 2: Cell line type) test. Post-hoc analysis was performed using Bonferroni test. P values under 0.05 were considered significantly different.

## RESULTS

Results show that the effects of 3,4-DHPEA treatment dose, cell type, and the interaction between the 3,4-DHPEA treatment dose and cell type were statistically significant (P<0.001) (Table I). In the follow-up one-way ANOVA test for PC3 cells, all possible pairwise comparisons among the 3,4-DHPEA treatment doses were significantly different (P<0.001, Bonferroni) except for zero and 10 uM pair (P=0.155). Likewise, all possible pairwise comparisons among 3,4-DHPEA concentrations for DU-145 cells were significantly different (P<0.001) except for zero and 10 uM pair (P=1.000). However, all pairwise comparisons among 3,4-DHPEA concentrations for 22Rv1 were significantly different (P<0.001). The 100 μM 3,4-DHPEA treatment for 24 h resulted similar level decrease (~99%) in viability of the DU-145 and 22Rv1 cell lines (Figure 1). Overall, 3,4-DHPEA treatment showed the greatest cytotoxic effect on the cell lines 22Rv1 and DU-145 by killing higher percentages of these cells compared to PC3 cell line (Figure 1).

Table I. Percent viability values compared to ethanol after 3,4-DHPEA treatment on human prostate cancer cell lines PC3 (*PTEN* -/-), DU-145 (*PTEN* +/-), and 22Rv1 (*PTEN* +/+) for 24 h. Values are given as mean±standard deviation.

| | PC3 | | | | DU-145 | | | | 22Rv1 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 3,4-DHPEA treatment (24 h) | 0 µM (EtOH) | 10 µM | 50 µM | 100 µM | 0 µM (EtOH) | 10 µM | 50 µM | 100 µM | 0 µM (EtOH) | 10 µM | 50 µM | 100 µM |
| Mean± StDev | 100.0± 1.0 | 93.5± 2.6 | 82.1± 4.8 | 65.4± 1.7 | 100.0± 2.2 | 102.1± 4.3 | 70.4± 2.0 | 1.2± 0.2 | 100.0± 4.3 | 77.5± 5.0 | 50.0± 4.9 | 0.4± 1.9 |
| P† value | <0.001 | | | | <0.001 | | | | <0.001 | | | |
| | Between cell types | | | | Between 3,4-DHPEA doses | | | | Interaction between cell types & 3,4-DHPEA doses | | | |
| P‡ value | <0.001 | | | | <0.001 | | | | <0.001 | | | |

† One-way ANOVA; ‡ Two-way ANOVA.

