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EXPERIMENTAL STUDY

# Increased ROS alters E-/N-cadherin levels and promotes migration in prostate cancer cells

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**ABSTRACT**

BACKGROUND: Increased ROS has been reported to cause a change in E- and N-cadherin levels, and consequently promotes migrative behaviors in pancreas and breast cancer cells. In this study, the effect of a sublethal dose of $H_2O_2$ on E- and N-cadherin levels, and on migrative behaviors of prostate cancer cells was investigated.

METHODS: To determine a sublethal concentration of $H_2O_2$ on cell proliferation and ROS production were examined using WST-1 and DCFH-DA assays, respectively. E- and N-cadherin protein and mRNA levels were investigated by western blotting and real-time PCR, respectively. The migrative potentials of the cells were examined by Cytoselect 96-well cell migration assay.

RESULTS: Treatment of the prostate cancer cells with a sublethal dose of $H_2O_2$ results in a decrease in E-cadherin and an increase in N-cadherin levels, at both mRNA and protein levels. However, inhibition of ERK using PD98059 abolishes the effects of $H_2O_2$. In addition, the cells that were treated with $H_2O_2$ have gained further migrative abilities compared to control cells, and this ability was repressed when PD98059 was used together with $H_2O_2$.

CONCLUSION: Increased ROS alters E- and N-cadherin levels in an ERK-dependent manner and thereby promotes the migrative abilities of PCa cells *(Fig. 3, Ref. 32)*. Text in PDF *www.elis.sk.*

KEY WORDS: prostate cancer, ROS, E-cadherin, N-cadherin, cell migration.

## Introduction

Prostate cancer (PCa) is one of the most common cancers diagnosed in men, worldwide (1). The disease develops and progresses in a multiple-step manner and many mechanisms are involved in these processes (2). Oxidative stress, which occurs with the excessive production and consequently accumulation of Reactive Oxygen Species (ROS) has important roles in these processes (3) and contributes to the development and progression of the disease by affecting the cell proliferation, genomic instability, and cell adhesion (4).

Alterations in the cell adhesion dynamics, such as cadherins, are directly associated with the migrative and invasive behaviors of cancer cells (5). E- and N-cadherin proteins are members of $Ca^{2+}$ dependent cadherin protein family and have crucial roles in the regulation of cell adhesion (5). E-cadherin molecules have important roles in the establishment and maintenance of functional adherence junctions in the epithelial tissues, which is directly contributing to the regulation of tissue integrity and cell polarity (6). Therefore, the E-cadherin level is crucial in the controlling of some cellular properties such as cell shape, cell division, or interaction with neighbour cells (7). On the other hand, E-cadherin level is also important in carcinogenesis, and loss or decrease in the E-cadherin expression has been reported in many cancers, including PCa cells, compared to normal counterpart tissues (8). Decreased E-cadherin level has been shown to cause the development of invasive and metastatic behaviors in the cancer cells (9). Indeed, the cellular level of E-cadherin is negatively associated with tumor cell motility, invasion, and metastasis processes (10). However, N-cadherin is associated with tumor aggressiveness (11), opposite to E-cadherin, and enhanced N-cadherin expression has been shown in various cancers including PCa (8, 11).

The purpose of this study was to investigate the effects of a sublethal dose of ROS on E- and N-cadherin levels, and cell migration in PCa cells. In this way, we aimed to obtain knowledge about the effects of increased ROS on the migration of PCa cells, since cancer cells produce more ROS compared to normal cells dependent on the elevated metabolic activity.

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## Material and methods

*Cell lines and culture conditions*

PC-3 and LNCaP PCa cells were obtained from ATCC (Rockville, Maryland, USA). The cells were grown in RPMI-1640 or DMEM/F12 supplemented with 5–10 % FBS, in a humidified incubator in a 5 % $CO_2$ atmosphere at 37 °C. $H_2O_2$, DCFH-DA, and PD98059 were purchased from SIGMA (San Diego, CA, USA).

*Measurement of ROS, proliferation, and migration*

A wide range of $H_2O_2$ concentrations consisting of 5, 10, 20, 50, 100, 250, and 500 µM was used to determine a non-lethal dose. The criteria for selecting the sublethal dose of $H_2O_2$ was that it could not inhibit cell proliferation but also induce ROS production by at least 80 % compared to control cells (referred to as 0 µM in the experiments).

