Commagene Journal of Biology

Yüksel & Deveci Özkan (2021) *Comm. J. Biol.* 5(1), 84-89.
DOI: 10.31594/commagene.895415

e-ISSN 2602-456X

Research Article / Araştırma Makalesi

The Role of Citrus Nobiletin on Oxidative Stress Levels and Superoxide Dismutase Activities in Metastatic Castration-Resistant Prostate Cancer

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Received: 11.03.2021 **Accepted:** 15.06.2021 **Published online:** 24.06.2021 **Issue published:** 30.06.2021

Abstract: Nobiletin (NOB) is a polymethoxylated flavone. It has multiple biologic activities that can modulate oxidative stress in many cancer types. However, there is no study in the literature that has examined the effects of NOB on oxidative stress levels in Metastatic Castration-Resistant Prostate Cancer (MCRPC) yet. Motivated from this gap, we investigated the impact of NOB on oxidative stress and superoxide dismutase (SOD) enzyme activities in MCRPC as a preliminary study. For this purpose, PC-3 and HUVEC cells were used to determine the effects of NOB on the amount of Malondialdehyde (MDA), hydrogen peroxide (H_2O_2), and proline as well as SOD enzyme activities. NOB potentially induced SOD enzyme activities but the level of MDA, H_2O_2, and proline decreased after incubation with NOB in PC-3 cells ($p<.05$ and $p<.001$ were considered statistically significant). Our results confirmed that NOB acted as a protective agent for cancer cells and could selectively regulate oxidant status in MCRPC cells. Consequently, these preliminary findings provide better insight into the role of citrus NOB on oxidative stress levels and antioxidant enzyme activities in MCRPC. Additionally, there is a need to elucidate the molecular mechanisms of this cytoprotective effect of NOB as a potential chemotherapeutic agent.

Keywords: Antioxidant effect, cancer, flavanoid, malondialdehyde, proline.

Metastatik Kastrasyona Dirençli Prostat Kanserinde Narenciye Nobiletin'in Oksidatif Stres Düzeyleri ve Süperoksit Dismutaz Aktiviteleri Üzerindeki Rolü

Öz: Nobiletin (NOB) polimetoksile bir flavondur ve birçok kanser türünde oksidatif stresi modüle edebilen çok sayıda biyolojik aktiviteye sahiptir. Bununla birlikte, literatürde, MCRPC'de Metastatik Kastrasyona Dirençli Prostat Kanserinde (MCRPC) NOB'nin oksidatif stres seviyeleri üzerindeki etkilerine dair henüz bir çalışma bulunmamaktadır. Bu nedenle, ön çalışma olarak MCRPC'de NOB'nin oksidatif stres ve süperoksit dismutaz (SOD) enzim aktiviteleri üzerindeki etkilerini belirlemeyi amaçladık. Bu amaçla, çalışmada NOB'nin Malondialdehit (MDA), hidrojen peroksit (H_2O_2) ve prolin miktarı ile SOD enzim aktiviteleri üzerindeki etkilerini belirlemek için PC-3 ve HUVEC hücreleri kullanıldı. NOB'un potansiyel olarak SOD enzim aktivitelerini indüklediği, ancak PC-3 hücrelerinde NOB ile inkübasyondan sonra MDA, H_2O_2 ve prolin seviyesinin azaldığı tespit edildi ($p<.05$ ve $p<.001$ istatistiksel olarak anlamlı kabul edildi). Elde edilen veriler, NOB'nin kanser hücreleri için koruyucu bir ajan olarak hareket ettiğini ve MCRPC hücrelerinde oksidan durumunu seçici olarak düzenleyebildiğini doğruladı. Sonuç olarak, bu ön bulgular, MCRPC'de turunçgil NOB'nin oksidatif stres seviyeleri ve antioksidan enzim aktiviteleri üzerindeki rolü hakkında daha iyi fikir vermektedir. Ek olarak, potansiyel bir kemoterapötik ajan olarak NOB'nin bu sitoprotektif etkisinin moleküler mekanizmalarının aydınlatılmasına ihtiyaç vardır.

Anahtar kelimeler: Antioksidan etki, flavonoid, kanser, malondialdehyde, proline.