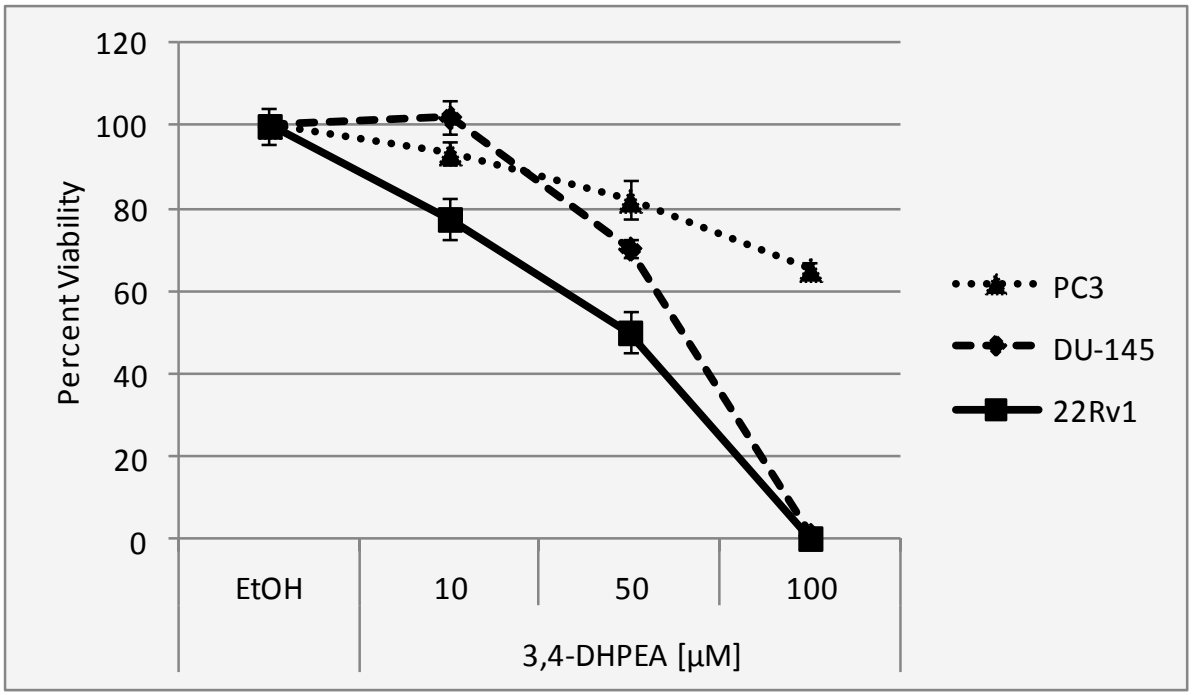


Figure 1. 3,4-DHPEA treatment on human prostate cancer cell lines PC3 (*PTEN* -/-), DU-145 (*PTEN* +/-), and 22Rv1 (*PTEN* +/+) for 24 h. Values are given as mean±standard deviation.

TPEN treatment, cell type, and the interaction between the TPEN dose and cell type were statistically significant (P<0.001) (Table II). The follow-up Bonferroni post-hoc test revealed that all possible pairwise comparisons among the TPEN concentrations applied were significantly different on PC3 cells (P<0.001, Bonferroni) except for the 0-4.5 and 6.0-6.5 µM pairs (P=1.000). Similar significant difference was observed among pairwise comparisons of TPEN concentrations on DU-145 cell line (P<0.001) except for the 0-4.5 µM pair and the pairs between 5.5, 6.0, and 6.5 µM (P>0.180). However, for the 22Rv1 cell line, all pairwise comparisons of TPEN doses were significantly different (P<0.001) except for those between 5.0 and 6.5 µM (P>0.929). Great cytotoxicity (~95%) was observed on all cell lines when treated with 6 or more µM TPEN for 24 h (Figure 2).

The DU-145 and 22Rv1 cell lines seemed to be more chemo-sensitive to TPEN than PC3, consistent with the data that shows higher cytotoxicity by TPEN at lower doses (5.0-5.5 µM) on DU-145 and 22Rv1 cell lines compared to PC3 (Figure 2). IC50 values for 24 h TPEN treatment were calculated to be 5.245 µM for PC3, 4.963 µM for DU-145, and 4.718 µM for 22Rv1 (Figure 2).

Table II. Percent viability values compared to DMSO after TPEN treatment on PC3 (*PTEN* -/-), DU-145 (*PTEN* +/-), and 22Rv1 (*PTEN* +/+) for 24 h. Values are given as mean±standard deviation.

| | PC3 | | | | | | DU-145 | | | | | | 22Rv1 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| TPEN treatment (24 h) | 0 µM (DMSO) | 4.5 µM | 5.0 µM | 5.5 µM | 6.0 µM | 6.5 µM | 0 µM (DMSO) | 4.5 µM | 5.0 µM | 5.5 µM | 6.0 µM | 6.5 µM | 0 µM (DMSO) | 4.5 µM | 5.0 µM | 5.5 µM | 6.0 µM | 6.5 µM |
| Mean± StDev | 100.0± 3.9 | 96.7± 3.2 | 76.9± 9.0 | 33.0± 11.9 | 6.8± 2.3 | 4.5± 0.6 | 100.0± 3.3 | 97.6± 3.5 | 46.1± 7.7 | 13.1± 5.0 | 3.7± 0.8 | 2.8± 1.0 | 100.0± 10.8 | 79,4± 10.4 | 11.1± 5.4 | 6.5± 2.3 | 3.8± 1.3 | 5.3± 1.2 |
| P† value | <0.001 | | | | | | <0.001 | | | | | | <0.001 | | | | | |
| | Between cell types | | | | | | Between TPEN doses | | | | | | Interaction between cell types & TPEN doses | | | | | |
| P‡ value | <0.001 | | | | | | <0.001 | | | | | | <0.001 | | | | | |

† One-way ANOVA; ‡ Two-way ANOVA.