DCFH-DA was used to examine the ROS production capabilities of used concentrations of $H_2O_2$ in PCa cells, as described previously (12, 13), and the fluorescence measurements were performed at 485 nm (Excitation) / 538 nm (Emission) using SpectraMax M2 microplate reader (Molecular Devices LLC, USA).

The WST-1 assay was used to examine the effects of the used $H_2O_2$ concentrations on PCa cell proliferation, as described previously (14), and the absorbances were measured at 450 nm with a SpectraMax M2 microplate reader (Molecular Devices LLC, CA, USA).

Cytoselect 96-well cell migration assay (Cell Biolabs Inc., USA) was used to examine the effects of the sublethal dose of $H_2O_2$ on migrative capacities of PCa cells, as described previously (15, 16), and the fluorescence intensities were measured at 480 nm (Excitation) / 520 nm (Emission) using SpectraMax M2 microplate reader (Molecular Devices LLC, USA).

*Western blotting and real-time PCR*

The protein lysates were extracted from the cells in the ice-cold RIPA buffer, containing protease and phosphatase inhibitor

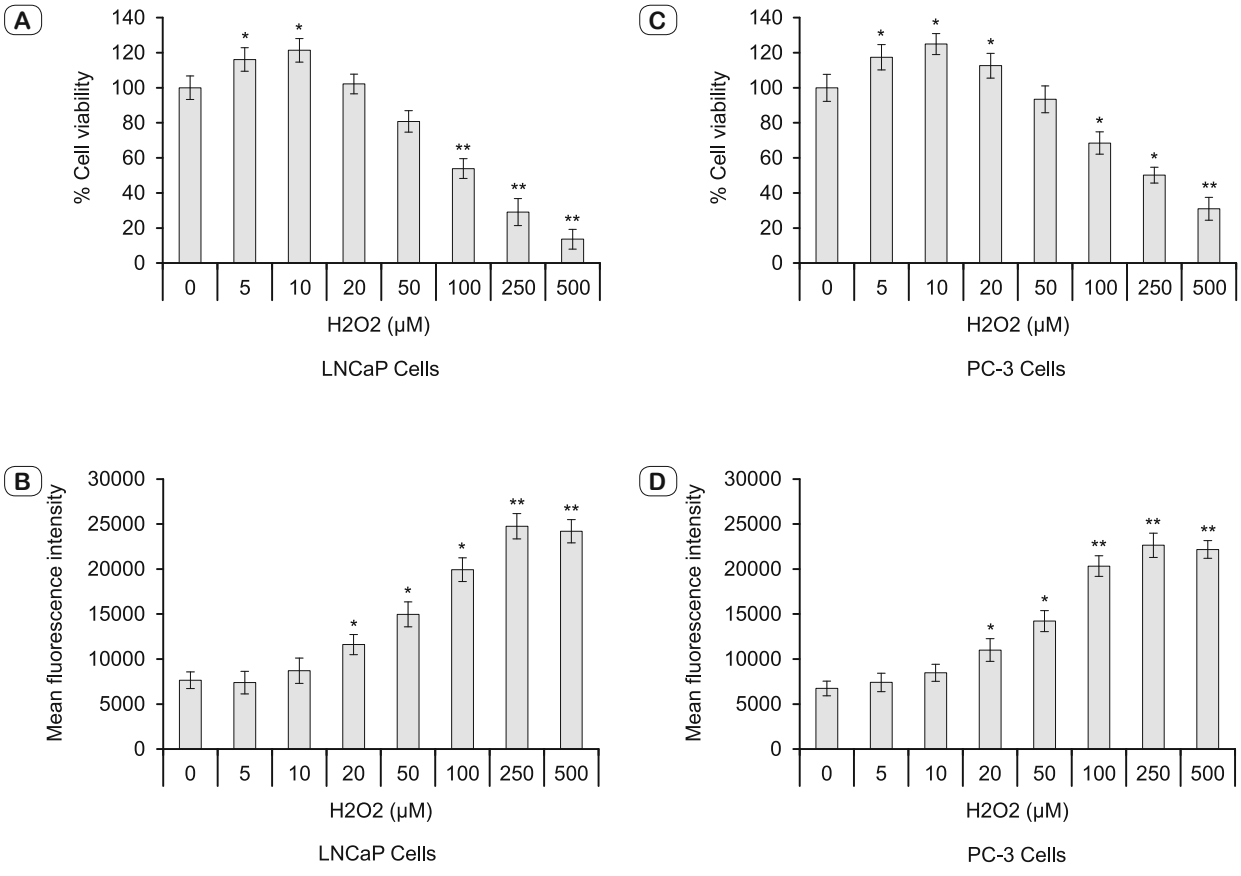


**Fig. 1. Determination of the sublethal dose of $H_2O_2$ in PCa cells. LNCaP (a and b) and PC-3 (c and d) cells were treated with 0, 5, 10, 20, 50, or 100 µM concentrations of $H_2O_2$ for 24 h, and then cell proliferation (a and c) and ROS levels (b and d) were measured by WST-1 and DCFH-DA assays, respectively. The absorbances obtained from WST-1 assay were converted to % values and were used to construct a graph representing % cell viability (a and c). The fluorescence values from DCFH-DA assay were used to construct a graph representing ROS levels (b and d). The data in the graphs represent the means of three independent results (± SEM), and the Student's t-test was used for statistical comparison. * $p < 0.05$ and ** $p < 0.005$.**

cocktails, and then were clarified by centrifugation at 12.000 X g for 10 min. The extracted lysates were subjected to 10% SDS-PAGEs (40 µg protein was loaded in each lane), and subsequently to western blotting for detection of the protein levels of E-cadherin, N-cadherin, pERK$^{(T202/Y204)}$, total ERK and β-Actin, as described previously (17, 18). All used antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA).