1. Introduction

In metastatic castration-resistant prostate cancer (MCRPC), therapies such as androgen ablation cannot be successful and oxidative stress has been regarded as one of the qualities of the aggressive disease phenotype (Shen & Abate-Shen, 2010). In particular, oxidative stress is related to MCRPC development, advancement, and response to therapy. Reactive oxygen species (ROS) are produced directly in damaged tissue in response to pro-inflammatory cytokines, growth factors, exposure to chemicals, and other stressors. Oxidative signals can regulate the expression of various cytokines and chemokines (TNFα, IL-1β, IL-6, IL-8). In inflammatory microenvironment conditions, the subsequent stimulation of ROS production causes DNA damage and increases mutation rate when it continues for a long time. This may induce oncogenic transformation in cancer-related genes and result in DNA damage, apoptosis, and genetic alterations of essential cellular proteins that regulate the cell cycle (Ma, 2010). To balance ROS-induced oxidative damage, cells have developed various antioxidant processes to sustain their genomic constancy such as enzymatic scavengers including superoxide dismutase (SOD), catalase as well as glutathione peroxidase, antioxidant enzymes involved in cell defence, DNA repair enzymes, and cellular mechanisms of genomic control. While SOD superoxide anions catalyze H_2O_2, catalase and glutathione peroxidase convert H_2O_2 into the water and the accumulation of peroxides is prevented (Acharya et al., 2010). In this context, current treatment options in MCRPC are low and oxidative stress plays a vital role in MCRPC development (Amaral et al., 2012). Therefore, it is essential to investigate the effects of alternative treatment strategies on oxidative stress markers in MCRPC.

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Nobiletin (NOB) is a bioactive polymethoxylated flavone (5,6,7,8,3',4'-hexamethoxyflavone) which was found in citrus peels and had multiple biologic activities (Bernini et al., 2011; Zheng et al., 2019; Dusabimana et al., 2019). Flavonoids exhibit anti-carcinogenic characteristics in vitro and reduce cancer risk by protecting cells from oxidation or inflammation by changing the level of sex hormones, reducing angiogenesis or cell proliferation, and regulating apoptosis (Gates et al., 2009). NOB is anti-apoptotic, anti-inflammatory, anti-tumor, and antioxidant and an effective inhibitor against human prostate cancer cells and melanoma cells (Kunimasa et al., 2010; Lee et al., 2013; Xiao et al., 2016; Huang et al., 2016; Surichana et al., 2018). Additionally, studies have shown that NOB has an anti-inflammatory effect and exhibit an inhibiting effect on tumor invasion, proliferation, and metastasis and inhibit the creation of reactive oxygen species (ROS) by increasing the superoxide dismutase (SOD) and glutathione (GSH) activity and reduction in the making of malondialdehyde (MDA) and H2O2-induced PC12 cells (Luo et al., 2008; Lu et al., 2010; Güney Eskiler et al.; 2018; Liu et al., 2019; Deveci Ozkan et al., 2020). In addition, researchers reported that nobiletin impeded cell proliferation both depending on the dose and duration and prevented cell cycle progression in G1 (Morley et al., 2007). Nobiletin can induce DNA damage that contributes to apoptosis by regulating polymerase activity in cancer cells (Zhang et al., 2020). It has been suggested that NOB may be a pathway that triggers pyroptosis associated with apoptosis by inducing IL-1β expression (He et al., 2015). NOB is hypothesized to inhibit metastasis by impeding the activity of activator protein-1, a dimeric protein, preventing DNA binding (Kawabata et al., 2005; Goh et al., 2019). Another suggestion proposes that NOB functions through the Nuclear Factor-kappa B route and differentiates gene expression via regulating its promoter regions (Xiong et al., 2015; Park et al., 2016). As a food ingredient, nobiletin may be a favorable new anti-prostate cancer functional food component that can generate ROS-mediated apoptosis in prostate cancer cells.