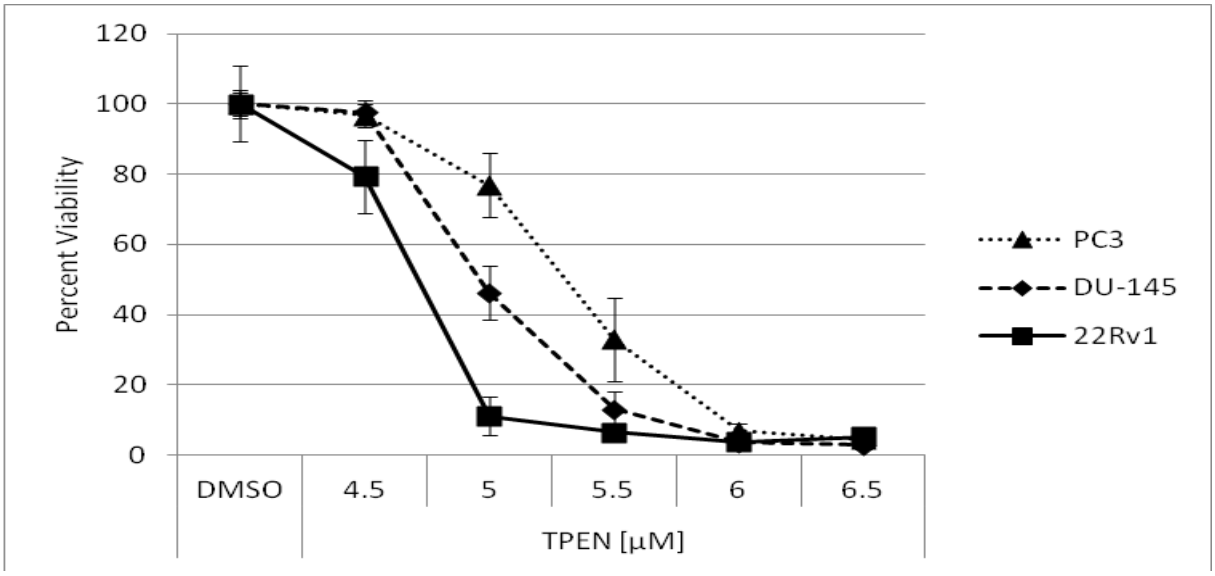


Figure 2. TPEN treatment on PC3 (*PTEN* -/-), DU-145 (*PTEN* +/-), and 22Rv1 (*PTEN* +/+) for 24 h. Values are given as mean±standard deviation.

The potential benefit of antioxidative effect of 3,4-DHPEA against the TPEN cytotoxicity was investigated in another experimental setup where 3,4-DHPEA was applied on the cell lines together with TPEN at IC50 doses on PC3, DU-145, and 22Rv1 cell lines. TPEN alone treatment was considered to be the negative control and the cell viability was adjusted to be 100% for comparison purpose. The 3,4-DHPEA treatment in the presence of TPEN at IC50 was still cytotoxic on all three cell lines in a dose-dependent manner (P<0.001 for all cell lines) (Table III). Along with the 3,4-DHPEA treatment dose, cell type, and the interaction in between them were statistically significant (P<0.001) (Table III). The post-hoc analysis showed that all possible pairwise comparisons among the 3,4-DHPEA doses were significantly different on PC3, DU-145, and 22Rv1 viabilities (P<0.001 for all 3 cell lines). The 100 µM 3,4-DHPEA treatment for 24 h in the presence of TPEN at IC50 resulted similar level decrease (~99%) in viability of the DU-145 and 22Rv1 cell lines (Figure 3). Overall, the co-treatment showed the greatest cytotoxic effect on the cell lines 22Rv1 and DU-145 by killing higher percentages of these cells compared to PC3 cell line (Figure 3).