Isolation of total RNA and conversion of mRNAs to cDNAs were performed as described previously (19). The gene expression changes in E- and N-cadherin were examined by a SYBR Green-based RT-PCR kit and the StepOnePlus Real-Time PCR system (Applied Biosystems, USA). The relative quantification method was used to calculate the abundance of E- and N-cadherin cDNAs, and GAPDH was used as the housekeeping gene, as described previously (19). The primer pairs are as follows and were designed using LightCycler Probe Design Software (LCPDS) 2.0.; E-cadherin (Genbank Accession No: NM_004360.5) forward: CTGAAGTGACTCGTAACGAC, reverse: ACGAGCAGAGAATCATAAGG; N-cadherin (Genbank Accession No: NM_001792.5) forward: TGATTATGACATTGATCCAAATGC, reverse: ATGATGGGAACTTCATAGATACC; GAPDH (Genbank Accession No: NM_002046.7) forward: CATTGCCCTCAACGACCACTTT, reverse: GGTGGTCCAGGGGTCTTACTCC.

## Results

*Determination of sublethal doses of $H_2O_2$ in PCa cells*

To determine a sublethal concentration of $H_2O_2$ on PCa cells, LNCaP and PC-3 cells were treated with $H_2O_2$ in the concentrations of 0, 5, 10, 20, 50, 100, 250, or 500 µM for 24 h, and then total ROS levels and cell proliferation were measured. The proliferation results have shown that although relatively low concentrations of $H_2O_2$ (5 and 10 µM) may cause a statistically significant increase in the LNCaP cell proliferation, 100, 250, and 500 µM concentrations of $H_2O_2$ cause a dramatic decrease in the proliferation (Fig. 1a). However, 20 and 50 µM concentrations of $H_2O_2$ couldn't change LNCaP cell proliferation in a statistically significant manner. In PC-3 cells, although the cell proliferation increases in the 5, 10, and 20 µM $H_2O_2$ treated groups, 100, 250, and 500 µM concentrations of $H_2O_2$ caused a decrease (Fig. 1c). However, cell proliferation has not changed in the 50 µM $H_2O_2$ treated cells, compared to the control cells. The ROS production results have shown that although 5 and 10 µM concentrations of $H_2O_2$ can't induce an increase in the ROS level, in both of the used cells, an increase in the ROS level begins at 20 µM concentration and then further increases at other, higher, used concentrations (Fig. 1b and d). Consequently, the effects of all used concentrations of $H_2O_2$ on cell proliferation and ROS production were evaluated according to the criteria explained above, in a combination, and it was decided to use a 50 µM concentration of $H_2O_2$ for subsequent experiments, in both of the cells.