No study has been conducted in the literature that shows the effects of NOB on oxidative stress levels and antioxidant enzyme activities in MCRPC. Therefore, in this study, we aimed to determine the effects of NOB on oxidative stress in MCRPC (PC-3) and control cells (HUVEC). To clarify if NOB affects oxidative stress, we measured the amount of Malondialdehyde (MDA), hydrogen peroxide (H2O2), and proline as well as SOD (superoxide dismutase) enzyme activities. Thus, it is aimed to obtain information that will contribute to the potential of NOB as a functionally unique and possible chemopreventive agent in inflammation-related tumor formation such as MCRPC.

2. Material and Methods

2.1. Cell culture and NOB treatment

In this study, the PC-3 (prostate cancer) cell line was used as MCRPC cells and human umbilical vein endothelial cells (HUVEC) were evaluated as a control cell line. All cells were bought from American Type Culture Collection (ATCC). PC-3 was cultured in RPMI-1640 (Thermo Fisher, USA) and HUVEC cells were grown in DMEM medium (Thermo Fisher, USA) supplemented with 0.1% penicillin and streptomycin, 10% FBS, and incubated at 37°C with 5% CO_2. The optimal treatment concentration and times for NOB in PC-3 and HUVEC cell lines were determined in our previous studies (Deveci Ozkan et al., 2020). Therefore, all cells were treated with NOB (80 µM for PC-3 and HUVEC, respectively) for 48 h in all experiments. Additionally, cells were grown in a culture medium without NOB used as a negative control.

2.2. Antioxidant enzyme activity and oxidative stress assays

2.2.1. Preparation of cell lysate

To determine the effects of NOB on antioxidant enzyme activity and oxidative stress level, the cells ($5x10^5$) were seeded six-well plates and treated with indicated concentrations (80 µM for PC-3 and HUVEC, respectively) of NOB for 48h. After incubation, the cells were treated with RIPA lysis and extraction buffer (Sigma Aldrich, USA), centrifuged for 10 min at 10.000 rpm at 4°C. After centrifugation, obtained supernatant was used for the analysis of antioxidant enzyme activity and oxidative stress.

2.2.2. Determination of Superoxide Dismutase (SOD) activity

SOD activity was established phytochemically according to the proposed method of Beauchamp and Fridovich (1971). SOD accelerates superoxide radicals' degradation (O^{2-}), H_2O_2, and molecular oxygen formed during oxidative energy production. SOD activity is determined by the inhibition of NBT (nitrobluetetrazolium) and formazan (blue crystals) formation. The blue-purple color formation from NBT with the effect of light is inversely proportional to the activity of the SOD enzyme. One unit of SOD activity was determined as the quantity of enzyme needed to reduce 50% of the NBT kept under light at 560 nm. Therefore, SOD enzyme activity was calculated according to the formula below.

%Inhibition = [(Blank OD – Sample OD)/Blank OD] x 100

2.2.3. Determination of Malondialdehyde (MDA) level

MDA level was verified by the proposed method of Ohkawa et al. (1979). According to this method, the thiocarboxylic acid (TBA) test, which accepts MDA as the final product of lipid peroxidation, was used. The formation of MDA content resulting from the TBA reaction is accepted as a lipid peroxidation measure. The amount of MDA in 1 ml of solution was calculated according to the formula below and the results were given as MDA (nmol/gram tissue) (Ananieva et al., 2002).

MDA (nmol/g): [(A532-A600)/155000] x 106

2.2.4. Determination of Proline Level

Proline level was determined by the proposed method of Myara et al. (1982). According to this method, the proline level was determined by measuring absorbance in a spectrophotometer. Absorbance values were obtained from an ultraviolet spectrophotometer (Shimadzu UV mini-1240 spectrophotometer) at 520 nm wavelength. Proline concentration was calculated as µmol/g according to the proline standard curve.

2.2.5. Determination of Hydrogen Peroxide (H_2O_2) level

H_2O_2 level was determined by the proposed method of

Jana and Choudhuri (1981). According to this method, the H_2O_2 level was determined by measuring absorbance in a spectrophotometer. Absorbance values were obtained from an ultraviolet spectrophotometer (Shimadzu UV mini-1240 spectrophotometer) at 410 nm wavelength. H_2O_2 concentration was calculated according to the proline standard curve.