## DISCUSSION

*PTEN* losses occur at late stage in PCa development. This may mean that PCa development is not a *PTEN*-dependent process and *PTEN* loss may be responsible from the late stage progression. In this study, three different PCa cells with different *PTEN* status were treated with TPEN to cause oxidative stress through zinc chelation. Next, it was aimed to investigate the

Table III. Percent viability values compared to ethanol after 3,4-DHPEA co-treatment with TPEN at IC50 doses on human prostate cancer cell lines PC3 (*PTEN* -/-), DU-145 (*PTEN* +/-), and 22Rv1 (*PTEN* +/+) for 24 h. Values are given as mean±standard deviation.

| | PC3 | | | | DU-145 | | | | 22Rv1 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| TPEN [μM] at IC50 | 5.245 μM | | | | 4.963 μM | | | | 4.718 μM | | | |
| 3,4-DHPEA treatment (24 h) | 0 μM (EtOH) | 10 μM | 50 μM | 100 μM | 0 μM (EtOH) | 10 μM | 50 μM | 100 μM | 0 μM (EtOH) | 10 μM | 50 μM | 100 μM |
| Mean± StDev | 100.0±1.0 | 88.7±4.9 | 76.1±1.7 | 48.2±5.5 | 100.0±4.9 | 80.1±4.3 | 46.3±4.6 | 0.9±0.2 | 100.0±6.0 | 60.1±7.0 | 25.9±4.9 | 0.2±1.9 |
| P† value | <0.001 | | | | <0.001 | | | | <0.001 | | | |
| | Between cell types | | | | Between 3,4-DHPEA doses | | | | Interaction between | | | |
| P‡ value | <0.001 | | | | <0.001 | | | | <0.001 | | | |

† One-way ANOVA; ‡ Two-way ANOVA.