*$H_2O_2$ treatment alters E- and N-cadherin levels in PCa cells*

Since the previous studies have reported that oxidative stress may cause a decrease in E-cadherin level and an increase in N-cadherin level in pancreas and breast cancers (20–22), the effects of a sublethal dose of $H_2O_2$ on E- and N-cadherin protein levels were investigated in PCa cells. PC-3 and LNCaP cells were seeded on 60 mm plates and were treated with $H_2O$ or a 50 µM concentration of $H_2O_2$ for 24 h. Total cell lysates were extracted and were used for western blotting. The results showed that using a sublethal concentration of $H_2O_2$ resulted in a decrease in E-cadherin protein and an increase in N-cadherin protein level, in both of the used cells (Fig. 2a). To examine whether $H_2O_2$ treatment-dependent alterations in E- and N-cadherin levels are in the mRNA level, PC-3 and LNCaP cells were cultured and treated, as described above. The total RNAs were extracted, converted to cDNAs, and consequently used for real-time PCR. The obtained results were in concordance with the results of western blotting and consequently demonstrated that a sublethal dose of $H_2O_2$ alters E- and N-cadherin levels at the transcriptional level (Fig. 2b).

It has been shown that oxidative stress causes a decrease in the E-cadherin level in an ERK-dependent manner, in breast and pancreas cancer cells (20–22). Therefore, it was investigated whether

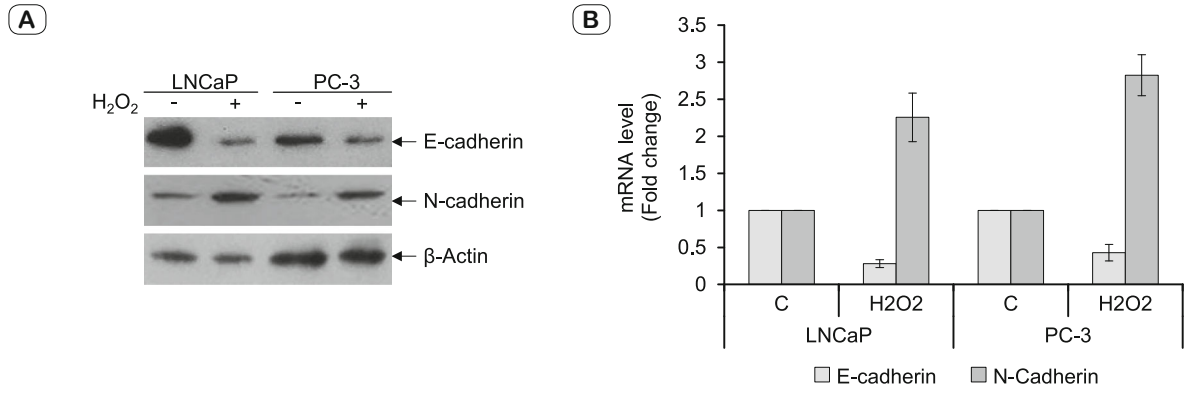


**Fig. 2. A sublethal dose of $H_2O_2$ causes a change in E-cadherin and N-cadherin levels in PCa cells. LNCaP and PC-3 cells were treated with a 50 µM concentration of $H_2O_2$ for 24 h, and then E- and N-cadherin protein (a) and mRNA (b) levels were examined by using western blotting and real-time PCR, respectively. β-Actin was used as a loading control in the western blotting experiment. In the real-time PCR experiments, the relative quantification method was used to calculate E- and N-cadherin mRNA levels and GAPDH was used as the housekeeping gene.**