2.3. Statistical analysis

Statistical analyses were carried out via Graph pad Prism v9.0 (Software, CA) and showed the mean ± standard deviation of three independent experiments. One-way analysis of variance (ANOVA) and Tukey's test was utilized to obtain multiple comparisons ($p<.05$ and $p<.001$ were taken as statistically significant).

3. Results

Our results showed that the SOD activities of the cells incubated with NOB for 24 and 48 h showed a significant increase in PC-3 cells (9.71 ± 2.04 and 9.36 ± 2.32, respectively) compared to the control cells not treated with NOB (7.98 ± 0.41 and 6.87 ± 0.13, respectively). Moreover, a similar significant increase was observed in HUVEC cells treated with NOB compared to the control group level in both treatment times (*$p<.05$, **$p<.001$, Table 1 and Fig. 1). As we expect that a significant decrease was observed in MDA levels of the PC-3 cells treated with NOB (0.19 ± 0.03) compared to the control (0.37 ± 0.06) for 48 h but a significant increase was determined in HUVEC cells treated with NOB (0.69 ± 0.28) compared to the control (0.60 ± 0.47) for 48h (*$p<.05$, **$p<.001$, Table 1 and Fig. 2). Additionally, a similar significant decrease was observed for in H_2O_2 concentration of the PC-3 cells treated with NOB for 24 and 48h (0.21 ± 0.08 and 0.34 ± 0.01, respectively) compared to the control (1.49 ± 0.05 and 0.12 ± 0.05, respectively) but a significant increase was determined in HUVEC cells treated with NOB compared to the control for both 24 and 48h (*$p<.05$, **$p<.001$, Table 1 and Fig. 3). Besides, the proline level of the cells incubated with NOB showed a statistically non-significant decrease in PC-3 and HUVEC cells compared to the control cells for 48h (Table 1 and Fig. 4). Therefore, according to our results, MDA and H_2O_2 levels were significantly lower whereas SOD activity was significantly higher in the MCRPC cells treated with NOB.

Table 1. Comparison of the oxidative stress levels and antioxidant enzyme activities according to the control group and NOB treated group in PC-3 and HUVEC for 24 and 48h.

Parameters		PC-3-Control Mean ± SD *(n=3)*	PC-3-NOB Mean ± SD *(n=3)*	HUVEC-Control Mean ± SD *(n=3)*	HUVEC-NOB Mean ± SD *(n=3)*
SOD (unit/mg)	24h	7.98 ± 0.41	9.71 ± 2.04*	8.36 ± 0.38	14.21 ± 1.29**
	48h	6.87 ± 0.13	9.36 ± 2.32*	3.38 ± 0.78	11.06 ± 0.86**
MDA (µM)	24h	0.53 ± 0.21	0.56 ± 0.15	0.42 ± 0.21	0.47 ± 0.08*
	48h	0.37 ± 0.06	0.19 ± 0.03*	0.60 ± 0.47	0.69 ± 0.28**
H_2O_2 (µM)	24h	1.49 ± 0.05	0.21 ± 0.08**	0.16 ± 0.05	0.21 ± 0.01**
	48h	0.12 ± 0.05	0.34 ± 0.01**	0.06 ± 0.01	0.10 ± 0.07**
Prolin (µM)	24h	9.46 ± 1.83	6.68 ± 0.83	4.27 ± 0.57	6.85 ± 1.18
	48h	11.22 ± 1.22	7.63 ± 1.46	7.98 ± 0.08	7.27 ±1.07**

The results are expressed as mean ± SD of eight experiments. *$p< .05$ and **$p< .001$ significantly different compared to the controls.

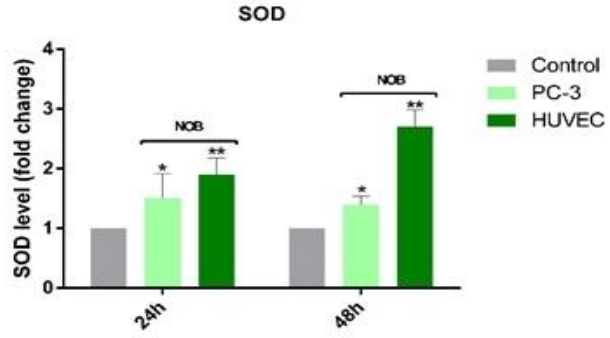

Figure 1. Relative enzyme activity level of SOD in PC-3 and HUVEC cells. To determine the enzyme activity level of SOD in PC-3 and HUVEC cells were treated with 80 mM NOB, for 24 and 48 h. *(*$p<.05$, **$p<.001$*, NOB: Nobiletin).