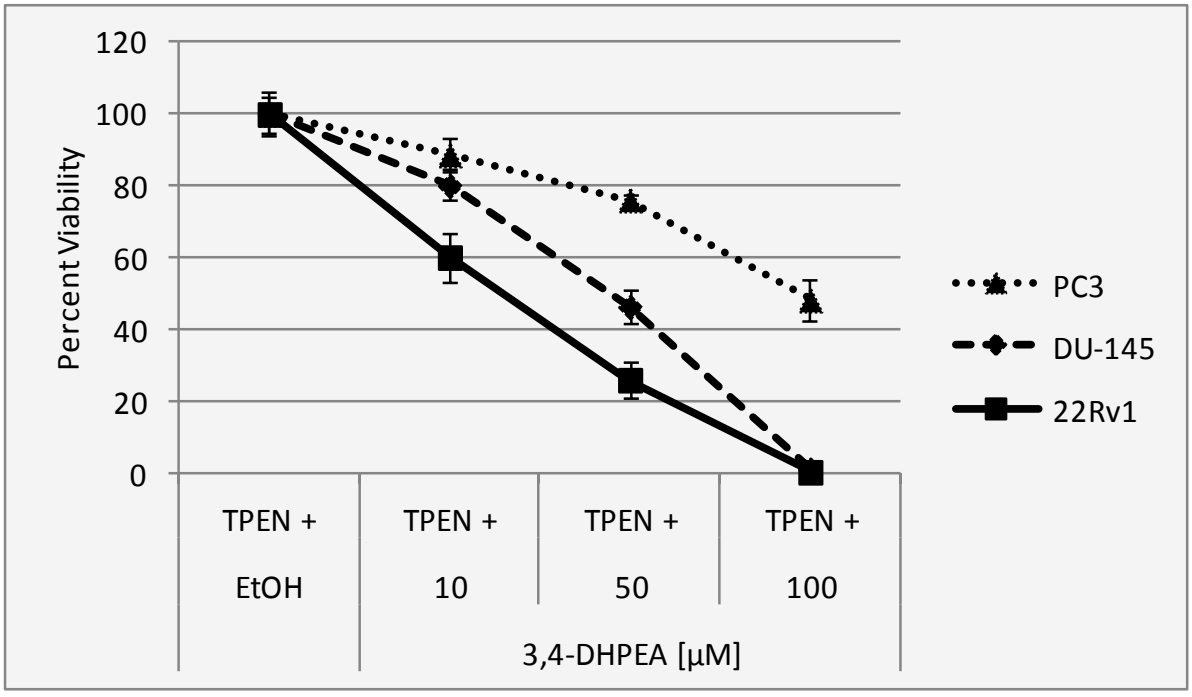


Figure 3. 3,4-DHPEA co-treatment with TPEN at IC50 doses on human prostate cancer cell lines PC3 (*PTEN* -/-), DU-145 (*PTEN* +/-), and 22Rv1 (*PTEN* +/+) for 24 h. PC3 was treated with 5.2 μM TPEN, DU-145 with 5.0 μM TPEN, and 22Rv1 with 4.7 μM TPEN. Values are given as mean±standard deviation.

potential chemopreventive effect of 3,4-DHPEA, the main phenolic compound in olive oil, on PCa cell lines to see whether *PTEN* status has a role in survival.

Effect of *PTEN* function on sensitization of cancer cells against exogenous agents have been the topic of several research efforts with mixed results. Kao et al. (2007) re-expressed the *PTEN* in *PTEN*-null U251 cell line and inhibited the DNA double strand break repairs, sensitizing the cells to ionizing radiation (16). In our results, *PTEN*-null PC3 cells were the most resistant against the cytotoxic effects of both 3,4-DHPEA and TPEN. DU-145 cell line with heterozygous *PTEN* was less resistant than PC3 and 22Rv1 was the most sensitive to 3,4-DHPEA and TPEN treatments. This result could be explained by the fact that PCa development might have followed different molecular pathways in the presence or absence of functional *PTEN* gene. Therefore, the cell lines used in this study might have developed different strategies to cope with the oxidative stress caused by TPEN treatment.

A recent study has showed that olive oil consumption is negatively related with the occurrence of breast and

gastrointestinal cancers (17). However, knowledge on olive oil and PCa relationship is not comprehensive. The phenolic content of olive oil is also another area where further research needs to be conducted (18). In this respect, the findings of the current study feed much needed information to the field. While there was no reported study elucidating the preventive effect of 3,4-DHPEA against the oxidative stress caused by TPEN in cancer cells, studies are available on the preventive effect of 3,4-DHPEA against the DNA damage in various cancer types induced by the $H_2O_2$ treatment (6-9). Mostly, the preventive effect of 3,4-DHPEA was observed when it was co-incubated with $H_2O_2$, suggesting that 3,4-DHPEA acts as a scavenger for $H_2O_2$ preventing DNA damage (19,20). The same effect was also observed when $H_2O_2$ was applied first and 3,4-DHPEA was applied after the removal of the $H_2O_2$ on PCa cells, which is consistent with the idea that 3,4-DHPEA enters the cells to revert the $H_2O_2$ effect. DNA preventive effect was positively correlated with the duration of 3,4-DHPEA treatment up to 6 h. However, 24 h long or high dose 3,4-DHPEA incubation unexpectedly lost the preventive effect, which could be explained by the oxidative effect due to the 3,4-DHPEA itself.

In another study, high dose (75-100 μM) of 3,4-DHPEA showed growth inhibitory effect in various cancer cell lines (17). Also it was suggested that the cell lines take up and metabolize 3,4-DHPEA at different effectiveness levels, which may be alter the amount of $H_2O_2$ the cells will be faced. The ability of the cells to modify the $H_2O_2$ will be of pivotal importance in setting up the sensitivity of the cells. As a result, PCa cell lines of LNCaP and PC3 were found to be less sensitive to the growth retardation effect of 3,4-DHPEA compared to the breast and colon cancer lines (17). The same hypothesis was also supported in another study where catalase added to the culture media to remove $H_2O_2$ lowered the antiproliferative effect of 3,4-DHPEA (21).

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