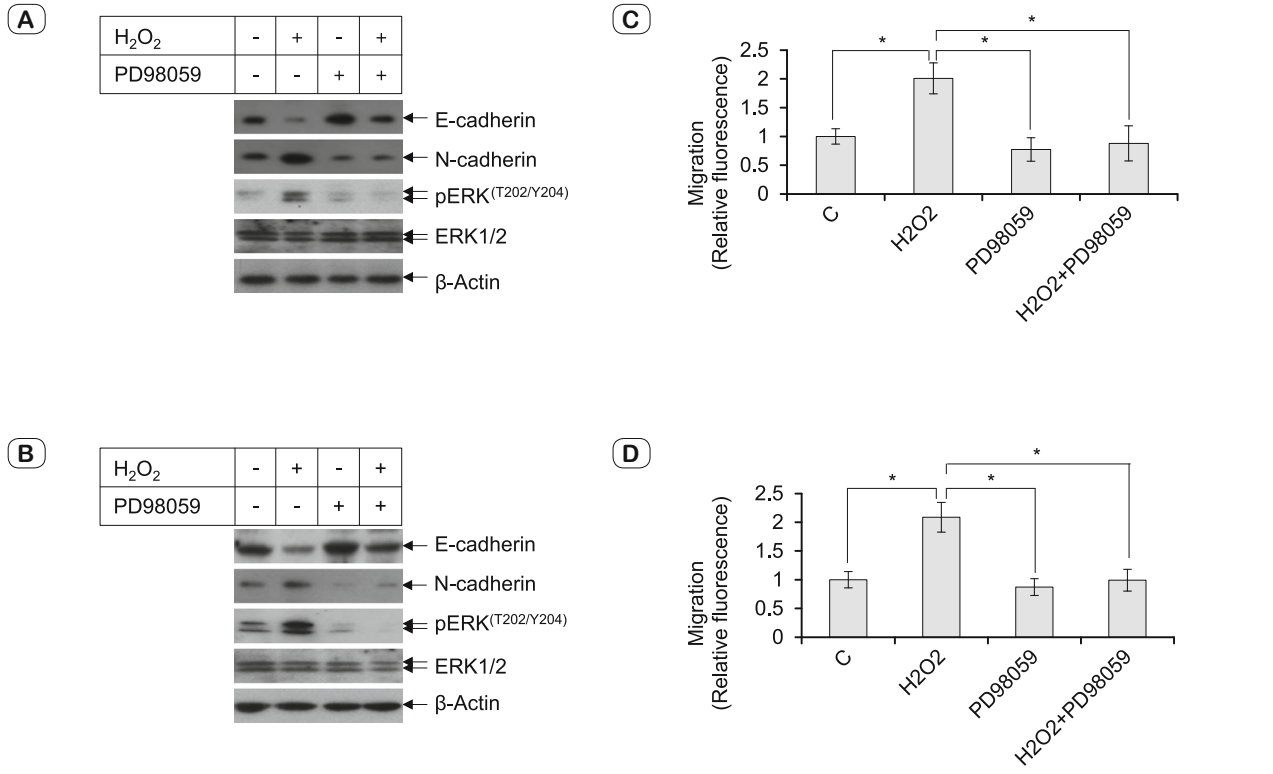


**Fig. 3. Inhibition of ERK restores $H_2O_2$-induced changes in E-cadherin and N-cadherin levels and consequently their increased migrative abilities of PCa cells. LNCaP (a) and PC-3 (b) cells were treated with a 50 µM concentration of $H_2O_2$ for 24 h, in the present or absence of ERK inhibitor PD98059, and then E-cadherin, N-cadherin pERK(T202/Y204), and total ERK levels were examined by western blotting. LNCaP (c) and PC-3 (d) cells were treated with a 50 µM concentration of $H_2O_2$ for 24 h, in the presence or absence of PD98059, and then the migrative abilities of the cells were measured using Cytoselect 96-well cell migration assay. β- Actin was used as a loading control in western blotting experiments. The fluorescence values obtained from the Cytoselect 96-well cell migration assay were normalized to the basal fluorescence signal of the control cells and graphs were constructed by using Microsoft Excel. The data in the graphs represent the means of three independent results (± SEM), and the Student's t-test was used for statistical comparison. * $p < 0.05$.**

changes in E- and N-cadherin levels caused by $H_2O_2$ treatment are ERK-dependent. The results have shown that $H_2O_2$ increases ERK activity in concordance with changes in E- and N-cadherin levels in both LNCaP (Fig. 3a) and PC-3 (Fig. 3b) cells. Furthermore, inhibition of ERK activity by PD98059 has mostly abolished the effect of $H_2O_2$ on E- and N-cadherin levels (Fig. 3a and b). Since the inhibition of ERK activity using PD98059 reversed the effects of $H_2O_2$ on E- and N-cadherin levels, the effects of PD98059 on cell migration were also investigated, in the presence or absence of $H_2O_2$. Indeed, the results have demonstrated that the migrative behaviors of PCa cells induced by $H_2O_2$ were inhibited by PD98059, in both LNCaP (Fig. 3c) and PC-3 (Fig. 3d) cells.