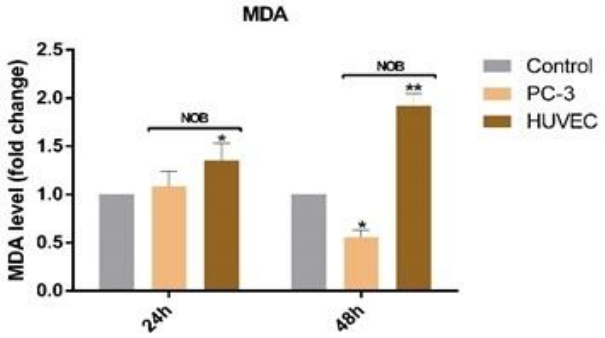

Figure 2. Relative level of MDA in PC-3 and HUVEC cells. To determine the level of MDA in PC-3 and HUVEC cells were treated with 80 mM NOB, for 24 and 48 h. *(*$p<.05$, **$p<.001$*, NOB: Nobiletin).

4. Discussion

In this study, the role of citrus nobiletin on oxidative stress levels and antioxidant enzyme activities was investigated in metastatic castration-resistant prostate cancer cells for the first time. Our preliminary findings demonstrated that NOB could modulate oxidative stress in PC-3 cells.

Oxidative stress is one of the inevitable consequences of aerobic life and increasing evidence indicates that the accumulation and formation of ROS and reactive nitrogen species play a significant role in various age-related diseases such as prostate cancer. Besides, if this oxidative stress that can cause damage to the cells is not repaired, the development of carcinogenesis will be inevitable over the years (Cooke et al., 2000; DeWeese et al., 2001; Cooke et al., 2003). Moreover, antioxidant enzymes' activities decrease when the balance of ROS-antioxidant is disturbed in prostate cancer cells (Zhou et al., 2006). Our study found significantly higher SOD (antioxidant enzyme) activity levels in NOB-treated PC-3 cells. This finding has supported that NOB has a potential protective role on the cells from oxidative stress by increasing the level of antioxidant enzymes.

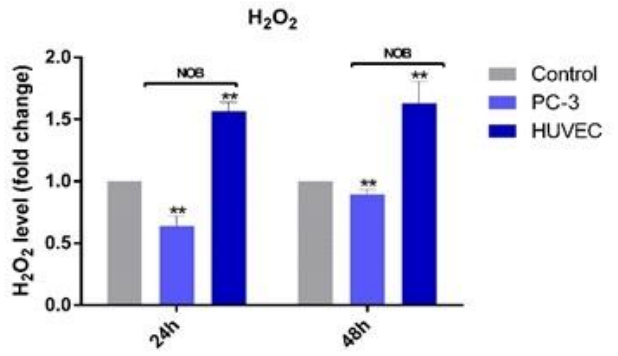

Figure 3. Relative concentration of H_2O_2 in PC-3 and HUVEC cells. To determine the concentration of H_2O_2 in PC-3 and HUVEC cells were treated with 80 mM NOB, for 24 and 48 h. *(*p<.05, **p<.001*, NOB: Nobiletin).

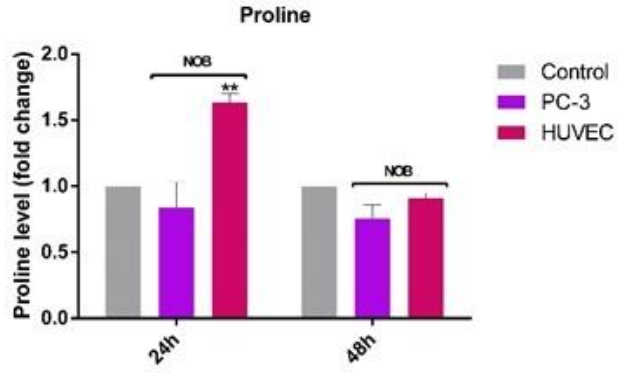

Figure 4. Relative level of proline in PC-3 and HUVEC cells. To determine the level of proline in PC-3 and HUVEC cells were treated with 80 mM NOB, for 24 and 48 h *(*p<.05, **p<.001*, NOB: Nobiletin).

Oxidation of lipid peroxidation is one of the most widely reported markers of oxidative stress as a contributor to cancer and MDA is the most frequently studied of these markers. In one study, Meng et al. (2017) showed that Cu^{2+} -induced intracellular ROS accumulation and increased MDA levels with apoptosis reduced by astaxanthin treatment and this might have a protective effect against Cu^{2+} -induced oxidative damage by reducing ROS and MDA levels. Similarly, our results revealed that NOB treatment decreased the MDA level in PC-3 cells compared to the control cells not treated with NOB.

Acute and chronic inflammatory cells such as prostate cancer produce superoxide, H_2O_2, and other ROS and recently it has been shown that oxidative stress is higher in prostate cancer cells compared to normal cells. In a study, the effect of doxorubicin, a chemotherapeutic drug, on H_2O_2 production in PC-3 cells was examined and an increase in intracellular H_2O_2 was observed as doxorubicin concentration increased (Wagner+ et al., 2005). However, according to the results of our study, as a substance with the potential of being a chemotherapeutic agent, NOB reduced the hydrogen peroxide level in PC-3 cells compared to the control cells not treated with NOB. On the contrary, our findings demonstrated that NOB increased the H_2O_2 level in HUVEC cells. Our results confirmed that NOB acted as a protective agent for cancer cells and could selectively regulate oxidant status in MCRPC cells. H_2O_2 activates the cellular or mitochondrial apoptotic pathway (Cho et al., 2015). Considering all these data, nobiletin reduced the cytotoxicity induced by H_2O_2, scavenging ROS, reducing MDA, especially in PC-3 cells, and restoring the activities of antioxidants (Lu et al., 2010; Malik et al., 2015). In this study, it can be assumed that the protection provided by nobiletin may be due to its antioxidant effect.

Extracellular matrix (ECM) proteins are a large source of amino acids that are likely to be discharged into the tumor microenvironment through the activity of matrix metalloproteinases and/or collagenases emitted by cancer cells, thereby affecting the metabolism of cancer cells. There are specifically Glycine and Proline, as a non-essential amino acid, among the ECM proteins. Additionally, many studies have demonstrated that proline metabolism impacted many pathways in the control of cancer cell plasticity and had the potential to be a prognostic marker and potential therapeutic target (Phang & Liu, 2012; Phang et al., 2015). Many cancer cells can use proline to produce ATP and ROS and proline oxidation had an essential role in cancer cells' survival (Phang, 2019; Huynh et al., 2020). Thus, our results showed that NOB treatment decreased the proline level in PC-3 cells but increased in HUVEC cells compared to those not treated with NOB. These findings suggested that NOB can modulate oxidant status through ECM and selectively regulate oxidant status in MCRPC cells, consistent with the literature. Nobiletin may be an efficient cytostatic anti-cancer agent. Inhibition of cell spread without stimulating cell mortality might be valuable in the treatment of tumors in a way that is less likely to generate cytotoxicity and mortality in non-tumor tissues.

5. Conclusion

The imbalance between antioxidants and oxidants occurs by decreasing the number of antioxidants or increasing the number of oxidants in the cell and can induce positive responses, including cellular proliferation or activation and negative responses as growth suppression and cell mortality. Thus, stress development studies have proven to be instructive for the development of cancer treatments and are useful in developing anti-cancer strategies. Therefore, these preliminary findings provide better insight into citrus nobiletin's role, as a cytoprotective and potential chemotherapeutic agent, on oxidative stress levels and antioxidant enzyme activities in MCRPC through by antioxidant properties of NOB.

Acknowledgements: We would like to thank Dr. Ozlem AKSOY (Kocaeli University) for providing the lab facilities

Ethics committee approval: Ethics committee approval is not required for this study.

Conflict of interest: The authors declare that there is no conflict of interest.

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