## Discussion

ROS are important molecules involved in the regulation of cellular physiology in both healthy and pathological conditions (4, 23). Although ROS are required for normal cellular functions at the physiological level, their excessive production, dependent on the high cellular activity, or deficiency in their destruction causes ROS accumulation in the cells (24). This event is referred to as oxidative stress and generally results in the damaging of cell components and consequently changing of cell behaviors (25). ROS are interesting molecules that can show opposite effects on the cells and tissues at low and high doses and although high levels of ROS may cause damage to the cells and tissues, low levels generally promote cell proliferation (4). Therefore, initially, a sublethal concentration of $H_2O_2$ was investigated that can induce ROS production by at least 80 % but also can't inhibit cell proliferation, compared to control cells, and consequently, a 50 µM concentration of $H_2O_2$ was found as a sublethal dose for PC-3 and LNCaP PCa cells.

The epithelial to mesenchymal transition (EMT) is an important process in which the cells gain further migrative and invasive potential, and is characterized by an increased N-cadherin and a decreased E-cadherin level (26). Indeed, it has been shown that loss or decrease in E-cadherin expression impairs the adhesions of primary prostate tumor cells (27), and leads to metastasizing to lymph nodes and bones in PCa (5). Furthermore, it is known that increased *Cdh2* expression confers further migrative abilities to PCa cells and thereby increases their metastatic potential (28). Since it has been shown that oxidative stress alters E- and N-cadherin levels in pancreas and breast cancers in previous reports (20–22),

we investigated the effects of the sublethal dose of $H_2O_2$, which was determined in the previous step, on E- and N-cadherin levels in PCa cells. Indeed, we have observed that $H_2O_2$ induced alterations in E-and N-cadherin levels are ERK dependent.

In the cancer cells, the total ROS level is generally higher compared to counterpart healthy cells, and increased ROS affects many cellular mechanisms, including cell migration (3, 29). Indeed, it has been shown that the extracellular redox state has been shown to regulate invasive behaviors of cancer cells (30). In concordance, elevated ROS level has been shown to be associated with increased migrative abilities in breast and pancreas cells (20–22). Furthermore, since our results have shown that a sublethal dose of $H_2O_2$ alters E- and N-cadherin levels, we examined the effects of the sublethal concentration of $H_2O_2$ on cell migration. We have seen that $H_2O_2$ further promotes migration behaviors of PCa cells and inhibition of the ERK pathway represses $H_2O_2$ induced cell migration, in correlation with changes in E- and N-cadherin levels.

In a previous study, it was shown that psoralidin (Pso) induces ROS production in PCa cells and causes a decrease in E-cadherin level (31). However, due to the fact that a sublethal dose hasn't been determined in the study, Pso treatment caused a prominent inhibition in cell proliferation and also induced apoptosis (31). Since E-cadherin undergoes cleavage into smaller fragments and consequently total E-cadherin level decreases in the apoptotic cells (32), it could be speculated that a decrease in the E-cadherin level may be related to cell death mechanisms. However, we have seen that a decrease in E-cadherin level is independent of death mechanisms when we used a sublethal concentration of $H_2O_2$ as a ROS source. Das et al have also reported that although treatment of the cells with Pso caused an increase in the ROS production, this event results in the inhibition of migrative and invasive behaviors of PCa cells (31). However, our results have shown that increased ROS level cause an increase in the migrative abilities in PCa cells. The differences between our results and the results of Das et al. may be related to the unknown effects of pso in PCa cells. Therefore, the molecular basis for this difference will be revealed with a better understanding of the cellular mechanisms that pso affects.

## Conclusion

In the present study, a sublethal dose of $H_2O_2$ was determined, and it was demonstrated that treatment of the PCa cells with the sublethal concentration of $H_2O_2$ results in diminishing of E-cadherin and enhancement of N-cadherin, at mRNA and protein levels. Additionally, it was shown that $H_2O_2$-treated PCa cells gain further migrative abilities, in concordance with the alterations of E- and N-cadherin levels. Furthermore, inhibition of ERK activity by using PD98059 along with $H_2O_2$ treatment repressed alterations in E- and N-cadherin levels as well as migrative abilities of the cells.

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